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ABOUT THE TRANSACTIONS

TPGM is acronymed for Transactions of the Persatuan Genetik Malaysia (or Genetics Society of Malaysia). The Transactions is the Society's scientific publication. It is published periodically and can be downloaded free from PGM website.

Genetics is a rapidly expanding field that has far reaching impact in almost every horizon of life, from agriculture to industry to medicine, and has made many invaluable contributions towards our well being. The Transactions is therefore a valuable publication to highlight new developments and findings encompassing these wide-ranging fields and disciplines.

Each issue comprises articles, reports and reviews which had been contributed to the Society's scientific activities. Selection of articles or contributions to be considered for publication in the Transactions will be done by the Society based on their scientific merit, and also their research and application potential.

Each issue is indexed and a cumulative index is planned for the future.

The Society gratefully acknowledges the outstanding contribution of members and non-members alike.

FOCUS OF THIS TRANSACTIONS

In this Transactions, focus is given to the science of genetics and its allied fields. In a nutshell, genetics is the study of genes, genetic variation, and heredity in living organisms. It is generally considered a field of biology, but intersects and converges with many other life sciences, and is now strongly associated with molecular biology, genomics and bioinformatics.

TPGM Number 10 is a compilation of selected papers from two tracks, namely Track on Plant Genetics and Track on Animal Genetics, contributed to the 13th Malaysia International Genetics Congress 2019 (MIGC13) with the theme "***Exploring Innovation in Genetics***" held on 19-21 November 2019 in Bangi, Selangor, Malaysia. Selected papers from Track on Human Genetics will be published separately.

TPGM Number 10 is now available for download from the Society's website.

PREFACE

The First National Congress on Genetics was held over 25 years ago in 1994. The 13th Malaysia International Genetics Congress (MiGC13) is the 13th edition of such Congress on Genetics. Underpinned by both successes and challenges of past Congresses, PGM vehemently strives to further internationalise this flagship scientific activity. The main aims of the International Congress are to create a platform from which researchers can reflect on advances made in the scientific fields of genetics, consider the best of contemporary research progress, and anticipate future developments. Realising the significance and powerful impacts that the field of genetics could benefit humankind, PGM is therefore very proud to organise MiGC13 on 19-21 November 2019.

ACKNOWELDGEMENTS

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We are indeed very thankful for the sincere cooperation and efforts by all the reviewers. We received constructive criticisms and many positive comments that lifted our spirits to keep working on the Transactions until completion.

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THE GENETICS SOCIETY OF MALAYSIA

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TPGM

Agronomic performance of ten selected potential kenaf mutant lines at Beseri, Perlis

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Abstract

Kenaf (*Hibiscus cannabinus* L.,) is a high value fiber plant commonly used as a raw material for various industries such as pulp, paper, furniture, construction and automotive. This plant was first introduced in Malaysia in the early 2000 as part of government effort to replace tobacco in tobacco cultivation areas nationwide. Malaysian government through The National Kenaf and Tobacco Board (NKTB) has initially allocated approximately 2000 hectares for smallholder to cultivate kenaf, and the planting areas is expected to increase to 10,000 by 2020. Among the research that have been carried out on kenaf in Malaysia were screening for locally-adapted varieties, development of good agronomic practices, mechanization of planting and harvesting processes as well as development of animal feed and bio-composite. In this project, gamma irradiation was used to induce genetic mutation in Kenaf V36 variety for the development of new varieties with improved traits such as high fiber yield and late flowering. The project which was started in 2015, has generated a number of mutant lines carrying these traits. This paper discusses agronomic performances of ten selected M5 mutant lines at a field plot in Beseri, Perlis. All these selected mutant lines produced higher fiber yield as compared to the control whilst one of them (mutant code: 36-21) also has an additional improved trait (high number of seed pods per plant). Based on their agronomic characters, these mutant lines have the potential to be introduced as new planting materials for kenaf in Malaysia.

Key words: Kenaf, *Hibiscus cannabinus* L., gamma irradiation, mutation breeding, mutant

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) is considered a herbaceous annual plant that belongs to Malvaceae family. Other well-known plants that are from the same genus are *Hibiscus rosa-sinensis* (bunga raya) and *Hibiscus sabdariffa* (roselle). It is characterized by a large, bell shaped, creamy yellow flower of about 8 to 10 cm in diameter with widely open petals (H'ng *et al*., 2009). Under normal condition, kenaf plants can grow up to 3 meter tall with an average stem diameter of approximately 2 cm. Its stem fiber is divided into two; bast or bark and core (Paridah 2011).

Kenaf is planted for its fiber in many countries including Malaysia. Besides fiber, kenaf can also be used as an animal feed as they have relatively high protein content. The crude protein content for the whole plant was between 8.99 to 16.23% and crude fiber content was between 16.71 to 37.43% (Jie *et al*., 2017)

Kenaf was first introduced into Malaysia in the early 2000, as part of the government effort to replace tobacco plants in Kelantan and Terengganu areas. Starting from a very humble beginning with only 0.4 hectare in 2004, the planting acreage has increased to approximately 1,140 hectare in 2011 and is expected to escalate to 10,000 hectare in 2020. Depending on soil and weather conditions, kenaf fiber yield in Malaysia can reach up to 20 tons per hectare in experimental plots, (Lembaga Kenaf and Tembakau Negara, 2013) but generally, the yield in plantation areas is still low which is around 5 to 10 tons per hectare. The highest yield achieved in plantation areas was about 9.8 tons per hectare from V36 (Basri *et al.*, 2014).

A number of research on kenaf has been carried out in Malaysia, mainly in the aspects of screening of suitable varieties adaptable to local weather, development of good agronomic practices, mechanization of planting and harvesting processes. One of the problems faced by kenaf industry in Malaysia is the lack of kenaf varieties that are suitable to Malaysian climate. Most areas are still planted with the same variety which is V36. New varieties which can perform well in local condition especially in terms of biomass yield are highly needed. Therefore, this project was formulated in order to develop new varieties of kenaf with good agronomic traits using mutation breeding technology. Among the traits required are high fiber and seed yield. The other characters that were also investigated in this project were photoperiod insensitive and late flowering or maturity as these are the most essential traits required for growing kenaf plants in a

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Table 1. List of ten best performed mutant lines at the experimental field plot in Beseri, Perlis.

No	Mutant Line	Irradiation Dose (Gy)	Main Characters
1.	ML5	300	High Fiber, palmate leaves
2.	ML9	300	High fiber
3.	ML10	300	High fiber
4.	36-3	300	Tall, high fiber
5.	36-8	300	Tall, high fiber
6.	36-11	300	Tall, high fiber plant
7.	36-20	800	High fiber
8.	36-21	800	Tall, high fiber, high seed pod number
9.	36-24	1300	Tall, high fiber
10.	36-25	1300	Tall, high fiber

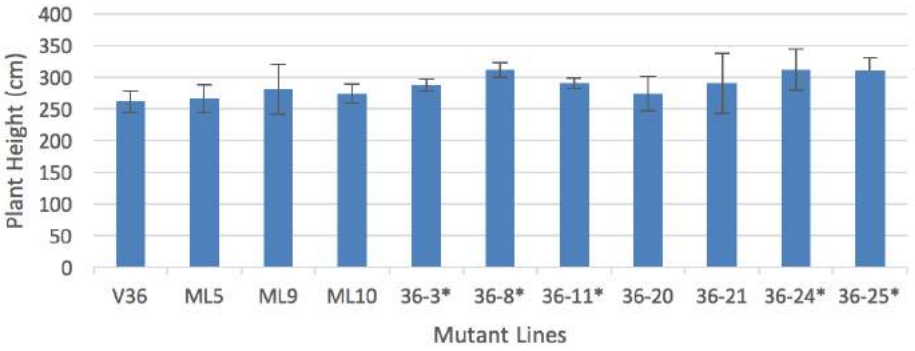

Figure 1. Average plant height recorded on mutant lines as compared to the control. Bars represent mean + SE.
* represents mutants that are significantly different from mother plant V36 meant at 5% level according to a Student's t-test.

tropical country like Malaysia (Hossain *et al*., 2011; Wong *et al*., 2008; Daud *et al*, 2012). Theoretically, if a plant can delay the reproductive (flowering) process, the energy that is meant for reproduction could instead be channeled to biomass development (especially stem).

Mutation breeding has long been established as a technique for developing new varieties with desirable traits. Since the first release of a mutant variety in 1930s, several outstanding successes have been achieved in this field. The mutation breeding technique has been used in many countries especially China, India, Japan and many others to improve crop varieties such as rice, wheat and barley which have been widely commercialized. Currently, a total of 3,308 new plant varieties have been developed worldwide through mutation breeding technique and registered under International Atomic Energy Agency (IAEA) Mutant Variety Database (http://www-mvd.iaea.org). There were also some reports on the success of generating new mutants of kenaf through mutation breeding (Balogun *et al*, 2009; Cook and Banuelos, 2011) but at present, none of these mutants are registered with the IAEA (http://www-mvd.iaea.org).

MATERIALS AND METHODS

Plant Materials

The initial planting materials (seeds of kenaf V36 variety) used in this project were provided by the National Kenaf and Tobacco Board (NKTB). Irradiations of the seeds (3 kg seeds for each selected dose; 0, 200, 300, 500, 800, 1000, 1300 Gy) were carried out in 2015. The mutated plants were then planted for five consecutive generations for selection of potential mutant lines. Ultimately, a total of 30 mutant lines (M5 generation) and one control (mother plant) were used in this field experiment to evaluate their agronomic performances.

Field Planting Of Mutant Lines

The experiment was carried out at the NKTB's field plot at Beseri, Perlis for the period of March to July 2018. The soil type of the plot was sandy loam and the climatic condition during this period was hot and humid with frequent rain. The experiment was conducted using direct seeding method with approximately 100 seeds per mutant line. Fertilizer, herbicide and pesticide treatments were carried out according to NKTB's standard practice (Lembaga Kenaf Dan Tembakau Negara, 2013). The mutant lines as

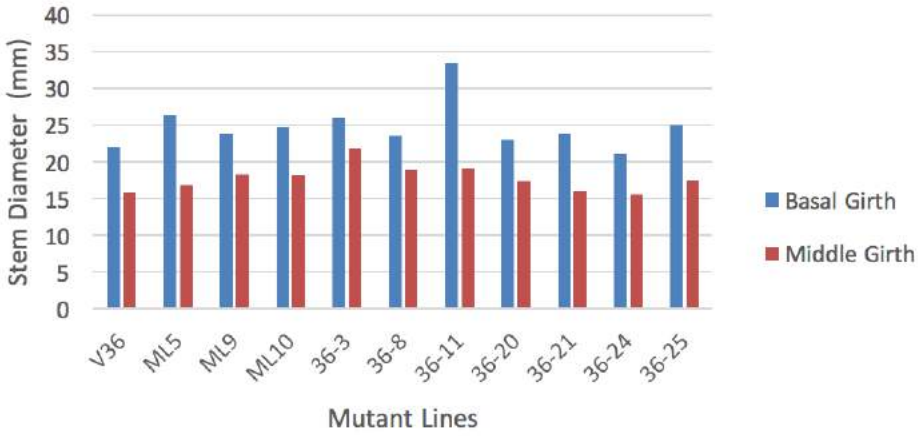

Figure 2. Basal and middle stem diameter for mutant lines and the control

Figure 3. Cross-section of the stems of a mutant line, 36-11 (left) vs. parent variety, V36 (right)

well as the control were grown until harvesting, approximately 135 days after planting.

Agronomic Characterization Of Mutant Lines

The agronomic data was collected during the harvesting period; (i) plant height (cm), (ii) bast fibre weight (g), (iii) basal and middle stem diameter (mm), (iv) number of pods per plant, (v) leaf morphology, (vi) flower morphology and (vii) percentage of seed maturity. The characters for the mutant lines were analyzed and compared with the control.

Data Analysis

Agronomic data were statistically analyzed using t-test analysis to compare differences between mutant lines and the control at 0.05 significant level using SAS ver. 9.2.

RESULTS AND DISCUSSION

Screening Of Mutants For Good Agronomic Characters

The main criteria for selection of new kenaf mutants was high fiber yield since it was the ultimate aim of the whole project. Based on the agronomic performance of the 30 mutant lines used in this study, a total of 10 mutant lines were observed to have the high yield characteristic as compared to the control. The details on selected mutant lines are given in Table 1. Most of these selected mutant lines showed an increase in plant height, which consequently contributed to overall increase in fiber yield. Other characteristics that were also considered during the screening were stem diameter/girth which may also positively affect the final yield of the fiber and the number of seed pods.

Plant Height

All the 10 mutant lines selected were found to be taller than the control, with the highest plant height was observed in mutant lines 36-8 and 36-26, both recorded the same height of approximately 311 cm as compared to approximately 261 cm for the control (Figure 1). Tall plants, in general, produced high fiber yield, along with other traits such as big stem size.

Basal and middle stem diameter

Another character that may affect fiber yield is stem size. In this experiment, the stem diameter was measured at the base (approximately 3 cm from the ground) and in the middle of the stem. The highest basal diameter measurement was recorded in mutant line 36-11 (33.42 mm) as compared to the control (21.96 mm). Other mutant lines that gave significantly higher stem diameter values

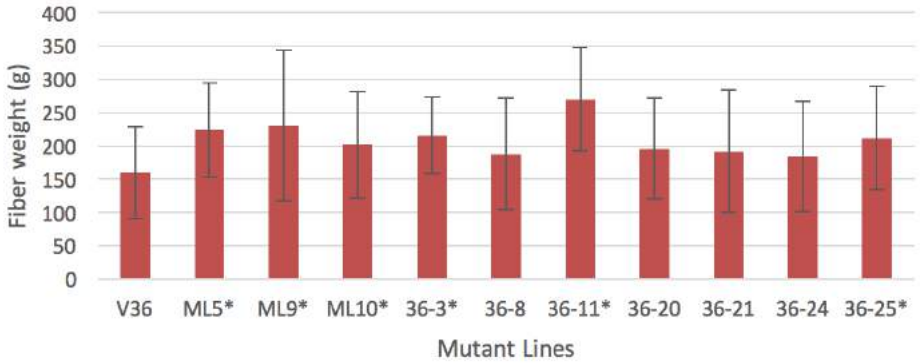

Figure 4. Average fiber weight of mutant lines as compared to the control (V36). Bars represent mean + SE.
* represents mutants that are significantly different from the control meant at 5% level according to a Student's t-test

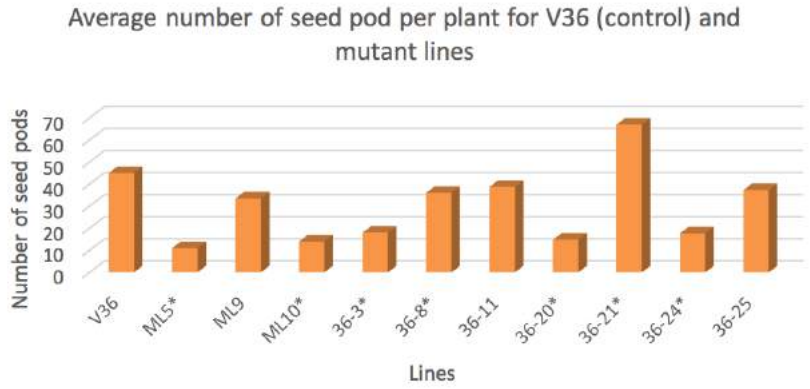

Figure 5. Average number of seed pods per plant for mutant lines and the control.
* represents mutants that are significantly different from the control meant at 5% level according to a Student's t-test

than the control were ML5, 36-3 and 36-25 (Figure 2). Figure 3 shows the cross-section of the stems of a mutant line 36-11 and the control demonstrate the difference in terms of stem size between the two plants.

Bast Fiber Weight

Kenaf fiber can be divided into two classes; bark or bast (outer) and core (inner). Bast fiber is the tough fiber and comprises approximately 25-30% of the whole plant. Core fiber, on the other hand, is a soft fiber and comprises of 70-75% of the total weight (Wong *et al*., 2008). In this experiment, bast fiber was separated from the core fiber using an automated kenaf skin peeler (fiber extracting machine). The weight of fresh bast fiber were recorded and analyzed. Mutant lines ML5, ML9, ML10, 36-3, 36-11 and 36-25 have significantly higher bast fiber weight than the control. The highest value recorded was 270 g (mutant 36-11) as compared to approximately 160 g for the control (Figure 4).

Number Of Seed Pods

Seed availability is very important for sustainable production of seed-propagated plants. Besides genetics, several other factors can affect seed production such as environmental conditions, cultural practices, handling and harvesting techniques (Basri *et al*., 2014). In this experiment, all mutant lines produced less seed pods as compared to the control, with the exception of mutant line 36-21 which was observed to produce a significantly higher number of seed pods per plant (Figure 5). The low number of seed pods might be due to a delay in flowering time which in turn delay the overall seed production. During harvesting time of 135 days after planting, only 60-70% of the seed pods from the mutant lines were

Figure 6. The difference in flower colour for the control variety (left) vs. ML5 mutant line (right)

already matured as compared to approximately 90% in the control plants. In most mutant plants, small seed pods were still congregated near the terminal end of the stems.

Flower Colour And Leaf Morphology

New flower colour and leaf morphology are not the main target traits in this project. However, one mutant line with high fiber yield (ML5) also exhibited other unique morphological traits in which the inner petal has yellowish colour as compared deep purple for the control variety (Figure 6), whilst the leaf was in palmate shape as compared to cordate for the control (Figure 7).

Selection Of Good Potential Mutant Lines

From this experiment, several mutant lines have been identified to have the potential to be further developed into new varieties for commercial use. Mutant lines 36-11, ML5 and ML9 were good candidates for high fiber yield varieties, whilst 36-21 has both characters; high seed pod number and good fiber yield.

Currently, these mutant lines which are in the M7 generation, are being screened at Universiti Putra Malaysia, Serdang, Malaysia. Several more tests are needed before these mutants can be certified as the new varieties and used by farmers in Malaysia. Among the tests to be carried out are multi-location trials (including on infertile lands previously used for tobacco plantation) and Distinctiveness, Uniformity and Stability (DUS) tests for plant variety registration. The development of these mutants is in line with the need from the beneficiary that requires a kenaf variety which can grow well and produce high fiber yield in local conditions.

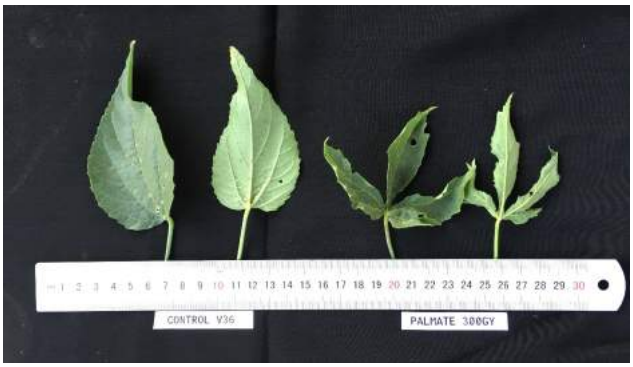

Figure 7. The difference in leaf morphology for the control variety (left) vs. ML5 mutant line (right)

CONCLUSION

Based on the field experiment in Beseri, Perlis, ten mutant lines have been found to produce higher fiber yield than the mother plant (control). Of these ten mutant lines, one line (36-21) which produced high yield of both fiber and seed can be developed into a new kenaf variety that can be planted for both fiber and seed production. Another three lines (36-11, ML5 and ML9) with high fiber trait are also good mutant candidates for fiber plantation in Malaysia.

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TPGM

Validation of similarity between brinjal T-64 and Bt brinjal event EE-1

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Abstract

Transgenic brinjal with insect resistance (Bt brinjal) was developed as an alternative to control large insecticide usage against fruit and shoot borers in brinjal crop. Bt brinjal termed as event EE-1, contains insect resistance gene (*Cry1Ac*) under the control of CaMV promoter and NOS terminator, with *nptII* gene as the selectable marker. Brinjal is one of the commonly consumed vegetables and has been continuously imported to meet the local market's demand in Malaysia. Therefore, genetically modified (GM) detection is crucial to determine brinjal identity for food safety concern. However, EE-1 brinjal reference material is not commercially available and the requirement of long process of documentation to get the material from the developer has limited our effort to develop event specific detection method for EE-1 brinjal in Malaysia. Meanwhile, Biosafety Department of Malaysia has provided potential EE-1 brinjal seed (T-64) to be tested. The brinjal seed was validated using polymerase chain reaction (PCR) and Sanger DNA sequencing for evidence of similarity with EE-1 brinjal. The PCR conducted has successfully amplified all the DNA fragments of EE-1 brinjal. DNA sequencing result revealed that both T-64 and EE-1 brinjal have high similarity score. The result confirms that T-64 brinjal is indeed EE-1 brinjal and could be used in developing event specific detection method for EE-1 brinjal.

Key words: LMO detection; Cry1AC gene, Bt brinjal

INTRODUCTION

Brinjal or eggplant (*Solanum melongena* L.) belongs to Solanaceae family is an important vegetable in South and South-East Asia (Gautam, 2019). It is beneficial for human health because of its high fiber and water content, rich in anti-oxidants, high in vitamins, proteins, calcium and phosphorus (Shaukat, 2018). Brinjal has been an important vegetable crop and is being grown extensively in India, Bangladesh, Pakistan, China and the Philippines as well as at the warm temperate zones, especially in Southern Europe and the Southern United States.

Despite its potential as commercial vegetable crop, brinjal is susceptible to many diseases and pests, including the eggplant fruit and shoot borer (EFSB, *Leucinodes orbonalis* Guenée). EFSB is a medium-sized moth (Lepidoptera: Crambidae) whose feeding larvae cause damage to the brinjal crop by boring into stems and fruits. Yield, therefore, can be affected either by severely damaged or destroyed fruits, or by damage to the developing plant. EFSB has been described as the most serious and destructive pest to brinjal crops (Shelton *et al*., 2018; Parimi & Zehr, 2009). Brinjal losses to EFSB in India lies anywhere between 5.3-54.8% of the total yield of crop (Andow, 2010). Farmers depend on broad spectrum of insecticide on brinjal crops to control the infestation but it is a costly practice and also rendering residues on the fruits.

In view of the pest and diseases serious problems, the genetically modified brinjal or known as Bt brinjal has been developed by India-based Maharashtra Hybrid Seed Company (Mahyco) to give resistance against lepidopteron insects, in particular the EFSB. The Bt brinjal registered as EE-1, was created by inserting a crystal protein gene (*Cry1Ac*) from the soil bacterium *Bacillus thuringiensis* into the genome of various brinjal cultivars. The insertion of the gene, along with other genetic elements such as promoters, terminators and an antibiotic resistance marker gene into the brinjal plant is accomplished using *Agrobacterium*-mediated genetic transformation.

Malaysia has imported brinjal from abroad to meet local market's demand (Suhana *et al*., 2016). Therefore, it is crucial to develop effective and accurate analytical methods for detection of this EE-1 brinjal in order to ensure compliance to Malaysia Biosafety Act 2007. Such method has been developed by Ballari *et al*., in 2012 from CSIR-Central Food Technology Research Institute, India. They had developed a single and

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Table 1. Primer sequences used in this study

Primer	Sequence	Amplicon size (bp)	Reference
35S Forward 35S Reverse	5'- GTGGTCCCAAAGATGGACCC -3' 5' –CCCTTACGTCAGTGGAGATATCACA -3'	111	(Ballari *et al.*, 2017)
nptII Forward *nptII* Reverse	5'- CTCACCTTGCTCCTGCCGAGA -3' 5' – CGCCTTGAGCCTGGCGAACAG -3'	215	(Kamle *et al.*, 2011)
NOS Forward NOS Reverse	5' – TTAAGATTGAATCCTGTTGCCG -3' 5' – TAATTTATCCTATCCTAGTTTGCGCGC -3'	192	(Ballari *et al.*, 2017)
18s Forward 18s Reverse	5' – TCTGCCCTATCAACTTTCGATGGTA -3' 5' – AATTTGCGCGCCTGCTGCCTTCCTT -3'	137	(Ballari *et al.*, 2017)
Cry1Ac Forward *Cry1Ac* Reverse	5' – ACAGAAGACCCTTCAATATC -3' 5' –GTTACCGAGTGAAGATGTAA-3'	654	(Cheema *et al.*, 2016)
EE-1 Forward EE-1 Reverse	5' – GGAGCTTCTCTTGATGGAGG -3' 5' – TGCGGTGATAATTGAATGCATC -3'	685	(Char & Ghandi, 2016)

multiplex PCR methods using both standard and real-time PCR (qPCR)to detect EE-1 brinjal. Molecular diagnostic method for EE-1 brinjal detection using primer pair to amplify the insertion locus region was also published in patent document WO2007/091277 by Char & Ghandi in 2016. The two research papers were the main references in this study.

Reference material plays an important role for quality control in GM detection to make certain of the GM element traceability. Lack of reference material caused by long process of documentation to obtain the material from the developer has limits the effort to develop event specific detection method for EE-1 brinjal in Malaysia. Hence, the focus of this study was to validate the brinjal seed sample provided by Biosafety Department of Malaysia for its compatibility as a reference material. The EE-1 positive brinjal would be useful for the development of event specific EE-1brinjal detection method in Malaysia.

METHODS

DNA Extraction

Seeds of brinjal (T-64) sample provided by Biosafety Department of Malaysia were ground and extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The absorbance ratio of ($A_{260/230}$) and ($A_{260/280}$) were used to determine the quantity and quality of total DNA using Nanodrop 2000 (Thermo Scientific, US).

