Short Research Article

Occurrence and distribution of antibiotic resistant bacterial genes in agricultural lands of Japan, Indonesia and Philippines

Abstract

The spread of Antibiotic-Resistant Bacteria (ARB) world-wide leads to difficult and prolonged treatment of diseases and causes significant damage to human health and the environment. In this study, the distribution of resistance genes was investigated in terms of country, plant species, and with/without manure. Fifty-five soil DNA libraries from Japan, the Philippines and Indonesia were amplified by PCR and electrophoresis was used to detect target bands of antibiotic resistance genes, which included three types of sulfonamide resistance, *Sul1*, *Sul2*, and *Sul3*; eight types of tetracycline resistance, *TetM*, *TetO*, *TetS*, *TetW*, *TetC*, *TetA*, *TetB*, and *TetL*; *blaTEM* for beta-lactam resistance; *ermB* for macrolide resistance; and *qnrA* for quinolone resistance, intl1 as integrons. The most characteristic results were obtained for plant species, and the difference between orchards and fields was found to affect the resistance genes. The distribution of genes was bimodal between Japan, which belongs to the temperate zone, and the Philippines and Indonesia, which belong to the tropical zone, and the differences in drug resistance genes were found to be due to differences in biome. The detection rate increased in soils with and without manure, but there were no significant differences in resistance genes other than *ermB*.

Key words: antibiotics, resistant bacteria, country, animal manure, drug

Introduction

Antibiotics are introduced to farm animals along with their food to treat or prevent the development of diseases. Drug-resistant bacteria, that are resistant to the antibiotics action, survive and multiply in the cattle. The proliferating drug-resistant bacteria migrate to the cattle manure and remain in the compost. The compost is used on farmland, and the drug-resistant bacteria also spread through it. Antibiotic-Resistant Genes are also leached into the soil around cattle farms, where they are spread into the environment by the movement of people and wildlife, by wind with soil particles, and by runoff as wastewater and groundwater (Li et al., 2017; Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014).

Human agricultural practices have a significant impact on soil ecosystems, and tillage is one of the most effective ways to significantly change the ecological environment (Mirsky et al., 2012). Therefore, the soil ecosystem is most stable in nature, without human intervention, while the least stable is in fields where annual plants are replanted every year, the soil is plowed, and compost is applied. In contrast, orchards grow fruit trees, which are perennial plants, so although fertilizers and other human interventions are required, the soil is tilled less frequently and the soil ecosystem is considered more stable than in fields (Meyer, Wooldridge, & Dames, 2015; Nesme & Simonet, 2015). It was assumed that the less stable the soil, the less likely that only certain bacteria will increase, so that drug resistance genes will be less likely to remain.

In recent years, as the worldwide population has increased, the number of resources consumed by humans has increased to the equivalent of 2.8 Earths, according to a 2014 study (Ecological Footprint., 2012019) the goal is to create a sustainable society (Tilman, Cassman, Matson, Naylor, & Polasky, 2002). The same is true for agriculture, where resource recycling and production efficiency are desired to meet the increasing population, while one of the problems is the residue of antibiotics and ARGs (antibiotic resistant genes) contained in animal manure in the soil and their leaching into the surrounding environment (De Vries et al., 2011; Li et al., 2017). An increase in the number of antibiotic resistant pathogenic bacteria due to the spread of drug resistance genes may reduce the options of antibiotics that can be used when infecting livestock and humans (Okubo et al., 2019; Zhao, Dong, & Wang, 2010).

In China, around 8,000 tons of antibiotics are administered annually as feed additives (Ben, Qiang, Adams, Zhang, & Chen, 2008). In the United States, 16,000 tons of antibiotics are administered annually. In the United States, 16,000 tons of antibiotics are used annually, and it is estimated that 70% of these are for prevention and growth promotion rather than for disease

treatment (Sarmah, Meyer, & Boxall, 2006). Prolonged exposure to the selection pressure of antibiotics causes bacteria in animals to acquire drug resistance genes. Large doses of administered antibiotics are rarely absorbed by animals (Sarmah et al., 2006).

