Assessment of Genetic Diversity of *Kauayantinik* (*Bambusa blumeana* Schultes f.) from Five Plantations in the Philippines through Transferability of SSR Markers

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Abstract

The assessment of the genetic diversity of *Kauayantinik* (*Bambusa blumeana* Schultes f.) plantations in selected provinces such as Pangasinan (PKT), Batangas (BKT), Cebu (CKT), Iloilo (IKT) and Bukidnon (BdKT) in the Philippines using microsatellites/SSR markers that previously identified some genome sequences of other bamboo and rice species was studied. 75% of the primers screened were able to cross-amplify *Kauayantinik* DNA. Out of the 64 loci detected, 35.9% was found to be polymorphic. Based on Nei-Li's similarity index, high similarity rate was observed suggesting a close relationship within populations. Pangasinan population was the most diverse source of *Kauayantinik* based on the expected heterozygosity within populations while Iloilo had the highest diversity rate (ca. 0.56 Nei-Li's dissimilarity coefficient)among populations. Iloilo population was also clustered singly based on the dendrogram generated. Data gathered gave information that the endemic bamboo, *Kauayantinik*, has rich genetic resources that can be used for genetic improvement of the species for economic and biodiversity purposes.

INTRODUCTION

Bambusa blumeana Schultes f., known as *Kauayantinik* in the Philippines, is a non-timber, woody plant belonging to the family of grasses (Poaceae). It is widely distributed at low and medium altitudes, settled areas throughout the Philippines and mostly occurring in southern China to the Malay Peninsula (Roxas 1998). Bamboo nowadays is an essential non-timber forest species in the world because of its adaptability, quick physical growth and development that result to high quality finished products, giving high profit to businessmen. After three to five years of its propagation, it can be sold in a hundred peso per cutting or seedling which is faster than any other woody forest species which may need longer years to be sold. However, with the continued and increasing demand for bamboo and non-timber products, massive logging and deterioration of these forest species occur, resulting to decrease in genetic resources and biodiversity in the country.

One of the solutions to the problem of continued deforestation is conducting analyses on the genetic diversity of the forest species to provide strategic and more effective implementation of tree conservation and reforestation. The data gathered from these studies could be useful for yield increase in trees, improvement of defense system and adaptation against pathogens and drastic changes in the environment.

Researchers are continuously giving efforts to determine the diversity of several forest species in the Philippines. Species such as Narra (*Pterocarpus indicus* Willd), Limuran (*Calamusornatus* var. *philippinensis* Becc.), Dao [*Draconto melondao* (Blanco) Merr. & Rolfe], Mahogany (*Swietenia macrophylla* King), Yemane (*Gmelina arborea*Roxb.) have preliminary studies on its status, diversity and survival (Delos Reyes, et.al 2013; Ambida, et.al 2012; Baja-Lapis and Santander 2003;

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Corpuzand Carandang 2012).These studies have involved the use of molecular markers to provide data on the polymorphism and characterization of the forest species, resulting to genetic diversity determination. Also, there are limited local literatures on the genetic diversity status of our native bamboos in the country that are currently cited. Using Simple Sequence Repeats (SSR) or Microsatellite Markers, genetic diversity within and among populations of the bamboos in the country, specifically, *Kauayantinik* may be determined.

This study aims to currently assess the genetic diversity of *Kauayantinik* (*Bambusa blumeana* Schultes f.), using SSR molecular markers. The specified objectives are:

1) to establish a protocol to isolate high quality genomic DNA (gDNA) from young leaf tissue of *Kauayantinik*;
2) to compare genetic variation within and among the different populations of the species;
3) to cross –amplify *Kauayantinik* with rice SSR markers for further genetic analysis; and
4) to measure and analyze the genetic distance and similarity and polymorphisms detected on the SSR bands of *Kauayantinik.*

REVIEW OF RELATED LITERATURE

Coming from the family of grasses, *Kauayantinik* (Figure 1)*,* is a non-timber, woody, perennial species widely used in agroforestry, landscape architecture, engineering and industry. Bamboo can help in climate mitigation by its use as energy source, distillation and gasification for production of charcoal, vinegar and oil (Dube 2008).Bamboo has a high carbon fixation rate, rapid growth rate and high adaptability to extreme environmental conditions, thus, it is considered as one of the important forest resource (Bhandawat, et al. 2014). In about three to five years after macropropagation, its young shoots may be sold as grown seedlings (Bareja 2010).

The finished products (e.g., furniture, tools and ornaments) of *Kauayantinik* provide business for entrepreneurs and can be a source of daily income to billions of people. The Philippine Forestry Statistics reported in 2013 that the prices of species of bamboo like *Bayog, Boho, Bulo* and *Kauayantinik* ranged from Php43.00 to 87.53 per piece. Currently, China is the top largest producer of bamboo in the world while the Philippines ranked as sixth. The bamboo industry worldwide is expected to exponentially increase up to 20 billion dollars in the upcoming years (Lobovikov et. al. 2005; De Vera 2012; Forest Management Bureau 2013).

There are many reported abundant sources of bamboo in the world. Among the 1,000 species of bamboo in the world, 62 of its species can be found in the Philippines. Out of the 62 species, 21 are endemic in the country, 13 were classified as climbers while 8 are erect (Bareja 2010).

Based on limited archeological and anthropological studies, bamboo was believed to be originated in China where it was first annotated based on recorded histories which were used for their daily use (Bamboo grove 2014). In some of its native cuisine, bamboos serve as their based ingredients in making their delicacies. The most well-known delicacy among the Filipino dishes is *Labong* with bamboo shoots as the main ingredient. Some of the native varieties of Labong include *Ginataang Labong*(with coconut milk and chilies) and *Dinengdengna Labong* (labong in fish anchovies with string beans, spinach, and sun-dried fish), according to Filipino Style Recipe (2012, 2013).

Currently, asexual reproduction is the mode commonly used on its propagation. The common methods included in the asexual reproduction of bamboo are: Clump Division, Basal Clump Division, Culm, Culm Cutting, Branch Cutting, Marcotting, Twig Cutting, Integrated Mass Propagation Technique, as well as offset or rhizome cutting and tissue culture (Bareja 2010; Roxas 1998).

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Figure 1.

Simple Sequence Repeats/ Microsatellite Markers

Molecular markers are important for plant improvement and involve the following applications: (1) determination of genetic diversity within and among populations, (2) genotype characterization and (3) marker-assisted selection (Porth and El-Kassaby 2014). In diversity screening, whole range of molecular techniques can be used to detect polymorphisms. Screening is categorized into non-PCR based (Restriction Fragment Length Polymorphism/RFLP), PCR-based (Random Amplified Polymorphic DNA/RAPD, Amplified Fragment Length Polymorphism/AFLP, Inter Simple Sequence Repeats/ISSRs, Single Sequence Repeats/SSRs and Single Nucleotide Polymorpism/SNPs) and targeted PCR and sequencing (Karp and Edwards 1997). PCR-based DNA markers such as SSRs and SNPs help in many desired characters simultaneously by back-cross populations and F2 generations (FAO/IEAE Division of Nuclear Techniques 2002; Mondini 2009).

PCR-targeted sequences involve procedures on designing primers to target specific regions of genome. Targeted amplified product can be compared to corresponding product from another individual and will detect differences in base pair lengths (Karp and Edwards 1997). Table 1 shows the comparison of markers and molecular techniques and corresponding uses.

Table 1. Comparison on the marker systems/molecular techniques (FAO/IEAE Division of Nuclear Techniques, 2002).

Marker/technique	PCR-based	Polymorphism (abundance)	Dominance
RFLP	No	Low-Medium	Co-dominant
RAPD	Yes	Medium-High	Dominant
SSR	Yes	High	Co-dominant
ISSR	Yes	High	Dominant
AFLP	Yes	High	Dominant
IRAP/REMAP	Yes	High	Co-dominant
Morphological	No	Low	Dominant/Recessive/Co-dominant
Protein/isozyme	No	Low	Co-dominant
STS/EST	Yes	High	Co-dominant/Dominant
SNP	Yes	Extremely High	Co-dominant
SCARS/CAPS	Yes	High	Co-dominant
Microarray		High	

SSRs, also known as microsatellites are short tandem repeats consisting of a repeat unit of 2-10 bp in length. These markers are used in diversity analysis, breeding, QTL mapping, genetic linkage map, gene tagging, map-based gene cloning and other biotechnology and genetic applications (Kahl 2001; FAO /IEAE Division of Nuclear Techniques 2002; Applied Biosystems, Inc. 2004).These markers are ideal for detecting differences between and within species of genes of eukaryotes (Farooq and Azam 2002). SSRs are also widely applied in studies of plant molecular genetics. They are abundant in genome, distributed codominant in nature, have high repeatability and transferability in cross-species applications. It is PCR-based and co-dominant nature make it a marker type choice in a variety of research fields (e.g., map construction and genetic variability identification).Strategies to use SSRs are time and cost-consuming, especially in development of enriched libraries and species-specific primers, thus, limiting the application of SSR in genomes with less information. One way to solve this is through cross amplification of SSR markers from genome with an abundant SSR markers in a target genome (Powell et. al 1996; Nayak and Rout, 2005; Chen, et.al. 2010; Miah, et.al 2013).

