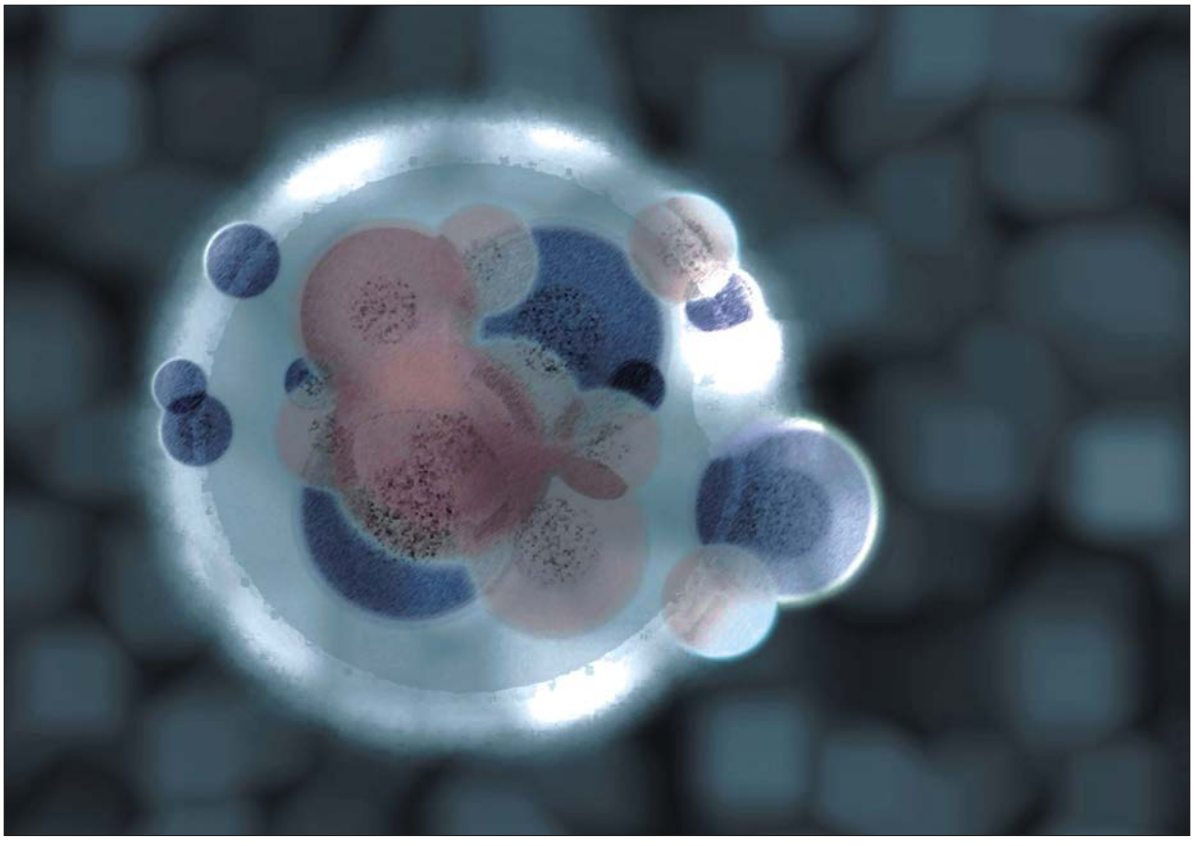

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A Comparison Between Aerial Mass Colors, Antibacterial Activities and RAPD Fingerprints of Soil Isolates of *Streptomyces*

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Abstract: This study compared the distribution of aerial mass colors, antibacterial activities and RAPD-based genomic patterns of 39 *Streptomyces* isolates harvested mainly from soil at the University Agricultural Park, Universiti Putra Malaysia. *Streptomyces griseus* ATCC 10137 strain was also included as a reference strain (n = 40). Based on ISP-2 media, the aerial mass colors observed were categorized as yellow (n = 15), grey (n = 9), brown (n = 7), white (n = 6) and others (n = 3). Antibacterial activities were assessed on Mueller Hinton Agar (MHA) and Tryptic Soy Agar (TSA) by perpendicular streak method against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella* sp. and *Enterococcus* sp. MHA demonstrated 15 isolates with broad spectrum antibacterial activities and 18 as non-broad spectrum. TSA gave lower proportions with 15 and 9 isolates respectively. Regardless of the test media used, a higher proportion of isolates with non-white color showed antibacterial activities to suggest a potential correlation. RAPD dendrogram (a composite of 3 random primers) also clustered majority of them but segregated those of white color, which showed less antibacterial activities, in a different cluster. Further validations involving more isolates are warranted to establish the findings.