Livestock manure is used as compost to improve soil fertility on farmland, but livestock manure contains drug- and antibiotic-resistant bacteria that have acquired drug resistance in their bodies and spread to farmland. Widespread antibiotics exert selective pressure on the bacterial community in agricultural land, and drug-resistant genes can be transmitted horizontally to indigenous bacteria, thus promoting the establishment of more antibiotic-resistant genes (Heuer & Smalla, 2007; Martinez, 2009).

Vegetables grown in soils with high concentrations of ARGs due to fertilizers may mediate the transfer of resistance genes from soil to humans and threaten human health (Berger et al., 2010; Heuer & Smalla, 2007; Marti et al., 2013). In addition, compost has been shown to contain large amounts of bacteria harboring antibiotic resistance genes and mobile genetic factors such as plasmids, transposons and integrons (Heuer & Smalla, 2007; Khan, Chao, Waqas, Arp, & Zhu, 2013).

Reviewing the use of animal manure is an unavoidable task to prevent the spread of antibiotic resistance genes. Drug Resistance genes exist in the form of resistant bacteria, which originally exist as a natural ecosystem, but are thought to be influenced by various human agricultural systems, such as fertilization, irrigation, and tillage (Akiba et al., 2015; Mirsky et al., 2012). Among these, manure application is thought to be particularly likely to affect drug resistance genes in soil because it mediates the movement of antibiotic-resistant bacteria and contains high concentrations of antibiotics in addition to carbon, which is used for bacterial growth. In fact, sulfonamide resistance genes increased in Korean paddy fields soils after prolonged manure application (Kim et al., 2017). Differences in water quality, physicochemical properties, and bacterial composition had different outcomes and impacts in rice soils and terrestrial soils (Chang Chien, Wang, Hsu, & Seshaiah, 2006; Chen et al., 2012; Ma et al., 2010). There were no studies that reported the effect of cultivation on drug resistance genes. It is possible that drug resistance genes are affected by the frequency of tillage in fields and rice fields where annual plants are grown and in orchards where perennial plants are grown.

This survey mainly investigated the distribution of resistance genes per country and plant at the moment, in order to combat the spread of antibiotic resistance genes from animal manure into the environment. Following this, the background and objectives are described, as well as the materials and methods used for data analysis, the methodology, and the results.

2.Materials & Methods

2.1 Sample Collecting and DNA Library

PCR was performed on soil DNA libraries from each site and plant to amplify the drug resistance target genes. Then, electrophoresis is used to identify the target band of DNA amplified by PCR and to identify the presence or absence of the drug resistance target gene in the sample.

The next step in the methodology is to consider the antibiotic resistance genes used in the experiment. Three Sulfanamide resistance genes, eight tetracycline resistance genes and one resistance gene for each beta-lactam, macrolide, quinolone and integron integrase were investigated.

Samples of DNA were selected from seven regions - Tohoku, Kanto, Chubu, Kansai, Chugoku-Shikoku, Kyushu, and Okinawa - so that each contained at least one species of fruit tree, grain, rice, and vegetable plant. DNA libraries from the Philippines and Indonesia are considered. Four banana samples were used as fruit trees and four soybean samples as cereals. The samples from Indonesia are from a University of Tadulako farm in Poso, Sulawesi. Eight cocoa samples were used as fruit trees.

A total of 55 samples were used in the experiment: 11 samples from Japanese orchards, 28 samples from Japanese fields and paddy fields, 4 samples from Philippine orchards, 4 samples from Philippine fields, and 8 samples from Indonesian orchards. Each sample was also checked for the use of animal manure.

The soil DNA libraries used were those that had been cryopreserved after extraction. Japan was selected to include at least one fruit tree, one field grain, paddy rice, and plant species from Tohoku, Kanto, Chubu, Kinki, Chugoku/Shikoku, Kyushu, and Okinawa, respectively. In the Philippines, four types of cereal (soybean) and four types of fruit trees (banana) from the University of the Philippines Los Baños field were used. In Indonesia, eight species of fruit trees (cocoa) from the University of Tadulako field near Poso, Sulawesi were used. The use of animal manure in each soil was confirmed by the soil sample donor. The country, plant and manure availability for each soil DNA library are presented in the part "results and discussion".