PCR amplification

PCR was conducted using exTen PCR Mastermix (1st BASE, Malaysia) with 1µL of T-64 extracted DNA and 0.5µL 10 Mm primers specific (18S rDNA, CaMV 35S promoter, NOS terminator, *nptII* gene, *Cry1Ac* gene and EE-1).Each primers sequences and expected amplicon size are listed in Table 1. Commercially available Certified Reference Material, MON531 cotton (AOCS0804-C) was used as positive control. PCR conditions included initial denaturation of 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 20s, annealing at 60°C (18S, 35S, NOS, *nptII*, EE-1) and 50°C (*Cry1Ac*), extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were analyzed using 1.5% agarose gel electrophoresis supplemented with 0.1% GelRed in 1 x TAE buffer for 33 min at 120 V. The PCR amplifications were viewed using AlphaImager MINI (Cell Biosciences, Australia). PCR product of EE-1 was eluted from the gel and purified using MinElute Gel Extraction Kit (Qiagen, Germany) following the manufacturer's protocol. The DNA fragment was prepared for cloning into a plasmid vector.

Plasmid construct and transformation into *Escherichia coli* Top10 competent cells

The DNA fragment of EE-1 was ligated into pCR® 2.1 and the recombinant (*EE-1∴pCR® 2.1*) was then transformed into TOP10 competent cells through heat shock method. Subsequently, SOC medium was added and shook at 37°C for 1 h. The transformants was spread on LB agar supplemented with 100 µg/mL ampicilin and 40 µL X-gal, and then incubated for overnight at 37°C. Single colonies that grew on the plate were isolated and cultured overnight in LB broth.

Purification and sequencing of plasmid DNA

Plasmid DNA of cultured bacteria was purified using QIAprep Spin Miniprep Kit (Qiagen, Germany) following the manufacturer's protocol. BigDye Terminator Sequencing Kit (Applied Biosystems, US) was used in cycle sequencing. The cycle sequencing thermal conditions included initial denaturing step of 1 min at 95°C, followed by 40 cycles at 95°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequencing reactions were purified by using BigDye XTerminator Purification Kit (Applied Biosystems, US) and run on the ABI 3130xl Genetic Analyzer (Applied Biosystems, US).

DNA Sequencing data Analysis

The raw sequence was edited and assembled using SEQUENCHER version 5.3 (Gene Codes Corporation, US). The

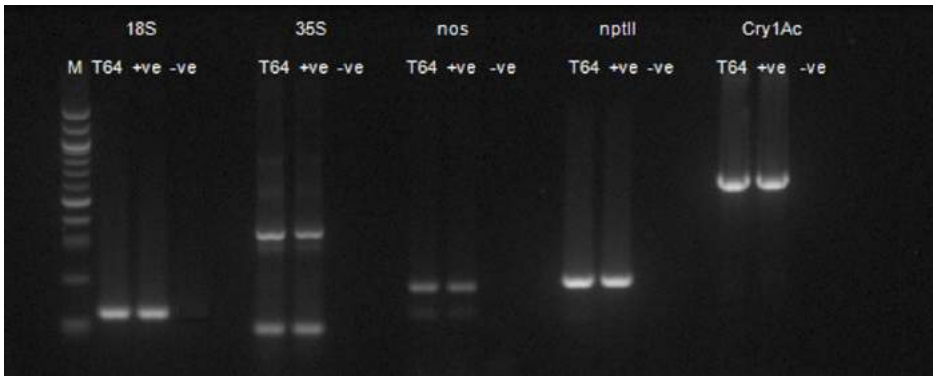

Figure 1. Agarose gel electrophoresis of T-64 amplification products using primer of 18S, CaMV 35S promoter, NOS terminator, *nptII* and *Cry1Ac* gene. T-64 represent T-64 DNA; +ve is MON531; -ve is negative control without DNA and M is 100 bp DNA ladder

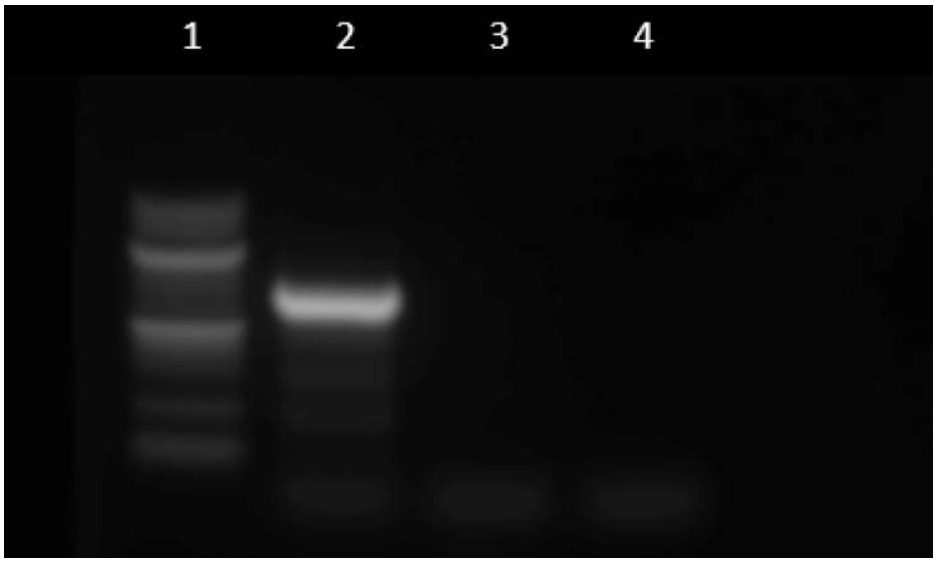

Figure 2. Agarose gel electrophoresis of PCR amplification product of T-64 brinjal. Lane 1, 100 bp ladder; lane 2, T-64 ; lane 3, MON 531 ; lane 4, control

identified sequence was compared to reference sequence using multiple sequence alignment tool of BLASTN with the default parameters. Reference sequence of EE-1 brinjal *Cry1Ac* gene was obtained from patent document WO2007/091277 (Char & Ghandi, 2016).

RESULTS AND DISCUSSION

PCR screening for EE-1 brinjal detection

PCR of T-64 shows amplification of 137 bp fragment when 18S rDNA eukaryote specific primer was used (Fig. 1). Based on gel electrophoresis result in Figure 1, CaMV 35S primer pair amplified two fragments, 111 bp and 374 bp corresponding to enhanced 35S promoter as mentioned earlier by Ballari *et al*., (2012). NOS terminator primer pair amplified a 192 bp fragment corresponding to Ballari *et al.*, (2012). Further, *nptII* gene primer pair amplified a 215 bp fragment and *Cry1Ac* gene primer pair amplified 654 bp fragment as expected. This corresponds to the positive control used in this study, MON531 cotton (AOCS0804-C) which is a Certified Reference Material, where the genetic elements construct consists of CaMV 35S promoter, enhanced 35S promoter, NOS terminator, *nptII* gene and *Cry1Ac* gene.

Event-specific PCR detection of T-64

Among four PCR-based methods of GM detection, event-specific is the most specific rather than screening, gene- and construct-specific (Ballari *et al*., 2012). Event-specific PCR refers

Figure 3. (A) Schematic diagram of recombinant construct in EE-1 brinjal adapted from Ballari *et. al* (2012). Arrows indicate location of EE-1 primer pair used to amplify EE-1 region. (B) Sequence showing the integration between *Cry1Ac* gene and T-64 brinjal genome. Arrows show forward and reverse primer. Underlined sequence refers to exogenous DNA sequence that consist of *Cry1Ac* gene, 7S terminator and T-DNA right border sequences. The shaded sequence refers to T-64 brinjal genome sequence

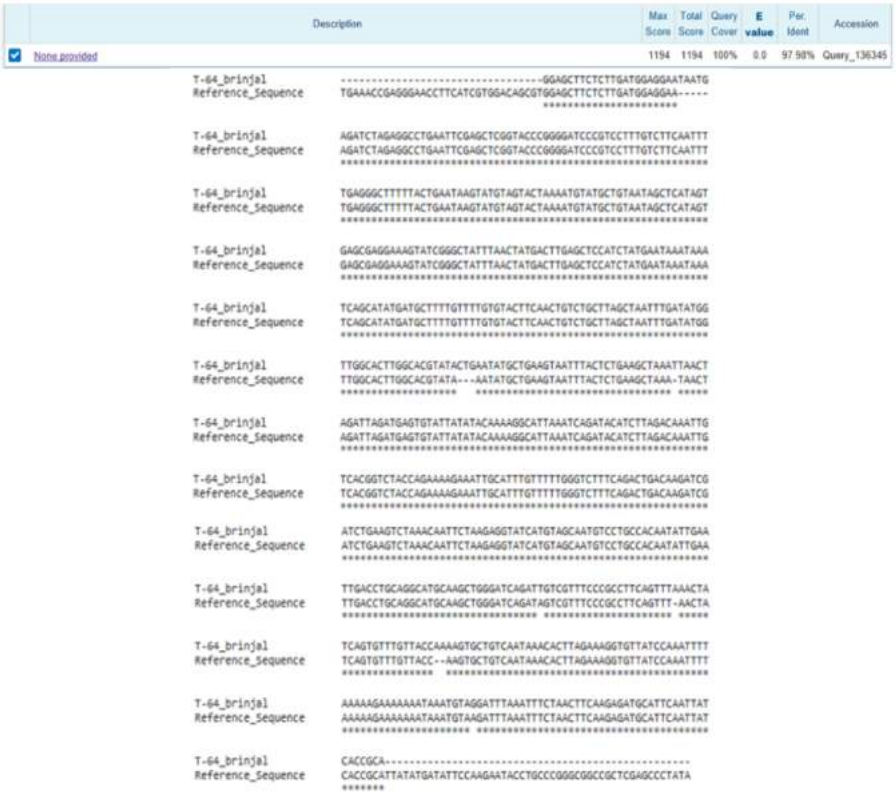

Figure 4. Alignment of T-64 brinjal sequence with EE-1 brinjal reference sequence using BLASTN alignment tool

to the amplification of integration junction between host genome and transgenic DNA sequence which promotes high specificity for GM detection. Thus, to reconfirm the preliminary screening results, T-64 was amplified using EE-1 event specific primers retrieved from patent document WO2007/091277. EE-1 forward primer targets the transgenic region of *Cry1Ac* sequence and EE-1 reverse primer targets flanking region of the brinjal DNA genome sequence. EE-1 primer pair generates expected amplification product of 685 bp for T-64 (Figure 2), but no amplification was found in MON 531 sample indicated the specificity of EE-1 event specific primers. Hence, T-64 is potentially an EE-1 Bt brinjal due to the amplification of EE-1 specific region.

DNA Sequence data analysis

According to Ballari *et al.,* (2012), recombinant construct of EE-1 brinjal contains two gene cassettes as illustrated in Figure 3A with both cassettes are flanked with CaMV 35S promoter (P-35S, P-E35S) at 5' end and terminator (T-NOS, 7S) at 3' end, respectively. In this study, cloning of PCR product between *Cry1Ac*gene and plant genome was performed prior to sequencing (Fig 3A). The sequence produced as shown in Figure 3B represent the complete 695 bp nucleotide sequence of the exogenous *Cry1Ac* gene and the T-64 brinjal genome which was amplified with EE-1 primer pair.

The sequence was aligned with EE-1 brinjal reference sequence of the patent document WO2007/091277 (Char & Ghandi, 2016) to further confirm the accuracy. Based on the alignment results, T-64 brinjal sequence shows 97.98 % identity to the reference sequence (Figure 4). T-64 brinjal sequence which incorporated the *Cry1Ac* gene flanked by the 7S terminator, T-DNA right border and brinjal genomic DNA match the reference sequence which confirm that T-64 brinjal is an EE-1 brinjal.

CONCLUSION

This study significantly shows that T-64 brinjal provided by Biosafety Department of Malaysia is an EE-1 brinjal. T-64 as an EE-1 brinjal was validated following the PCR screening method reported by Ballari *et al.* (2012) using primer pairs of targeted elements present in EE-1 brinjal which are CaMV 35S promoter, NOS terminator, *nptII* gene and *Cry1Ac* gene.T-64 was further confirmed as EE-1 brinjal when the integration region between exogenous *Cry1Ac* gene with the brinjal genome was also amplified in PCR using specific EE-1 primer. In addition, sequencing was also carried out to further verify the EE-1 brinjal. Alignment of the T-64 brinjal sequence with the reference sequence generated high percentage of similarity. This finding strengthens the evidence to certify T-64 brinjal as an EE-1 brinjal that could be used as a reference material for developing brinjal EE-1 detection method.

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TPGM

Diversity and genetic potential of Indonesia pea (*Pisum sativum* L.) landrace based on morphological traits in lowlands

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Abstract

Pisum sativum is an important vegetable for biofortification of Selenium, Zinc, Fe, Mg, and a number of important minerals that can be used to improve public health. This plant has long been cultivated in some highlands in Indonesia. Landrace can be formed from cultivation of plants in local geographical. Pea selection was an attempt to obtain adaptive lines in the lowlands of the tropics. The research objective was to study diversity and genetic potential of Indonesia peas landrace based on morphological characters. Research was conducted in greenhouse of Seed and Nursery Industry, Agrotechnopark, Universitas Brawijaya, Jatikerto (340 m asl), Malang from February to May 2019. The study used sixty-one lines from Berastagi (Karo), Cilawu (Garut), Bandungan (Semarang), Parakan (Temanggung), Ngadisari (Probolinggo), and Junrejo (Batu). Planting each line is carried out in a single row. Characters variability was analyzed using principal component analysis. Pea lines diversity was analyzed by agglomerative hierarchical clustering. The research found that there were first eleven principal components having an eigenvalue >1 contributing to total variability of 76.67%. Characters that high variability in each principal are plant, stem, leaf, leaflet, stipule, petiole, flower, peduncle, pod, seed, and cotyledon. Based on morphological characters, diversity of pea lines is divided into 6 groups. Grouping clearly shows origin of different geographic regions. Influence of the region is very strong in forming genetic differences through morphological variability. Indonesia pea landrace has high genetic potential to be developed as a superior and adaptive cultivar in the tropical lowlands.

Key word: Indonesia; *Pisum sativum*; numerical taxonomy; landrace; lowlands

INTRODUCTION

Pea is a legume crop with high nutritional potential and active compounds that are beneficial to health (Dahl *et al*., 2012; Malcolmson *et al*., 2014). Apart from being a food source, this plant is also used as a source of animal feed (Bilgili *et al*., 2010). Pea is a legume plant that has the potential to be developed to meet the nutritional needs of the community. Seeds contain 22-25% protein, complex carbohydrates, and fiber content, as well as a variety of vitamins, minerals, and phytochemicals which make it a valuable source for human consumption and animal feed, and dried pods rank third for the most developed bean species in the world (Poblaciones *et al*., 2013; Smýkal *et al*., 2012). Pea has a strong ability to absorb and accumulate Selenium and has been proposed as a biofortification source for increasing Se consumption. Se is a micronutrient that acts as an antioxidant, anticancer, and antiviral and is important for humans and animals (Poblaciones *et al*., 2013). Pea also has the ability to provide Fe, Zn, and Mg (Amarakoon *et al*., 2012) so that it can be used as a potential food-based solution to global micronutrient malnutrition. Peas has become an important commodity for diet and healthy life because it contains fiber, protein, starch, useful phytochemical, antibacterial, antidiabetic, antifungal, anti-inflammatory, antihypercholesterolemia, antioxidant, anticancer, active compounds including alkaloids, flavonoids, glycosides, isoflavones, phenols, phytosterols, and folic acids, protease inhibitors, tannins, and are rich in apigenin, hydroxybenzoic, hydroxycinnamic, luteolin, and quercetin, all of that have been reported contributing to remedial properties including anti-carcinogenesis properties (Rungruangmaitree and Jiraungkoorskul, 2017). In some zones of the world, this crop is important component in agroecology systems (Gopinath *et al*., 2009). Total production in the world fluctuates from 10-12 million tons, with Canada as the main producer, followed by the United States, India, Russia, France and China (Gixhari *et al*., 2014a).

In Indonesia, pea have been popular for many years. However, some of the peas are not met characterization. Pea are widely distributed in some region in Indonesia, including Java and Sumatra. This crop is usually cultivated in the highlands. Pea are largely cultivated in the areas of Berastagi (Karo Regency), Cilawu (Garut Regency), Bandungan (Semarang Regency), Parakan (Temanggung Regency), Ngadisari (Probolinggo Regency), and Junrejo (Batu City). Plant parts consumed are young pods (peas), seeds during the pod phase

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Tabel 1. The origin and number of lines tested in the study

Regency/City	District	Count	Code
Karo	Berastagi	5	BTG
Garut	Cilawu	14	GRT
Temanggung	Parakan	22	TMG
Semarang	Bandungan	8	SMG
Batu	Junrejo	6	Batu
Probolinggo	Ngadisari	6	Bromo

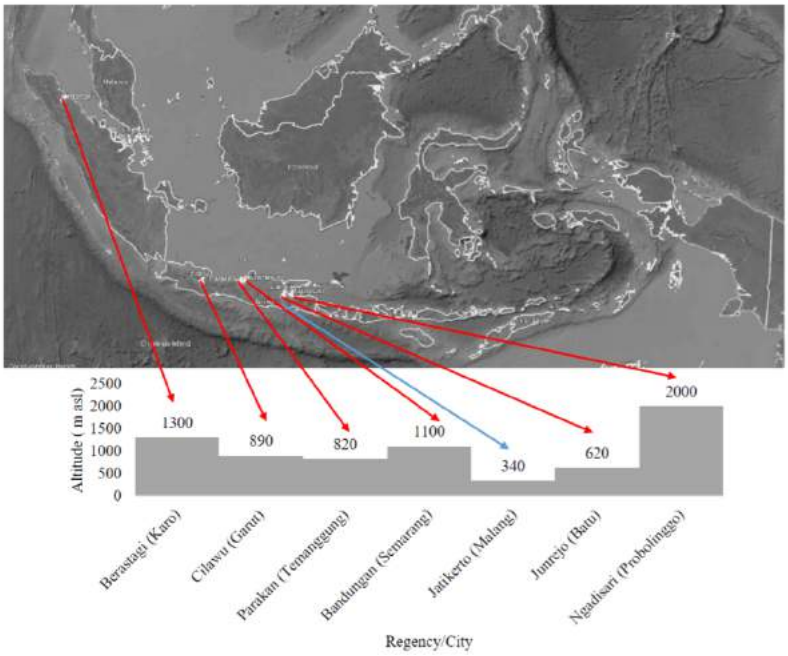

Figure 1. Distribution of Indonesian pea landrace (red arrows) and genetic diversity trial sites in the lowlands (blue arrows)

just before physiological maturity (peas, green peas), and dry seeds (dry peas). Nevertheless, information about characters variability and genotypes diversity of peas is still limited. Some farmers usually only suggest genetic differences in peas based on morphology in stems, leaves, flowers, fruits, and seeds to determine a local name. Many local names reflect genetic diversity and adaptation to environmental conditions.

Peas are vary in the character of the pod and seed that usually adjusted for the purpose of consumption and processing as well as the smells (Malcolmson *et al.*, 2014). Character variability in peas has been extensively studied in morphological and agronomic characters which show a wide diversity and serve as a basis for selecting superior genotypes (Avci and Ceyhan, 2013; Barcchiya *et al*., 2018; Gudadinni *et al*., 2017; Pal and Singh, 2013; Singh *et al*., 2017). The selection of pea germplasm that has been successfully cultivated by farmers from wild crops can play an important role in genetic improvement (Gixhari *et al*., 2014b). Genotype identification and evaluation of population genetic variation are important in the protection of genotypes and plant breeding programs (Khan *et al*., 2016).

Improving the quality and quantity of peas requires an effort to improve the genetic quality of the crop by implementing a plant breeding program (Bhuvaneswari *et al*., 2017). One of the effective ways is to filter out the existing pea germplasm through characterization activities. In order for the crop to spread over large areas of planting, it is necessary to screen plants in the lowlands. The lines selected from previous study are evaluated in the lowlands to determine the lines diversity and characteristic variability of crop originating from

Table 2. Eigenvalues, variability, cumulative variability, and principal components for 51 morphological traits of 61 lines of peas in lowland

Parameters and characters	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Eigenvalue	12.91	4.82	3.74	2.75	2.52	1.88	1.60	1.52	1.24	1.20	1.10
Variability (%)	28.07	10.49	8.13	5.97	5.47	4.08	3.48	3.29	2.70	2.60	2.39
Cumulative %	28.07	38.56	46.69	52.66	58.13	62.20	65.69	68.98	71.68	74.28	76.68
Plant: anthocyanin coloration	**0.77**	0.47	-0.07	0.23	0.27	-0.06	0.00	0.16	0.01	0.00	-0.01
Stem: anthocyanin coloration of axil	0.41	**0.75**	0.18	0.17	0.31	-0.11	-0.12	0.00	0.09	0.00	-0.05
Stem: fasciation	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stem: length	-0.31	0.21	-0.34	0.32	-0.33	0.22	0.19	0.12	-0.19	0.14	-0.07
Stem: number of nodes up to and including first fertile node	**-0.64**	0.05	0.29	0.07	0.26	0.15	0.03	0.20	-0.02	-0.01	0.09
Foliage: color	0.39	-0.42	-0.39	0.01	-0.31	-0.21	-0.29	-0.23	0.02	0.03	0.06
Foliage: intensity of color	0.43	-0.26	-0.54	0.13	-0.11	-0.25	-0.38	-0.21	0.01	0.16	0.04
Leaf: leaflets	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leaf: maximum number of leaflets	-0.52	0.19	0.01	0.25	-0.28	0.35	-0.16	-0.03	0.12	-0.07	0.10
Leaf: size	**-0.77**	0.19	-0.35	-0.04	-0.07	0.02	-0.10	0.05	0.03	0.07	-0.01
Leaflet: length	-0.36	0.22	-0.48	-0.16	-0.06	0.22	-0.13	0.30	-0.16	-0.35	0.06
Leaflet: width	**-0.65**	0.25	-0.31	-0.12	0.13	0.07	0.02	-0.15	0.01	0.13	-0.06
Leaflet: position of broadest part	0.17	-0.03	-0.39	-0.12	0.17	0.20	0.33	-0.03	0.08	**0.61**	0.20
Leaflet: dentation	-0.21	0.24	0.22	0.25	-0.33	0.32	-0.36	-0.03	-0.16	0.26	-0.31
Stipule: length	**-0.60**	0.30	-0.53	-0.06	-0.09	0.04	0.12	-0.03	0.13	-0.02	-0.02
Stipule: width	**-0.79**	0.18	0.07	-0.14	0.01	0.09	-0.03	-0.20	0.00	0.01	-0.20
Stipule: size	**-0.74**	0.01	-0.35	0.12	0.01	-0.06	0.03	0.07	0.16	-0.09	-0.23
Stipule: length from axil to tip	-0.58	0.31	-0.56	0.01	-0.16	0.04	0.10	0.06	0.05	-0.16	-0.08
Stipule: length of lobe below axil	-0.42	0.20	-0.26	0.12	0.39	0.15	0.14	0.14	0.10	0.24	-0.28
Stipule: flecking	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stipule: density of flecking	-0.35	-0.14	0.16	0.44	-0.19	-0.42	0.12	0.34	0.14	-0.18	0.05
Petiole: length from axil to first leaflet or tendril	**-0.63**	0.31	-0.21	0.01	-0.23	-0.04	-0.16	0.06	-0.01	0.01	0.29
Time to flowering	-0.43	0.35	-0.07	-0.26	0.13	0.18	-0.30	0.03	0.20	0.16	0.18
Plant: maximum number of flowers per node	**-0.66**	-0.16	0.30	0.18	0.31	0.18	0.07	0.27	-0.05	0.02	0.04
Flower: color of wing	**0.77**	0.47	-0.07	0.23	0.27	-0.06	0.00	0.16	0.01	0.00	-0.01
Flower: color of standard	**-0.77**	-0.47	0.07	-0.23	-0.27	0.06	0.00	-0.16	-0.01	0.00	0.01
Flower: width of standard	-0.27	0.19	-0.32	-0.10	-0.14	-0.16	0.49	0.05	-0.38	0.05	0.33
Flower: shape of base standard	-0.28	-0.11	-0.04	-0.25	-0.08	-0.03	-0.10	0.17	**0.73**	0.12	0.10
Flower: width of upper sepal	-0.53	0.22	0.05	-0.10	-0.03	0.08	-0.05	0.08	-0.08	0.03	0.21
Flower: shape of apex of upper sepal	0.20	0.07	-0.12	0.22	-0.12	-0.35	-0.37	0.34	-0.26	0.50	0.02
Peduncle: length of spur	-0.36	**0.66**	0.34	0.08	0.24	0.08	0.04	-0.02	-0.12	-0.04	0.00
Peduncle: length from stem to first pod	**-0.72**	0.26	0.04	-0.12	-0.17	-0.28	0.02	-0.08	-0.25	0.00	-0.09
Peduncle: length between first and second pods	**-0.61**	-0.40	0.05	0.19	0.20	-0.04	-0.09	0.21	-0.11	0.17	0.05
Peduncle: number of bracts	-0.31	**0.68**	0.23	0.07	-0.17	-0.20	-0.05	-0.28	0.05	0.01	-0.21
Pod: length	**-0.64**	0.23	-0.06	0.22	0.17	-0.49	-0.11	0.04	0.09	-0.03	-0.08
Pod: width	**-0.80**	-0.17	0.14	0.02	0.14	-0.08	-0.12	-0.13	-0.07	0.04	-0.11
Pod: parchment	-0.49	-0.39	-0.04	-0.14	0.43	-0.02	-0.38	0.08	-0.17	-0.07	0.01
Pod: thickened walls	0.28	-0.11	0.09	0.57	-0.21	0.55	-0.15	-0.01	0.06	-0.07	0.13
Pod: shape of distal part	0.32	-0.24	-0.16	0.51	0.19	0.32	0.04	-0.27	-0.02	-0.05	0.03
Pod: curvature	-0.03	0.34	-0.02	-0.17	0.50	-0.09	-0.12	-0.31	0.02	-0.04	0.44
Pod: color	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pod: intensity of green color	0.34	-0.08	0.22	-0.40	-0.23	0.13	-0.01	0.57	0.14	0.05	-0.06
Pod: number of ovule	0.05	0.39	-0.31	0.46	-0.16	-0.18	-0.09	0.09	0.16	-0.18	0.16
Immature: intensity of green color	0.31	0.29	0.38	-0.54	-0.28	-0.14	0.03	0.12	-0.03	0.05	-0.09
Seed: shape	**0.83**	-0.08	-0.27	0.13	0.16	-0.01	-0.03	0.17	-0.06	-0.02	-0.06
Seed: wrinkling of cotyledon	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Seed: color of cotyledone	**-0.76**	-0.51	0.05	0.16	0.25	-0.10	-0.04	0.07	-0.03	-0.04	0.03
Seed: hilum color	0.30	-0.01	-0.51	-0.35	0.47	0.11	-0.11	-0.01	-0.01	-0.11	-0.28
Seed: color of testa	-0.32	0.33	**0.65**	0.05	-0.08	0.05	-0.18	-0.12	0.02	0.11	0.22
Seed: weight	-0.34	-0.20	0.23	0.41	-0.01	-0.21	0.42	-0.19	0.26	0.19	-0.08
Cotyledon	**-0.76**	-0.51	0.05	0.16	0.25	-0.10	-0.04	0.07	-0.03	-0.04	0.03