Key words: *Streptomyces*, soil, aerial mass, antibacterial activities, random amplified polymorphic DNA

INTRODUCTION

Streptomycetes are filamentous Gram-positive bacteria that give the musty odor of soil. They belong to a complex group of actinomycetes that produce various bioactive metabolites of commercial value such as antibiotics. *Streptomycetes* have been intensively isolated, leading to characterization of more than 3000 species since 1970s (Kim *et al*., 2004; Guo *et al*., 2008). Due to such huge species diversity, traditional methods utilizing biochemical tests and morphological examinations for identification of these organisms are laborious and time-consuming (Cook and Meyers, 2003; Guo *et al*., 2008). Polymerase chain reaction (PCR)-based methods have been very useful to rapidly and accurately characterize the identity of bacteria at molecular level, particularly through the sequence homology of 16S rDNA (Taddei *et al*., 2006; Arasu *et al*., 2009; Li *et al*., 2009). PCR-Random Amplified Polymorphic DNA (RAPD) allows the rapid differentiation of bacterial isolates based on whole genomic pattern which may facilitate the discovery of unique genetic markers in association with the bacterial genus and species (Welsh and McClelland, 1990; Williams *et al*., 1990). The technique has been used in several studies on *Streptomyces* whereby the potential of RAPD to discriminate the isolates, as well as to identify potential genetic probes for genus and species detection was shown to be promising (Mehling *et al*., 1995; Malkawi *et al*., 1999; Roberts and Crawford, 2000). Later, Gharaibeh *et al*. (2003) used the technique to correlate RAPD pattern with various phenotypic features of *Streptomyces*. Such approach may identify evidence at genetic level on the interrelation among the diverse phenotypes for potential manipulation. Nevertheless, the description would be limited to the experimental conditions used in the respective studies that may not coincide with other studies using different cultivation approaches.

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Due to the high potential of *Streptomycetes* as antibiotic producing organisms, this study was undertaken to assess the phenotypic and genomic features of our local isolates mainly from soil of the University Agricultural Park (UAP), Universiti Putra Malaysia (UPM). In view of the lack of standardization in the cultivation methods of *Streptomyces*, this study was preliminarily conducted to characterize the morphological characteristics and antibacterial activities of the isolates utilizing some common commercial media. For genomic typing, the isolates were subjected to RAPD analysis and the outcomes were compared with the phenotypic properties of the isolates for any potential correlation.

MATERIALS AND METHODS

Isolation and identification: Thirty-nine isolates of *Streptomyces* strains were isolated from eight patches of soil, each collected at a different site and labeled with an alphabet; five from various locations around UAP (center; F, northeast; D, east; E, southeast; B and C and south; H), one from a site further south of UAP outside university's periphery (off-campus; I) and one from a far off-location (off-state; A). Isolates, taken from a same patch of soil, were selected to have at least an obvious difference in their morphological appearances such as color of aerial mass, substrate mycelium, spore and appearance of colony. The isolates were labeled with an alphabet representing the sampling site followed by a number to indicate the identity of the isolate. *Streptomyces griseus* ATCC 10137 was also included, giving a total of 40 strains for analysis in this study. All isolates were confirmed as *Streptomyces* based on odor, morphological characteristics and 16S rRNA gene sequence homology (Duraipandiyan *et al*., 2010). Morphological characterization on aerial mass, substrate mycelium, pigment and spore production of matured cultures on ISP-2 (International *Streptomyces* Project-2) agar media (Difco, USA), were done as described by Shirling and Gottlieb (1966) and Taddei *et al.* (2006). Bacterial stocks were preserved in Actinomyces broth (BBL, USA) with 15% glycerol and kept at -80°C. The 16 rDNA sequences of the isolates including *S. griseus* ATCC 10137 strain are available at the GenBank database (http://www.ncbi.nlm.nih.gov) with accession numbers JN116247 to JN116248 and JN566155 to JN566192.