2.2 PCR analysis and agarose gel electrophoresis

The reaction solution was a 25 $\mu\ell$ mixture containing 1 $\mu\ell$ of genomic DNA, 12.5 $\mu\ell$ of Takara's Emerald Amp MAX PCR Master Mix, 2 $\mu\ell$ of each of the forward and reverse DNA primers, 7. 5 $\mu\ell$ of sterile distilled water containing 25 $\mu\ell$ of mix; PCR amplification proceeded

with an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and finally extension at 72°C for 7 min. The primers for the target genes were the sulfonamide resistance genes (*Sul1*, *Sul2*, *Sul3*) (Phuong Hoa et al., 2008), the tetracycline resistance genes (*TetM*, *TetO*, *TetS*, *TetW*, *TetC*, *TetA*, *TetB*, *TetL*) (Y. H. Kim et al, 2007; de Vries et al., 2011), the β-lactam resistance gene (*blaTEM*) (Colom et al, 2003) macrolide resistance gene (*ermB*) (Knapp et al., 2010) quinolone resistance gene (*qnrA*) (Kehrenberg, 2006), and Integron gene (*intl1*), which aids in horizontal gene dissemination (Barraud et al, 2010) A total of 15 primers were used: 7$\mu\ell$ PCR products were stained with Loading 0.5$\mu\ell$ and Midori Green 0.5 $\mu\ell$ dye and placed next to a 100 bp λ marker for band size confirmation and subjected to 0.8% Agarose gel electrophoresis was performed. After electrophoresis, the agarose gel was irradiated with UV light and photographed to visually confirm band detection; the presence of the target gene was confirmed and recorded by comparing the detected band size with reference to the λ marker and the photograph of the previously confirmed positive control.

2.3 Data Analysis

For each sample, the ranking of country, plant species, and presence/absence of target gene detection was presented using a heat map. To determine the effect of country, plant species, and compound on the presence or absence of target genes, significant differences were calculated for each resistance gene using the Kruskal Wallis test with SPSS ver 20. To examine the similarity of each sample, Genes software was used to calculate correlations based on the presence or absence of resistance genes, and a principal coordinate analysis was performed (Cruz, 2013).

Results

The Sulfanamide 1 gene was found in large numbers in fruit trees from Japan and the Philippines. Tetracycline O, C, A and β-lactam were also abundant in Japanese fruit trees. On the other hand, tetracycline B was found in many fields, but rarely in fruit trees. These differences may be influenced by the plants grown. Tetracyclines W and A, circled in green, were rarely found in the Philippines and Indonesia, while macrolides and integron integrase were found in greater numbers than in Japan, suggesting that they may have been influenced by the country.

The correlations between the detection of ARGs by plant species, country and compound availability are calculated using the Kruskal Wallis test. By plant, significant differences were identified in seven species. By country, four species had a significant difference of 90% or more. Only the macrolides had a significant difference of 90% or more in the presence or absence of

animal manure. Cereals and vegetables, are distributed in a close area, indicating similar trends in drug resistance genes. The fruit trees plotted with green triangles were distributed over a much larger area and showed a different trend than the cereals and vegetables. It can be seen that the Japanese fruit trees show a rather specific trend. The analysis of principal correlations for each sample, classified by country shows that the Philippines and Indonesia were concentrated in close proximity, while Japan was widely dispersed.

In general, resistance genes were detected most frequently in fruit trees, with a particular concentration in Japanese fruit trees. Resistance genes were detected in 37% of plots in fruit trees as a whole, compared to 27% in cereals and 29% in vegetables. Looking at resistance genes individually, *Sul1*, *TetO*, *TetC*, *TetA* and *blaTEM* were detected with particular frequency in Japanese fruit trees. In contrast, *TetB* was detected mostly in Japanese cereals and vegetables, and almost none in others. Looking at countries, 33% of the resistance genes were detected in Japan, 30% in the Philippines, and 25% in Indonesia. Analyzing the resistance genes individually, *TetW* and *TetA* were detected less frequently in the Philippines and Indonesia, while *ermB* and intl1 were detected more frequently. *TetM* and *TetL* were universally abundant, while *Sul2*, *Sul3* and *qnrA* were generally present, but were detected at low levels.