Bold numbers indicate factor loading values > 0.6

Table 3. Descriptions of Indonesian pea landrace agronomy characters planted in the lowlands

Characters	Minimum	Maximum	Average	Stdev	CV(%)
Flowering day (dap)	29	47	35.16	4.96	14.12
Harvesting day (dap)	56	79	62.64	5.12	8.18
Plant height (cm)	96.20	235.08	161.30	31.81	19.72
Pod number per plant	2.25	16.75	7.95	2.94	37.02
Pod weight per plant (g)	1.94	12.50	5.98	2.18	36.38
Pod length	4.45	9.42	5.91	0.78	13.15
Seed number per pod	3.00	6.00	4.28	0.63	14.83
Seed number per plant	8.50	56.25	29.00	10.48	36.13
Seed weight per plant (g)	1.68	10.67	5.19	1.87	36.02
Weight of 100 seed (g)	14.56	24.55	18.03	1.97	10.92

CV = coefficient of variation, stdev = standard deviation

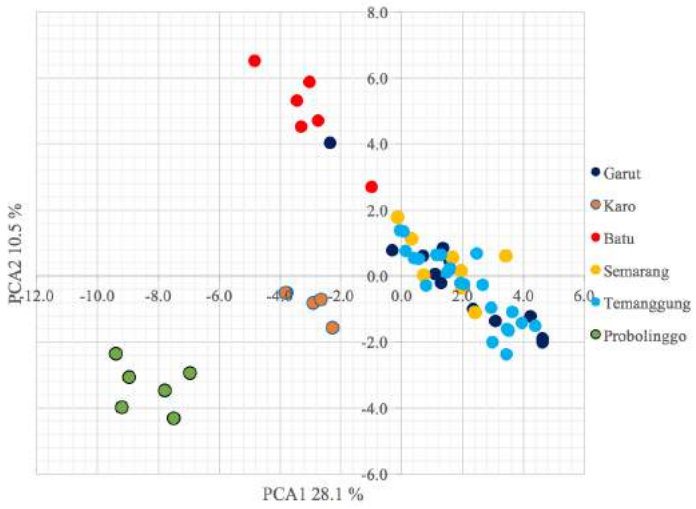

Figure 2. Two-dimensional graph of first two factor score 61 peas lines from Karo, Garut, Semarang, Temanggung, Batu, and Probolinggo based on 51 morphological characters described in the lowlands

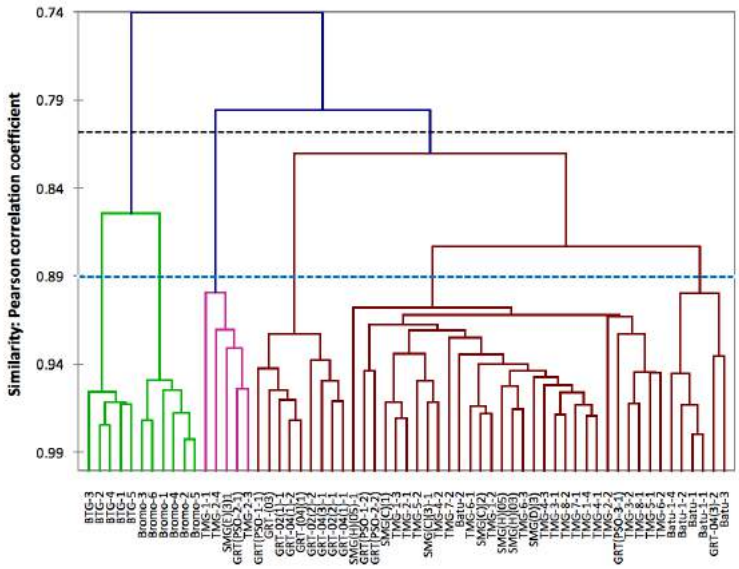

Figure 3. Genetic diversity of 61 peas lines derived from Berastagi (Karo, code: BTG), Cilawu (Garut, code: GRT), Bandungan (Semarang, code SMG), Junrejo (Batu: code Batu), and Ngadisari (Probolinggo, code: Bromo) based on 51 morphological characters in lowland

different regions. Assessment of lines diversity and character variability is important to be expected in relation to selection and the use of peas as a candidate for varieties and material for enhancing the genetic capacity (Ahmad *et al*., 2014; Ali *et al*., 2007; Habtamu, 2013; Iqbal *et al*., 2017; Singh *et al*., 2017). The objective of this study is to study the diversity and genetic potential of Indonesian local peas based on morphological characters planted in lowland areas.

MATERIALS AND METHODS

Research was conducted from February to May 2019 at the greenhouse of Seed and Nursery Industry, Agrotechnopark, Universitas Brawijaya, Jatikerto (340 m asl), Malang. The study used sixty-one lines separated from landrace origin from Berastagi (Karo Regency), Cilawu (Garut Regency), Bandungan (Semarang Regency), Parakan (Temanggung Regency), Bromo (Ngadisari, Probolinggo Regency), and Junrejo (Batu City) (Table 1). Each lines was planted in a single row plot. In each plot eight plants were planted. Observations were made on the morphological characteristics of each plant in the plot. *Pisum sativum* characterization was performed using the criteria of the International Union for the Protection of New Varieties of Plants (UPOV) (TG 7/9 *Pisum sativum*).

Morphological character variability was analyzed using principal component analysis (PCA) based on the type of Pearson correlation coefficient (n). The component meaningful if eigenvalue has a value >1. The characters in each principal component are contributing to the total variability if the value of the loading factor > 0.6 (Peres-Neto *et al*., 2003). Lines diversity was analyzed by agglomerative hierarchical clustering (AHC) to grouping based on similarity Pearson correlation coefficient and unweighted pair group average as a fusion criterion. Analysis of data using Microsoft® Excel 2007/XLSTAT Version 2009.3.02.

RESULTS AND DISCUSSION

Lines are obtained from pea-producing areas in Indonesia and have been cultivated by farmers for generations. Separation of lines from each area of origin is based on seed physical characteristics, including shape, size, and seed color. Seeds are planted individually. In the following season seeds from a plant are then planted in a row. Because pea are self-pollinating plants, one row of plants shows a homogeneous appearance and is called a line. The origin of ercis region of each line and altitude are shown in Figure 1.

Generally seeds from the same area or from different regions have different physical characteristics that are easily distinguishable visually. This is a high genetic potential to form high yielding varieties from local population. The presence of peas in tropical highland areas can be used to select plants that are adaptive in the lowlands. This selection effort was initiated by measuring characters variability and lines diversity obtained as a basis for selection.

Principal component analysis (PCA) was performed for 51 morphological traits of peas genotypes as indicated in Table 2. Out of forty-three, first eleven principal components showed eigenvalue >1. These three components explain 76.7% of the variation in the data.. The first eleven principal components were given due importance for the further explanation. PC1 had eigenvalue 12.91 with the highest variability of 28.07 %. PC2 had eigenvalue 4.82 with a variability of 10.49 %. PC3 had eigenvalue 3.74 with variability of 8.13 %. PC4 had eigenvalue 2.75 with a variability of 5.97 %. PC5 had eigenvalue 2.52 with variability of 5.47 %. PC6 had eigenvalue 1.88 with variability of 4.08 %. PC7 had eigenvalue 1.60 with variability of 3.48 %. PC8 had eigenvalue 1.52 with variability of 3.29 %. PC9 had eigenvalue 1.24 with variability of 2.70 %. PC10 had eigenvalue 1.20 with variability of 2.60 %. PC11 had eigenvalue 1.10 with variability of 2.39 %. Each component can be determined characters that contribute to the total variability which is indicated by a loading factor > 0.6. The larger absolute value of loading factor, more important the corresponding variable is in calculating the component. Although total variability is determined by firt eleven principal components but based on factor loading only PC1, PC2, PC3, PC9, and PC10 has characters with high variability.

PC1 was more related to plant: anthocyanin coloration, stem: number of nodes up to and including first fertile node, leaf: size, leaflet: width, stipule: length, stipule: width, stipule: size, petiole: length from axil to first leaflet or tendril, plant: maximum number of flowers per node, flower: color of wing, flower: color of standard, peduncle: length from stem to first pod, peduncle: length between first and second pods, pod: length, pod: width, seed: shape, seed: color of cotyledon, cotyledon. In PC2 was related to stem: anthocyanin coloration of axil, peduncle: length of spur, peduncle: number of bracts. PC3 correlated with, seed: color of testa. PC9 was more related to flower: shape of base standard. PC10 exhibited leaflet: position of broadest part. The positive and negative sign on factor loading values indicates the influence of character on variability.

In the PCA and AHC, agronomic character data is converted into data categories according to the International Union for the Protection of New Varieties of Plants (UPOV) (TG 7/9 *Pisum sativum*). Agronomic character is a very important characteristic of each pea line planted in the lowlands because it is a selection criterion. In this study some agronomic characters are displayed descriptively with respect to harvest time and yields obtained (Table 3). The pea population from Indonesia planted in the lowlands has a flowering day range of 29 dap to 47 dap with an average of 35 dap, and has harvesting day between 56 dap to 79 dap with an average of 62 dap. Plant height between 96.2 cm to 235.08 cm with an average of 161.30 cm. Pod numbers per plant are between 2.25 and 16.75 with an average of 7.95. Pod weight per plant is between 1.94 g and 12.50 g with an average of 5.98 g. Pod lengths are from 4.45 cm to 9.42 cm with an average of 5.91 cm. Seed number per pod between 3 to 6 with an average of 4.27. Seed number per plant is between 8.50 and 56.25 with an average of 29.00. Seed weight per plant is between 1.68 g and 10.67 g with an average of 5.19 g. Weight of 100 seeds ranging from 14.56 g to 24.54 g with an average of 18.03 g. Based on the coefficient of variation the lowest variation is found in the harvesting day, while the highest variability is found in the number of pods per plant.

PCA biplot first two factor score of morphological characters indicated the presence of peas grouping by origin collection location (Figure 2). This is very possible because the influence of the regional origin environment is very strong in forming genetic differences. Lines originating from different regions show diversity to form specific lines grouping which shows that local germplasm has the great genetic potential to be developed as superior and adaptive varieties in the tropical. Lines derived from Berastagi, Bromo, and Batu indicate a clear genetic separation based on morphological character. This divergence indicates that farmers who have inherited genetic material have been able to explicitly form distinct populations. The addition of pure line selection to the physical characteristics of different seeds has increased the diversity of peas in Indonesia. While lines separated from the population of Garut, Temanggung, and Semarang do not show any specificity for the region. However, these lines also express themselves differently from the lines derived from the Batu and Probolinggo.

Batu, and Probolinggo based on 51 morphological characters described in the lowlands

Peas lines were also classified into different clusters on the basis of all morphological traits. The similar genotypes were classified into the same cluster based on their various morphological traits studied. Cluster analysis is used to determine the genetic proximity between lines using the morphological properties of a plant because morphological properties can be used to identify and describe species-level kinship (Saragih *et al*., 2018). Study of genetic divergence among the plant materials is an important tool to the plant breeders for an efficient selection of the diverse parents for their potential use in a peas breeding program for the improvement of the peas production in lowlands.

Cluster analysis of 61 peas lines based on 51 morphological characters performed in the lowlands resulted in genetic divergence. The similarity coefficients generated in the dendrogram range from 0.74 to 1. At the dendrogram cutoff with a coefficient of 0.8, there are three major groups of lines. But this distance does not separate the lines based on the region's origin (Figure 3). At a similarity distance deduction of 0.89, it formed 6 clusters in which the lines were divided based on the area of origin of the original population. Most noticeable are the pea lines originating from Garut, Semarang, and Temanggung although some of these lines still show a mixing of the original region. The greater the value of the coefficient of similarity, the higher the similarity that these strains have. On the contrary, the smaller the value of the similarity coefficient, the lower the degree of similarity that these lines have. Peas lines were also classified into different clusters based on their various morphological traits. Lines having the same characteristics were classified into the same cluster.

CONCLUSION

Characters of Indonesian pea landrace planted in the lowlands have a high variability. Based on principal component analysis revealed that the first eleven principal components accounted for 76.6% of the total variation. The character of each component that contributes to variability were plant, stem, leaf, leaflet, stipule, petiole, flower, peduncle, pod, seed, and cotyledon. In agronomic characters the lowest variability was found in the harvesting time and the highest variability was found in the number of pods per plant. The lines based on studied traits were classified in 6 groups based on similarity Pearson correlation coefficient 0.89. The lines show unique characteristics based on the region of origin. It was shown that Indonesia pea landrace has the great genetic potential to be developed as superior and adaptive varieties in the tropical lowlands.

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TPGM

Preliminary study on polyembryonic Harumanis mango (*Mangifera indica*) seedlings

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Abstract

Harumanis mango (*Mangifera indica*) are classified as polyembryonic, which enable each seed to produce several seedlings. Identification of zygotic and nucellar seedlings is important for maintaining genetic characters which ensures field uniform performance of rootstocks. However, the seedlings from each seed differ in terms of vigour, plant size or height depending on whether they are nucellar or zygotic in origin. Therefore, this preliminary study was to evaluate the interactions between growth parameters on vigor of seedlings related to the nucellar or zygotic origin at seedbed stage. The experiment was conducted by collecting of Harumanis fruits at MARDI Sintok, Kedah to obtain the seeds. Fifty-five mature fruits of Harumanis mango was surface sterilized before sown in seedbag. The observations were recorded after one-month germination of seedlings. The data was analyse using SAS Statistical software (SAS 9.4) and Pearson's Correlation Coefficient was carried out to confirm the interaction between germination sequence, leaf number, plant height, stem diameter and leaf area on vigor of seedlings. In this study, there were positive or negative correlation at ($p \leq 0.05$) between germination sequence, leaf number, plant height, stem diameter and leaf area on growth of Harumanis seedlings. Furthermore, the germination sequence showed negative correlation with leaf number, plant height, stem diameter and leaf area. These result indicate that the first emergence of seedling not always nucellar or zygotic. However, leaf number showed positive correlation with plant height ($r = 0.60$), stem diameter ($r = 0.53$) and leaf area ($r = 0.44$). In term of plant height, there were strongly positive correlation with stem diameter ($r = 0.77$) and leaf area ($r = 0.63$). These results also indicate that the most vigorous seedling are not always nucellar or zygotic. The identification of seedlings derived from nucellar or zygotic by morphological criteria is not possible or difficult to be confirmed. Thus, the use of molecular markers in the future is necessary to make the distinction for production of good quality planting materials.

Key words: Harumanis, seedling, polyembryonic, nucellar, zygotic

INTRODUCTION

Mango (*Mangifera indica* L.) belongs to the family Anacardiaceae is an important tropical fruit, which is being grown in more than 100 countries of the world (Alam *et al*., 2006; Ram *et al*., 2012). As recorded in 2017, there is about 6048.29 hectare of mango cultivation in Malaysia with 16,912.59 metric tons production (DOA, 2017). There are several varieties of mango grown in Malaysia; the better known cultivars are Golek, Masmuda, Maha 65, Chok Anan, Nam Dok Mai, Sala and Harumanis (DOA, 1995). Harumanis among them, the most popular clone, which was registered as MA128 on May 28, 1971 by the Department of Agriculture, Malaysia (DOA, 1995). The fruit shape of Harumanis is oblong, has a prominent beak, the skin colour is green with a little bit of glossy and will turn to yellowish green when ripen, the fruit size varies ranging from 300 to 650 gram, has 16-17°Brix and the flesh is yellowish to orange colour and sweet smell (Mohd Mokhtar, 2014). Harumanis mango is one of the famous fruit that have high economic demand and potential for Malaysia export business especially the Perlis State in Malaysia (Farook *et al*., 2013).

Usually, mango can be propagated by seeds or by grafting (Ram *et al*., 2012). However, propagated by seeds is not true to type. Harumanis mango are classified as polyembryonic, which enable each seed to produce several seedlings (zygotic and nucellar) (Mohd Asrul *et al*., 2018). Identification of zygotic seedlings (referred to as off type) from nucellar seedlings (true to type) is important for maintaining genetic homogeneity which ensures field uniform performance of rootstocks (Rao *et al*., 2008; Simon *et al*., 2010). In polyembryonic cultivars, nucellar seedlings can be identified by the uniformity in the colour of the emerging leaves that are not present in zygotic seedlings (Zakaria *et al*., 2002). However, only one embryo is of zygotic in origin which then degenerates or becomes weak and stunted seedling (Litz, 1997). Seedling detection, whether zygotic or nucellar, and separation at the seedbed stage before transplanting into nursery polybags is important to minimize the number of abnormal (crooked) seedling rootstocks or zygotic seedlings (Zakaria *et al*., 2002; Simon *et al*., 2010). With these facts in view, the present investigation was undertaken to appraise the interaction among growth parameters of Harumanis mango seedlings after germination.

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MATERIALS AND METHODS

Plant Materials And Growth Conditions

The experiment was conducted by collecting of Harumanis fruits at MARDI Sintok, Kedah to obtain the seeds. Fifty five mature fruits of Harumanis Fifty-five mature fruits of Harumanis mango was surface sterilized before sown in seedbeg. The seedbed was shaded with black netting and watered daily. Seedlings germinated from each seed were labelled with different colour of tag according to sequence of germination.

Measurement Of Growth

At 30 days after germination of seedlings, plants were sampled at random for determination of plant height, stem diameter, leaf number and leaf area (Figure 2). Measurement of plant height was taken from the surface of the soil in the seedbed to the highest shoot tip by using a measuring tape. Stem diameter was measured at the lowest part of stem using Electronic Digital Caliper (Model SCM DIGV-6) while the leaf number was manually counted based on fully expanded leaves. Leaf areas were measured and recorded as total leaf area per plant using automatic leaf area meter (MODEL LI-300, LI-COR).

Statistical Analysis

The data was analyzed using SAS Statistical Software (SAS 9.4) and Pearson Correlation Coefficient was carried out to determine the interaction between growth parameters.

RESULTS AND DISCUSSION

Correlation Analysis Of Growth Parameters Of Harumanis Seedlings

In this study, there were significant correlation at ($p \leq 0.05$) between germination sequence, leaf number, plant height, stem diameter and leaf area on growth of Harumanis seedlings. Furthermore, the germination sequence showed negative correlation with leaf number ($r = -0.38$), plant height ($r = -0.44$), stem diameter ($r = -0.46$) and leaf area ($r = -0.28$). According to Zakaria *et al*. (2002), the first seedling that emerged is not always the most viable or vigorous in growth. These result indicate that the first emergence of seedling not always nucellar or zygotic. However, leaf number showed positive correlation with plant height ($r = 0.60$), stem diameter ($r = 0.53$) and leaf area ($r = 0.44$). These result showed that the increase of leaf number with increase of plant height, stem diameter and leaf area. Same result obtained by Shaban (2010). They found that number leaf was correlated positively with leaf area and plant height and leaf area for zebda mango seedlings from Egypt. In term of plant height, there were strongly positive correlation with stem diameter ($r = 0.77$) and leaf area ($r = 0.63$). The stem diameter also had strong positive correlation with leaf area ($r = 0.66$). Rocha et al. (2004) found that the seedlings from each seed differ in terms of vigour, plant size or height depending on whether they are nucellar or zygotic in origin. Zakaria et al. (2002) and Muralidhara et al. (2015) suggested that the removal of seed coat may be gave superior response in all initiation of plant height, stem diameter, number of leaves per plant and leaf area. At the same time, the different response growth of seedlings produced after germination and emergence that may be cause by competition between seedlings for nutrient uptake, light and space.

CONCLUSIONS

Based on the results, leaf number showed significant positive correlation with regard to plant height, stem diameter and leaf area. More significant results showed positive correlation studies of plant height and stem diameter. Hence, the preliminary results of this study are providing useful information in future to identify the genetic origin, zygotic or nucellar of seedlings from Harumanis mango polyembryonic by using molecular technique, relating it to the seedling vigor for production of good quality planting materials.

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Table 1. Correlation analysis of growth parameters for Harumanis mango seedlings

	Germination Sequence	Leaf Number	Plant Height	Stem Diameter	Leaf Area
Germination Sequence	1.0000	-0.37894 *	-0.43721 *	-0.45441 *	-0.28156 *
Leaf Number		1.00000	0.59770 *	0.53223 *	0.43671 *
Plant Height			1.00000	0.76701 *	0.62909 *
Stem Diameter				1.00000	0.66499 *
Leaf Area					1.00000

* Correlation is significant at 0.05 level of probability

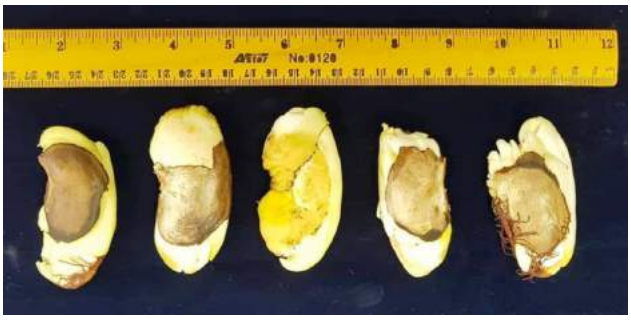

Figure 1. Seeds of Harumanis mango after remove the seed coat

Figure 2. Seedlings of Harumanis Mango after 30 days of germination

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TPGM

Polymorphism information content and heterozygosity values of simple sequence repeat markers in 'Harumanis' and other mango cultivars

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Abstract

'Harumanis' is a mango cultivar that is exclusively cultivated in Perlis for its high commercial value and market potential. Most farmers and consumer rely on morphological characteristics to differentiate 'Harumanis' from other mangoes for sapling and fruit purchasing purpose. Sellers greatly exploited this situation by substituting 'Harumanis' with other cheaper mangoes such as 'Sala', 'Susu' and 'Tong Dam' since they have almost similar morphological characteristics. Despite the relative ease of use and rapidity, identification by morphological marker is inefficient as this marker compounded with the problem of low stability as it is highly affected by environmental factors. A significant genetic improvement in 'Harumanis' can be accelerated by using molecular-based approaches like Simple Sequence Repeat (SSR) as it has high association with expressed genes and directly contributing to a phenotype. Here, we described a set of Simple Sequence Repeat (SSR) markers which can be applied to discriminate 'Harumanis' and other mango cultivars. Sixteen DNA samples of different mango varieties including 'Harumanis', 'Tong Dam' and 'Sala' were obtained from different locations in Perlis. A total of 39 SSR markers were examined using PCR and PAGE. We then performed genetic statistical analyses to determine heterozygosity values and polymorphism information content (PIC). From the 39 SSRs screened, 17 SSRs produced clear and reproducible bands. These SSRs were then selected for subsequent downstream analyses. A total of 443 alleles from 17 SSR loci were detected in the 16 mango samples, with an average number of alleles at 2.65 per locus. The PIC values ranged from 0.16 to 0.61. SSR 7, LMMA 10 and LMMA 8 were considered as high performing markers as they attained PIC values higher than 0.5. In addition, it was found that the observed heterozygosity (Ho) was higher than the expected (He), which is indicative of high genetic variability in the samples. The SSR markers identified in this study are useful for evaluating genetic variability of 'Harumanis' as well as for comparison with other cultivars towards the development of DNA profiles of the mango.

Key words: 'Harumanis', simple sequence repeats (SSR), polymorphism information content, heterozygosity

& INTRODUCTION

In Malaysia, there are numerous variety of mango grown, among the well-known cultivars are Golek (MA 162), 'Masmuda' (MA 204), 'Maha' (MA 165), 'Chok Anan' (MA 224), 'Nam Dok Mai' (MA 223), 'Sala' and 'Harumanis', the most popular clone which was registered as MA128 by the Department of Agriculture, Malaysia (Ariffin, Sah, Idris & Hashim, 2015) 'Harumanis' has emerged as the main choice among consumers due to its distinctive properties of fragrant odor, a delicate flavor and fine texture. The Perlis government also took the ownership of the 'Harumanis' mango (MA 128) as it can only be found in Perlis, Malaysia and in Surabaya, Indonesia (Zakaria, Saim, Osman, Haiyee & Juahir, 2018).