Antimicrobial screening: Perpendicular streak method was used for rapid antimicrobial assessment of *Streptomyces* cultures using Mueller Hinton Agar (MHA) (Merck, Germany) and Tryptic Soy Agar (TSA) (Merck) as test media. The *Streptomyces* isolates were inoculated in a single streak down the middle of the test media and incubated at 28°C for seven days. Then, a single streak of each test organism; two reference strains: *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 and two laboratory strains of clinical origin: *Salmonella* sp. and *Enterococcus* sp., were inoculated in perpendicular against the earlier streak on the agar medium and incubated at room temperature for 24 h (Duraipandiyan *et al*., 2010). Growth inhibition of the test bacteria was observed by absence or presence of inhibition zone from the streak of *Streptomyces* culture.

Genomic DNA extraction: Culture containing mycelia and spores was taken from single colonies on ISP-2 agar medium and suspended in tris-EDTA buffer. Genomic DNA of the isolates was extracted using the GF-1 bacterial DNA extraction kit (Vivantis Tech., Malaysia). Concentration and quality of DNA extract was checked by using the Biophotometer (Eppendorf, Germany).

PCR-RAPD: Three random RAPD primers (OPERON Tech., USA), 10-mer long each, were used in this study in separate experiments; OPA-02: 5'-TGCCGAGCTG-3', OPA-09: 5'-GGGTAACGCC-3' and OPA-10: 5'-GTGATCGCAG-3' (Gharaibeh *et al*., 2003). The reaction was carried out in a 50 μL reaction volume containing the following reagents: 10 μL of 5X i-PCR RED Master Mix (i-DNA Biotech., Singapore), 0.2 μL of primer, 38.8 μL of nuclease free water and 1 μL of template DNA. The thermal cycling conditions were as follows: 3 min at 95°C for initial denaturation, 40 amplification cycles with each comprising 30 sec at 95°C for denaturation, 30 sec at 36°C for annealing and 1 min at 72°C for extension and a final extension at 72°C for 7 min in TPersonal Thermocycler (Eppendorf, Germany). Upon purification, the PCR amplification products and VC 1 kb DNA ladder (Vivantis Tech.) were respectively mixed with EZ-Vision™ One DNA dye (AMRESCO, USA) at 5:1 v/v, resolved by electrophoresis in 1.2% agarose gel and viewed under gel documentation system. PCR reaction for each primer was repeated three times for assuring the reproducibility.

DNA fingerprint analysis: DNA electrophoretic patterns were analyzed using BioNumerics gel analysis software version 6.1 (Applied Maths, Belgium) on the basis of presence or absence of DNA band. RAPD dendrograms based on each and composite of all primers were then generated by the software using the Dice coefficient (Dice, 1945) and UPGMA (Unweighted Pair Group Method of Arithmetic means) cluster analysis. The RAPD clustering patterns were further analyzed in relation to sampling sites and other phenotypic properties of the isolates.

Statistical analysis: The distribution of phenotypic properties of the isolates and their potential associations

were analyzed in a 2×2 contingency table using chi-squared (χ^2) and Fisher's exact test with significant level set at $p<0.05$.