Transferability of Molecular Markers in Plant Species

With less time for marker development on species and scarcity on the developed marker on the species selected for study, cross-amplification or transferability of markers from one species to another is done on several plant diversity analyses. This is also done to confirm the classification and taxonomic groupings of the species within the genus. Choosing a marker for plant molecular analyses is objective dependent. For example, RAPD, RFLP or AFLP can be used when a species with similar accessions is to be differentiated. On the other hand, neutral markers like DNA micro-array or isozyme markers can be used if few environmentally physiological characters in a species are to be distinguished (Farooq and Azam 2002).

The microsatellite markers developed in genomic libraries were used for mapping as this can be featured to the high level of polymorphism, abundance in their genomes, reproducibility and codominant nature (Gupta 2000; Squirrell 2003; Zeid et.al 2010). Transferability of molecular markers, specifically SSRs, have been used for baseline studies for the species with limited or no developed marker for genetic and molecular analyses. Examples of marker transferability for initial species studies were done in pearl millet (Yadav et. al 2008), bottlebrush and clove (Rai et. al 2013).

High percentage of marker transferability may be useful in genetic variability from different species in breeding programs. Other studies of cross-amplification were done in *Allium*, *Hevea*, *Eucalyptus,*

Oryza, Zostera, Cucumis, Glycine, Trifolium, Brassica,Vitis, Cajanus, Festuca and *Arachis*. These several studies were suggest that marker transferability is useful for establishment of new markers to species with limited to no genetic analysis studies (Lee et al 2011; Mantello et al 2012; Rossetto 2001; Cui et. al 2008; Saha et. al 2006; Chen et. al 2010).

Marker transferability studies are also done in bamboo species since little information on their sequences are established.Bhandawatet. al (2014)have developed and cross-transferred microsatellite markers in *Dendrocalamuslatiflorus*and related bamboo species. Interspecies and intergenera cross-transferability rates resulted to 92.7% and 84.5%, respectively.Dong et.al (2012) also studied *Dendrocalamus*sinicus using 16 novel microsatellite markers which yielded to 53.6-81.3% cross-transferability rate to other *Dendrocalamus*species. In *Phyllostachys*species, RAPD, RFLP, AFLP and complementary DNA SSR markers were used to detect genetic diversity and transferability of markers (Lin, et.al 2014; Ding 1998; Friar and Kochert 1991, 1994; Hodkinson et.al 2000). Molecular markers were also developed and cross-amplified in and across bamboo species. Nayak and Rout (2005) isolated and characterized microsatellites in *Bambusaarundinacea*and cross-amplified these markers to other *Bambusa* species and bamboo genus (e.g., *Dendrocalamus, Dinochlea, Arundinaria, Cephalostachyum*). Allelic diversity ranged from 0.128-0.789.

Rice markers were also cross-amplified in different species, e.g., C4 perennial grass *Miscanthus sinensis* (Yu et. al. 2013), seashore paspalum, finger millet, bermudagrass (Wang et. al. 2005), and sugarcane (Banumathi, et.al. 2010). Of these studies, average to high levels of transferability (39% - 78.2%) were observed which are useful for genetic analysis and germplasm evaluation.

Molecular and Genetic Studies on Bamboo

The genetic studies on bamboo were started using morphological markers. Physical attributes such as floral and vegetative parts of the bamboo was used for its identification and characterization. Usage of morphological markers initiated in the early 1890s by recording the bamboos observed in the Old World British-mapped areas (Gamble 1896). After an almost half-century, a taxonomic identification key was made on the 22 reported bamboo species. Size, texture, shape of the sheath and their blades of the culm sheath was used as the primary physical characteristics for the differentiations of the current-recorded bamboo during those times (Chatterjee and Raizada 1963).The latest updated morphological marker-based for bamboo was studied based on its vegetative and floral parts. Twenty-eight key vegetative and floral characters were the recent studies tested for possible more accurate identification of bamboo (Naithani et. al 2003; Clayton et. al 2013). However, the usage of morphological based markers became unpopular in the recent years because of its huge limitations. The reproductive cycle of bamboo was reported to be too long for it takes 3 to 120 years for the flowering stage of bamboo occurs (Janzen 1976). Environmental factors such as nature disturbances, light, carbon dioxide concentrations and distribution of soil nutrients, as well as the variation on the different developmental stages, may influence the research for more accurate taxonomic studies using the morphological characters of bamboo (Kalia et. al 2011).

With the invention of electrophoresis set-up and other protein-based assays, bamboo was one of the timely used plant species subjected for biochemical marker-based analysis. Using isozymes extracted from its leaves, polymorphism was detected among the five bamboo genera analyzed in the study. While a more accurate method was discovered for characterization of bamboo, annotations of the isozyme loci and slow massive development of specific protein detection using staining reagent systems gave a limitation for the biochemical marker to be used as a standard assay for taxonomic studies of bamboo. Also, influence of environmental interaction and developmental variation of proteins made isozyme marker analysis outdated for further diversity analysis (Heng et. al., 1996)

With the development of DNA or molecular markers, numerous genetic diversity studies were published around the world; it also ledto the first ever bamboo genome project which could help to

unify the researchers in the DNA analysis of bamboo. Changtragoon and Laphom (2009) assessed the genetic diversity of *Bambusabambos* using SSR markers and yielded to 66.7 to 88.89% polymorphism.

A whole genome sequence for bamboo was successfully drafted using the *Phyllostachysheterocycla* species of bamboo commonly called as moso. A 2.05 Gb of DNA sequences of bamboo was consolidated and organized by Zhao, Peng and Fei et. al (2014) to pioneer the first ever web-based bamboo genome database called Bamboo GDB. The full genomic sequence done covers 95% of the genomic region of bamboo where 31, 987 genes were annotated using cDNA and Deep RNA sequencing data. While the first published full genome sequence of *Phyllostachysheterocycla* needs further studies for possible use as a model for all bamboo species. According to the study, whole genome duplication occurred in the bamboo 7-12 millon years ago. It was also found that the majority of the genome duplication generated is involved on the shoot development of bamboo. This result suggested that whole genome duplication event may be attributed to the evolution of the rate of development of its shoots (Peng et. al. 2013). No specific type of shoot development whether it was faster or longer growth was indicated in the study. Nevertheless, this study, including the formation of a bamboo genome database made a first committed step towards a more central genomic resources and well-coordinated bioinformatics analysis of bamboo.

MATERIALS AND METHODS

Chemicals, Enzymes, Equipment and Kits

Solids. Agarose, bromophenol blue (BPB), cetryl methyl ammonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), polyvinylpyrrolidone (PVP), sodium acetate (Sigma), sodium chloride and Trizma base were used.

Liquids. Chemicals Acetic acid, chloroform, ethyl acetate, ethyl alcohol, hexane, hydrochloric acid,isoamyl alcohol , isopropanol, liquid nitrogen, β-mercaptoethanol, phenol, sterile nanopure water and RNAse solution were used.

Enzyme and Kits. DNA Taq polymerase (Kapa Biosystems), 10X PCR buffers + magnesium chloride (MgCl2) (Kapa Biosystems), 10 mMdeoxyribonucleotide triphosphates (dNTPs) were used.

Equipment. Micropipettes**,** Centrifuge, water bath, gel documentation system (Bio-Rad), agarose gel electrophoresis set-up (CBS Scientific), PCR thermocycler (Bio-Rad and Veriti®, Applied Biosystems), Nanodrop 2000® spectrophotometer (Thermo Scientific) were used.

Selection of *Kauayantinik* (*Bambusablumeana*Schultes f.)Samples

Representing the three major geographical islands in the Philippines (Luzon, Visayas and Mindanao), five populations of *Kauayantinik* located from the provinces of Pangasinan (PKT), Batangas (BKT), Cebu (CKT), Iloilo (IKT) and BdKT (Bukidnon) were identified for the study. Forty plant materials from each population were selected to be used for the collection of young and healthy leaves. Healthy physical check-up of each plant material was the basis of the selection. The collected samples were used as source of the DNA samples for analysis. Figure 2 shows the locations of the five plantation sources of the bamboo leaves in the Philippine map.