Fruit dealers have taken this opportunity to gain fraudulent profit by substituting the Perlis premium 'Harumanis' mango with cheaper mangoes varieties such as 'Tong Dam', 'Sala', 'Arumanis' and 'Susu'. The high price and fame of 'Harumanis' have turn out to be the subject of economic counterfeiting involving replacement and misjudgement. The existence of other mangoes which have identical attributes with Harumanis such as the skin shade and fruit size had aggravated the situation as these may mislead the consumers. Media has also continually substitute the Perlis premium 'Harumanis' mango with cheaper mangoes such as 'Tong Dam', 'Sala', 'Arumanis' and 'Susu' which are referred as unauthentic 'Harumanis' (Zakaria *et al*., 2018).

Thus, a powerful tool need to be implemented to discriminate between the original and unauthentic 'Harumanis' mangoes. Identification tags known as markers is one of the best solution for this problem. Among the three types of markers which are morphological, biochemical and molecular markers, DNA markers (one of the type of molecular marker) has been selected as it is not affected by environmental factors and the developmental stage of the plant (Winter & Kahl, 1995). DNA markers are known for their rapid and extensive uses in the study of genetic diversity, identification of redundancies in germplasm collections, tests of accession stability and integrity, and resolve the taxonomic relationships (Ariffin *et al*., 2015).

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Table 1. List of 16 mango accessions with their classification and origin

Accession no	Cultivar	Species	Origin
TD 1	'Tong Dam'	*M. indica*	Thailand (Pattani)
PA 1	'Pedal Ayam'	*M. indica*	Thailand (Pattani)
SALA GS1	'Sala'	*M. indica*	Malaysia (North)
HM 1	'Harumanis'	*M. indica*	Malaysia (North)
HM 2	'Harumanis'	*M. indica*	Malaysia (North)
HM 11	'Harumanis'	*M. indica*	Malaysia (North)
HM 12	'Harumanis'	*M. indica*	Malaysia (North)
HM 21	'Harumanis'	*M. indica*	Malaysia (North)
SALA GS 2	'Sala'	*M. indica*	Malaysia (North)
SALA AA1	'Sala'	*M. indica*	Malaysia (North)
SALA AA3	'Sala'	*M. indica*	Malaysia (North)
HM 22	'Harumanis'	*M. indica*	Malaysia (North)
HM 31	'Harumanis'	*M. indica*	Malaysia (North)
HM 32	'Harumanis'	*M. indica*	Malaysia (North)
HM 41	'Harumanis'	*M. indica*	Malaysia (North)
HM 42	'Harumanis'	*M. indica*	Malaysia (North)

Table 2: The sequence and details of the primer pairs

Locus	GenBank Acc. No	Sequence 5' to 3'	Annealing T (°C)	Size (bp)
MiSHRS-18	AY942819	F: AAACGAGGAAACAGAGCAC R: CAAGTACCTGCTGCAACTAG	55.9	100-150
MiSHRS-39	AY942829	F: GAACGAGAAATCGGGAAC R: GCAGCCATTGAATACAGAG	52.8	300-450
MIAC-4	AB190347	F: CGTCATCCTTTACAGCGAACT R: CATCTTTGATCATCCGAAAC	53.6	100-250
MIAC-5	AB190348	F: AATTATCCTATCCCTCGTATC R: AGAAACATGATGTGAACC	44	100-120
mMiCIR003	AJ635165	F: GATGAAACCAAAGAAGTCA R: CCAATAAGAACTCCAACC	53.2	350-550
mMiCIR005	AJ635167	F: GCCCTTGCATAAGTTG R: TAAGTGATGCTGCTGGT	47	150-200
MMiCIR008	AJ635170	F: GACCCAACAAATCCAA R: ACTGTGCAAACCAAAAG	55.5	200-250
mMiCIR0020	AJ635182	F: GACTTGCAGTTTCCTTTT R: TCAAGAACCCCATTTG	47.6	250-300
LMMA4	AY628376	F: AGATTTAAAGCTCAAGAAAAA R: AAAGACTAATGTGTTTCCTTC	49.6	250-300
LMMA7	AY628379	F: ATTTAACTCTTCAACTTTCAAC R: AGATTTAGTTTTGATTATGGAG	41	300-350
LMMA8	AY628380	F: CATGGAGTTGTGATACCTAC R: CAGAGTTAGCCATATAGAGTG	53.6	300-400
LMMA10	AY628382	F: TTCTTTAGACTAAGAGCACATT R: AGTTACAGATCTTCTCCAATT	46	200-250
LMMA11	AY628383	F: ATTATTTACCCTACAGAGTGC R: GTATTATCGGTAATGTCTTCAT	49.8	350-650
MillHR36	EF592216	F: TCTATAAGTGCCCCCTCACG R: ACTGCCACCGTGGAAAGTAG	42.9	300-400
MillHR17	EF5922197	F: GCTTGCTTCCAACTGAGACC R: GCAAAATGCTCGGAGAAGAC	57.4	400-450
MillHR12	EF592192	F: GCCCCATCAATACGATTGTC R: ATTTCCCACCATTGTCGTTG	52.6	200-300
MillHR06	EF592186	F: CGCCGAGCCTATAACCTCTA R: ATCATGCCCTAAACGACGAC	58.1	100-150

Of the various approaches of DNA markers, simple sequence repeats (SSRs) or also known as microsatellites has emerged as the marker of choice for plant breeding applications and fingerprinting purposes in most plant species (Miah *et al*., 2013) due to its high polymorphism, co-dominancy and reproducibility. SSR marker has been identified as a superior molecular tool in elucidating the genetic diversity (Zhang *et al*., 2015) and this is proven as it has been used in many previous mango genetic diversity research. The approach has been useful for integrating the genetic, physical and sequence-based physical maps in plant species, and simultaneously have provided the breeders and geneticists with an efficient tool to link phenotypic and genotypic variation (Varshney, Graner & Sorrells, 2005). This further can be employed in DNA fingerprinting for individual identification of cultivars or rootstock for different horticultural purposes, such as breeder's right, identification of pollen parents and determination of genetic relatedness. This study was undertaken to identify high potential SSR markers and to evaluate the selected SSRs ability for assessment of genetic diversity in 'Harumanis' and other mangoes, specifically on 'Tong Dam' and 'Sala' from different locations.

METHODS

Plant Materials

Information of 16 mango accessions used in this study, which belong to M. indica species were listed in Table 1. The collection of each mango leaf was performed at different locations which mainly from Perlis, Malaysia (Chelong Balik Bukit, Paya Kelubi, Alor Ara Timur, Santan, Simpang Empat), Institute of Sustainable Agriculture Technology, Perlis Malaysia and Pattani, Thailand. The samples were then stored in -20°C chest freezer and analysed at Chemical Engineering Technology Laboratory, Faculty of Engineering Technology, UniMAP, Perlis.

Genomic Dna Extraction

Matured healthy leaves of all accessions were selected for genomic DNA extraction.The genomic DNA extraction was performed according to modified cetryl trimethyl ammonium bromide (CTAB) method based on the protocol described in previous study (Doyle, 1990). The purity and concentration of DNA were measured using 1% agarose/TBE gel electrophoresis (Dillon *et al*., 2013). After electrophoresis, the DNA was visualized under ultraviolet (UV) light (Trans Illuminator) using compact, automated Gel Doc™ EZ Gel Imager and Image Lab 6.0 software. DNA was quantified using a DeNovix DS-11 spectrophotometer and the DNA was diluted to 50 ng/µL as the final working concentration used for 25-µL polymerase chain reactions (PCRs). The extracted DNA were then stored in -20°C.

PCRAmplification And Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

Thirty nine previously published microsatellite markers were selected for this study. Those markers were selected as previously published by (Viruel, Escribano, Barbieri, Ferri & Hormaza, 2005), (Ravishankar, Mani, Anand & Dinesh, 2011), (Shamili, Fatahi & Inaki Hormaza, 2012), (Kumar, Ponnuswami, Nagarajan, Jeyakumar & Senthil, 2013), (Begum *et al*., 2013a), (Begum *et al*., 2013b), (Begum *et al*, 2014), (Dillon *et al*., 2013) and (Tsai *et al*., 2013). All microsatellite markers are reported to produce clear polymorphic amplification patterns. PCR reactions (25 µl) were performed in a Thermal cycler (BioRad C-1000) as per protocol suggested by Promega GoTaq Green Master Mix. A 25µl reaction mixture contains approximately 12.5 µl PCR Master Mix, 2X , 0.25 µl forward and reverse primer, 1 µl of DNA template of 50ng to 80ng and 11.2 µl nuclease free water. The PCR was performed with an initial denaturation at 95 ◦C for 2 min followed by 20 cycles of 30 s denaturation at 95 ◦C, 30 s annealing at different temperature based on the primer pair and 30 min extension at 72 ◦C, followed by a final extension for 5 min. The amplified products were separated and visualized on a 15% polyacrylamide gel followed by ethidium bromide, EtBr, staining. The 20 bp and 50bp DNA ladder were loaded onto the gel for size estimation of the PCR products. Alleles size were visually scored in bp as indicated in Figure 1 for each genotype. Amplification was repeated with each primer to confirm the reproducibility of the results (Ramakrishnan, Antony Ceasar, Duraipandiyan, Al-Dhabi & Ignacimuthu, 2016).

Genetic Statistics

The data were used to estimate major allele frequency (MAF), allele number, gene diversity, expected heterozygosity, heterozygosity, polymorphic information content (PIC) and inbreeding coefficient using Power Marker v3.0 (Liu & Muse, 2005). Heterozygosity is simply the proportion of heterozygous individuals in the population. At a single locus it is estimated as:

$$\hat{H}_l = 1 - \sum_{u=1}^{k} \tilde{P} \qquad (1)$$

Gene diversity, often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different. An unbiased estimator of gene diversity at the lth locus is:

$$\hat{D}_l = (1 - \sum_{u=1}^{k} \tilde{p}_{lu}^{\,2})/(1 - \frac{1+f}{n}), \text{ where } f \text{ is the inbreeding coefficient,} \quad (2)$$

Polymorphism information content (PIC) was calculated using the formulae of (Botstein, White, Skolnick & Davis, 1980). It is estimated as:

$$\widehat{PIC}_l = 1 - \sum_{u=1}^{k} \tilde{p}_{lu}^{\,2} - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}_{lu}^{2}\tilde{p}^{2} \qquad (3)$$

A dendrogram was constructed based on SSRs with 1000 bootstrapping values using PAST ver 3.23 software with an unweighted pair-group method with arithmetic average (UPGMA) based on the Jaccard's similarity coefficient (Jaccard 1908).

RESULTS AND DISCUSSION

Statistical Analysis Of Genomic SSR Markers

39 SSR primer pairs were used for the initial screening of the 16 mango samples. Out of 39 primer pairs, 17 of them (Table 2) were selected for further analysis based on the amplification of the DNA. The sequences of these 17 primer pairs are shown in Table 1. Ineffective amplification shown by some primer pairs caused them to be excluded in the study as they may be attributed to the presence of introns in the amplified sequence, a lack of primer specificity, variation in the number of repeated motifs, or assembly errors (Varshney, Graner & Sorrells, 2005b).

Table 2 on the other hand shows the performance of the 17 selected primer pairs. The 17 SSR primer pairs produced a total of 443 amplified products, of which 216 (48.76%) are polymorphic, 224 are monomorphic and another three are unique across all 16 mango samples. The observed sizes of PCR products of the current study ranged from 100 to 650bp. Primer pairs of LMMA11 showed the highest PCR product sizes (350 to 650bp), followed by MiIIHR17 (400 to 450bp) and MiSHRS-39 (300 to 450bp). MiSHRS-18, SSR 8 and MIAC-5 produced the smallest PCR product sizes which are 100 to 150bp and 100 to 120bp respectively.

The number of alleles detected vary from 2 (LMMA 7, 8, 11, MIAC 4, MiSHRS-18, MMiCIROO20, MiIIHR-36 and 17) to 3 (LMMA 4, MISHRS 39, MIAC 5, MMiCIROO3, 5, 8 and SSR 8) and 4 (LMMA 10 and SSR 7). The 17 SSR primer pairs (loci) revealed a total of 45 alleles with average number of alleles per primer pair is 2.65 (Table 3). Earlier, (Kumar, Ponnuswami, Nagarajan., Jeyakumar & Senthil., 2013) and (Begum *et al*., 2013b) reported similar numbers of alleles range (2 to 4) with average allele per locus of 2.70 and 3.08 respectively.

A total of three unique alleles were detected at three SSR loci. The 'Pedal Ayam' (PA1) mango accession reported a unique allele with the size of 300 bp at locus MMICIR008, whereas 'Sala' (SGS1) mango accession produced two unique alleles with the sizes of 160 bp and 360 bp at loci SSR 8 and LMMA 4. The unique alleles could be used as markers to distinguish between 'Harumanis' with 'Pedal Ayam' and 'Sala' genotypes. Major allele frequencies ranged from 0.38 (MIAC 5) to 0.91 (MIAC 4) with the mean of 0.63.

Polymorphic SSR Among *M. indica* L. Isolates

Identification Of Polymorphic Markers

The PIC value which represents the relative information of each marker ranged from 0.16 for marker MIAC 4 to 0.61 for marker LMMA 10. The value describes the intra-population diversity and characterizes the degree of polymorphism in each locus. Most of the loci indicated moderate discriminatory power as the PIC value were in the range of 0.25 and 0.5 except for SSR 7, LMMA 10, MIAC 5 and MIAC 4. The polymorphic SSRs identified indicating moderate discriminatory power with a PIC mean value of 0.41. SSR 7, LMMA 10 and LMMA 8 are considered as highly polymorphic markers for identification and genetic diversity estimation of mango as the SSRs attained PIC values of higher than 0.5, while MIAC 4 with a PIC value of 0.16 was characterized as a low discriminatory polymorphic SSR marker as it is below 0.25. These findings indicated that those selected SSRs primer pairs are useful for inside population studies e.g. to trace marker trait association as the PIC value average (0.41) is within the range of 0.4 to 0.5, whereas lower PIC values (0.0–0.1) of single alleles are usually more useful for studies of evolution or genetic drift (Silvertown & Doust 1993). The PIC values of each locus were comparable to those reported by Begum, which were between 0.25 to 0.56 in 'Panchadarakalasa' mango (Begum *et al*., 2013a), 0.02 to 0.82 in 'Cherukurasam' mango, and 0.03 to 0.72 in 'Cherukurasam' mango cultivars (Begum *et al*, 2014).

Gene Heterozygosity SSR Of *M. indica* L.

Observed heterozygosity Ho obtained in this study are in the range of 0.00 (SSR 8) to 1.00 (LMMA 7, MISHRS 18 and MMICIR0020). Gene diversity or expected heterozygosity He values are found to be highest in primer LMMA 10 (0.67), followed by MIAC 5 (0.66) and SSR 7 (0.62). 10 out of 17 selected SSR primers recorded higher gene diversity values than the average value (0.47). The mean value of observed heterozygosity (Ho = 0.57) is higher than expected heterozygosity (He = 0.47), resulting in negative value of inbreeding coefficient mean or known as fixation index F.

Expected He and observed heterozygosity Ho values were compared using the fixation index F, which reported an average over all the SSR loci of -0.30 with values between −0.39 (MMICIR003) and 1.00 (SSR 8). 14 of the SSR loci fixation index (F) are negative in values indicating an excess of heterozygotes in the genotypes except for LMMA 4, MIAC 5 and SSR 8. The heterozygote excess observed could be caused by the outcrossing breeding system and low effective number of population size of the accession studied, meanwhile the positive fixation index F probably because of some parameters such as null alleles, inbreeding phenomenon, homozygosity, and self-compatible genotypes (Maghuly, Borroto, Ruthner, Pedryc & Laimer, 2005).

Cluster Analysis And Genetic Relationships Of *M. indica* L.

Cluster analyses and relationship among 10 'Harumanis' accession native to five locations in Perlis and six control samples ('Sala', 'Pedal Ayam' and 'Tong Dam') were evaluated. The analyses indicated high genetic similarity among 'Harumanis' accessions as all of the ten accessions were clustered in one group (HM1, HM2, HM11, HM12, HM21, HM22, HM31, HM32, HM41 and HM 42) and the second group gathered allanother six mangoes (SALA GS1, SALA GS2, SALA AA1, SALA AA3, PA and TD). This result shows that microsatellite marker is also effective for cultivars discrimination as indicated in previous study by (Honsho, Nishiyama, Eiadthong, and Yonemori, 2005.) and not only based on localities (Eiadthong, Yonemori, Sugiura, Utsunomiya & Subhadrabandhu, 1999). This division suggest that different cultivars probably had different genomic component, meanwhile the cluster of 'Harumanis' in one group proposed that those accessions probably had common genomic components that originated from other mother trees as the accessions not subdivided according to the localities but according to their cultivars (Begum *et al*., 2013b). The clustering analyses able to express the true potential of the

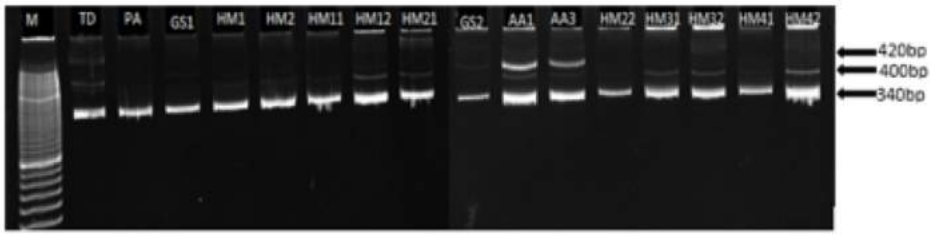

Figure 1. PCR amplified patterns by MiSHRS-39primer in sixteen mango cultivars. #Lanes M; 20bp ladder, TD, 'Tong Dam', PA, 'Pedal Ayam', GS1, 'Sala Guar Sanji 1', HM 1, 'Harumanis 1', HM 2, 'Harumanis 2', HM 11, 'Harumanis 11', HM 12, 'Harumanis 12' , HM 21, 'Harumanis 21', GS2, 'Sala Guar Sanji 2', AA1, 'Sala Alor Ara Timur 1', AA3, 'Sala Alor Ara Timur 3', HM 22, 'Harumanis 22', HM 31, 'Harumanis 31', HM 32, 'Harumanis 32', HM 41, 'Harumanis 41' and HM 42, 'Harumanis 42'. The arrows indicate the position of scoreable markers

Table 3. Summary of genetic diversity of 16 accessions of *Mangifera indica* L., using 17. Microsatellite markers. Major Allele Frequency, number of genotype, number of allele, PIC: Polymorphism Information Content, H_{exp}: expected heterozygosity, H_{obs}: observed heterozygosity and fixation index calculated per locus

NO	Marker	Major.Allele.Frquency, M_{Af}	Genotype No	Allele No	Gene Diversity, H_D	Heterozygosity, H_E	PIC	f
1	LMMA10	0.47	5	4	0.67	0.88	0.61	-0.28
2	LMMA4	0.84	3	3	0.27	0.25	0.25	0.11
3	LMMA7	0.50	1	2	0.50	1.00	0.38	-1.00
4	LMMA8	0.72	2	2	0.40	0.56	0.32	-0.36
5	LMMA11	0.75	2	2	0.38	0.50	0.30	-0.30
6	MIAC4	0.91	2	2	0.17	0.19	0.16	-0.07
7	MIAC5	0.38	2	3	0.66	0.63	0.59	0.09
8	MISHRS18	0.50	1	2	0.50	1.00	0.38	-1.00
9	MISHRS39	0.63	3	3	0.53	0.75	0.47	-0.38
10	MMICIR003	0.66	3	3	0.49	0.69	0.41	-0.39
11	MMICIR005	0.63	3	3	0.53	0.75	0.47	-0.38
12	MMICIR008	0.60	3	3	0.53	0.69	0.44	-0.28
13	MMICIR0020	0.50	1	2	0.50	1.00	0.38	-1.00
14	SSR7	0.50	5	4	0.62	0.81	0.54	-0.29
15	SSR8	0.69	3	3	0.46	0.00	0.40	1.00
16	MIIHR-36	0.72	2	2	0.40	0.56	0.32	-0.36
17	MIIHR17	0.78	2	2	0.34	0.44	0.28	-0.25

17 selected SSR markers as it able to efficiently discriminate the 'Harumanis' accessions with the other six controls.

CONCLUSION

Molecular markers have been used for many years in different applications mainly genotype, gene identification and genome mapping. Our finding demonstrates the effectiveness of the SSR marker collection used for differentiation of selected mango cultivars. Comparable results with other previous reports confirmed the potential of analyzed SSR markers for 'Harumanis' as well as other mango cultivars genotypes identification and characterization.

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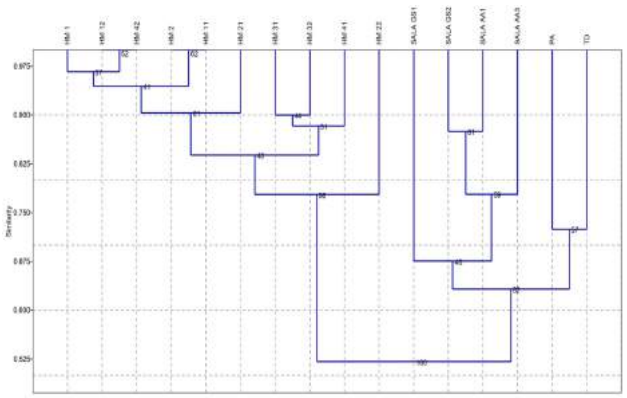

Figure 2. UPGMA cluster analysis showing the diversity and relatedness among 10 'Harumanis' and six accessions of other mangoes using 17 microsatellite markers

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TPGM

Identification of Agrodyke-induced genes and pathways potentially associated with defense response in *Acacia* hybrid against *Ceratocystis* infection

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Abstract

In Malaysia, the productivity of *Acacia* plantations is being threatened by *Ceratocystis* wilt disease. Up to date, the molecular mechanisms regulating defense responses of *Acacia* towards *Ceratocystis* are not yet fully known. Currently, the application of an organic plant booster, named Agrodyke, has been claimed to increase soil fertility and improve plant defense mechanism against pests and pathogens. In this study, a comprehensive transcriptome analysis was performed on treated (Agrodyke-sprayed) and non-treated of infected *Acacia* hybrid at four time-points (1, 5, 10 and 15 days after inoculation) in order to identify differentially expressed genes (DEGs) and pathways in response to Agrodyke treatment. Across all of the times tested, a total of 483,519 unique transcripts were obtained by *de novo* assembly of 302.11 million paired-end clean reads using Trinity pipeline. About 1,425 DEGs were identified after the Agrodyke treatment in *A*. hybrids. Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, majority of these DEGs were involved greatly in biosynthesis of antibiotic, phenylpropanoid, flavonoid, phenylalanine, starch and sucrose metabolism as well as metabolism of xenobiotics by cytochrome P450. Interestingly, genes associated with defense response were found to be up-regulated in treated *A*. hybrid upon 24 hours of treatment, indicating a quick response of the host tissue towards the Agrodyke. These findings demonstrate that Agrodyke play an important role in enhancing plant defense response in *A*. hybrid against *Ceratocystis* infection. The identified genes could be further studied and exploited to develop *Ceratocystis*-resistant *Acacia* varieties.

Key words: Transcriptome, plant-pathogen interaction, disease resistance, Agrodyke treatment, fertilizer

INTRODUCTION

Acacia is an important forest tree species which belongs to the family of *Leguminosae. Acacia mangium* and *Acacia auriculiformis* are two of the most common plantation tree species that are widely planted in Malaysia, Indonesia and Vietnam. The establishment of *Acacia* plantation in Malaysia began around 1980 with an estimated plant area of about 310,000 hectares (FAO, 2002), making them the most widely cultivated species of plantation compared to the other forest species such as *Tectona grandis*, *Gmelina arborea* and *Paraserianthes falcataria*. The naturally-crossed *Acacia* hybrids (*A. mangium* x *A. auriculiformis*) have great potential as raw materials for pulp, paper and furniture industry due to their superior growth, longer wood fibres and better pulp quality. Also, they have many attractive traits for tree improvement such as enhanced growth, form, disease resistance and adaptability (Wong *et al*. 2011).

According to Lee *et al*. (2018), *Ceratocystis* wilt disease has emerged as a devastitating threat to *Acacia* plantation, especially *A. mangium* and some of the *A.* hybrids. The *Ceratocystis* attack *Acacia* trees by entering the host through small openings or wounds caused by animals like elephant, wood-boring insects and unsound silviculture practice (Mohd Farid *et al*. 2017). They would then kill the host cells and tissue to derive nutrition from dead cells, proliferate within the vascular tissue, disrupt water translocation and cause the typical wilt symptoms (Van Wyk *et al*., 2007). Other symptoms of this disease include canker, blister lesions, gummosis and vascular discoloration of the woody tissues which would lead to tree death within six months after infection (Tarigan *et al*., 2011). Additionally, studies by Mohd Farid *et al*. (2017) has reported that *C. fimbriata* killed *Acacia* seedlings within five weeks after inoculation process.