RESULTS

Based on growth on ISP-2 medium, the varieties of aerial mass color among the 40 isolates were categorized as yellow (n = 15), grey (n = 9), brown (n = 7), white (n = 6), black (n = 1), orange (n = 1) and peach (n = 1) with *S. griseus* ATCC 10137 strain having a white color. Color of substrate mycelium, spore and soluble pigment were also observed but not analyzed for simplicity. In the antibacterial assay, 83% of the isolates showed antibacterial activity on MHA to at least one of the test bacteria. However, that on TSA was only 60% of the isolates. *S. griseus* ATCC 10137 strain showed antibacterial activity against all four test bacteria on both media. Table 1 shows the detail on distribution of the aerial mass color-categories and antibacterial activities of the isolates based on both MHA and TSA as the test media, while Table 2 summarizes the aerial mass color-grouping to show only the significant correlation. By classifying the test bacteria into gram-positive and gram-negative groups respectively, with MHA as the test media, 15 isolates showed antibacterial activities by inhibiting either one or both members in both test bacterial groups; referred as broad spectrum, while 18 isolates inhibited either one or both in only either one of the two bacterial groups; referred as non-broad spectrum. Those without any antibacterial activity against the four test bacteria were referred as non-antibacterial isolates. With TSA as the test media, 15 isolates were of broad spectrum but only nine of non-broad. By taking the isolates with broad and non-broad antibacterial activities as a single group in comparing with those without any antibacterial activity, statistical analysis found a significant association between isolates with aerial mass of non-white and white color-categories in relation to antibacterial activities of the isolates on MHA (χ^2: p = 0.001, Fisher's exact test: p = 0.005) but not on TSA ($p>0.05$). The analysis is stipulated in Table 2 whereby a high percentage of isolates with antibacterial activity on MHA is of non-white color-category (91%) as compared to isolates with white color, which are only 33%. Although that on TSA was not found to be significant, a high percentage is also observed for isolates with non-white color-category (65%) to show antibacterial activities against 33% of the isolates with white color.

In the RAPD analysis, the three random primers were able to generate consistent DNA fingerprint pattern in the repeated experiments except primer OPA-02 on three strains (F3, I13 and *S. griseus* ATCC 10137) that gave no amplification. With the exception of the three strains, majority of RAPD patterns were different with number of bands ranging from one to 11 for all primers and size between 350 bp and 1.5 kb for OPA-09 and OPA-10, while 450 bp and 2.5 kb for OPA-02. RAPD pattern generated by OPA-02 had band ranging from one to three for about 50% of the isolates but that for the other two primers had more than three bands for more than 80% of the isolates. *S. griseus* ATCC 10137 had three and eight bands for primer OPA-09 and OPA-10 respectively. OPA-02 identified a similar RAPD fingerprint pattern for two isolates, while OPA-09 and OPA-10 segregated 10 and 20% of the isolates into two and three groups, respectively sharing similar RAPD fingerprint among members of the respective groups. None of the group member with a common RAPD pattern as generated by one primer turned out to have a similar pattern among the group members by the other respective primers. The isolates also differed either in their species name as identified by the Blast analysis based on gene 16S rRNA, or phenotypic properties (e.g. aerial mass color and antibacterial activity).

In Fig. 1, the dendrogram shows a scattered distribution of the isolates indicating the discriminatory potential of the composite analysis based on the three

Table 1: Distribution of aerial mass color-categories in relation to antibacterial activities of the isolates

		Aerial mass				
Test media	Antibacterial pattern	Yellow n = 15	Grey n = 9	Brown n = 7	White n = 6	Others n = 3
MHA	B	4	6	3	2	-
n = 40	n = 15	(27)	(67)	43	(33)	
	NB	10	3	3	-	2
	n = 18	(67)	(33)	(43)		(67)
	N	1	-	1	4	1
	n = 7	(6)		(14)	(67)	(33)
TSA	B	4	5	3	2	1
n = 40	n = 15	(27)	(56)	(42)	(33)	(33)
	NB	4	2	2	-	1
	n = 9	(27)	(22)	(29)		(33)
	N	7	2	2	4	1
	n = 16	(46)	(22)	(29)	(67)	(33)

Values in brackets are percentage, others: Black, orange and peach; n = 1 each, B: Broad spectrum, NB: Non-broad spectrum, N: Non-antibacterial isolates, MHA: Mueller Hinton agar, TSA: Tryptic soy agar

Table 2: White and non-white color-categories in relation to antibacterial activities of the isolates