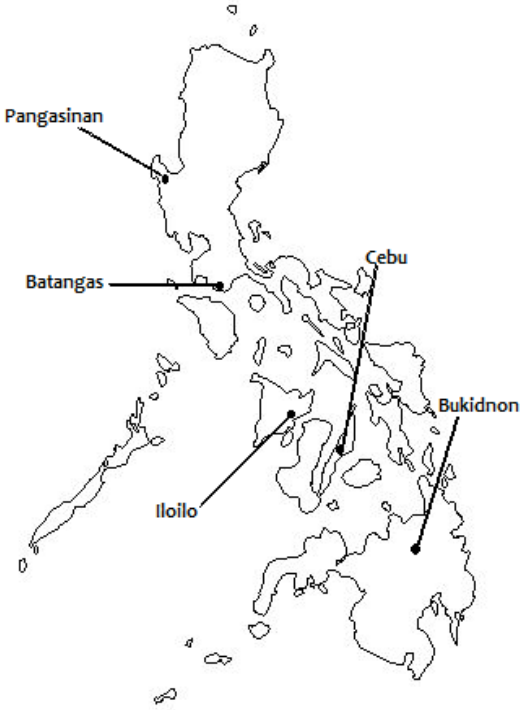

Figure 2

Place of Study

The research was conducted at the Forest Molecular Laboratory of the Ecosystems Research and Development Bureau (ERDB), College, Laguna, Philippines from January to November 2014.

Preparation of Leaf Materials

Young and healthy leaf samples obtained from the different *Kauayantinik* populations were washed with warm water to remove possible pesticide residues, dirt and other unnecessary materials. The leaf samples, gathered from each individual of every population, were placed into sealable plastic bags and stored in the biofreezer (-80°C) before DNA extraction. Figure 2 shows the diagram of the processes done on the leaf materials in this study.

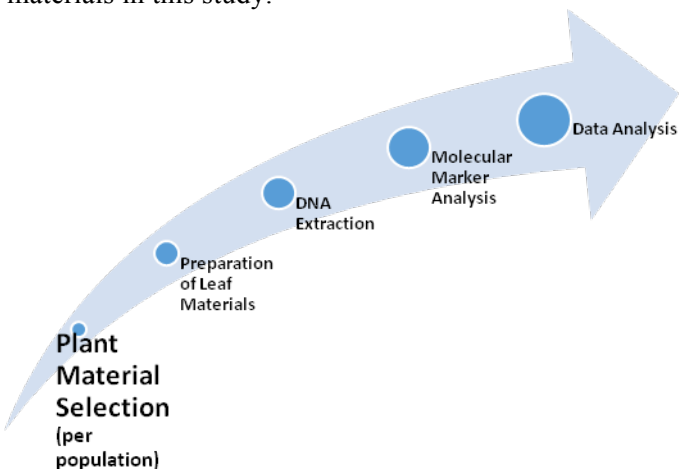

Figure 3

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DNA Extraction

The genomic DNA (gDNA) was extracted from the young and healthy leaf samples of *Kauayantinik* using Doyle and Doyle (1990) CTAB (CetylTrimethyl Ammonium Bromide)extraction method with few minor modifications by (Delos Reyes, et. al 2013). CTAB DNA extraction method has been commonly used for it increases the yield of the DNA isolated by improving the separation process between the polysaccharides and gDNA (Center for Plant Molecular Biology-Osmania University, 2013). One DNA sample from each selected individual of every population was prepared. About 200 mg of leaf tissue was processed for homogenization by grinding using mortar and pestle and by adding liquid nitrogen. The homogenized leaf was transferred into 2.0 ml microcentrifuge tubes. The homogenized leaf in the microcentrifuge tubes were added with 1.2 ml pre-heated CTAB extraction buffer (Table 2) and was mixed thoroughly. The solution sample was allowed to incubate in a water bath at 65°C for one hour. The incubated solutions were cooled at room temperature and were spun at 11000 rpm for 10 mins. About 800-1000 µl of the supernatant was transferred again to 2.0 mL microcentrifuge tubes and was added with 1uL of a 40ug/mL stock solution of RNAse A to remove possible RNA contaminants. The sample was incubated again at 37°C for 30 mins. One volume of chloroform: isoamyl alcohol (24:1) was then added to the supernatant for prevention of foaming, and denaturation and precipitation of contaminating proteins. The tubes were placed in a refrigerated centrifuge (28°C) spun at 13000 rpm for 5mins and shaken for 15 mins. After centrifugation, a separation of upper aqueous and bottom layer which contains chloroform and the precipitated proteins were observed, this was discarded and the clear upper aqueous phase layer was pipetted and transferred to a clean 1.5mL tube. The chloroform extraction was then repeated and the aqueous phase layer was pipetted again. A 1/10 volume ammonium acetate and 2 volumes of cold absolute ethanol was added to the tube to lower the solubility in water and consequently precipitated the DNA of the sample. The tubes were incubated at -80°C for an hour. After incubation, the tubes were spun at 13000 rpm for 10mins at 4°C. The supernatant was discarded. The DNA pellet was washed and decanted and finally washed again with 500 mL ice cold 70% ethanol and dried for 15 minutes under the fumehood. The pellet was resuspended in 50 µl TE buffer. The DNA was stored at 4°C in freezer after centrifugation at 13000 rpm for 10 mins.

Table 2. Preparation of components and volumes of CTAB extraction buffer.

Component	[Stock]	[Final]	Volume needed for 14 mL buffer
Tris-HCl, pH 8.0	1M	0.122 M	17.08 mL
NaCl	3M	1.707 M	79.660 mL
EDTA, pH 8.0	0.5M	0.025 M	7.00 mL
CTAB	-	2.440 %	3.416 g
PVP 40	-	1.219 %	1.7066 g
β-mercaptoethanol	-	-	0.026 mL
SNP Water	-	-	36.620 mL

Quantification and Quality Check of gDNA Samples

*Absorbance method.*Absorbance method was the most common technique on checking the quality, quantity and concentration of DNA. The concentration of the gDNA samples was assessed by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc.). NanoDrop 2000 Spectrophotometer uses ulta-violet lamp to identify and measure the value of the light absorbed by the DNA at 260/280 and 260/230 nanometers. Also, only small amount of the gDNA were only needed to test its purity. Only 1 µL of the gDNA samples were pipetted and loaded to the said spectrophotometer. The absorbance, concentration and purity of the gDNA were read and recorded. A 1.8 – 2.0 ratio of gDNA samples was considered a pure gDNA.

*Computation of the DNA Yield.*DNA yield (µg/g) = DNA concentration x total sample volume

Agarose gel electrophoresis (AGE) method. Agarose gel electrophoresis (AGE) method was also done on the determination of the integrity of the DNA. AGE method visualizes the DNA. The distinct and intense bands of DNA assured its purity. Five microliters (5µL) of 1+kb DNA ladder, 0.2 µL gDNA were loaded into a 1.0% (w/v) agarose gel (added with 5 µL of SYBR Safe stain) at 100 V for 30 mins. The gDNA samples were mixed first with 0.3 µL of 1.5X Bio-Rad loading dye buffer.

Molecular Marker Analysis

Primer screening. Simple Sequence Repeats (SSR) markers were the primers used for amplifying some flanking regions in the genome of *Kauayantinik*. Microsatellites from bamboo and rice used by Nayak and Rout (2005), Chen et. al (2010) and by Samaco, et.al (2012) were the markers used in this study. The 8 bamboo markers, previously used for *Bambusaarundinacea,* were designed by Nayak and Rout (2005) while the remaining 72 rice markers was derived from the work of McCouch et. al (2002) which was also used by Chen et. al (2010), and Samaco, et. al (2012) in their bamboo and rice genetic study respectively.

PCR Amplification. Polymerase Chain Reaction for the amplification of the DNA samples against the SSR markers were performed using the optimized PCR profile of the University of Missouri (2001) (Table 3). Dubcovsky (2011) was the formulated PCR reaction mixed used for the PCR cocktail (Table 4).