The interaction between plants and pathogen comprises a range of mechanisms that determine the outcome of the interaction, for example compatible will lead to susceptibility while incompatible will lead to resistance. Plants have developed structural, chemical and protein-based

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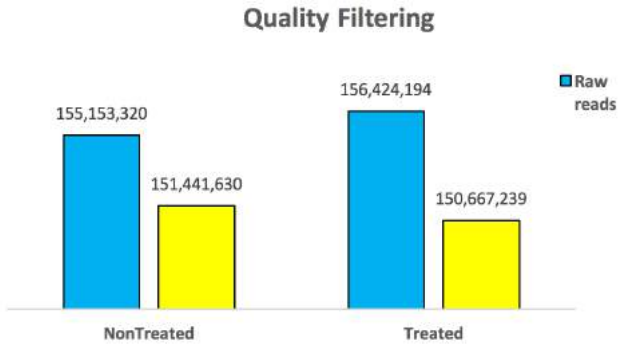

Figure 1. Summary of raw and clean reads obtained in treated and non-treated samples

Table 1. Overview of the *de novo* assembly

Attributes	Value
No. of transcripts	156,557
Average length (bp)	1,032
GC-content (%)	40.76
N50 transcript (bp)	1,846
Largest transcript (bp)	17,150
Smallest transcript (bp)	183

defenses to detect invading organisms and stop them from causing extensive damage. Understanding on how plants defend themselves from pathogens is essential in order to develop highly disease-resistant plant species. Plants react to pathogen infection by activating certain responses that have been implicated as mechanisms of disease resistance (Loon *et al*., 2006) which include hypersensitive reaction, synthesis of phytoalexins and increased production of lignin (León & Montesano, 2013).

Up to present, there are many studies on plant defense mechanisms using transcriptome approach since it provides enhanced detection compared to conventional approach like microarray and northern blot analysis (Birch & Kamoun, 2000). Transcriptome sequencing, commonly known as RNA-Seq has higher sensitivity to discover differentially expressed genes (DEGs), including the identification of novel stress responsive genes (Bezier *et al*., 2002) and detection of gene expression. In the past few years, transcriptome sequencing has been successfully used for plant-pathogen interaction investigations in *Acacia mangium* (Nur Nabilah *et al*., 2018), black pepper (Hao *et al*., 2016), palm oil (Goh *et al*., 2014; Ho *et al*., 2016), banana (Bai *et al*., 2013) and rice (Kawahara *et al.*, 2012). This technology is often used as more genes can be sequenced in a shorter time with lower cost, provides a better estimation of absolute expression levels and increases understanding of plant stress response (Nibedita & Jolly, 2017). Substances that increase plant immunity and defense response have a potential to be used as an alternative to pesticides, which are less hazardous to the environment. The application of an organic plant booster with conditioning effect called Agrodyke has been claimed to increase soil fertility and enhance plant defense mechanism against pests and pathogens. The scientific explanation of Agrodyke application as organic plant booster is essential as it will finally contribute in the production of safe food to the society. Although Agrodyke has been reported to be effective in increasing plant defense response towards pathogen, it has not been shown to protect *Acacia* against *Ceratocystis* infection until now.

Previous studies by Nur Nabilah *et al*. (2018) mainly focused on studying the molecular mechanism of susceptible *A. mangium* infected with *Ceratocystis manginecans*. The study demonstrated the induction of ten key enzymes associated with phenylpropanoid pathway such as phenylalanine ammonia-lyase (PAL), peroxidase (POD), cinnamyl alcohol dehydrogenase (CAD), caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), cinnamate 4-hydroxylase (C4H), phenylalanine/tyrosine ammonia-lyase (PTAL), caffeic acid O-methyltransferase (COMT), O-hydroxycinnamoyl transferase (HCT) and 4-Coumarate-CoA ligase (4CL) has increased *A. mangium* resistance to *Ceratocystis* infection.

This present study was conducted in order to investigate the impact of Agrodyke treatment on the expression of genes in infected *A.* hybrids treated at different time-points (1, 5, 10 and 15 days post-inoculation (dpi)). The results generated in this study were expected to provide better understanding and information on the Agrodyke-mediated host defense against *Ceratocystis* infection in *A.* hybrids and also to formulate effective strategies to protect plants by using resistance inducer. Besides, the knowledge of genes and pathways involved in the induced resistance is useful in order to understand the mechanism of action of Agrodyke on plants.

METHODOLOGY

Preparation of plant materials and *Ceratocystis* inoculations

Eight healthy *Acacia* hybrid clone M5 (*Acacia mangium Acacia auriculiformis*) with 1–1.5m height seedlings, kindly prepared by FRIM Pathology group were used as study materials. The prepared seedlings were located at shade house, re-potted and left for a month to harden and acclimatize before being used in pathogenicity test. All the seedlings were inoculated with 5-mm fungal plug of *Ceratocystis fimbriata* on their stem approximately 15-cm from the ground. For the treated group, four out of eight seedlings were pre-sprayed on the soil and stem with Agrodyke suspension prior inoculation. After that, Agrodyke suspension was sprayed periodically for every three days until day-12. Another four seedlings were sprayed with distilled water and acted as negative controls (untreated group). Subsequently, all the seedlings were left over a period of 1, 5, 10 and 15 days after inoculation. This method has been registered under FRIM Invention Disclosure (FRIM ID) with reference number FRIM ID 43/2019. Stem samples from eight seedlings were collected at each sampling time point, then were immediately frozen in liquid nitrogen and stored in -80°C freezer until used for RNA extraction.

Table 2. Pairwise comparison of differentially expressed genes (DEGs) between treated (T) and non-treated (NT) groups for each time-point (1, 5, 10 and 15 days post-inoculation (dpi))

	Number of differentially expressed genes (DEGs)			
	T_day1 vs NT_day1	T_day5 vs NT_day5	T_day10 vs NT_day10	T_day15 vs NT_day15
Up-regulated	106	402	59	159
Down-regulated	32	20	578	69
Total	**138**	**422**	**637**	**228**

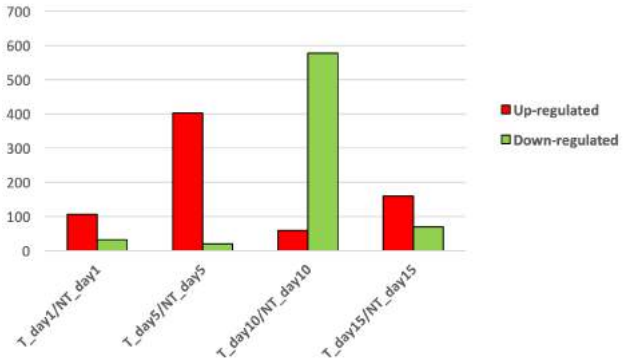

Figure 2. Comparison of the number of up- and down-regulated DEGs between treated (T) and non-treated (NT) groups

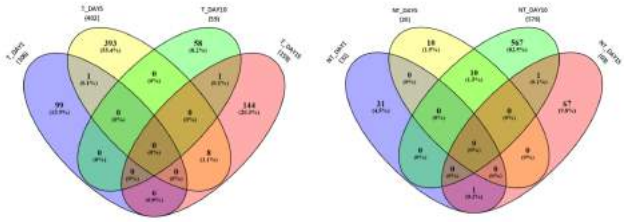

Figure 3. Venn diagrams of overall up- and down-regulated transcripts during Ceratocystis infection across four time-points (Day1, Day5, Day10 and Day15). (A) Number of up-regulated transcripts in treated (T) group; (B) Number of down-regulated transcripts in non-treated (NT) group

RNA extraction and transcriptome sequencing

Total RNA from treated and untreated stems were extracted using combination method of RNeasy Plant Mini Kit (Qiagen, USA) and Fruit-mate™ (Takara, Japan). The RNA integrity and quantification of the extracted RNA were determined using Agilent 2100 BioAnalyzer (Agilent Technologies, USA) with the minimum RNA integrated number (RIN) value of 8 (Bharudin *et al*., 2014). The cDNA libraries were generated using Illumina TruSeq RNA Sample Preparation Kit Protocol and sequencing was performed on Illumina HiSeq 4000 platform at Beijing Genomics Institute (BGI).

Pre-processing analysis and *de novo* transcriptome assembly

The quality of the obtained raw reads of treated and untreated *A.* hybrids was evaluated using FastQC tool (Andrews, 2010) and trimming process was done using SolexaQA++ (Cox *et al*., 2010) to filter sequences with a low quality value of < QV20 and discard sequences with < 50 bp in length. The reads were then screened for contamination by aligning onto phiX library using Bowtie2 tool (Langmead & Salzberg, 2012). *De novo* transcriptome assembly of the cleaned paired-end reads was performed using Trinity pipeline (Grabherr *et al*., 2011).

Differential expression analysis and quantification of gene expression levels

The Fragments per Kilobase of transcript per Million fragments mapped (FPKM), a normalized quantitative procedure for gene expression of RNA-Seq data was employed to measure the expression level of each assembled transcript. The filtered reads of treated and untreated *A.* hybrids were mapped back to the assembled transcripts using Bowtie2 (Langmead & Salzberg, 2012), followed by transcript abundance estimation process using RNA-seq by Expectation Maximization (RSEM) software package (Li & Dewey, 2011). Next, the differentially expressed genes (DEGs) were identified using EdgeR with default parameters (Robinson *et al*., 2010). Transcripts showing Benjamin-Hochberg False Discovery Rate (FDR) < 0.01 were considered as significant. Additionally, significant transcripts with the log fold-change value (logFC) > 2 were marked as up-regulated transcripts while logFC < -2 as down-regulated transcripts.

Functional annotation classification of differentially expressed genes (DEGs)

The DEGs were annotated using BLASTX alignment against protein databases NCBI non-redundant (nr) with cut-off E-value < 0.00001. For functional annotation of DEGs, the BLAST results were imported into Blast2GO PRO software (Gotz *et al*., 2008) to obtain Gene Ontology (GO) term for the transcripts, thus describing the biological process, molecular function and cellular components categories. The DEGs sequences were also aligned to the Cluster of Orthologous Groups (COGs) protein database (Tatusov *et al*., 2013) to determine and classify possible functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG), a pathway-based analysis, was then

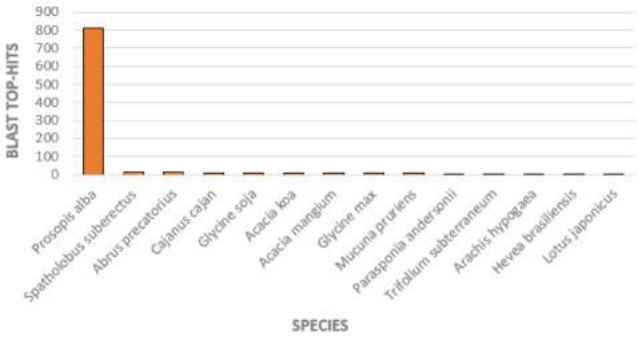

Figure 4. Species distribution of transcript sequences of differentially expressed genes (DEGs)

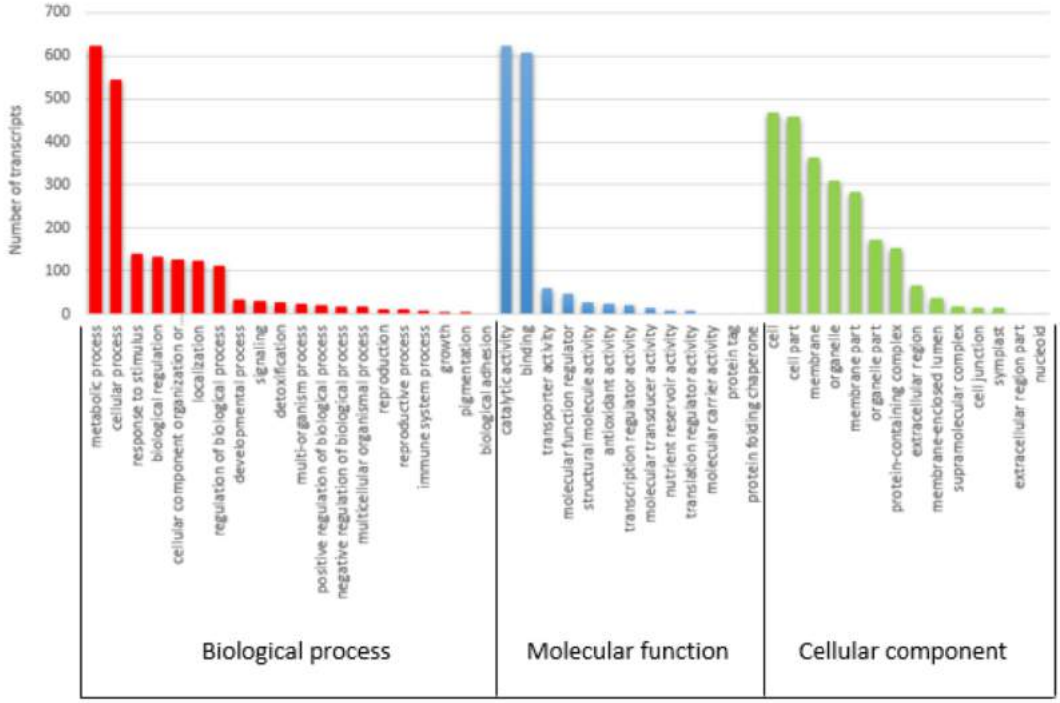

Figure 5. Functional annotation of the differentially expressed genes (DEGs) using Gene Ontology (GO) terms

performed to retrieve pathway information for all the annotated sequences (Kanehisa and Goto, 2000).

GO and KEGG pathway enrichment analysis of DEGs

The Blast2GO PRO software was used to identify GO enriched terms by implementing standard parameters (Fisher's exact test) while KEGG Orthology Based Annotation System (KOBAS 2.0 software) (Xie *et al.*, 2011) was used to identify statistically significantly enriched pathways in response to Agrodyke treatment. Benjamini-Hochberg FDR correction (adjusted p-value < 0.05) was used as thresholds in order to define significantly enriched KEGG pathways.

RESULTS

Transcriptome sequencing and *de novo* assembly

To generate the transcriptome of A. hybrid, eight cDNA libraries were prepared from four treated samples and four untreated samples at four different time-points (1, 5, 10 and 15 days post-inoculation (dpi)) which were then subjected to paired-end sequencing using the Illumina HiSeq 4000 platform. After filtering adapter sequences and discarding low-quality reads using SolexaQA++, a total of 302.11 million high-quality paired-end reads from 311.58 million raw reads with a mean length of 100 bp were obtained (Figure 1). Using the Trinity program, the *de novo* transcriptome assembly of the remaining high-quality reads yielded 156,557 transcripts with N50 length of 1,846 bp. The basic statistics of the libraries were summarized in Table 1.

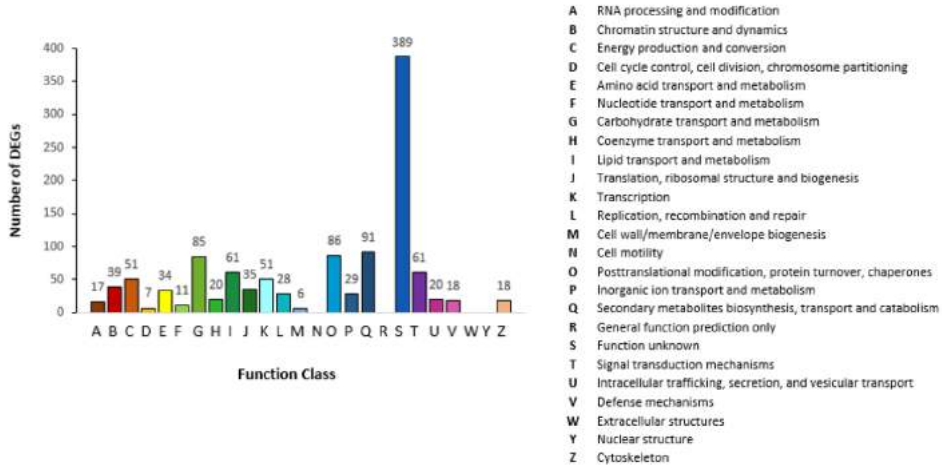

Figure 6. Clusters of Orthologous Groups (COG) functional distribution of the *Acacia hybrid-Ceratocystis* transcripts treated and non-treated with Agrodyke

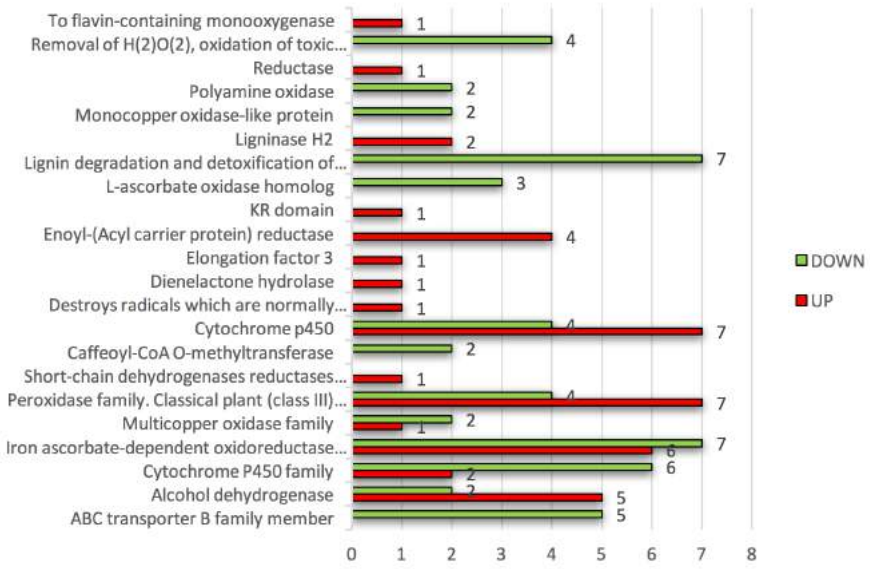

Figure 7. Gene regulation in secondary metabolites cluster based on COG database classification

Differentially expressed genes responsive to Agrodyke treatment in *Acacia* hybrid

A total of 1,425 differentially expressed genes (DEGs) were identified in response to Agrodyke treatment, of which 138 were between T_day1 vs NT_day1 (106 up-regulated and 32 down-regulated), 422 between T_day5 vs NT_day5 (402 up-regulated and 20 down-regulated), 637 between T_day10 vs NT_day10 (59 up-regulated and 578 down-regulated) and 228 between T_day15 vs NT_day15 (159 up-regulated and 69 down-regulated). It showed that the total number of DEGs was the highest in group T_day10 vs NT_day10 compared to the other groups. Across all of the time-points tested, a total of 726 (50.9%) and 699 (49.1%) DEGs were up-regulated and down-regulated, respectively after Agrodyke treatment. This result showed that the response of Agrodyke-treatment involved more gene activation than suppression. The numbers of the DEGs for each time-points were presented in Table 2 and Fig. 2.

In addition, among the up-regulated genes at all time-points, about 95.6% of the genes were uniquely activated by the Agrodyke of which 99 at T_Day1, 393 at T_Day5, 58 at T_Day10 and 144 at T_Day15 (Figure 3A). The result showed that the genes were up-regulated the most at T_Day5 but the least at T_Day10.

In contrast, about 96.6% out of the down-regulated genes were uniquely repressed by the Agrodyke with the

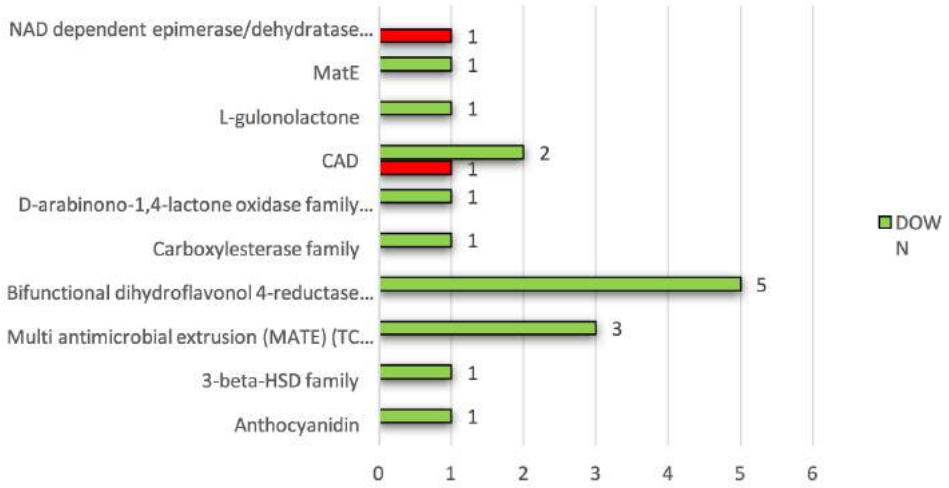

Figure 8. Gene regulation in defense mechanism cluster based on COG database classification

highest number of genes at NT_Day10, followed by NT_Day15, NT_Day1 and NT_Day5 (Figure 3B). This analysis showed that the DEGs were responsive differently towards Agrodyke treatment at certain time. The results also suggested that the Agrodyke treatment probably acted the best up until five days after *Ceratocystis* infection but became inefficient after prolonged usage.

Functional analysis of Agrodyke-responsive DEGs

To find the homologous sequences of Agrodyke-responsive DEGs, 1,425 transcripts were searched against the NCBI non-redundant (NR) protein database using Blastx with a cut-off E-value of 10^{-5}. From Blast searches, *Prosopis alba* was the most frequent species in blast hits with 815 transcripts, followed by *Spatholobus suberectus* (17 transcripts), *Abrus precatorius* (12 transcripts), *Cajanus cajan* (11 transcripts), *Glycine soja* (10 transcripts), *Acacia koa* (10 transcripts) and *Acacia mangium* (10 transcripts) (Figure 4).

Functional gene ontology (GO) classification of 1,425 Agrodyke-responsive DEGs was conducted using Blast2GO PRO. Based on Figure 5, a total of 47 GO terms were significantly enriched and assigned to biological process, molecular function and cellular component category. Within the biological process category, GO terms 'metabolic process', 'cellular process', 'response to stimulus', 'biological regulation' and 'biogenesis' were the most highly represented groups. While for the molecular function category, most of the terms belonged to the 'catalytic activity', 'binding', 'transporter activity', 'molecular function regulator', 'structural molecule activity' and 'antioxidant activity'. GO terms 'cell', 'cell part', 'membrane', 'organelle' and 'membrane part' were the most enriched groups in the cellular component category.

All the DEGs were also aligned to the COG database (Tatusov *et al*., 2003) to predict and classify their possible functions. A total of 1,157 DEGs were classified into 21 COG functional categories. "Function unknown" category (389, 33.62%) was the predominant cluster followed by "Secondary metabolites biosynthesis, transport and catabolism" (91, 7.87%), "Posttranslational modification, protein turnover, chaperones" (86, 7.43%) and "Carbohydrate transport and metabolism" (85, 7.35%). 0 gene/transcript was assigned to the categories "Nuclear structure", "Extracellular structures", "Cell motility" and "General function prediction only". Notably, 91 and 18 genes were respectively annotated to "Secondary metabolites biosynthesis, transport and catabolism" and "Defense mechanism", indicating that several conserved proteins were associated with defense and biosynthesis of secondary metabolism pathways in response to *Ceratocystis* infection and Agrodyke treatment. Hence, the identification of genes in these pathways is essential in order to elucidate the defense mechanisms in *A.* hybrids. The results of COG functional annotation of the DEGs are showed in Figure 6.

Further analysis on the secondary metabolite and defense mechanism clusters was carried out based on COG database classification. Out of 1,425 transcripts that were assigned through COG database, 91 transcripts were clustered in "Secondary metabolites biosynthesis, transport and catabolism" with 41 up-regulated and 50 down-regulated transcripts, respectively (Figure 7). In this cluster, the highest number of up-regulated transcripts were 'Peroxidase family' and 'Cytochrome p450' with 7 transcripts each, followed by 'Iron ascorbate-dependent oxidoreductase family' (6 transcripts) and 'Alcohol dehydrogenase' (5 transcripts). While the highest number of down-regulated transcripts were 'Lignin degradation and detoxification of lignin-derived products' and 'Iron ascorbate-dependent oxidoreductase family' with 7 transcripts, followed by 'Cytochrome P450 family' (6 transcripts) and 'ABC transporter B family member' (5 transcripts).