		Aerial mass	
Test media	Antibacterial pattern	White n = 6	Non-white n = 34
MHA	B/NB	**2**	**31**
n = 40	n = 33	**(33)**	**(91)**
	N	**4**	**3**
	n = 7	**(67)**	**(9)**
TSA	B/NB	2	22
n = 40	n = 24	(33)	(65)
	N	4	12
	n = 16	(67)	(34)

Values in brackets are percentage, bold figures show significant correlation at $p<0.05$. B: Broad spectrum, NB: Non-broad spectrum, N: Non-antibacterial isolates, MHA: Mueller Hinton agar, TSA: Tryptic soy agar

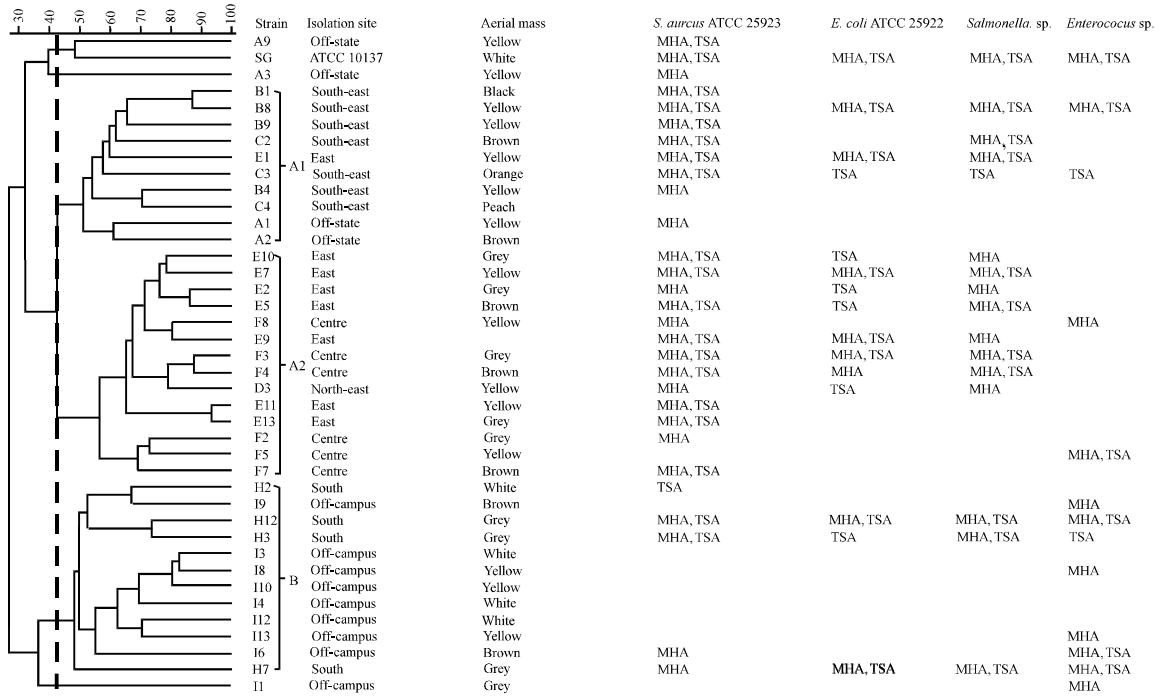

Fig. 1: RAPD dendrogram based on a composite analysis in association with isolation sites, aerial mass color-categories and antibacterial activities of the isolates against four test bacteria on two different test media; MHA and TSA. The indicated major clusters; A1, A2 and B are at the similarity matrix of 42%. *S. griseus* ATCC 10137 is labeled with SG

Table 3: Distribution of aerial mass color-categories and antibacterial activities of the isolates according to RAPD clusters

						Antibacterial activity					
	Aerial mass					MHA			TSA		
RAPD cluster n = 36	Y n = 13	G n = 8	B n = 7	W n = 5	Ot n = 3	B n = 14	NB n = 15	N n = 7	B n = 14	NB n = 8	N n =14
A1	5	-	2	-	3	3	5	2	5	1	4
n = 10	(38)		(28)		(100)	(21)	(33)	(29)	(36)	(13)	(29)
A2	5	5	3	1	-	8	6	-	6	6	2
n = 14	(38)	(63)	(44)	(20)		(58)	(40)		(43)	(74)	(14)
B	3	3	2	4	-	3	4	5	3	1	8
n = 12	(24)	(37)	(28)	(80)		(21)	(27)	(71)	(21)	(13)	(57)