Table 3. PCR Components per reaction mixture for SSR marker amplification from *Kauayantinik* genomic DNA (University of Missouri 2001)

COMPONENT	CONCENTRATION	VOLUME (µL)
Sterile nanopure water (SNPW)	-	5.896
10 X PCR Buffer + $MgCl_2$	1x	1
10 mMdNTPs	0.01 mM	0.01
10µM Forward Primer	1.00 µM	1
10µM Reverse Primer	1.00 µM	1
Taq Polymerase (5.0 Units/ µL)	0.02 U	0.004
***Kauayantinik*Genomic DNA**	-	1
Final Volume	-	10

Table 4. PCR conditions for the SSR marker amplification from *Kauayantinik* genomic DNA (Dubcovsky 2011)

STEP	STEP NAME	TEMPERATURE (°C)	TIME (min.)
1	Initial Denaturation	95.0	1
2	Denaturation	65.0	1
3	Annealing	55.0	1
4	Extension	72.0	1.5
5	Repeat 2 TO 5 (29 X)		
6	Final Extension	72.0	4
7	Hold	10.0	∞

Visualization of the PCR products/SSR bands

Amplified PCR products were analyzed using the agarose gel electrophoresis method with 3% agarose gel mixed with 10uL of SYBR Safe stain with the run set up at 135V for 30 minutes. The PCR product (10 uL) was run with a 5 uL 10 bp DNA ladder (Kappa Biosystems). The PCR products were mixed first with 3 µL of 6X Kappa Biosystems loading dye buffer.

Data Analysis

Scoring of bands/genotype. The SSR bands amplified from the *Kauayantinik*gDNA were scored for further genetic analysis. The number of bands (alleles) in each marker were identified and marked for presence across each DNA samples of every population. Binary data format (1, 0) was applied to represent "1" for a presence of a band while "0" for its absence, "9" on the other hand was used to indicate the missing data.

Measuring and displaying the genetic relationship. Biostatistics/bioinformatics called AFLPSurv 1.0 (Vekemans 2002) and Numerical Taxonomy and Multivariate Analysis System Version 2.1(NTSYSpc 2.1) (Rohlf 2004) were used. AFLPSurv 1.0 (Vekemans 2002) analyzed the number of loci, number of polymorphic loci (P or PLP), expected heterozygosity or Nei's genetic diversity (H_j) and its variance components, and average gene diversity within populations or samples (H_w) and its variance components. The population genetic structure was also computed by AFLPSurv 1.0 (Vekemans 2002) to analyze the genetic relationship within the species and populations simultaneously. A dendrogram generated from the software NTSYSpc 2.1 (Rohlf 2004) was constructed to display the genetic relationship among all Bamboo population samples. Nei-Li's dissimilarity index and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was the method used for measuring and clustering the genetic dissimilarity/distances, respectively.

RESULTS AND DISCUSSION

Quantification and Quality Check of gDNA Samples

Absorbance method and Agarose gel electrophoresis (AGE) observation method were done to check and assess the DNA quantity, purity and integrity. About 1.0 µl of the resuspended DNA was loaded in NanoDrop 2000 to read the absorbance at 260/280 nanometers. Table 4 and Figures 4-8 show the mean absorbance ratio and electrophoretic image of the extracted DNA of the different populations, respectively. Batangas population has the highest DNA Concentration reading while the Iloilo has the most approximately best quality of genomic DNA (gDNA) extracts (Table 4). Iloilo population recorded 1.90 A260:280 ratio. The universal good gDNA quality has an absorbance reading (A260:280) of 1.8. In this study, an absorbance reading (A260:280) of 1.8-2.0 was considered a good quality and indicator of pure DNA extracts.

The absorbance method results were also consistent with the data gathered for gDNA visualization using AGE. Distinct bands indicating good quality of gDNA was observed among the different populations of *Kauayantinik*.

Table 5. Mean Absorbance readings (260:280), concentration (ng/µl), absolute quantity (ng/µl) of genomic DNA from Pangasinan (PKT), Batangas (BKT), Cebu (CKT), Iloilo (IKT) and Bukidnon (BdKT) population samples of *Kauayantinik*.

DNA Sample ID	**Total Volume** (µL)	**Concentration Range of the Samples** (ng/ µL)	**Mean Concentration** (ng/ µL)	**Mean Absolute quantity** (ng/µl)	**Absorbance** (260:280)	**Total Weight Plant Sample** (g)	**Mean Yield** (µg/g)
Pangasinan	100	71.60-449.00	149.62	85.67	1.92	2	14.96
Batangas	100	72.60-546.00	167.43	76.1	1.93	2	16.74
Cebu	100	82.150-474.00	184.14	32.68	1.96	2	18.41
Iloilo	100	64.95-268.90	142.77	45.17	1.90	2	14.28
Bukidnon	100	90.55-512.80	180.318	28.53	1.91	2	18.00

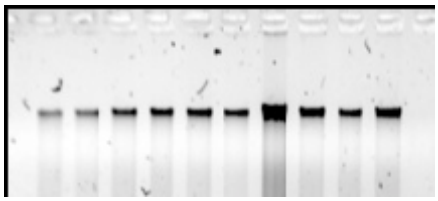

Figure 4.

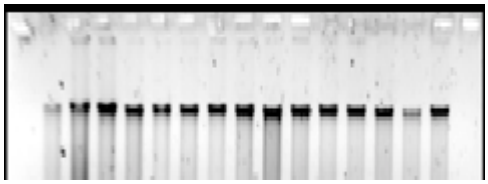

Figure 5.

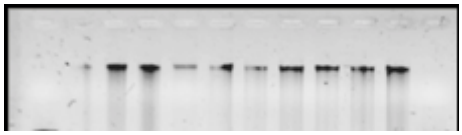

Figure 6.

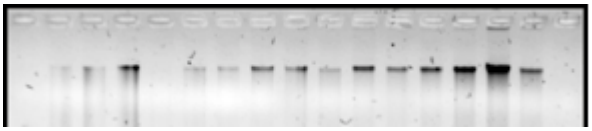

Figure 7.

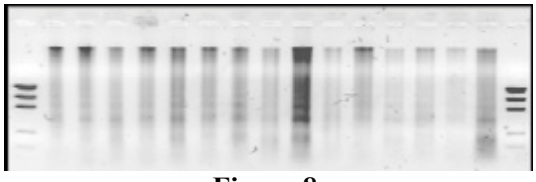

Figure 8.

Molecular Marker Analysis

Out of the 80 primers screened, 50 SSR markers were successfully cross-amplified with the gDNA of Kauayantinik. A transferability rate of 75% was recorded suggesting that SSR primers specific for genetic analysis of rice may cross-amplify the gDNA within its relatives in the family of Poaceae. This theory may further be supported by the draft whole genome study on moso bamboo which reported that 85% of the bamboo genes were collinear when aligned to rice or sorghum homologues (Peng et. al. 2013). Further genome analysis should be done to determine the correlation of cross amplification and colinearity of gene homologues between bamboo and rice genome.

Possible regional divergence occurred between rice and bamboo during evolution may be the reason on the failure of some rice SSR markers to amplify against the K. tinikgDNA(Chen et. al. 2010). The transferability rate (75%) in this study was higher than the reported cross-amplification of SSR rice markers to other bamboo species by Chen et. al. (2010) which may be possible due to lower detected loci in this study as compared to the other bamboo species. The transferability rate in this study is also higher than the study of Sharma et. al. (2008) against rice SSR markers (44.9%) but equally the same on the usage of sugarcane SSR markers (75%).

Out of the 64 loci detected, 35.9% was found to be polymorphic. The 50 SSR markers, positively screened for cross amplification of KauayantinikgDNA, was used for PCR amplification of the populations (Table 6). The electrophoretic images result of the Pangasinan (PKT), Batangas (BKT), Cebu (CKT), Iloilo (IKT) and Bukidnon (BdKT) against the 50 SSR screened markers were analyzed for further genetic analysis. Figures 9a-f showed the electrophoretic images of the PCR product yielded from KauayantinikgDNA and some of the primers used in this study. The loci detected (64 alleles) in this study was a slightly lower from the 69 alleles identified by Torres et. al. (2009) on their study on *Guaduaangustifolia* bamboo species.

Table 6. List of SSR primers and their sequences positively screened to cross-amplify *Kauayantinik*DNA.