In total, there were 18 transcripts that were mapped into defense mechanism cluster, with 2 and 16 up-regulated and down-regulated transcripts, respectively (Figure 8). In this cluster, two transcripts were up-regulated in response to Agrodyke treatment, namely cinnamyl-alcohol dehydrogenase (CAD) and NAD dependent epimerase/dehydratase. The highest down-regulated transcripts were 'Bifunctional dihydroflavonol

Table 3. Distributions of differentially expressed genes (DEGs) in KEGG pathway database classification

Category	Sub-category	DEGs with pathway annotation
Metabolism	Amino acid metabolism	97
	Biosynthesis of other secondary metabolites	193
	Carbohydrate metabolism	220
	Energy metabolism	54
	Glycan biosynthesis and metabolism	5
	Lipid metabolism	55
	Metabolism of cofactors and vitamins	99
	Metabolism of other amino acids	30
	Metabolism of terpenoid and polyketides	18
	Nucleotide metabolism	63
	Xenobiotics biodegradation and metabolism	80
Organismal systems	Immune system	8
Genetic information processing	Translation	2
Total transcripts		924

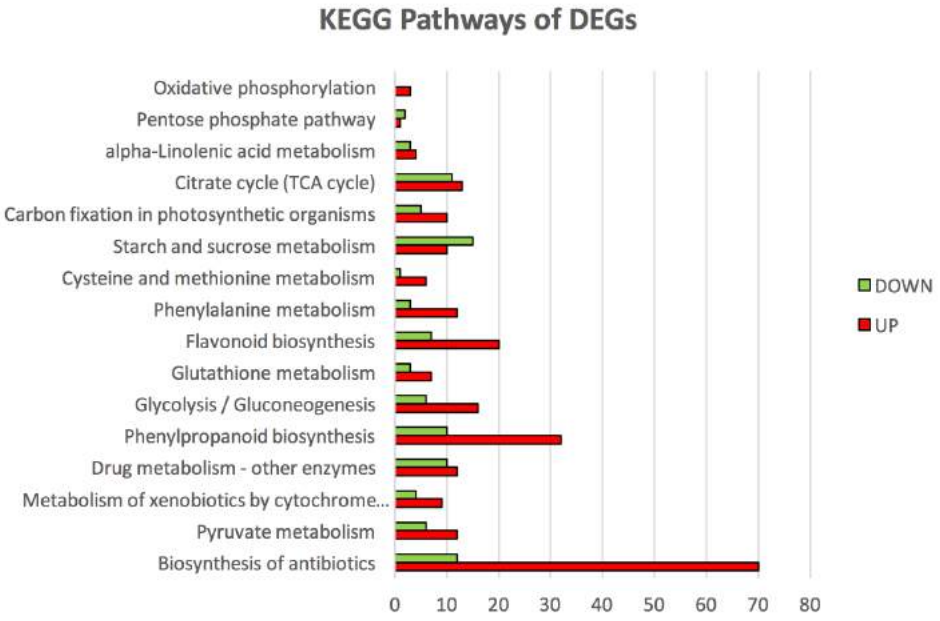

Figure 9. 16 out of 107 KEGG pathways with significant differential expression of genes

4-reductase flavanone' with 5 transcripts and 'Multi antimicrobial extrusion (MATE) (TC 2.A.66.1) family' with 3 transcripts.

KEGG enrichment of candidate pathways in response to Agrodyke treatment

According to sequence homology searches using Blastx against KEGG pathway database, 924 out of 1,425 DEGs were assigned to 107 KEGG pathways under three main categories in KEGG which includes Metabolism, Organismal systems and Genetic information processing (Table 3). As shown in Table 3, the most abundant DEGs were classified in Metabolism category with 914 transcripts, followed by Organismal systems (8 transcripts) and Genetic information processing (2 transcripts). There were 11 sub-categories under the Metabolism category. Most of the DEGs were found in 'Carbohydrate metabolism' sub-category (220 transcripts), followed by 'Biosynthesis of other secondary metabolites' (193 transcripts), 'Metabolism of cofactors and vitamins' (99 transcripts), 'Amino acid metabolism' (97 transcripts) and 'Xenobiotics biodegradation and metabolism' (80 transcripts). Notably, both Organismal systems and Genetic information processing and categories consists of one sub-category which was 'Immune systems' (8 transcripts) and 'Translation' (2 transcripts), respectively.

Figure 9 illustrated 16 out of 107 KEGG pathways discovered for all DEGs, with the most dominant pathways were in biosynthesis of other secondary metabolites, namely biosynthesis of antibiotics (82 transcripts), phenylpropanoid biosynthesis (42 transcripts) and flavonoid biosynthesis (27 transcripts). Also, majority number of the upregulated DEGs involved in these three protective activities pathways.

Table 4. The significantly enriched KEGG pathways of DEGs between treated and non-treated *A*cacia hybrids

Sub-category	Pathway	Number of genes	Enzymes in Pathway	Number of transcript
Biosynthesis of other secondary metabolites	Flavonoid biosynthesis (KO00941)	8	ec:2.3.1.133, ec:5.5.1.6, ec:1.3.1.77, ec:1.1.1.219, ec:1.14.11.9, ec:1.17.1.3, ec:2.3.1.74 & ec:2.3.1.170	27
	Phenylpropanoid biosynthesis (KO00940)	6	ec:1.1.1.195, ec:3.2.1.21, ec:2.3.1.133, ec:1.2.1.44, ec:6.2.1.12, ec:1.11.1.7	42
Carbohydrate metabolism	Starch and sucrose metabolism (KO00500)	13	ec:3.2.1.21, ec:3.2.1.4, ec:3.1.3.12, ec:3.2.1.26, ec:3.2.1.39, ec:3.2.1.48, ec:2.4.1.18, ec:3.2.1.20, ec:2.7.7.27, ec:2.7.1.1, ec:2.7.7.9, ec:2.4.1.12	25
	Citrate cycle (TCA cycle) (KO00020)	8	ec:1.3.5.1, ec:1.1.1.37, ec:4.1.1.32, ec:4.1.1.49, ec:2.3.3.8, ec:6.2.1.4, ec:2.3.3.1 & ec:6.2.1.5	24
	Galactose metabolism (KO00052)	6	ec:3.2.1.26, ec:2.7.7.64, ec:3.2.1.20, ec:2.7.1.1, ec:2.7.7.9, ec:1.1.1.21	7
Xenobiotics biodegradation and metabolism	Drug metabolism - other enzymes (KO00983)	6	ec:2.5.1.18, ec:3.6.1.23, ec:2.7.1.21, ec:2.3.1.5, ec:3.1.1.1 & ec:1.17.4.1	22
	Metabolism of xenobiotics by cytochrome P450 (KO00983)	3	ec:2.5.1.18, ec:1.14.14.1 & ec:1.1.1.1	13
Metabolism of other amino acids	Glutathione metabolism (KO00480)	4	ec:2.5.1.18, ec:1.11.1.15, ec:1.17.4.1 & ec:1.1.1.49	10
Lipid metabolism	Fatty acid elongation (KO00062)	1	ec:2.3.1.16	5
Glycan biosynthesis and metabolism	Other glycan degradation (KO00511)	2	ec:3.2.1.25, ec:3.2.1.24	4

Conversely, starch and sucrose metabolism as well as pentose phosphate pathway were amongst the significantly enriched pathway for down-regulated DEGs. There was also no down-regulated transcript observed in oxidative phosphorylation pathway. As overall, there were more up-regulated transcripts compared to down-regulated transcripts in majority KEGG pathways.

Additionally, by referring to Table 4, it was observed that out of 107 KEGG pathways discovered for all DEGs, only 10 pathways were significantly enriched with DEGs ($P \leq 0.05$ and FDR < 0.05). They were flavonoid biosynthesis, phenylpropanoid biosynthesis, starch and sucrose metabolism, citrate cycle (TCA cycle), galactose metabolism, drug metabolism - other enzymes, metabolism of xenobiotics by cytochrome P450, glutathione metabolism, fatty acid elongation and other glycan degradation.

DISCUSSION

In the present study, a transcriptome analysis was performed in order to investigate the impact of Agrodyke treatment on the expression of genes in infected *A*. hybrids treated at different time-points (1, 5, 10 and 15 days post-inoculation (dpi)). The results showed that Agrodyke treatment significantly influenced the gene expression compared to non-treated *A.* hybrids as it involved more gene activation than suppression. Importantly, a significant impact was observed at all investigated time-points, including 1 dpi, indicating a fast response of the host tissue towards the Agrodyke.

The similarity searches of DEGs against COG database found that majority DEGs were under "Function unknown" category. These transcripts could be candidates of novel genes for future studies. Other than that, the GO enrichment analysis revealed the involvement of genes mainly in the metabolic process and catalytic activity. These results were in accordance with the KEGG analysis where Agrodyke treatment works entirely on the metabolic pathways of the infected *A.* hybrids. Most of the enriched pathways were involved in metabolism category such as secondary metabolites biosynthesis, carbohydrate metabolism, xenobiotics biodegradation and metabolism, metabolism of other amino acids and lipid metabolism. According to Wenping *et al*. (2011), genes within similar pathways commonly cooperate together in order to carry out certain biological functions.

The KEGG pathway analysis revealed that most of the upregulated genes involved in biosynthesis of antibiotics. Antibiotics are phytochemicals that are known to have antimicrobial and antiviral properties in plants (Wink & Schimmer, 2010). Considering to this properties, the activation of genes in this pathway might be one of the main mechanisms of action of the Agrodyke. Apart from that, phenylpropanoid and flavonoids pathways were also significantly enriched in response to the Agrodyke treatment. Phenylpropanoids exhibit antimicrobial activity which play major role as chemical and physical barriers against pathogen infection. Also, they act as signal molecules in local and systemic plant defense mechanism. The phenylpropanoid pathway is linked to flavonoid biosynthetic pathway which also plays important role in play resistance (Treutter, 2006). The overproduction of phenylpropanoid and flavonoids by the plant is considered to be a part of specific antimicrobial defense system (Wink et al., 2010). Therefore, the activation of genes like peroxidase (POD, EC:1.11.1.7), cinnamyl-alcohol dehydrogenase (CAD, EC:1.1.1.195) and O-hydroxycinnamoyl transferase (HCT, EC:2.3.1.133) in phenylpropanoid pathway and chalcone isomerase (EC:5.5.1.6), flavanone (EC:2.3.1.74) and 4-reductase (EC:1.1.1.219) in flavonoid pathway reconfirms their important involvement as plant defense response.

Interestingly, this study discovered that genes associated with defense response were highly up-regulated while genes related to growth and development process were down-regulated upon Agrodyke treatment. Starch and sucrose metabolism and pentose phosphate pathway have important functions in plant growth and development. However, in this study, more genes are identified to be down-regulated in these pathways upon the treatment of Agrodyke. This finding supports a view that there is a trade-off between growth and defense response as secondary metabolites and structural reinforcement are physiologically constrained in dividing and enlarging cells since they divert resources from the production of new leaf. In the present study, it is suggested that Agrodyke probably plays a central role in controlling resource allocation between the competing process of defense and growth.

In addition, more up-regulated genes were identified in cysteine and methionine metabolism and oxidative phosphorylation pathway, indicating their crucial role in oxidation resistance in plants (Bin *et al.*, 2017). Furthermore, these pathways are important for producing cellular energy which results in the activation of the host defense mechanisms and suppression of the pathogen colonization of the host tissue. The results also showed an overall high expression of defense genes involved in xenobiotic metabolism in *A.* hybrids treated with Agrodyke. Xenobiotics are foreign chemical contaminants that can be absorbed and accumulated in plant cells (Sandermann, 1992). The plant induces the expression of several genes involved in xenobiotics metabolism in order to detoxify those components. The activation of genes responsible for plant detoxification, specifically cytochrome P450 and glutathione S-transferases (GSTs, EC:2.5.1.18) suggested *A*. hybrid response to the Agrodyke treatment.

CONCLUSION

In conclusion, the results of the present study provide a comprehensive overview of the impact of Agrodyke on the gene expression of treated *A.* hybrids, highlighting the induction of multiple metabolic responses. In particular, GO analysis and pathway mapping of the DEGs showed the induction of important defense pathways such as phenylpropanoid biosynthesis pathway, flavonoid biosynthesis pathway and metabolism of xenobiotics by cytochrome P450. Besides, the identified genes associated with defense response like POD and CAD could provide a resource for further developing resistant varieties of *Acacia* varieties against *Ceratocystis* infection.

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TPGM

Identification of WRKY transcription factor expressed in root of Tongkat Ali: Preliminary study

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Abstract

Tongkat Ali or scientifically known as *Eurycoma longifolia* is one of the most well-known herbal medicines in Southeast Asia. Tongkat Ali extract especially from root has widely been used in pharmaceutical industries and various secondary metabolites have been isolated. Due to their beneficial effects to health, biosynthesis of secondary metabolite has been a prime focus of research. Many transcription factors (TF) has been identified to play major roles in regulating secondary metabolite biosynthesis pathways at the transcriptional level. Among TFs frequently reported to regulate plant secondary metabolism biosynthesis are the families of WRKY. Here we report 10 WRKY-related TF which expressed in root of Tongkat Ali. The TFs were deduced from transcriptome analysis of 10- and 1-year-old Tongkat Ali root. RNA from both root were sequenced using Illumina Hi-Seq 2000 technology and the sequencing data was assembled using SOAP*denovo* software. 60,753 non-redundant unigenes were finally generated. BLASTX search analysis showed that 60 unigenes were similar to WRKY TF and 33 unigenes were up-regulated in root of 10-year-old tree. Out of 33 TFs, 17 were

Key words: *Eurycoma longifolia*, transcriptome, secondary metabolites

INTRODUCTION

Tongkat Ali (*Eurycoma longifolia)* is a well-known medicinal plant with significant economic value in Malaysia. It is a slow growing herbal plant under the family of Simaroubaceae and native to Malaysia, Indonesia, Vietnam, Myanmar, Loas and Thailand (Rehman *et al*., 2016). The root extract of Tongkat Ali has been reported to exhibit different types of pharmacological effects such as anti malaria (Al-Adhroey *et al*, 2010; Wernsdorfer *et al*., 2009), aphrodisiac (Wahab *et al*., 2010), anti cancer (Tee *et al*., 2005), ergogenic (Muhamad *et al*., 2010) and toxicity (Razak *et al*., 2011). This plant species is also reported to consist of several classes of secondary metabolites and the major among them are the quassinoids, canthin-6-one, beta-carboline alkaloids, squalene derivatives, tirucallane-type triterpenes and biphenylneolignans (review by Bhat and Karim, 2012).

Plant secondary metabolites are compounds that play roles in interaction of the organism with its environment. These compounds are often involved in plants protection against biotic or abiotic stresses. Plant secondary metabolites are highly diverse in their chemical structures and are classified into three major classes which are terpenoids, alkaloids and phenolics. Biosynthesis of plant secondary metabolites went through a complex network of enzymatically catalysed metabolic pathways. Several families of TFs have been identified as regulators of plant secondary metabolism. Among TFs frequently reported to regulate plant secondary metabolism biosynthesis are the families of WRKY, AP2/ERF, MYB and bHLH (Misra *et al*., 2014; Yang, 2012; Vom Endt, 2002).

WRKY domain-containing genes comprise one of the largest TF families that have to date only been found in plants. It is characterized by highly conserved WRKYGQK motif and usually followed by zinc-finger motif, located at the N-terminus (Rushton *et al*., 2010). The WRKY domain is approximately 60 amino acid residues in length and has potential DNA binding activity. Numerous WRKY TFs from a wide range of plant species have been characterized and shown to participate in growth, development, metabolism and response to environmental cues (Chi *et al*., 2013; Schluttenhofer and Yuan, 2015; Phukan *et al*., 2016; Chen *et al*., 2017; Jiang *et al*., 2017). Since 2007 accumulation of evidence suggests that certain WRKYs regulate the production of valuable natural products by regulating metabolite biosynthesis genes (Kato *et al*., 2007; Ma *et al*., 2009; Suttipanta *et al*., 2011). The first structure of WRKY domain was published in 2005. Based on the arrangement of zinc-finger motif and number of WRKY domain, WRKY TFs are classified into three major groups and further sub-groups (Rushton *et al*, 2010). Group I members typically have two WRKY domains and a C2H2 zinc-finger motif whereas Group II members have a single WRKY domain and a C2H2 zinc-finger motif. According to the pattern of their zinc-finger motif, Group II members can be further divided into II(a)-(e) by the presence of ten additional structural motifs that are conserved among subsets of WRKY family members. A single WRKY domain and a C2HC zinc-finger motif characterize Group III WRKY TFs. Group III WRKY TFs are divided into two types on the basis of pattern of zinc-finger motif. The structure of zinc-finger motif for subgroup III(a) is C-X7-C-X_{23}-HXC (X for any amino acid), while that of subgroup III(b) is C-X7-C-Xn-HXC (n ≥ 24).

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Table 1. List of 17 Tongkat Ali transcriptome unigenes annotated having similarity to WRKY transcription factor with their read per kilobase mapped (RPKM) expression in root of 10 and 1 year old tree

Gene ID	Gene length	10 year-old root RPKM	1 year- old root RPKM	Nr- E value	Nr-annotation
Unigene12199	971	85.7325	22.246	3E-125	WRKY transcription factor 65 (*Citrus clementina*)
Unigene13804	572	5.8677	1.9876	5E-58	WRKY27; transcription factor (*Citrus sinensis*)
Unigene14905	841	33.1074	1.659	2E-130	WRKY transcription factor 31 (*Citrus clementina*)
Unigene18724	642	22.3107	1.6904	2E-73	WRKY1 transcription factor 40 (*Citrus clementina*)
Unigene18953	642	30.3366	1.4489	3E-36	WRKY transcription factor 70 (*Citrus clementina*)
Unigene29906	1398	7.1686	0	3E-134	WRKY transcription factor 31 (*Citrus clementina*)
Unigene35055	1441	8.8246	1.7214	2E-124	WRKY transcription factor 53 (*Quercus suber*)
Unigene37171	1061	10.8267	1.2176	1E-103	WRKY transcription factor 53 (*Citrus clementina*)
Unigene37175	820	11.1262	0.063	2E-64	WRKY transcription factor 50 (*Citrus clementina*)
Unigene38695	1064	15.3723	0.5828	6E-98	WRKY transcription factor 31 (*Citrus clementina*)
Unigene38714	872	8.728	0.0593	8E-73	WRKY9; transcription factor (*Citrus sinensis*)
Unigene53601	521	55.0752	0.1984	6E-36	WRKY transcription factor 40 (*Citrus sinensis*)
Unigene54689	928	3.8205	0.111	1E-90	WRKY transcription factor 22 (*Citrus sinensis*)
Unigene54709	945	19.159	2.515	4E-149	WRKY transcription factor 47 (*Citrus sinensis*)
Unigene54755	998	13.3575	0.3625	8E-114	WRKY transcription factor (*Citrus maxima*)
Unigene55000	1867	16.3566	1.1072	0	WRKY transcription factor 31 (*Citrus clementina*)
Unigene5766	1353	40.2844	17.722	4E-78	WRKY transcription factor 48 (*Citrus sinensis*)

WRKY TFs are unique to plants regulating various physiological functions therefore any changes at transcriptional level would alter diverse functions in plants including secondary metabolite biosynthesis. In this study, few WRKY TF that were highly expressed in 10 year old Tongkat Ali root were identified and characterized based on their WRKY domain. The TFs were classified according to methods utilized by other studies.

MATERIALS AND METHODS

Plant materials

Roots of ten year-old Tongkat Ali was collected from Tongkat Ali plot, Bukit Hari, Forest Research Institute Malaysia (FRIM) and one year-old Tongkat Ali was from the nursery of Kepong Botanical Garden, Forest Research Institute Malaysia (FRIM). Root of Tongkat Ali harvested from one and 10 year-old were immediately put in liquid nitrogen until further processing. For longer storage, the roots were kept at -80°C.

RNA isolation and quantification

RNA isolation from the roots of Tongkat Ali were carried out using RNeasy Plant Mini Kit (Qiagen) according to protocol outlined by the supplier. Sample was ground into powder in a pre-chilled DEPC-treated mortar in the presence of liquid nitrogen. QIAshredder spin column was used to filter the cell-debris and RNeasy spin column was used to trap all the RNA. The RNA was then eluted with 50 ul of RNase-free water and kept at 4°C or -80°C for longer storage. Agilent RNA 6000 Nano Chips run on Agilent 2100 bioanalyzer (Agilent Technologies) were used to assess the RNA quality. Sample preparation and apparatus set-up to determine the RNA quality was carried out according to the apparatus manual.

Illumina sequencing

The total RNA with absorbance 260/280 nm ratio of ~2.0 and RIN number more than 8.0 was chosen for Illumina Hi-Seq 2000

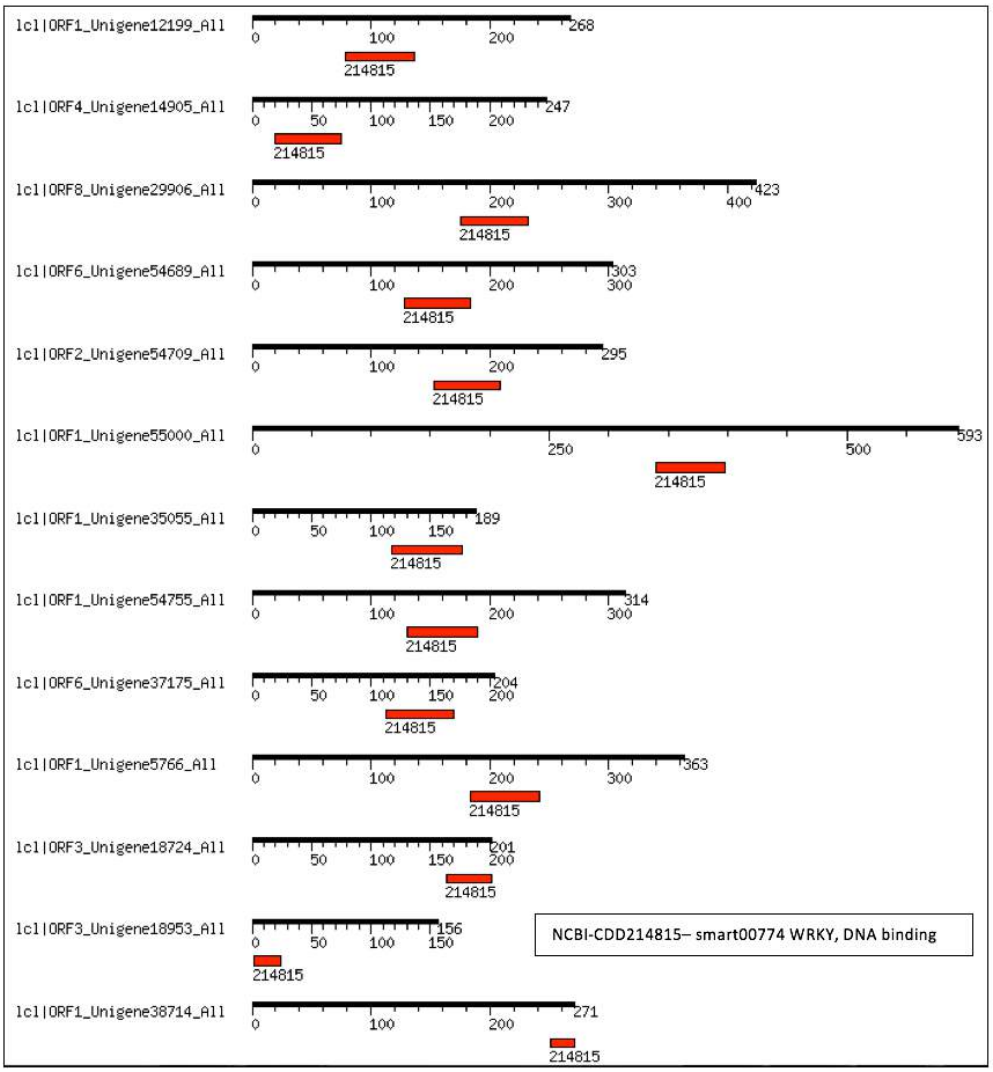

Figure 1. Schematic diagram of WRKY proteins conserved motifs in Tongkat Ali. The red box represents the motif of NCBI-CDD214815 – smart00774 WRKY, DNA binding using MOTIF Search software

sequencing. Paired end cDNA library of 100 bp sequencing reads were generated from each RNA samples using mRNA Sequence assay. The sequencing and library preparation were out sourced to Beijing Genome Institute (BGI), China.

Transcriptome sequencing data analysis

The raw sequence data was quality checked and trimmed. All the quality reads were *de novo* assemble using SOAP*denovo* program (Li *et al*., 2010). Briefly, the clean reads were overlapped to each other to formed contigs and the contigs were joined together to form scaffolds based on pair-end information. Finally, the paired-end reads were re-used to fill the scaffold gaps to obtain unigenes. The unigenes from the two libraries were combined to generate non-redundant unigenes. Unigenes sequences were aligned by Blast X (an E-value <1.0e^{-5} was used as the cut-off) to the public protein databases NCBI's non-redundant databases (NR). Unigenes encoded for WRKY TF were selected for analysis. ORFfinder (www.ncbi.nlm.nih.gov/orffinder) and MOTIF search (www.genome.jp/tools/motif) software were used to search for the open reading frame and protein sequence motif, respectively of each unigenes.

Identification of differentially expressed genes (DEGs)

Reads per kilobase per million mapped reads (RPKM) was used to normalize the mapped reads (Mortazavi *et al*., 2008). The ρ values and $\log_2$ of each TF were calculated and the DEGs were identified at threshold of $|\log_2| > 2$ and FDR $\leq$ 0.001. This

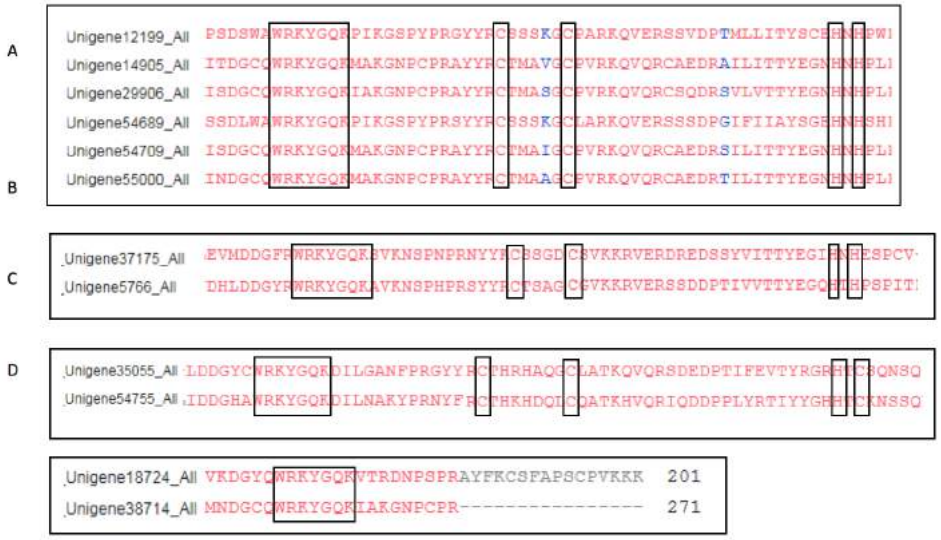

Figure 2. Multiple sequence alignment analysis of 12 selected WRKY domain. The WRKY motif (WRKYGQK) and the characteristic residues for the zinc-finger motif (C2H2 or HC) are marked by boxes

method is used to calculated gene expression and could be directly used to compare the difference of gene expression between samples.