Significant differences were calculated using the Kruskal Wallis test, and those with a significant difference of 90% or more were considered significant. In the plant species, two resistance gene types with 90% or more significant difference were *TetO* and *TetL*, three resistance gene types with 95% or more significant difference were *Sul1*, *TetA* and *ermB*, and two resistance gene types with 99% or more significant difference were *tetB* and *blaTEM*, resulting in seven resistance gene types with significant difference in total. In the country, three genes (*TetA*, *TetB* and *ermB*) and only one gene (*intl1*) showed more than 95% and 99% significant differences, respectively, indicating that a total of four resistance genes were significantly different. In the presence/absence of animal manure, only *ermB* showed more than 99% significant difference.

The distance between each plant species attribute and country was widely distributed among plant species, with cereals and vegetables being very close, and fruit trees ranging from near until far from the former. By country, the Philippines and Indonesia were concentrated nearby, while Japan was widely dispersed. The fruit tree sample was further compared between Japanese fruit trees and tropical fruit trees from the Philippines and Indonesia combined, with Japanese and tropical fruit trees forming distinct populations.

Discussion

Finally, the plant and country were correlated with many types of resistance genes. Plant species: orchards, which are not cultivated, may be more prone to the growth of antibiotic-

resistant bacteria than fields, and resistance genes may be more likely to remain. Regarding the countries, the Philippines and Indonesia are tropical, and rainfall and soil quality are relevant. And considering the possibility that the antibiotics used are different from those used in Japan.

There is a possibility that the resistance genes have spread to fields where the manure was not added due to human and material traffic. Concluding that plant species and country showed significant differences in several resistance genes. The reason for the correlation by plant species is the orchards that are not cultivated are more conducive to the growth of soil microorganisms than fields, and drug-resistant bacteria may have grown in the same way, leading to residual antibiotic resistance genes (Basulira, Olet, & Alele, 2019). The reasons for the correlation between the countries may be that there is a gap in the biological community between the temperate zone of Japan and the tropical zone of the Philippines and Indonesia, due to differences in climate and soil properties, and the fact that the type and rate of antibiotics used in each country may be different.

The presence or absence of compost did not differ significantly in correlation with the presence or absence of most resistance genes. This may be due to the original presence of antibiotic resistance genes in soils not fertilized with compost, or the spread of resistance genes due to human and material traffic. In the classification of plant species, many ARGs were detected, especially in fruit trees, while cereals and vegetables showed a similar distribution.

Cereals and vegetables showed a similar distribution. As a reason for this difference, it was observed that fruit trees are perennial plants, while cereals and vegetables are annual plants. Orchards that grow perennial crops do not use tillage, which is one of the main agricultural practices. Compost may contain residues of antibiotics fed to livestock (Boxall et al., 2003; Sarmah et al., 2006; Witte, 2000), and their selection pressure would be higher if there was no change in the soil environment due to tillage. In addition, as the number of microorganisms in the soil increases in no-till and tillage (Kanazawa, 1995), antibiotic-resistant genes may also increase with the growth of the resistant bacteria, especially in situations where antibiotic selection pressure is applied, which increases the likelihood that they will remain in the soil (Li et al., 2017; Segawa et al., 2013).

Regarding the country classification, the Japanese crop had a high variance, while the Philippines and Indonesia had a low variance. As a reason for this difference, it was observed that Japan belongs to the temperate zone, while the Philippines and Indonesia belong to the tropical zone. When forest soils are converted to new agricultural land, organic carbon and organic nitrogen tend to decrease for a certain period of time in temperate soils before it stops decreasing and creates a new equilibrium (Liu, Su, Li, Lang, & Huang, 2018). In contrast, tropical soils experience rapid reductions in organic carbon and organic nitrogen, as well as physical erosion

due to precipitation, and microbial biomass declines rapidly (Srivastava, Ahmed, Kumar, & Mohandas, 2012). The fact that more antibiotic-resistant genes were detected in Japanese fruit trees, in particular, compared to tropical fruit trees, may be due to the fact that tropical soils have a lower microbial biomass than Japanese soils, and drug-resistant bacteria may have been less likely to proliferate. However, it is possible that the sample data used in this study was biased because it was collected from one organization in the Philippines and Indonesia, while in Japan the samples were collected nationwide. More detailed and accurate data could be obtained by collecting samples from a wider area, such as farms from different organizations in the Philippines and Indonesia.