Primer	Sequence		Reference
	Forward	Reverse	
Ba10	GGTGGGGTCTAGCACCTAAG	TACCAGCATGTAACGGTCGG	Nayak and Rout (2005)
Ba14	AGAGTTAAGGAAGCCAGGTC	GTCTAGTAGCTGCTCAACTC	
Ba18a	TATCTCGACCTCCCCTTGCT	GTCTAGAAGCAGGGAGGTAG	
Ba18b	CCAGGTCGTTTCACTGCTC	ACAACGGTAGAGTTCACTCG	
Ba20	TTGATTGCCCTACTCTGTCG	TCAACGGTGGATGACCTAGG	
Ba25	GTGAGATGGGCTGGGCAG	GCTCCGATCTGTCAGTTTAC	
Ba58	TCCGAAGCACACTCATGAAG	TTCTACTATGCGCTAACTGC	
RM437	ACACCAACCAGATCAGGGAG	TGCTCGTCAATGGTGAGTTC	McCouch et. al. (2002)
RM327	CTACTCCTCTGTCCCTCCTCTC	CCAGCTAGACACAATCGAGC	
RM205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	
RM462	ACGGCCCATATAAAAGCCTC	AAGATGGCGGAGTAGCTCAG	
RM514	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	
RM468	CCCTTCCTTGTTGTGGCTAC	TGATTTCTGAGAGCCAACCC	
RM36	CAACTATGCACCATTGTCGC	GTACTCCACAAGACCGTACC	
RM60	AGTCCCATGTTCCACTTCCG	ATGGCTACTGCCTGTACTAC	
RM489	ACTTGAGACGATCGGACACC	TCACCCATGGATGTTGTCAG	
RM249	GGCGTAAAGGTTTTGCATGT	ATGATGCCATGAAGGTCAGC	
RM233B	CCAAATGAACCTACATGTTG	GCATTGCAGACAGCTATTGA	

RM480	GCTCAAGCATTCTGCAGTTG	GCGCTTCTGCTTATTGGAAG
RM153	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG
RM440	CATGCAACAACGTCACCTTC	ATGGTTGGTAGGCACCAAAG
RM7286	CAGAACAATTCGACCGCTTC	GGCTTGAGAGCGTTTGTAGG
RM589	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG
RM7121	GGAGATGGCACACGTCAAAC	AGGATCCCGTTTTGTAGCAG
RM531	GAAACATCCCATGTTCCCAC	TCGGTTTTTCAGACTCGGTC
RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
RM251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
RM214	CTGATGATAGAAACCTCTTCTC	AAGAACAGCTGACTTCACAA
RM269	GAAAGCGATCGAACCAGC	GCAAATGCGCCTCGTGTC
RM105	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC
RM167	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC
RM3	ACACTGTAGCGGCCACTG	CCTCCACTGCTCCACATCTT
RM151	GGCTGCTCATCAGCTGCATGCG	TCGGCAGTGGTAGAGTTTGATCTGC
RM210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG
RM18	TTCCCTCTCATGAGCTCCAT	GAGTGCCTGGCGCTGTAC
RM10	TTGTCAAGAGGAGGCATCG	CAGAATGGGAAATGGGTCC
RM6085	GGTGAGAGATGGCTAAAGCG	CATCGCCTCTAGCACCTCC
RM518	CTCTTCACTCACTCACCATGG	ATCCATCTGGAGCAAGCAAC
RM270	GGCCGTTGGTTCTAAAATC	TGCGCAGTATCATCGGCGAG
RM286	GGCTTCATCTTTGGCGAC	CCGGATTCACGAGATAAACTC
RM332	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCACCC
RM138	AGCGCAACAACCAATCCATCCG	AAGAAGCTGCCTTTGACGCTATGG
RM13	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG
RM417	CGGATCCAAGAAACAGCAG	TTCGGTATCCTCCACACCTC
RM82	TGCTTCTTGTCAATTCGCC	CGACTCGTGGAGGTACGG
RM296	CACATGGCACCAACCTCC	GCCAAGTCATTCACTACTCTGG
RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
RM52	CTACTCGCGCGTGGAGTT	TGTCTTACTGGTGAAGCTGG
RM230	GCCAGACCGTGGATGTTC	CACCGCAGTCACTTTTCAAG

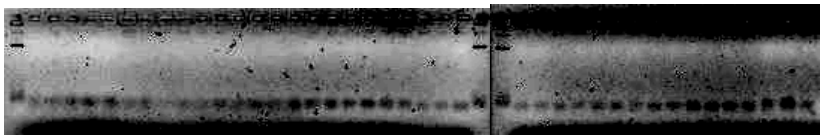

Figure 9a.

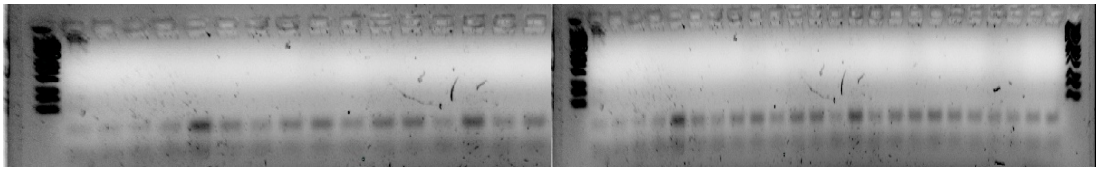

Figure 9b.

Theme: Biology, Morphology and Taxonomy

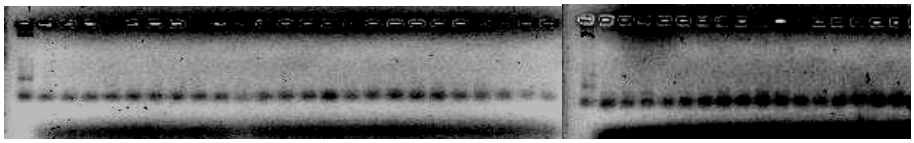

Figure 9c.

Figure 9d.

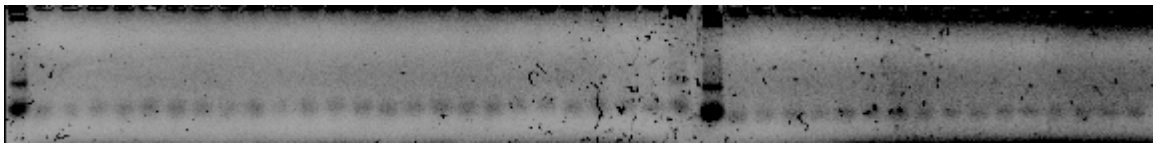

Figure 9e.

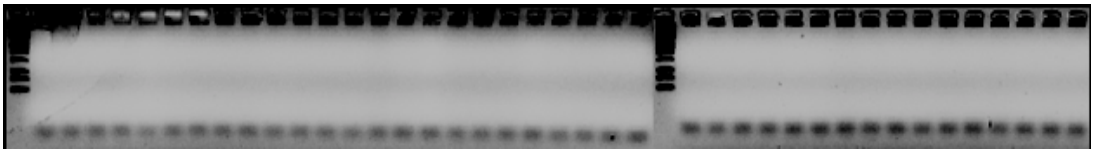

Figure 9f.

Data Analysis

The electrophoretic image of the five populations against the 50 SSR primer pairs were subjected to data analysis using biostatistics and bioinformatics tools for genetic diversity analysis.

Table 7 shows the percent polymorphic loci and expected heterozygosity of the five populations of *Kauayantinik* computed using AFLPSurv 1.0 (Vekemans 2002). Percent Polymorphic Loci (PLP) was defined as the number of polymorphic loci over the total number of loci:

$$P = n_{pj}/n_{total}$$

*P is the proportion of polymorphic loci, n_{pj} is the number of polymorphic loci and n_{total} is the total number of loci.

Table 7. Intrapopulation genetic diversity of Pangasinan, Batangas, Cebu, Iloilo and Bukidnon populations.

Population	N	Number of loci	Number of polymorphic loci	Percent Polymorphic Loci (PLP)	Expected heterozygosity (Hj)
Pangasinan	40	64	23	35.9 %	0.13959
Batangas	40	64	17	26.6 %	0.01997
Cebu	40	64	9	14.1 %	0.01739
Iloilo	40	64	13	20.3 %	0.02152
Bukidnon	40	64	8	12.5 %	0.01837

Based on the PLP and expected heterozygosity values, all populations were observed to be diverse. In this study, PLP with a positive value was considered as an indication of diverse status. Among the five populations, Pangasinan exhibited the most diverse within each population followed by Iloilo, Batangas, Bukidnon and Cebu. The highest PLP (35.9%) generated from the Pangasinan population

of this study was lower than the highest PLP (88.89%) calculated from the province of Sa Kauw&Surathtani of Thailand. Changtragoon and Laphom (2009) performed genetic diversity of *Bambusabambos*, a relative genus of *Bambusablumeana* in their country. Higher PLP may be attributed to lower SSR markers used in *Bambusabambos*.