Values in brackets are percentage, Y: yellow, G: grey, B: brown, W: white, Ot: others (black, orange and peach: n = 1 each), B: broad spectrum, NB: Non-broad spectrum, N: Non-antibacterial isolates, MHA: Mueller Hinton agar, TSA: Tryptic soy agar

random primers. The isolates are however clustered at 42% similarity matrix in two major clusters, named as super cluster A and B. Cluster A is further clustered in two sub-clusters named as A1 and A2, while cluster B stands in a single group. Sub-cluster A1 consists of 10 isolates and majority (70%) of them are from southeast of UAP. Although two off-state isolates are present in cluster A1, both are branched away from the rest of the cluster's members. Cluster A2 contains 14 isolates with about the same distribution of isolates from east and center of UAP. On the other hand, majority of isolates (67%) in cluster B (n = 12) are isolates from off-campus while the rest are from south of UAP. Four isolates from soils of non-university's origin are excluded from all major clusters.

The composite also identifies the members of the respective major clusters to be in harmony in term of their aerial mass color-categories and antibacterial activities. Table 3 shows the detail on the distribution of the isolates by excluding fours isolates not included in the major clusters (n = 36). A distribution pattern of aerial mass color-categories is observed with white mostly sorted into cluster B (80%), while others mostly distributed in cluster

A; yellow (76%), grey (63%) and brown (72%). In relation to antibacterial activity of the isolate, that on MHA and TSA were analyzed separately and patterns of distribution were compared (Table 3). Based on MHA as the test media, only two isolates in super cluster A (n = 24) are non-antibacterial while the rest are either broad or non-broad antibacterial isolates. In cluster B (n = 12), the proportion of non-antibacterial isolates is higher (42%) as compared to that in cluster A (8%). On TSA, although the antibacterial activity was observed to be lower than that on MHA, a similar pattern follows whereby the proportion of non-antibacterial isolates in cluster B is higher (67%) as compared to those in cluster A (25%). Only three isolates had uncommon color as of the other isolates (black, orange and peach) and were grouped together in the analysis; all are in cluster A1 and show antibacterial activities except one with peach color.

DISCUSSION

In the assessment of antimicrobial activity, MHA and TSA have been the test media of choice due to their wide availability and capacity to allow growth of many types of test bacteria and diffusion of antimicrobial compounds (Sahm and Torres, 1988; Dalsgaard, 2001). As for *Streptomyces*, an earlier preliminary study had shown a good growth of some representative *Streptomyces* isolates on these two media and thus were assumed to be able to provide sufficient growth for exhibiting visible antibacterial activity of the isolates in this study (Zin *et al*., 2011). Meanwhile, the four test bacteria included in this study are those associated with common normal flora and opportunistic pathogens that inhibitory effect against them would be of a significant interest. Based on this experimental design, it was observed that for some *Streptomyces* isolates, an inhibitory activity was observed on one media but not on the other and a higher proportion of isolates exhibited antibacterial activities on MHA than TSA. This suggested that MHA may be better than TSA in assessing the isolates for antibacterial properties. Further statistical analysis also identified a potential correlation between aerial mass color-categories and the antibacterial activities. Thus, to a certain extent, those with white color-category in this study could be considered as less likely to be antibacterial isolates. The antimicrobial metabolites could be harvested in broth culture and weighted to quantitatively establish the impact of media on production of antimicrobial compound but such approach would be tedious for screening purposes involving many isolates. The perpendicular streak method used in this study, although it is rather qualitative, generated reading based on absence and presence of the inhibition zone to rapidly justify the antibacterial status of the isolates.