Regarding the presence or absence of animal manure, more drug resistance genes were detected in the population without animal manure, but significant differences were found only for *ermB*, with no significant differences in other antibiotic resistance genes. There are several previous studies showing that fertilization with animal manure spreads and maintains antibiotic resistance genes in agricultural land (Stanton, Humphrey, & Stoffregen, 2011; Udikovic-Kolic et al., 2014). In the present experiment, the presence or absence of antibiotic resistance genes was detected, so the results were calculated in terms of the number of types of, not the quantity present.

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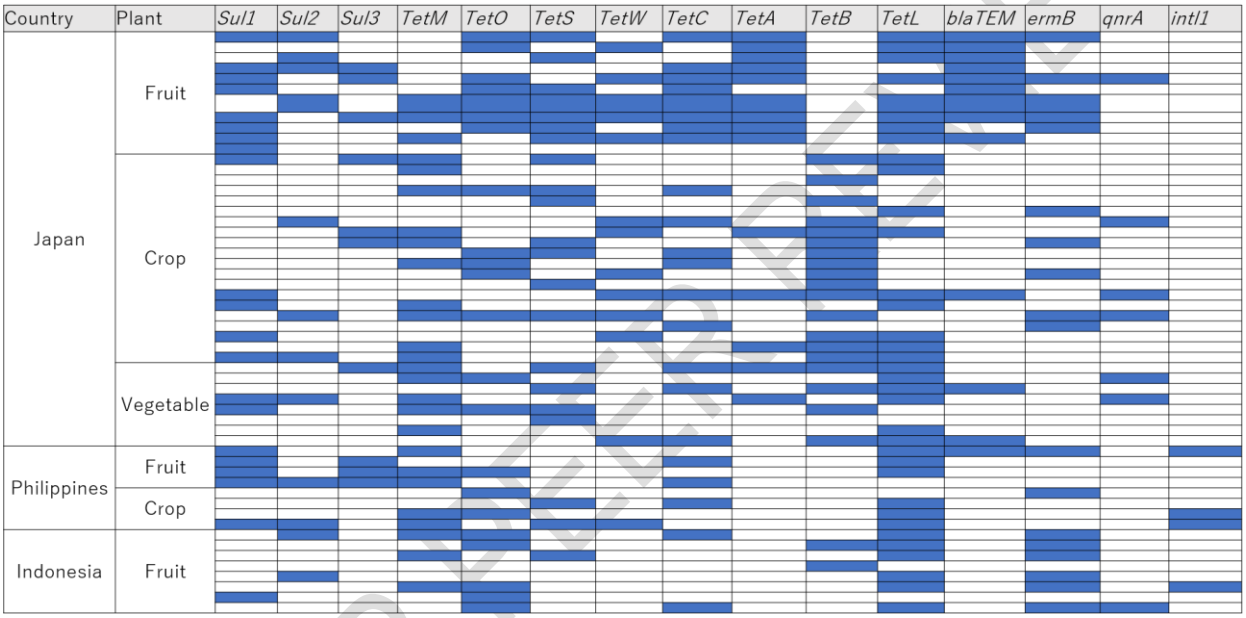
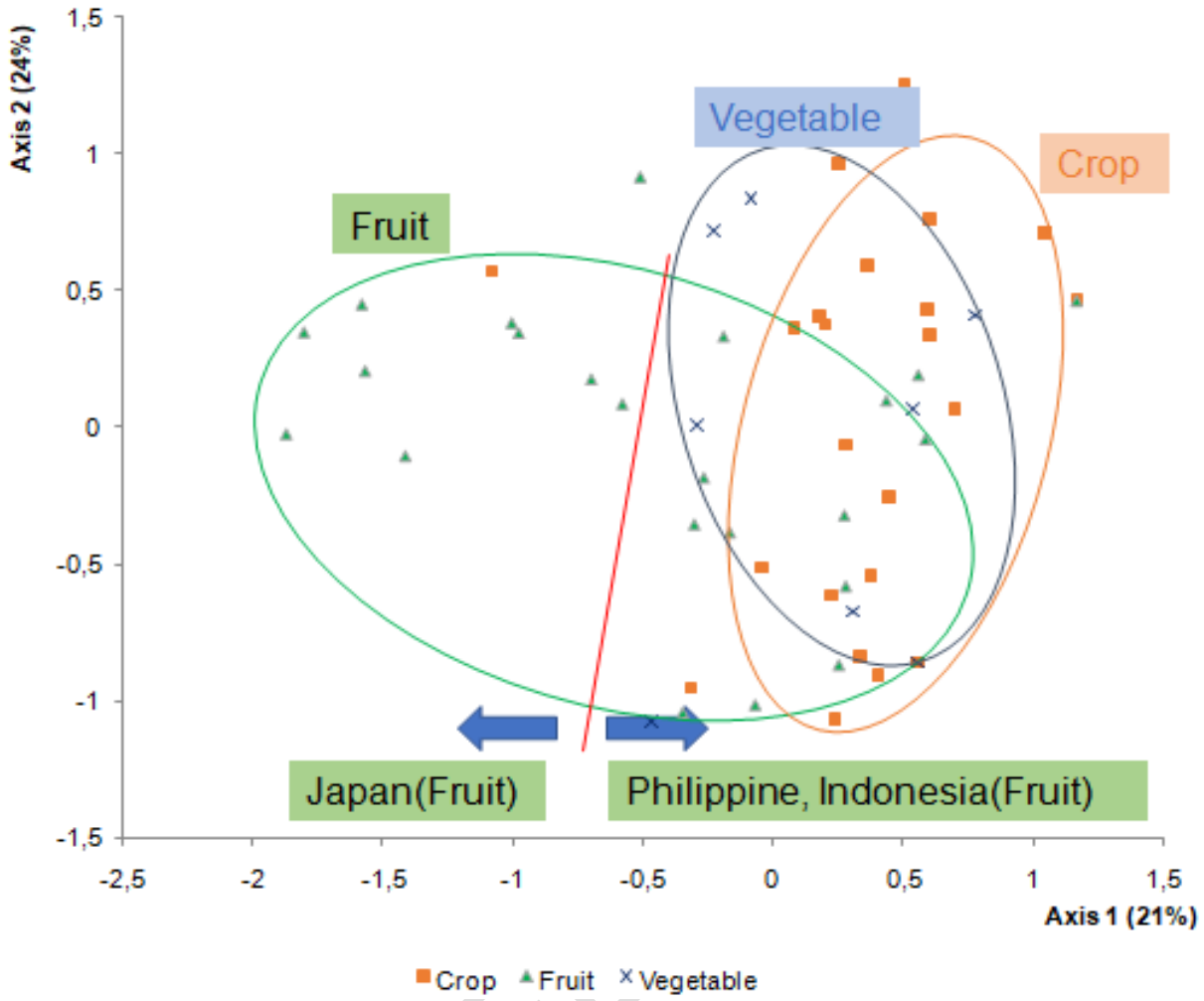