Statistics of population genetic structure were also computed by AFLPSurv 1.0 (Vekemans 2002). Total gene diversity (H_T), average gene diversity within populations (H_w), average gene diversity among populations in excess of that observed within populations (H_b), and Wright's Fixation Index (F_{st}) were also included in determining the statistics of population genetic structure. Tables 8 and 9 show the genetic diversity among and within populations, and the genetic distances and Wright's fixation indices of paired populations, respectively.

Table 8. Genetic diversity among and within populations values (Lynch and Millligan 1994).

N	Ht	Hw	Hb	Fst
5	0.3519	0.0984	0.2535	0.7196

Table 9. Genetic distance and Wright's fixation index of paired populations (Lynch and Millligan 1994).

Paired populations	Genetic distance	Fst
Pangasinan - Batangas	0.1711	0.4839
Pangasinan – Cebu	0.1493	0.5742
Pangasinan – Iloilo	0.6129	0.7721
Pangasinan – Bukidnon	0.3023	0.7017
Batangas – Cebu	0.1906	0.6241
Batangas – Iloilo	0.5255	0.7694
Batangas – Bukidnon	0.2939	0.7203
Cebu – Iloilo	0.5872	0.8270
Cebu – Bukidnon	0.3048	0.7881
Iloilo – Bukidnon	0.2647	0.7271

Wright's fixation index indicates the proportion of the total gene diversity that occurs among as opposed to within populations or the degree of gene differentiation among populations in terms of allele frequencies (Lynch and Milligan 1994; IPGRI and Cornell University 2003). Results in Table 8 show that the low Hb and high Fst values suggested a high genetic variation among populations.

On the other hand, results in Table 9 indicated that Pangasinan and Iloilo were the most distant while Pangasinan and Cebu were the most genetically similar populations. Cebu-Iloilo paired population had the highest Fst value which suggested a high differentiation value among paired populations. The Fst results in this study were higher than the Fst value obtained by Changtragoon and Laphom (2009), which is similar to this study. In line with this, the study focused on comparing the diversity status of five plantations geographically represented by the three major islands of the Philippines. Most of the published diversity analysis through SSR markers focused on phylogenetic relationships and clone identification (Sharma et. al. 2008; Chen et. al. 2010). So far, few literatures have been published focusing on the endemism of a bamboo species in a defined geographic location (Changtragoon and Laphom 2009; Torres et. al. 2009; Yang et. al. 2012).

A dendrogram generated from the software NTSYSpc 2.1 (Rohlf 2004) was constructed to display the genetic relationship among all Bamboo population samples. Nei-Li's dissimilarity coefficient and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was the method used for measuring and clustering the genetic dissimilarity/distances respectively.

Results showed that 56% was the average genetic dissimilarity among the bamboo population. Low genetic dissimilarity was observed within each population (Figures 10-14). The generated genetic dissimilarity of all Bamboo populations was approximately ranged from 12% to 56% (Figure 15).

Figure 16 shows that the samples within each population were very genetically similar. Therefore, close genetic relationship was exhibited within each population.

At most 0.56 Nei-Li's dissimilarity coefficient, only two distinct clusters were observed. These were Iloilo and the clustered group composed of Pangasinan, Batangas, Cebu and Bukidnon (Figure 15). The results on Figure 16 suggested that Iloilo has a 56% genetic variation among the other population, highest genetic diversity rate among the populations. The genetic variation (ca. 0.56 Nei-Li's dissimilarity coefficient) reported in this study is higher than the UPGMA tree generated for *Dendrocalumusmembranaceus*in China (ca. 0.09 Nei-Li's dissimilarity coefficient). Yang et. al. (2012) reported *D. membranaceus*in China is declining also in terms of the availability of genetic resources for *D. membranaceus*bamboo species.

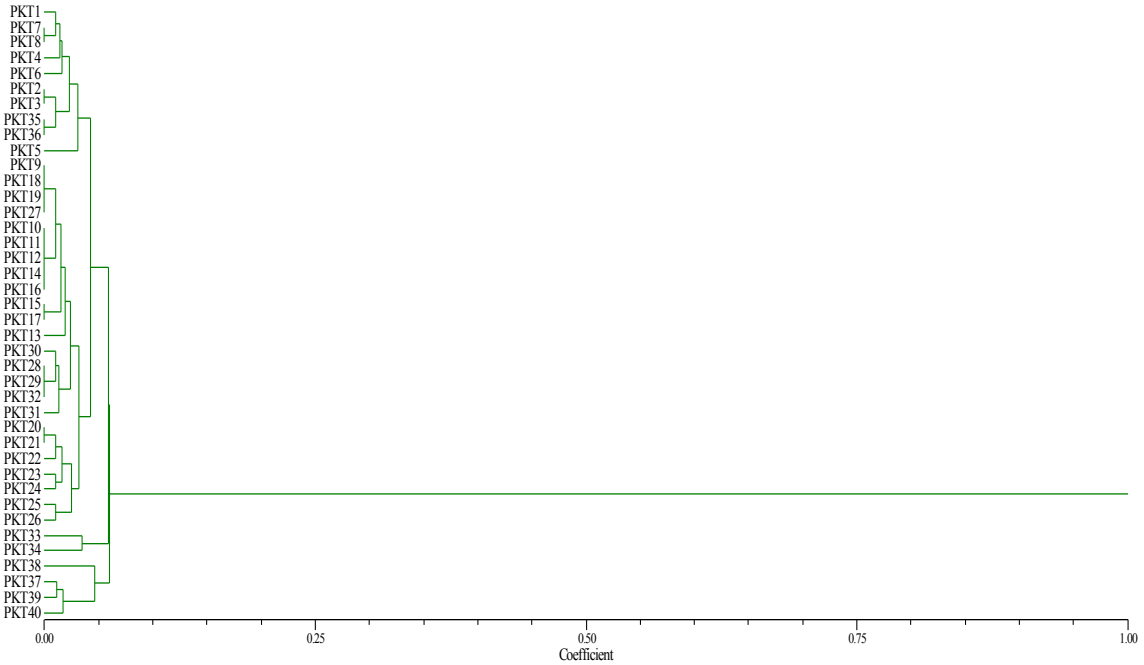

Figure 10.

Theme: Biology, Morphology and Taxonomy

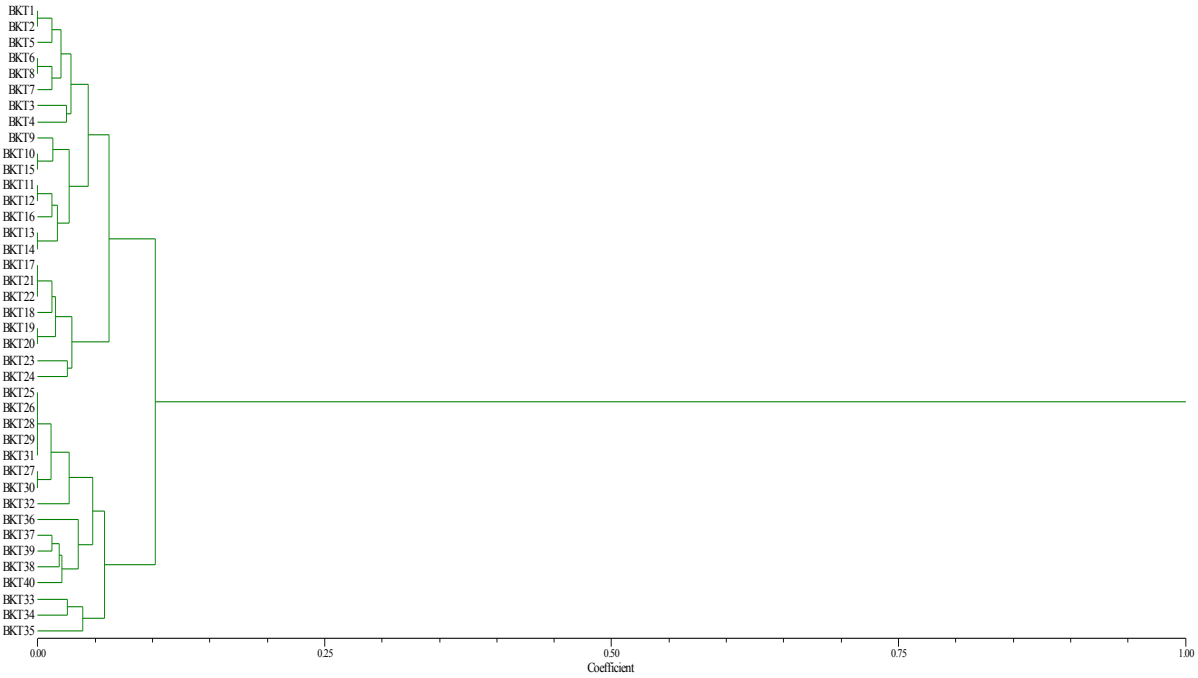

Figure 11.