The reliability of the relationship between aerial mass color and antimicrobial properties in this study would be affected if groups of related isolates (clone) were present, which was possible as more than one isolate were selected from a single patch of soil. To minimize the problem, isolates from a particular soil sample were selected to differ in one or more in their morphological characteristics so that the potential of having similar isolates would be reduced. The three random RAPD primers (OPA-02, -09 and -10), citing their frequent use in discriminating various organisms including *Streptomyces* (Yu *et al*., 1997; Gharaibeh *et al*., 2003; Naffa *et al*., 2006; Nomura *et al*., 2006; Yaqoob *et al*., 2007), eventually differentiated majority of the isolates as indicated by their different RAPD fingerprint patterns. The reproducibility of the primers was also confirmed in repeated experiments. Although a few isolates shared a similar RAPD fingerprint by the individual primers, the tendency of the isolates to also show a common pattern when tested with a different primer was not observed to rule out potential clones (Louise and Craig, 2001). Consistently, discrepancies of the isolates with regard to either 16S rDNA-Blast's results or phenotypic properties further indicated that those isolates were possibly different. Nevertheless, primer OPA-02 resulted in less number of bands for half of the total isolates as compared to the other two primers and also failed to detect in a few isolates. Thus, a composite rather than a single primer was used in the subsequent analysis for a better resolution (Sigurdsson *et al*., 1995; Louise and Craig, 2001; Gharaibeh *et al*., 2003; Larrasa *et al*., 2004; Cheah *et al*., 2008). In the later analysis, an obvious improvement was then observed that each isolate was branched in its own lineage pattern.

In an attempt to correlate the genomic pattern with the aerial mass color and antibacterial activity of the isolates, a dendrogramatic clustering pattern based on the composite analysis was possible when the similarity level was compromised at 42% (Fig. 1). Taking the analysis on the two phenotypes as independent, the non-white isolates mentioned earlier to be associated with antibacterial activities were mostly clustered in cluster A while white in cluster B. Consistently, regardless of the test media used, cluster A happened to harbor a higher number of isolates with antibacterial properties as compared to cluster B. Gharaibeh *et al*. (2003) also conducted RAPD analyses using the same random primers and showed that based on the composite analysis, there was a clustering pattern according to type of sporophore and melanin production of their isolates. This study also pointed a clustering pattern for aerial

mass color and antibacterial activity among the 40 isolates. A further scrutiny on these linkages is however needed for potential exploitation. To our knowledge, such comparative studies at both phenotypic and genotypic level for *Streptomyces* spp. have not been widely available. It could be due to the complexity of the spp. themselves that problems still persist to find suitable media that can stably and simultaneously express the various phenotypes of *Streptomyces* isolated from various different origins.

As a whole, UAP is a promising site to search for potential *Streptomycetes*, but the issue on the media escalates the challenges in exploring the diversity and useful traits of *Streptomyces*. Preliminarily, this study presents an inference analysis for association between aerial mass color, antibacterial and genotype but subjected to some limitations. Apparently, those observations were only a deduction based on limited growth media and test bacteria. Moreover, the sample size was only 40 isolates to be largely representing the diverse *Streptomyces* population, particularly the color-variety of the aerial mass that was not evenly distributed. Thus, the description and association between aerial mass color and antibacterial property require more validations. For a standardization purpose, this study used ISP-2 agar medium for describing the colonies of the isolates. An earlier analysis on representative isolates indicated the ability of this media to exhibit discrete morphological traits in different isolates over those on MHA and TSA which were quite homogenous (Zin *et al*., 2011). Thus, it was not known the impact of using different media on the outcome of the aerial mass color and its potential connection with the antibacterial activities among the isolates in this study as the later was conducted on MHA and TSA. In addition, the antibacterial status of the remaining isolates not detected to be exhibiting any antibacterial activity was still ambiguous as it could turn out differently if other media were used. As for the RAPD analysis, despite its rapidity, it carries the drawback in the chemical complexities of the reaction itself that variation may be expected when different RAPD primers, molecular reagents, PCR running protocols and thermocycler are used (Li *et al*., 2009). Therefore, further improvements are warranted involving a wider coverage of isolates, locations, phenotypic traits and microbiological media, as well as the use of other random primers or means of genomic typing for a better reliability and comparability of data.

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