Fig. 1 - Distribution of fruit, grain, and vegetable soils and resistance genes in Japan, the Philippines, and Indonesia.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Fruit
Japan(Fruit)
Philippine, Indonesia(Fruit)
Crop
Fruit
Vegetable

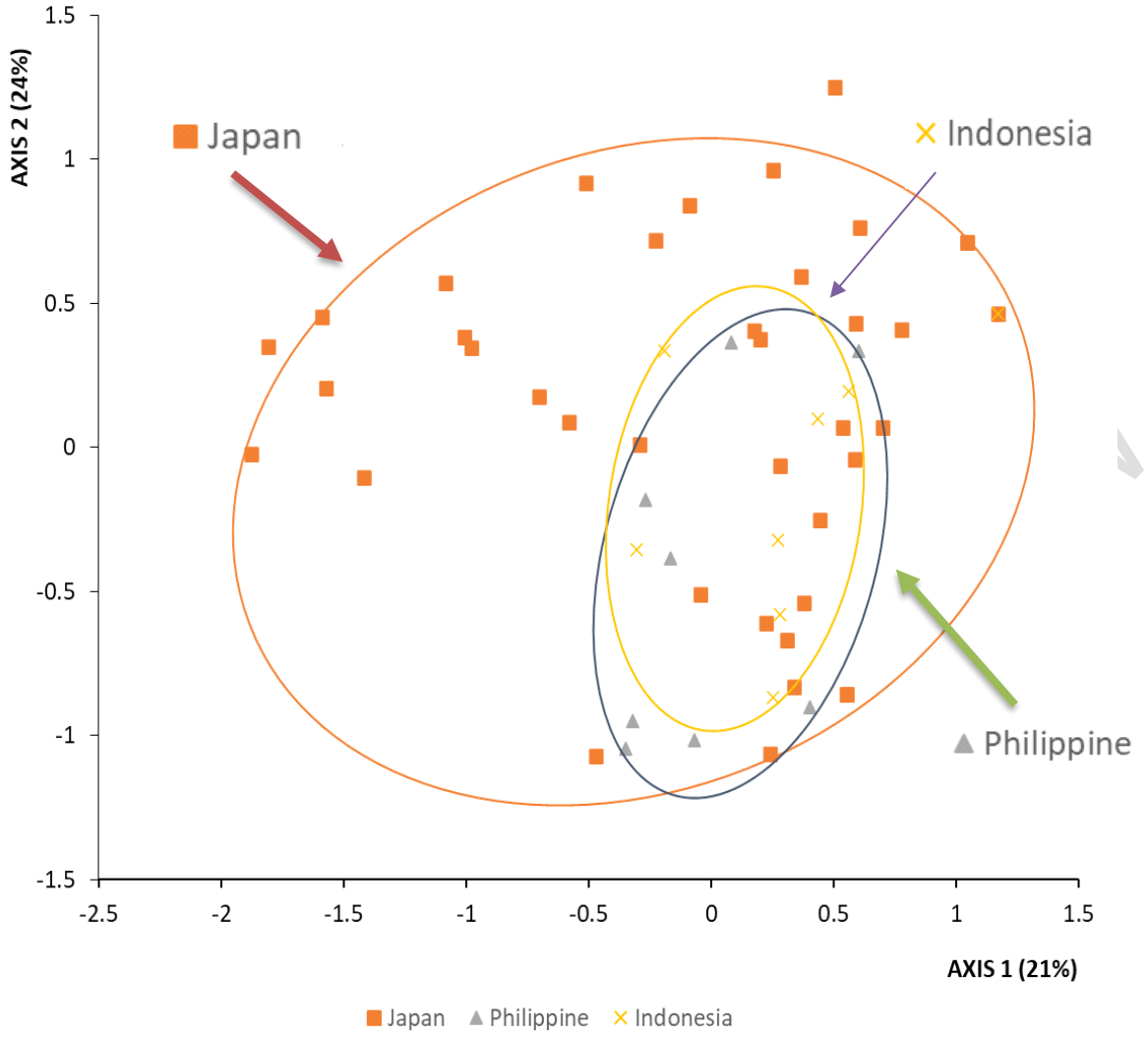

Fig. 2 Principal Coordinate Analysis (PCoA) to analyse the ARB according to the plant species (A), country (B).

Table 1. Significant difference between plant, country, and compost fertilization and detection of resistance genes (Kruskal Wallis Test)

	Sul1	*Sul2*	*Sul3*	*TetM*	*TetO*	*TetS*	*TetW*	*TetC*	*TetA*	*TetB*	*TetL*	*blaTEM*	*ermB*	*qnrA*	*intl1*
Plant	*	n.s.	n.s.	n.s.	z	n.s.	n.s.	n.s.	*	**	z	**	z	n.s.	n.s.

	Sul1	*Sul2*	*Sul3*	*TetM*	*TetO*	*TetS*	*TetW*	*TetC*	*TetA*	*TetB*	*TetL*	*blaTEM*	*ermB*	*qnrA*	*intl1*
Country	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	*	n.s.	**

	Sul1	*Sul2*	*Sul3*	*TetM*	*TetO*	*TetS*	*TetW*	*TetC*	*TetA*	*TetB*	*TetL*	*blaTEM*	*ermB*	*qnrA*	*intl1*
Manure	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.

(n.s. – Non significant ; z - p<0.1 ; * - p<0.05 ; ** - p<0.01)