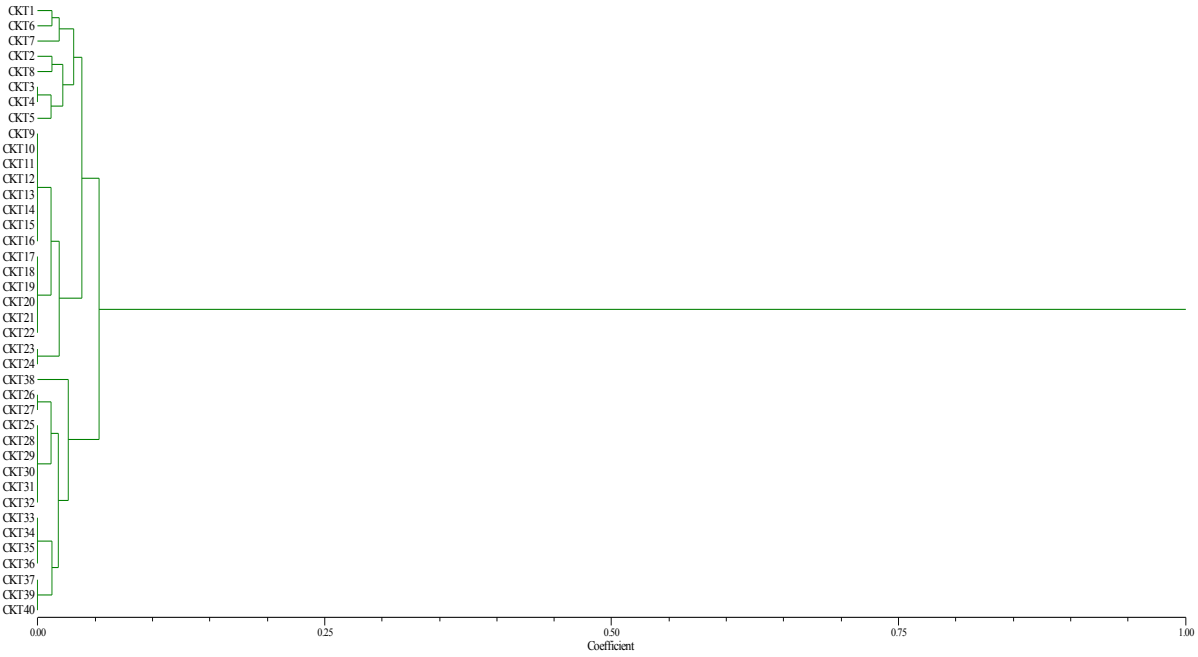

Figure 12.

Theme: Biology, Morphology and Taxonomy

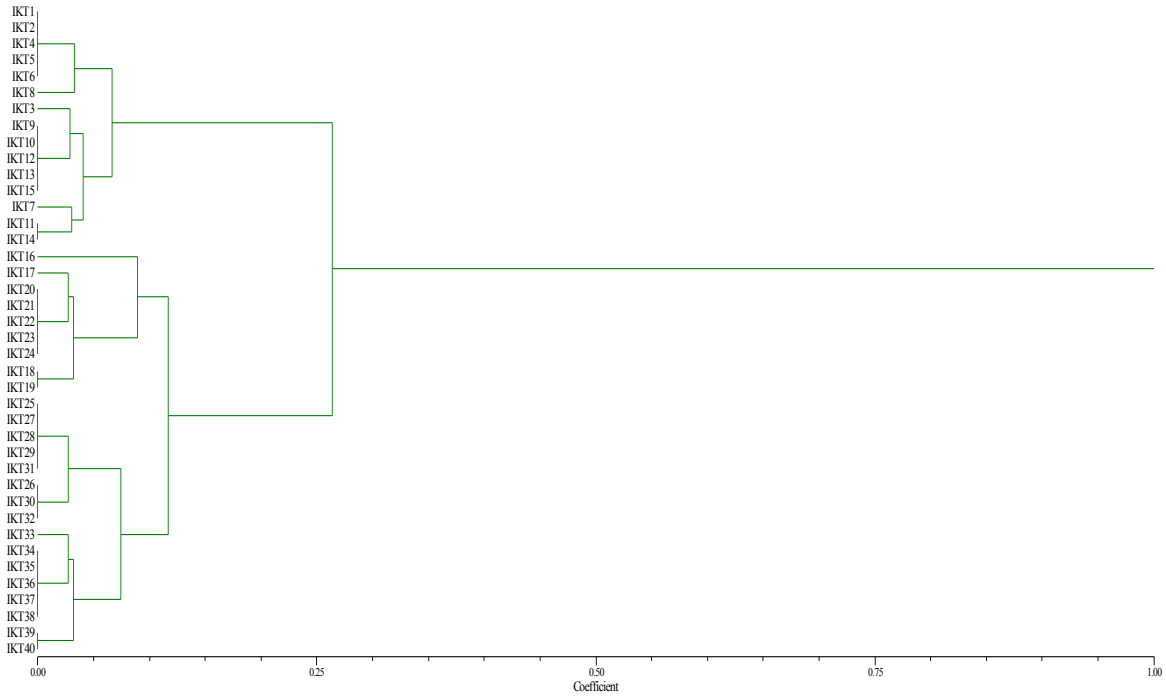

Figure 13.

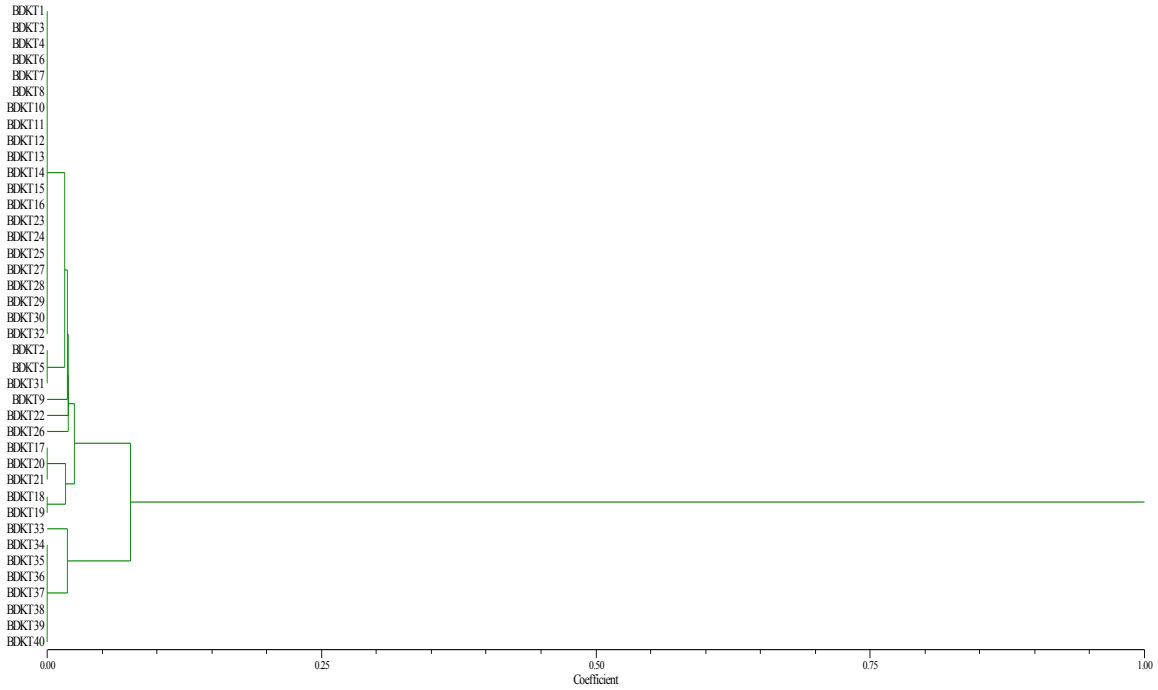

Figure 14.