RESULTS AND DISCUSSION

Output of sequencing and de novo assembly

Mature root of Tongkat Ali has been reported to contain high yield of various chemical compounds. Hence, in this project transcripts of 10-year-old Tongkat Ali root were compared with transcripts of 1 year-old Tongkat Ali root. Illumina Hi-Sequence DNA technology together with bioinformatics analysis of 1 year-old root generated 41,857 unigenes, while 81,907 unigenes were obtained from 10-year-old root. Assembled unigenes of the two roots were combined for non-redundant sequence and referred as reference transcriptome of Tongkat Ali. In total, 60,753 unigenes were obtained from the reference transcriptome with an average length of 629 bp and N50 of 921 bp.

Identification of WRKY genes in the Tongkat Ali transcriptome

BlastX search to non-redundant (Nr) of NCBI revealed that 967 unigenes of Tongkat Ali were annotated as putative transcription factors. The most abundant family was the MYB transcription factor (112 unigenes), followed with, WRKY (60 unigenes), bHLH (54 unigenes), AP2/ERF (52 unigenes), GRAS (52 unigenes) and bZIP(50 unigenes). In recent years accumulating evidence indicates that WRKYs also play an essential role in secondary metabolism production by regulating the related biosynthesis gene. Thus WRKY related genes in Tongkat Ali were analysed in this study. Number of WRKY TF has been reported contains several members in different plant species including 72 in Arabidopsis (Eulgem *et al*., 2000), more than 100 in rice, poplar and soybean (Berri *et al*., 2009; Jiang *et al*., 2014; Schmutz *et al*., 2010), and 45 in barley (Mangelsen *et al*., 2008). Currently, there was no WRKY TF of Tongkat Ali has been reported and in this study we identified 60 potential WRKY TFs expressed in root of Tongkat Ali. Out of 60 unigenes, only 33 were up-regulated in root of 10 year-old, and for preliminary study, 17 unigenes which were equal or more than 500 bp in size were chosen for further analysis (Table 1). Most of the unigenes were similar to the genus of Citrus, this is in-line with study reported by Nazirah *et al*. in 2018 stated that Citrus was the most similar species to Tongkat Ali at molecular level.

Tongkat Ali WRKY motif analysis

To better understand the similarity and diversity of WRKY motif composition among the 17 unigenes, the WRKY motifs in each unigenes was predicted using MOTIF Search software. The analysis revealed that the WRKY domain (motif 214815) was present in 13 out of 17 unigenes (Figure 1). Even though the other four unigenes were annotated as WRKY transcription factor by BLAST analysis but the WRKY motifs of the unigenes (Unigene37171, Unigene38695, Unigene53601 and Unigene13804) were not detected by MOTIF Search software. This might be due to DNA sequence of the unigenes which required further analysis for verification.

Protein sequence of WRKY domain

Sequence analysis of 13 identified WRKY unigenes demonstrated the deduce amino acid numbers were from 156 – 593. Protein sequence analysis revealed that all of WRKY proteins have the conserved motif "WRKYGQK" except unigenes18953 that having unfamiliar conserved motif. Even

though amino acid sequence of WRKY protein in few studies reported been replaced by WRRY, WSKY, WKRY or WKKY (Xie, 2005), but none of the amino acid sequence similar to unigene18953. All the 12 unigenes consist of one conserved motif "WRKY", indicating none of them were belong to group I. WRKY domain of Unigene18724 and unigene38714 were truncated at C-terminal of WRKY domain, therefore conserved zinc finger motif could not be identified (Figure 2[D]). However as these unigenes possess typical conserved sequence they were retained for further analysis to verify their WRKY domain.

Most proteins with one WRKY domain are belong to group II. Group II members have a single WRKY group and a C2H2 zinc-finger motif. Based on zinc-finger motif, this group is further divided into five subgroups (IIa-IIe). In this study, unigenes12199, 14905, 29906, 54689, 54709 and 55000 were predicted belong to group II(b) as their zinc finger motif type was $CX_5CX_{23}HXH$ (Figure 2[A]). While, Unigene37175 and 5766 were predicted belong to group II(c) as the characterization of the zinc finger motif showed as $CX_4CX_{23}HXH$ (Figure 2[B]). Zinc finger motif of Unigene35055 and 54755 exhibited $CX_7CX_{23}HXC$ type which belongs to WRKY domain group III(a) (Figure 2[C]).

WRKY TF roles in regulating metabolite biosynthetic gene for the production of valuable natural products have been reported in few studies (Kato *et al.*, 2007; Ma *et al*., 2009; Suttipanta *et al*., 2011). WRKY TFs of cotton have been identified to regulate sesquiterpene cyclase at the pathway leading to the production of gossypol, an antifeedant phytoalexin (Xu *et al*., 2004). The production of antimalarial drug artemisinin in *Artemisia annua* was also reported doubled by overexpressing of its WRKY TF (Han *et al*., 2014). These reports provide evidence that WRKY TFs could be used to engineer metabolite or secondary metabolite pathways and was also suggested that could control critical rate-limiting in metabolic pathways (Schluttenhofer *et al.*, 2015).

WRKY TFs identified from Tongkat Ali were potentially involve in regulation of its secondary metabolic pathways which was reported to produce in wide range especially quassinoids and alkaloids. However further characterization on each WRKY TF should be carry out in detail to identify which were the responsible genes in Tongkat Ali secondary metabolite production. Understanding their functions would provide ways to successfully reengineer this medicinal plant to provide continuous and higher yielding materials for pharmaceuticals industry purposes.

CONCLUSION

Despite being highly sought after medicinal plant in Malaysia, very limited numbers of study have been reported in secondary metabolites biosynthesis of Tongkat Ali. We have analysed transcriptomics data from root of Tongkat Ali to identify genes involved in its secondary metabolites production. Among interesting expressed genes identified were WRKY TFs. Few studies in other plant species have reported the role of WRKY TF in secondary metabolite production, thus WRKY TF of Tongkat Ali were worth to further analysed. As a preliminary result we report on identification of twelve WRKY TFs of Tongkat Ali base on their conserved WRKY domain. All the unigenes have WRKY motif with conserved WRKYGQK amino acid sequence and follow with either C2H2 or C2HC zinc finger motif. Six of the WRKY TFs were belong to group II(b), while two were belongs to group II(c) and another two were in group III(a). All these WRKY TFs were up-regulated in 10 year-old root which consist higher yield of secondary metabolites. However further analysis is required to characterized function of each WRKY TF.

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TPGM

Early nursery growth measures indicate successful transfer of desirable traits to 3rd cycle derivatives from oil palm originally prospected from Nigeria

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Abstract

Breeding for short palms has been a key focus for oil palm breeders over the years for its advantage in longer economic life cycle and ease for mature harvesting. With continuous difficulties to find competent tall palm harvesters each year and the industry ambition to incorporate automation in harvesting, work on developing short planting material has been gaining momentum. In Malaysia, the Nigerian Prospection Material (NPM) of the Malaysia Palm Oil Board (MPOB) is known to contain palms of small size and stature. Noh et al. in 2014 mentioned that genetic variability in the NPM population is still high, giving ample scope for further selection. Deli dura x NPM pisifera progenies were reported by Arolu *et al*. (2018) to be short with average annual palm increment of <30cm/year, which is 33-60% less than the typical range in annual height increment of 45-75cm/year for current commercial DxP planting material. Derivatives of the original NPM, i.e. 2nd cycle NPM, had been planted at IOI Group's Mamor Estate in Kluang, Johor, in 1996. To conserve the material for future breeding, palms were selected from a family exhibiting small stature to produce 3rd cycle NPM. We report early nursery growth measurement in this 3rd cycle NPM, showing that desirable traits of small size and stature have been successfully transmitted from the selected 2nd cycle parents. Nursery measures such as these are rarely if ever done in the normal course of oil palm breeding. These measures are relatively simple to carry out, and if done, could become useful early indicators of successful conservation or transmission of desirable growth traits in breeding populations.

Key words: Progeny trial, breeding strategy, genotype, environment

INTRODUCTION

With the acquiring of Pamol Plantations and its germplasm materials in 2003, IOI had inherited the most widely known and extensively evaluated short Nigerian Prospection Materials (NPM). This material was initially prospected by Malaysia Palm Oil Board MPOB at Nigeria in 1973 (Marhalil *et al*., 2017). The prospected Nigerian materials were planted and evaluated in a field genebank at MPOB Kluang, Johor in 1975/76 and has been widely distributed to other agencies ever since.

Genetic variability in the NPM population is still high which gives ample scope for further selection (Noh *et al*., 2014). Despite the poor fruit characters of this population, the value of this material hinge at its outstanding vegetative traits (Seng *et al*., 2015). The DxP derived from this population was reported by Arolu *et al*. (2018) to be short with average annual palm increment of <30cm/year, which is 33-60% less than the typical range in annual height increment of 45-75cm/year for current commercial DxP planting material.

In 1996, second generation of NPM was planted in Mamor Estate in Kluang, Johor. With the purpose of further breeding and conservation, several dura palms from a short family in this second generation NPM trial were selected and were either selfed, sib crossed or outcrossed with breeding material from other origins to produce the third generation of NPM populations.

A pre-nursery stage vegetative measurement analysis was therefore conducted to study the differences in growth patterns at pre-nursery stage between the following three groups of crosses (Table 1): inbred third generation NPM dura (selfed and sib-crossed), outcrossed third generation NPM dura (crossed out to other origins), and inbred Deli dura as control.

METHODS

All the seedlings were subjected to IOI Group standard operating procedure practices for pre-nursery. Sowing was conducted in IOI-Pamol Research Kluang Pre-nursery using elevated pot tray system. Sowing date for each treatment was recorded.

Seedling height, diameter of stem base, total relative leaf area and number of green leaves per plant were measured using the methods of Corley and Breure (1981) at 45 days, 75 days and 105 days after sowing.

All the data collected were subjected to frequency distribution analysis, rate of growth analysis and analysis of variance (ANOVA) using Statistical Analysis System (SAS) package.

RESULTS AND DISCUSSION

Table 2 shows results of ANOVA for vegetative measures at the end of the study period (i.e. 105 days after sowing). Differences

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Table 1. List of crosses and number of seedlings involved in the study

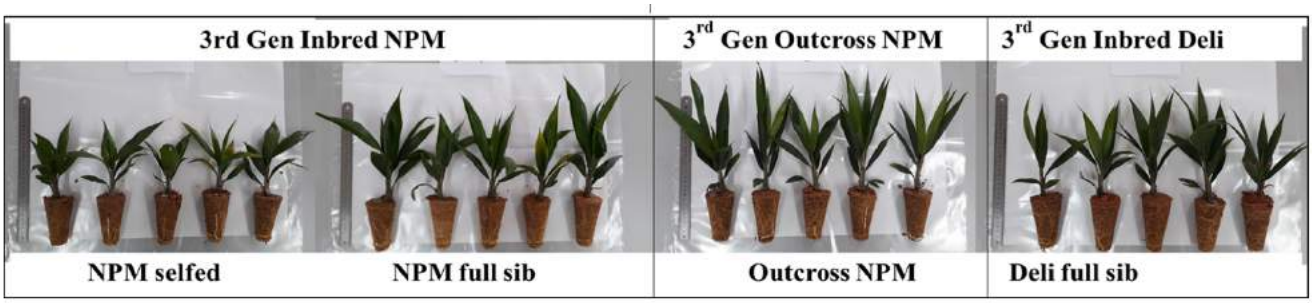
Treatment	Crosses	Number of seedlings
Treatment 1	3rd Gen NPM[1] inbred	200
Treatment 2	3rd Gen NPM outcross	100
Control	Deli inbred	50

[1] – NPM = Nigerian Prospection Material

Table 2. ANOVA results for vegetative measures at 105 days after sowing

Population	Number of green leaves /plant		Total relative leaf area (cm2)		Height (cm)		Stem diameter (cm)	
	Means	C/V (%)	Means	C/V (%)	Means	C/V (%)	Means	C/V (%)
3rd Gen Deli Dura inbred (control)	5.3 ± 0.2 *b*	15	153.6 ± 14.3 *c*	33	11.4 ± 1.0 *a*	31	1.4 ± 0.1 *b*	16
3rd Gen NPM Dura inbred	5.7 ± 0.1 *a*	16	200.9 ± 10.5 *b*	38	8.0 ± 0.4 *c*	35	1.4 ± 0.1 *b*	16
3rd Gen NPM Dura outcross	5.9 ± 0.1 *a*	11	283.9 ± 15.0 *a*	27	10.2 ± 0.7 *b*	36	1.5 ± 0.1 *a*	13
Trial Mean	**5.7 ± 0.1**	**15**	**218.5 ± 8.9**	**39**	**9.1 ± 0.4**	**38**	**1.4 ± 0.1**	**15**
ANOVA	*		*		*		*	

Means followed by the same letter are not significantly different at probability of 95%. (*) significantly different with at least 95% probability
1 – NPM = Nigerian Prospection Material
2 –ANOVA = Analysis of variance



NPM = Nigerian Prospection Material

Figure 1. Randomly selected seedlings of each treatment at 105 day after sowing

between treatments were statistically significant for height, number of green leaves per plant, total relative leaf area and trunk diameter.

The height of the 3rd generation inbred NPM dura was significantly lower compared to both 3rd generation outcrossed NPM dura and inbred Deli dura as shown in Figure 1. This is despite the 3rd generation inbred NPM Dura having significantly more number of green leaves per plant and higher total relative leaf area compared to the tallest Deli dura inbred which is the control for this study.

The same parent of the 3rd generation inbred NPM dura when outcrossed to a tall palm of another origin produced progeny that showed hybrid vigour, with significantly taller plant height, higher total relative area, and greater stem diameter. Number of greens leaves per plant was also higher though not statistically significant at the 95% level of probability.

It is interesting to note in this study that 3rd generation inbred NPM dura are significantly shorter without the expense of lower number of green leaves per plant. The mean total green leaves produced by this material at 105 days after sowing was 5.7 leaves (Table 2), or an average of 1.6 green leaves per plant per month which is 60 % higher compared to the leaf production by normal oil palm seedlings grown in pre nursery under best management practices (BMP) as reported by Donough *et al*. (2014) which average at 1 leave per month.

Although the Deli dura line had been domesticated by oil palm breeders much earlier since the exploitation of four palms in Bogor Botanical Garden in 1848 (Rajanaidu *et al*., 2013) in comparison to the utilization of NPM which started only in 1973 (Marhalil *et al*., 2017), the low height trait is not common in the former since the selection pressure in Deli dura

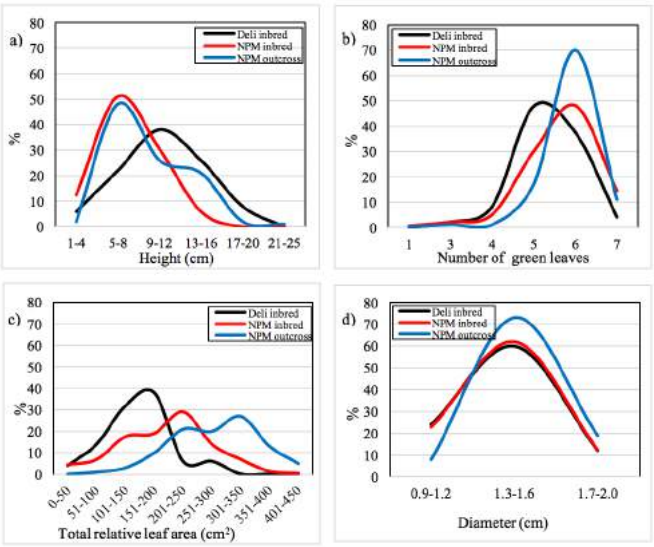
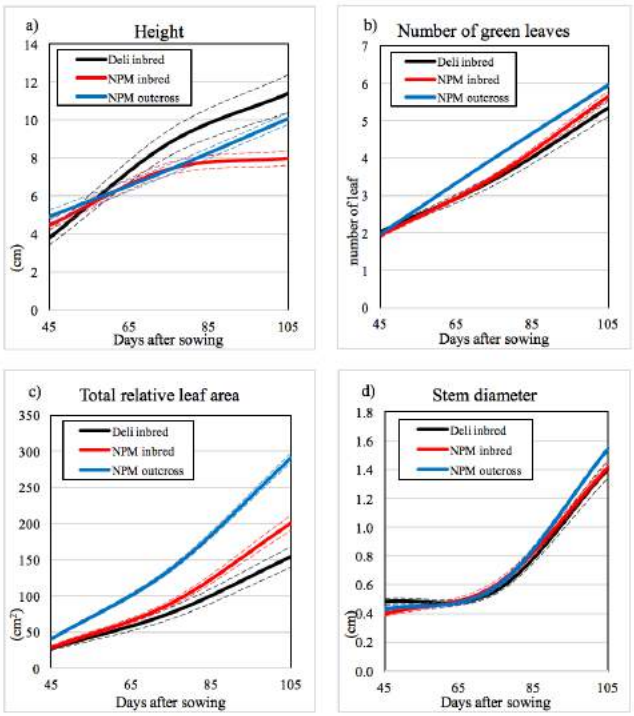


Figure 2. Frequency Distribution for a) height, b) number of green leaves, c) total relative leaf area and d) stem diameter for three population groups at 105 days after sowing



The broken lines of the same colour indicate the upper and lower confidence limit of 95% probability

Figure 3. Growth rate over 60 days (45 to 105 days after sowing) for a) height, b) number of green leaves, c) total relative leaf area and d) stem diameter for three population groups

population is mostly on the fruit characters especially mesocarp thickness and oil content in mesocarp as reported by Mathews *et al*. (2007).

Figure 2 shows the frequency distribution for all the vegetative measures at the end of the study period (i.e. 105 days after sowing). In terms of height, most individuals in the 3rd generation inbred NPM dura population skewed towards the shorter end of the height group while inbred Deli dura had almost a normal distribution.

Outcross of other origin into the NPM dura population had resulted in many of the outcrossed progeny that have inherited the shortness, as illustrated in Figure 2a. This early indication signals that there is possibility of introducing the short trait into other origins.

Figure 2b shows that both 3rd generation inbred NPM dura and 3rd generation outcrossed NPM dura had almost the same peak in the frequency distribution graph for the number of green leaves per plant while the control inbred Deli dura population peaks at slightly lower category of number of green leaves per plant. The trait of high number of green leaves for the NPM material continue to appear even after outcross into another origin.

Relative leaf area, and to a lesser extent stem diameter, appear to show hybrid vigour in the 3rd generation outcrossed NPM dura with more individuals having higher values compared to the 3rd generation inbred NPM dura values as shown in Figures 2c and 2d, respectively.

Figure 3 shows the rate of growth for all the vegetative measures across the studied period (i.e. from 45 days to 105 days after sowing). In terms of height (Figure 3a), the growth for the 3rd generation inbred NPM dura showed the slowest rate While the 3rd generation outcross NPM dura showed an intermediate growth rate. Taken together with the earlier frequency distribution for height, our data suggest that there exist a degree of variation for height in the outcross population into 2 or more groups.

For the other 3 parameters, there appeared to be hybrid vigour in growth rate, particularly for relative total leaf area (Figure 4c).

CONCLUSION

At the end of the pre-nursery stage, the 3rd generation inbred NPM dura palms were significantly shorter in height compared to outcrossed NPM dura and inbred Deli dura (control) despite having significantly more number of green leaves per plant, larger relative leaf area and comparable stem diameter to the control.

NPM dura population outcrossed to another origin which is not known for low height trait produced progeny with high variation in height which most of them skewed toward the shorter level.

This study gives early indication of successful transfer of low height trait to the 3rd generation NPM dura population from its predecessor. And it also indicates the possibility to transfer the shortness trait to other taller origins. Similar phenotypic growth measures in the main nursery and eventually in the field will still be needed to confirm these early observations.

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TPGM

Simple sequence repeat identification and marker development for Kopyor mutant and normal coconut based on NGS genome data

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Abstract

Kopyor coconut is a natural coconut mutant from Indonesia having abnormal endosperm phenotype. Kopyor coconut is highly looked for in Indonesia and it has high economic values. The abnormal endosperm is soft and peel off from the shell. The mutant phenotype is governed by a single or most probably two loci having recessive allele. Kopyor characters is not an easy character to breed because of its recessive nature. Therefore, developing molecular marker associated with kopyor coconut would be beneficial. This study aimed to develop SSR markers using kopyor coconut and normal coconut NGS genomic data, use the developed markers to evaluate the genetic diversity and identify the markers associated with the Kopyor characters. Partial coconut genomic data have been generated from Kopyor and Normal coconuts, the raw reads were assembled using De novo assembly, and the assembled genomic data were used to identify the SSR sequence library. We have identified as many as 4,500,078 and 5,797,081 scaffolds with the scaffold sizes of 100 and 180kb for Kopyor and Normal coconuts, respectively. Thousands of SSR sequences were identified from the assembled genome data and some of them were successfully used to design SSR primers. Validation of designed primers to generate SSR markers were conducted and the validated primers were used to evaluate genetic diversity of Indonesian coconuts. The ultimate goals of this research were to identify SSR markers associated with Kopyor mutant phenotypes which can be used to support Kopyor coconut breeding program. Progress of this research and update of its results will be presented in the conference.

Key words: Coconut mutant, abnormal endosperm, SSR markers, genome sequences, genetic diversity

INTRODUCTION

Coconut (*Cocos nucifera* L.) is an important tropical fruit and oil crop which is continuous fruiting perennial and adapted in the humid to sub-humid coastal tropics (Wu *et al*. 2018). Coconuts are monocots, possessing a diploid genome with 16 pairs (2n = 2x = 32) of chromosomes (Suriya 2016). Coconut is usually called as 'tree of life' because have a multitude of uses besides oil and its importance in sustaining the life of the people who grow them.

Kopyor coconut has a unique characteristic compared to other coconuts which is the endosperm formation is not solid. The endosperm is soft and crumbly, detached from the shell, that is forming flakes filling up the shell (Novarianto *et al*. 2018). The kopyor coconut is natural mutant and the phenotype is genetically inherited from parents to their progenies (Sukendah 2009). The uniqueness of kopyor coconut makes it has high economic value. However, kopyor coconut and normal coconut only can be distinguish by seen the endosperm, both are very difficult to distinguish by phenotype especially in seedlings phase.Coconuts are difficult crops to breed. The long generation interval, the cross-pollinating breeding behavior of tall coconuts, the lack of a viable vegetative propagation method, the low number of seeds produced per palm, and the massive stature of the palm are the most important constraints in coconut breeding (Perera 2010). However, molecular biology techniques have been faintly used in the assessment of genetic resources and for the improvement of important agronomic and quality traits in *Cocos nucifera*.

For about two decades molecular techniques have been used to enhance the ability of coconut-breeding programs in several countries to assess the genetic diversity of local and global germplasm collections and to develop genetic linkage maps (Suriya 2016). Some molecular marker techniques have been developed over the years, using RAPD (Randomly Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter- Simple Sequence Repeat), gene-based SSR (Simple Sequence Repeats) markers, and SNPs (Single Nucleotide Polimorphism) (Dumhai *et al*. 2019). Among them, SSR have shown promising results for germplasm evaluation and molecular aided breeding (Tabkhkar *et al*. 2012). Unfortunately, markers capable of providing high-resolution information regarding kopyor mutant and normal coconut genome have not been readily available.

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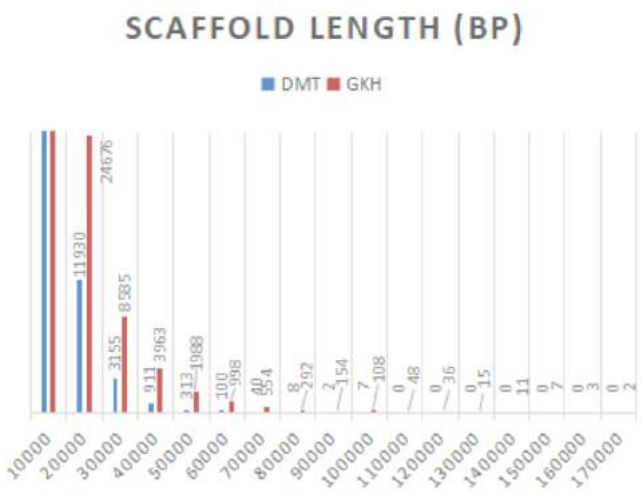

Figure 1. Scaffold length of normal coconut (DMT) and kopyor coconut (GKH)

Table 1. Next Generation Sequencing (NGS) and de novo assembly result summary from partial coconut (*Cocos nucifera*) genome sequences

NGS and *de novo* assembly summary	Number
Scaffold sequences number	5,797,081
Scaffold length (bp)	1,566,950,994.30
Shortest scaffold (bp)	100
Longest scaffold (bp)	102,316
Mean length of scaffold (bp)	270.3
N50	3,306

SSRs molecular marker are microsatellites with tandem DNA repeats (Singh *et al*. 2013). SSRs are repetitive DNA sequences that consist of 1–6 bp motifs widespread across both prokaryotic and eukaryotic genomes (Grover *et al*. 2012). They are widely distributed throughout the eukaryotic genomes in coding and non-coding regions. SSR markers are generally co-dominant and highly polymorphic, and are widely used for constructing genetic maps, biodiversity analysis and quantitative trait locus mapping (Singh *et al*. 2013). SSR markers can also be used for estimating the degree of relatedness of individuals or groups and for the detection of inter-varietal polymorphisms (Stepien *et al*. 2007).