Theme: Biology, Morphology and Taxonomy

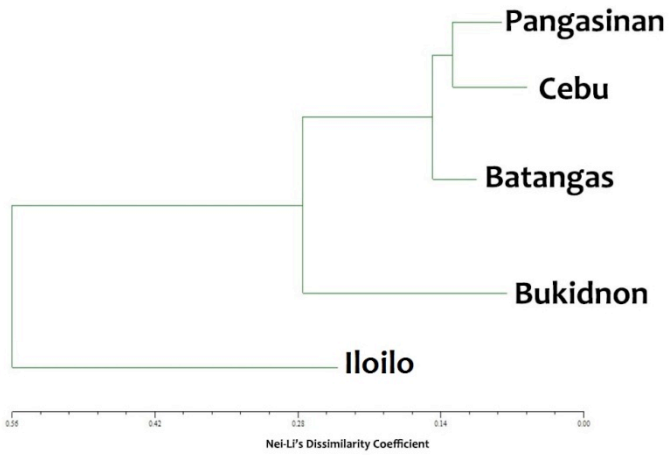
Pangasinan
Cebu
Batangas
Bukidnon
Iloilo
0.56
0.42
0.28
0.14
0.00
Nei-Li's Dissimilarity Coefficient

Figure 15.

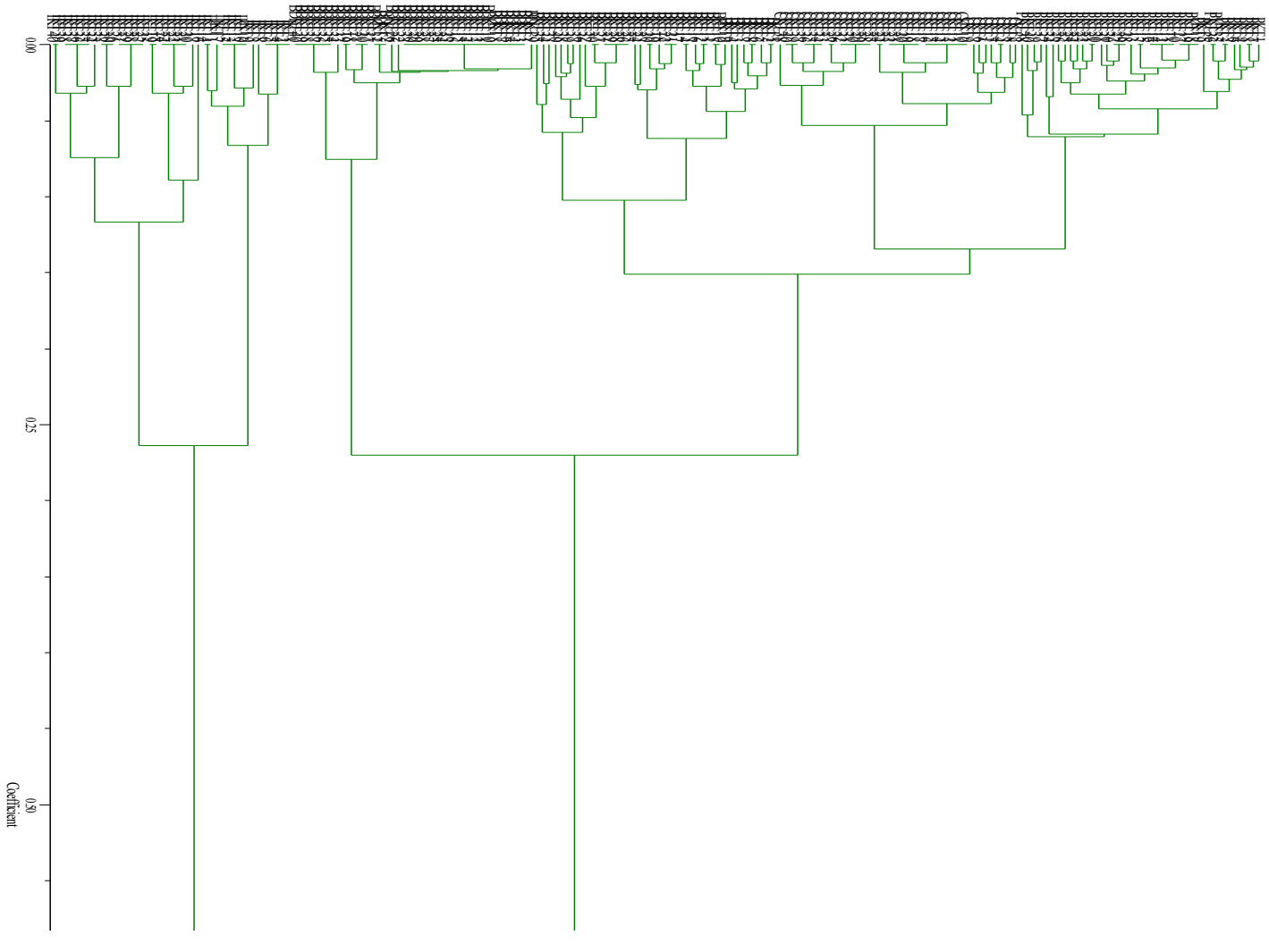
0.00
0.25
0.50
Coefficient

Figure 16.

SUMMARY AND CONCLUSION

The study aims to assess the genetic diversity status of *Kauayantinik*in the Philippines through SSR marker analysis. Its specific goals were to establish a protocol for the DNA isolation of *Kauayantinik*, to cross –amplify *Kauayantinik* with rice SSR markers for further genetic analysis and to measure and toanalyze the genetic distance, similarity and polymorphisms detected on the SSR bands of *Kauayantinik*.

A total of 40 individuals of each *Kauayantinik* population were randomly selected to harvest its young leaves for optimizing a DNA extraction procedure. The genomic DNA isolated from the populations of *Kauayantinik* was quantified and checked for its quality through absorbance and agarose gel electrophoresis method. High quality DNA, with an absorbance ratio of 1.8-2.0 and had an observable distinct and intense band, has been used for the molecular marker analysis through PCR amplification of the SSR bands. The SSR bands were scored in a binary data format of which "1" represented the presence of bands and "0" indicated its absence. The binary data of SSR bands were further analyzed for genetic analysis by measuring and studying the detected polymorphisms, geometric distance and genetic similarity. NTSYSpc, bioinformatics software, was used to generate a dendrogram or phylogenetic tree for determining the genetic similarity and detected polymorphisms among and within the populations. Nei-Li's similarity coefficient and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) were the methods used for measuring and clustering the genetic similarity/distances, respectively.

Theme: Biology, Morphology and Taxonomy

Based on the results of the study, it can be concluded that *Kauayantinik* from Pangasinan, Batangas, Cebu, Iloilo and Bukidnon populationscan yield a high quality of genomic DNA (gDNA) using the optimized modified CTAB DNA extraction method. The *Kauayantinik*gDNA had a high transferability rate to cross amplify the SSR markers from rice, a relative from the family of Poaceae. This high cross-amplification rate further strengthened the idea that rice SSR markers may isolate fragments ofDNA that can be used for assessing its genetic diversity. The low genetic similarity generated from the *Kauayantinik* populations clearly states high observable polymorphisms among the geographic locations of bamboo. These polymorphisms also indicate that *Kauayantinik*, an endemic bamboo species in the Philippines has rich genetic resources which can further be used for genetic improvement of the species for economic purposes.

Kauayantinik (*Bambusablumeana*) populations that exhibited high genetic diversity based on expected heterozygosity and high average genetic dissimilarity (based on Nei-Li's index) may be suggested to be used for possible future implementations of *in situ* conservation efforts in the Philippines to preserve its rich genetic resources.

RECOMMENDATIONS

With the many applications of bamboo in the country, it has a lot of potentials that may be discovered through biochemical or gene discovery researches, thus, the initiatives on the molecular study of native & endemic bamboos in the Philippines is established. Here are some of the recommendations that may further improve this study:

Polyacrylamide gel electrophoresis (PAGE) method should also be used to counter-check the bands detected from the agarose gel electrophoresis (AGE) method. PAGE has a higher resolving power than AGE because it produces more porous beads which may further increase the separation rate between the markers with low and high molecular weight. This increased separation between the markers low and high molecular weight may also increase the bands detected which has not possible been seen from the AGE.

A more dedicated SSR markers designed for flanking some fragment genome sequences for bamboo should be used to correctly represent each of the chromosome of bamboo. Representation of each bamboo chromosome augments the accuracy and precision of the genetic diversity analysis within and among the *K. tinik* populations. Also, exon-based SSR markers should be generated to map the important genes that have economic importance for bamboo. Through exon-based SSR markers, some gene-encoding enzymes involved in the cellulose biosynthesis of bamboo may be isolated which are responsible for the strengthening of its culm.

Extracting and cloning the PCR products of the SSR bands should be done to improve the dendrogram generated by the study. The random fragments flanked out from the genome sequence of *K. tinik*, once undergone DNA sequencing should be compared through multi-sequences pairwise alignment.

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