Next-generation sequencing (NGS) is one of the recent developments in DNA sequencing technology that have offered a new avenue to acquire large genome sequences of non-model crops, mine SSR marker sequences and develop the required primers to generate SSR markers (Zalapa *et al*. 2012). As one of the powerful next-generation sequencing tools, Illumina HiSeq2000 has proven to be robust and efficient with much higher throughput and much lower cost (Feng *et al*. 2012).

In this study, we aimed to identify and develop new SSR markers from genome sequences of kopyor mutant and normal coconut using the PHOBOS application. The raw data are from NGS using Illumina HiSeq2000. Following de novo assembly of the raw reads, the genomic sequence data were subsequently used to mine SSR sequences, design appropriate primers and develop specific SSR markers for kopyor mutant and normal coconut.

MATERIAL AND METHODS

NGS And Genome Assembly

Total DNA was isolated from leaf samples of normal coconut and kopyor coconut using the standard CTAB method modified for DNA isolation from palm leaves (Maskromo *et al*. 2016, Novero *et al*. 2012, Pesik *et al*. 2015, 2017, Tinche *et al*. 2014). We performed sago palm genome sequencing of DNA extracted from young leaves using the Illumina GAIIx instrument. Paired-end genomic library construction (2 × 72 bp) was conducted with a commercial Nextera XT Index with TruSeq Dual Index Sequencing Primer Box kit, following the manufacturer's

Table 2. Distribution of perfect SSRs in genomic sequences of coconut (*Cocos nucifera*)

Motifs length	Number of loci identified	Mean of repeat number	Cumulative length (kb)	Density (SSR/Mb)*
Di-	8,003	9.9	157,915	5.11
Tri-	3,446	5.6	58,017	2.20
Tetra-	3,093	4.3	53,566	1.97
Penta-	2,286	3.5	40,210	1.46
Hexa-	2,739	3.0	49,901	1.75
Total	19,576	26.4	359,609	12.49

*Density of SSR was calculated using ratio between the number of SSR loci over the identified total scaffold length (1,566.95)

Table 3. Frequency of dinucleotide and trinucleotide motifs in genomic sequences of coconut (*Cocos nucifera*)

Dinucleotide		Trinucleotide	
Motifs	Frequency (%)	Motifs	Frequency (%)
AG	46.74	AAT	29.34
AT	38.70	AAG	27.83
AC	13.62	AGG	11.33
CG	0.94	ATC	8.57
		ACC	6.01
		CCG	5.06
		AAC	4.88
		AGC	4.91
		ACT	1.16
		ACG	0.90

protocol. After quality trimming of raw reads with Trimmomatic, we used Ray software (https://github.com/sebhtml/ray) for de novo genome assembly. Subsequently, we used the assembled normal coconut genome sequences for SSR mining and marker development.

SSR Sequence Mining From KOPYOR And Normal Coconut Genome Scaffold

We used the assembled scaffold data to search for di-, tri-, tetra- and hexanucleotide repeats of SSR loci of at least 20 bp lengths. We used Phobos software (http://www.ruhr-uni-bochum.de/ecoevo/cm/cm_phobos.htm) to mine SSR motifs from the assembled genome sequence. Identified SSR loci were then grouped into either perfect or imperfect SSRs and designated as either class I or II SSRs. SSR locus density was determined based on the frequency of SSR loci and the total length of contigs containing SSRs. We also evaluated the motif length, loci numbers, mean repeat numbers and densities for the selected repetitive motifs.

SSR Primer Design And Marker Development

To design SSR primers, we selected 100 of the total class I SSR loci with a minimum of 10-fold coverage from the outputs of Phobos software. The scaffold containing selected SSRs were used for SSR primer design using Primer3-Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The parameters for SSR primer design included 200–600 bp amplicon size, 18 bp optimum primer size, 50°C–60°C primer

Table 4. List of sequences of SSR primer pairs

No.	Primer	Primer sequences (5'-3')	Motifs
1	scaffold157925_cov38	F: TTCACATGGTCGAATACAAA	AC
		R: TGAAAGGGACTGGTAAAGAA	
2	scaffold91208_cov38	F: GTGGTTTTCACCTAATTCCA	AC
		R: GTGGTTTTCACCTAATTCCA	
3	scaffold53767_cov39	F: ACAATTTGCAGCTATGGTTT	AC
		R: TCTCTCTGTTTCATCCATCC	
4	scaffold77143_cov37	F: TCGGGACTTAAACAAAAAGA	AT
		R: TTTGAATGAGAGCTTCTGGT	
5	scaffold265623_cov37	F: ACCCTTCTACTATTTTCCTTGT	AT
		R: TTTTTGCTCCCAAACTCTAT	
6	scaffold569263_cov40	F: GTCCAAATTCGACCAATCTA	AT
		R: GTGGACTGCAATGTCTCTCT	
7	scaffold69558_cov38	F: TAACATGGGATTGTCCTTTC	AG
		R: TGCGAACCTAATTTAAGACC	
8	scaffold619407_cov40	F: CTCTTGTTGGGTGGTATCAT	AT
		R: TGTTCCTAGTTTTCCTCGAT	
9	scaffold926705_cov37	F: CAGGTTGTTACTGGTGGTCT	AC
		R: AAAACCACCAACCATAACAG	
10	scaffold1130327_cov39	F: GCTTCAAACTTCCTTCACAG	AC
		R: TTGGAAGAAATTTTGGAAGA	
11	scaffold38227_cov40	F: GCATAGGTCATAGGCTGAAC	AC
		R: ATAGCAGTCCCCAACACTTA	
12	scaffold245936_cov39	F: ATGTAAGGAAGGGGAAAAAG	AC
		R: CTTGAATTTGGATGGTTCAT	
13	scaffold586646_cov39	F: TCTACACCGGCAATAAGTTT	AT
		R: ATTGCAGACCGGTATTTATG	
14	scaffold209105_cov40	F: CTCTCTATCTTGGCAACCAT	AG
		R: GCATTCTGCCTCAGTAAATC	
15	scaffold1088912_cov40	F: TGCAGAAACCAGTTATTCCT	AC
		R: AAAATCCCATAAAATGTCCA	
16	scaffold271239_cov38	F: ACCACGTACACATTGCACTA	AG
		R: TCCAGAAGTCTCTTCAGCAT	
17	scaffold10991_cov38	F: ATCAAAATTGAAGGTCAGGA	AT
		R: CCCATCGACAGAGATGTACT	
18	scaffold408260_cov36	F: GACAGCTTGACAAGGAGAAC	AC
		R: GTTCTCCTTGTCAAGCTGTC	
19	scaffold41_cov39	F: GCAGTTCCACAAATTTCTTC	AC
		R: AAATTTGTGGGATGAAGATG	
20	scaffold569674_cov37	F: ATGGTTTGGATCTTGATTTG	AC
		R: GATTGTCAGCTTCGAGTTTC	

melting temperature (Tm) and 40%–60% primer GC content. Once the SSR primers were identified, we performed synteny analysis on selected contigs containing SSR loci. Synteny analysis was performed on coconut (*Cocos nucifera*) chromosome sequences available from the NCBI website to evaluate their probable position distributions in the coconut genome.

RESULTS

NGS And Assembly Of *Cocos nucifera* Genome

In this study we use two type of coconut, normal coconut (DMT = Dalam Mapanget) and kopyor coconut (GKH = Gejah Kopyor Hijau). The raw reads of partial genome sequence data was generated using the Illumina GAIIx paired-end NGS system. From de novo assembly, the longest scaffold in DMT was 102,316 bp and in GKH was 177,450 bp (Figure 1). The primer was designed from DMT genome data then the genome data was analyzed. Results of total nucleotide composition analysis indicated that thymine was the most frequent base (T = 31.2%), followed by adenine (A = 30.9%), guanine (G = 18.9%) and cytosine (C = 18.7%). The percentage of GC content in partial genome sequences of normal coconut was approximately 37,7%. A total of 5,797,081 scaffold was identified following the de novo assembly (Table 1). The minimum length of the assembled scaffold was 100 bp and the maximum scaffold

length was 102,316 bp. The average length of the assembled scaffold was 270.3 bp, whereas that of N50 was 3,306 bp (Table 1).

SSR Sequence Mining

We identified 19,567 loci of perfect SSRs from the partial genome data of normal coconut (Table 2). Dinucleotides (8,003; 40.88%) were the most frequently found types among perfect SSR motifs, then following by trinucleotides (3,446; 17.60%), tetranucleotides (3,093; 15.79%), hexanucleotides (2,739; 13.99%), and pentanucleotides (2,286; 11.67%) were the least frequently found (Table 2). The cumulative length of the SSR-containing scaffold was 157,915 kb for dinucleotide and 40,210 kb for pentanucleotide SSRs, while the SSR loci densities were 5.11 loci/Mb for dinucleotides and 1.46 loci/Mb for pentanucleotides (Table 2). The frequency of SSR cumulative length decreasing from dinucleotide to pentanucleotide, while hexanucleotide was more longer than pentanucleotide (Table 2). The total density was 12.49 SSR/Mb, whereas dinucleotide density was 5.11 SSR/Mb and pentanucleotides was 1.46 SSR/Mb (Table 2). The mean repeat number for dinucleotides was 9.9, whereas that for pentanucleotides was 3.5 (Table 2).

Among the four dinucleotide repeat motifs found in the coconut genome (AC, AG, AT or CG), the AG repeat was most common (46.74%), whereas the CG repeat was least common (0.94% of the total number of SSRs) (Table 3). For trinucleotides, the AAT repeat (29.34%) was most common, whereas the ACG repeat was least common (0.9% of the total SSR) (Table 3).

SSR Design Primer

To design primers, we randomly selected 150 dinucleotides of class I SSR loci. However, for 33 (22%) of these randomly selected loci, we could not design the flanking primers because of either unsuitable flanking sequences or Tm constraints. We designed flanking primers for 117 (78%) selected loci. Next, we selected 20 primers each from 20 longest scaffold.

DISCUSSION

Our genomic analysis revealed a comprehensive genome of the normal coconut and kopyor coconut using Illumina sequencing technology, contributed a new and significant set of sequence reads, and provided a set of NGS genome data through de novo assembly. Our analysis also identified 19,576 genomic SSRs in the normal coconut genome. These findings not only provide valuable information for genetic diversity, linkage mapping, gene/QTL mapping, and marker-assisted selection in cowpea but also show promise in the search for informative molecular markers in a crop plant with few genomic resources (Chen *et al*. 2017).

We compared scaffold length of normal coconut and kopyor coconut. The longest scaffold in normal coconut (DMT) genome data was 102,316 bp and in kopyor coconut was 177,450 bp. However, the number of scaffold in DMT was 5,797,081 and in GKH was 4,500,078. Kopyor coconut (GKH) is a natural mutant, by seeing the number of scaffold from both DMT and GKH, kopyor coconut might be has a deletion in their sequences. Deletion means when a part of chromosome is deleted (Mahdieh and Rabbani, 2013).

The efficiency of SSR development was often limited because of short or missing flanking regions (Sharopova *et al*. 2002). We selected 150 dinucleotides of class I SSR loci from normal coconut genome to design their flanking primers. However, we couldn't found flanking region of 33 dinucleotides. We designed 117 flanking primers, and 20 primers had been choosen from the longest scaffold. These flanking primers from the longest scaffold are expected to get high quality amplification product when it used as PCR primer. Motifs from designed primers was AT, AG, and AC, which the frequency each of them was 46.74%, 38,70%, and 13,62%. We are not designed primers from CG motif because of the frequency was too low (0.94%).

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TPGM

Wild *Ipomoea* species inhabiting coastal area of Malaysia

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Abstract

Ipomoea is one of the largest genus in the family of Convolvulaceae. The genus consists of 600 – 700 species worldwide in the tropical and subtropical region. In Malaysia, it has been reported that nearly 40 species of *Ipomoea* which can be found in the highland, wetland and coastal zones. Inventory survey was conducted to determine the diversity of Ipomoea species in Malaysia from 2015 until 2019. From the survey, a total of 17 species of *Ipomoea* were found. One species was found in the highland, 12 species were found in lowland (especially near the swampy area), one species was found in both highland and lowland and 3 species inhabit along the coast. The species that were found along the coastal area were namely *Ipomoea pes-caprae* (L.) R. Br., *Ipomoea imperati* (Vahl) Griseb and *Ipomoea littoralis* (Blume). *Ipomoea pes-caprae* was found in every state in Malaysia excluding Sarawak, with striking purple to pinkish purple corolla with obcordate leaves and truncate base. The leaf has not lobed length varied from 3 to 9 cm and 3 to 10 cm wide. The seed is pubescent, dark brown and broad ovate shaped covered in pale brown capsule. *Ipomoea littoralis* can be found in 5 states including Johor, Perak, Terengganu, Pahang and Sabah, while *Ipomoea imperati* can be found in Johor, Kedah, Terengganu and Kelantan. Both have unique leaves that varies in shape. The leaf of *I. imperati* varies from hastate to reniform in shape with 3 to 5 lobes, while *I. littoralis* has reniform to cordate leaf shape with only 1 lobe. *Ipomoea imperati* has small leaf ranged from 2 to 4 cm long and 1 to 2 cm wide. The corolla is white with yellow throat with semi-stellate limb, while the seed is pubescent, dark brown in colour and ovate in shape. *Ipomoea littoralis* leaves range from 1 to 8 cm long and 1 to 7 cm wide. The flower is pink in colour with darker pinkish throat and pentagonal limb. The seed is glabrous brown and ovate shaped but rarely found. The study revealed there were no affinities between these three species by comparing polygonal diagram of the studied characters.

Key words: Wild *Ipomoea*, coastal area, morphological variation

,INTRODUCTION

Ipomoea species can be mostly found in sandy area. *Ipomoea* is one of the largest genus in the Convolvulaceae family. The genus consists of 600 – 700 species (Austin & Huaman 1996) which inhabit the tropical and subtropical region. As reported by Ooststroom 1940, there were 40 species of *Ipomoea* species found throughout Malaysia. Out of these 40 species reported, six species inhabit the coastal area namely; *Ipomoea gracilis* R. Br., *Ipomoea maxima* (L. f.) Sweet, *Ipomoea pes-caprae* (L.) R. Br., *Ipomoea imperati* (Vahl) Griseb., *Ipomoea digitata* L. and *Ipomoea violacea* L (Ooststroom, 1940). While, *Ipomoea littoralis* Blume, *Ipomoea pes-caprae* (L.) R. Br. and *Ipomoea imperati* (Vahl.) Griseb were 3 species that resides the sandy beaches area especially in coast of Sabah and Peninsular Malaysia. *Ipomoea pes-caprae* (beach morning glory) or known as beach morning glory is the most frequent species that can be found in all coast of Peninsular Malaysia and Sabah. Ipomoea species reported to have medicinal values. As claimed by Mohd Zahari *et al.* (2016), *I. pes-caprae* can be used to detect metal pollution at beach area. It is also can be used as medicine to treat jellyfish dermatitis (Chan *et al*. 2016). *I. littoralis* had always been confused with *I. gracilis* a species endemic to Australia (Austin, 1991) and belongs to the aquatic genera (Jana & Mondal, 2017). *I. imperati* species distribution is in pantropical and Mediterranian also can be found in the Malay Peninsular and Philipine islands. Before *I. imperati* name was used, it is widely called as *I. stolonifera* and had been confused with *I. littoralis* due to its difference in many descriptions and floras (Valva & Sabato 1983). This paper present the update of the *Ipomoea* species diversity in Malaysia including the coastal area.

Materials and Methods

An inventory of wild Ipomoea species was conducted throughout Malaysia including Sabah especially along the coastal, swampy area and highland. This project was part of the collaboration research with The Royal Botanic Gardens, Kew from 2015 until 2019. All of *Ipomoea* species were collected randomly, identified and referred using 'The Convolvulaceae in

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Morphological key

A simple identification key based on leaf lobe, seed pubescent, leaf shape, limb shape and flower colour of targeted species is shown in Key 1 below.

1. Leaf lobe present			*I. imperati*
1. Leaf lobe absent			
	2. Seed pubescent		
		3. Leaf obcordate, Emarginate central lobe, Rounded limb, purple to pinkish purle flower	*I. pes-caprae*
		4. Leaf reniform to cordate, one leaf lobe, central lobe obtuse, Limb pentagonal, pinkinsh flower	
	2. Seed glabrous		*I. littoralis*

Key 1. Morphological key of *Ipomoea pes-caprae, Ipomoea imperati* and *Ipomoea littoralis*

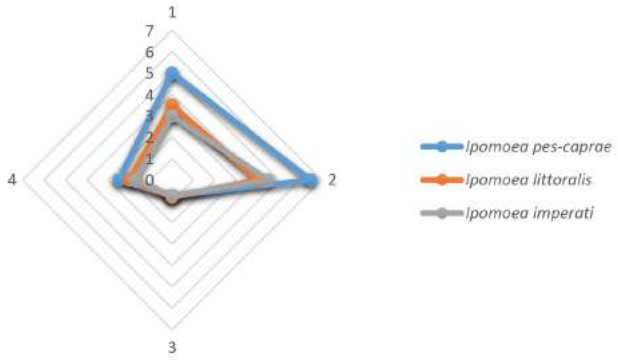

Figure 1. Polygonal representation of the floral parts measurements of *Ipomoea pes-caprae, Ipomoea littoralis* and *Ipomoea imperati*

Malaysia III' and 'The Flora of Thailand'. Polygonal representation of floral and foliar characteristics were constructed. All herbarium samples were prepared and deposited in the MARDI Herbarium (MDI), Royal Botanic Gardens, Kew and Universiti Malaysia Sabah Herbarium (BOHR).

RESULT AND DISCUSSION

As 4 years span of this collaboration project, there were 17 species of *Ipomoea* found in Sabah and Peninsular Malaysia. The *Ipomoea* species that were found were; *Ipomoea aquatica* Forssk, *Ipomoea batatas* (L.) Lam., *Ipomoea cairica* (L.), *Ipomoea campanulata* L., *Ipomoea carnea* Jacq. spp. *fistulosa, Ipomoea imperati* (Vahl) Griseb., *Ipomoea indica* (Burm.) Merr., *Ipomoea littoralis* Blume., *Ipomoea obscura* (L.) Ker Gawl.., *Ipomoea nil* (L.) Roth., *Ipomoea purpurea* (L.) Roth., *Ipomoea pes-caprae* (L.) R. Br., *Ipomoea pes-tigridis* L., *Ipomoea quamoclit* L., *Ipomoea sagittifolia* Burm. f., *Ipomoea tricolor* Cav.and *Ipomoea triloba* L.

Out of all species of *Ipomoea* reported occurred in Malaysia, only three *Ipomoea* species grew in the coastal area, namely *Ipomoea pes-caprae* (L.) R. Br., *I. littoralis* Blume and *I. imperati* (Vahl) Griseb. Botanical descriptions with reference to taxonomic characters are given below.

***Ipomoea pes-caprae* (L.) R. Br.**

Perennial creeper, stem prostrate along the seashores, rooting at the nodes. The leaf is green and obcordate with truncate base, no lateral lobes. The shape of central lobes is emarginated with 3 – 9 cm long and 3 – 10 cm wide. The petiole is glabrous with 2 – 10 cm long. Flower is funnel form, 4 to 6 cm long and 6 to 7 cm wide. Limb shape is rounded with purple to pinkish purple colour and darker pink throat. Sepal is glabrous, ovate to elliptic with obtuse apex. The length of sepal is ranged from 0.5 to 1 cm. Pedicel is 2 to 3 cm long. The style and stigma is pale pink in colour. Seed broad ovate, dark brown and densely covered with hair.

***Ipomoea imperati* (Vahl) Griseb**

Perennial creeper of the seashore and rooting at the nodes. The leaf is green that varies in shape from hastate to reniform. 3 or 5 leaf lobe with moderate and deeply type. 2 to 4 cm long with 1 to 2 cm wide. Petiole glabrous with 1 to 2.5 cm long. Flowers is white with bright yellow throat with 2 to 4 cm long and 4 to 5 cm wide. Limb shape is semi-stellate, ovate sepal and acute apex. Sepal glabrous with 0.5 to 1 cm long. Pedicel is 1.5 to 2 cm long. Pale yellow colour of stigma and style. Seed ovate, dark brown and densely covered with pale brown hair.

***Ipomoea littoralis* Blume**

Perennial creeper and twiner and rooting at the nodes. The leaf got no lateral lobe with green reniform to cordate leaf shape. It has one lobe with obtuse central lobe. The leaf range from 1 to 8 cm long and has 1 to 7 cm wide. The petiole is glabrous with 0.5 to 5 cm long. Flowers is pinkish with pentagonal limb shape. The flower is 3 to 4 cm long with 3.5 to 4.5 wide. Sepal is glabrous, elliptic with acute to obtuse apex

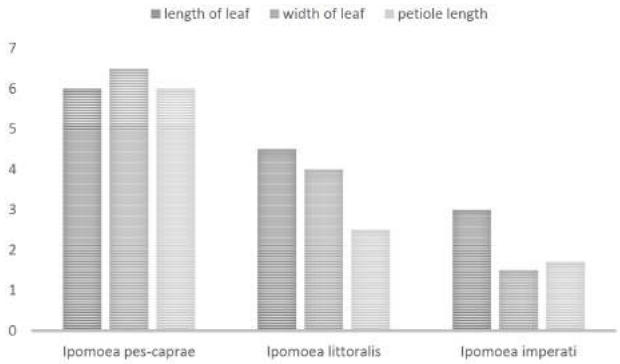

Figure 2. Table diagram representation of the foliar parts measurements of *I. pes-caprae, I. littoralis* and *I. imperati*

Plate 2. *Ipomoea imperati*: (a) Growth habit (b) Inflorescence

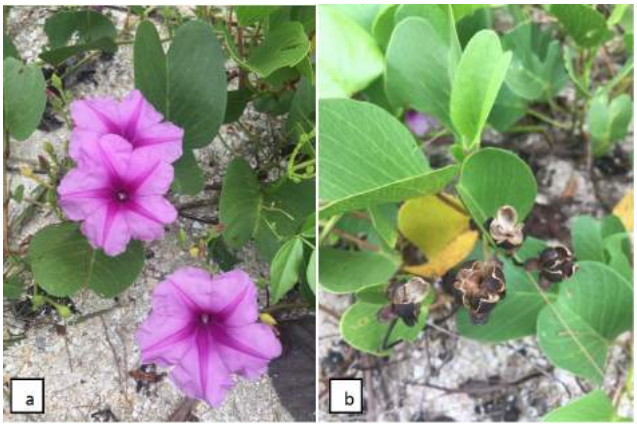

Plate 1. *Ipomoea pes-caprae*: (a) Inflorescence, (b) Fruits and seeds

Plate 3. *Ipomoea littoralis*: (a) Growth habit (b) Inflorescence

and 0.6 to 1 cm long. Pedicel is 1.5 to 2.5 cm long. Stigma and style are pale purple in colour while seed is glabrous brown with ovate shape.

According to the result shown in the table, the presence of the leaf lobe separated one species, *I. imperati* from the rest of two species. Meanwhile, *I. pes-caprae* and *I. littoralis* are separated according to the hair presence at their seed which *I. pes-caprae* seed are fully covered with hair while, *I. littoralis* seed are glabrous. They were little difference between this morphological key compared to the other researcher key. Morphological key as mention in the Flora of Thailand were separated by the leaves shape, leaves segments and sepal shape. The result showed their morphological difference plays the main part in classification of each species in *Ipomoea*. As suggested by Austin (1979) and Rabei and Abdel Khalik (2012) that stated the morphological characters of leaves and seeds used as one of taxonomic importance.

Variation in floral and foliar measurements

Measurements of morphologically important characters showed that these 3 species varied in both floral and foliar parts. These variations were confirmed by using polygonal representation of the floral parts shown in Figure 1 and foliar parts measurements shown in Figure 2.

Based on Figure 1, it shows that *I. pes-caprae* had the most distinct value compared to other species on three different categories and showing no different on one specific category. Figure 2 showing the differences between 3 categories; leaves length, leaves width and petiole length. The result shows that *I. pes-caprae* had the most significant value compared to *I. imperati* and *I. littoralis*.

CONCLUSION

Research shown that only three species were discovered to reside the coastal area; *I. pes-caprae, I. littoralis* and *I. imperati*. There were many things to be discovered for the future uses including its micromorphology, phenology, molecular study and others since there were few findings and limited information related to this genera done in Malaysia.

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TPGM

Preliminary study of paternal effect on the characters of 'Musang King' durian (*Durio zibethinus* L.) fruit from cross-pollination

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Abstract

The aim of this research was to study the paternal effect of 'Musang King' Durian (*Durio zibethinus* L.) fruit characters from cross-pollination with selected popular durian clones. The study was conducted at Top Fruits Sdn Bhd durian orchard in Parit Sulong, Batu Pahat, Johor. Four conducted treatments were assisted cross-pollination using pollen from durian clone 'D24', 'D168', 'D190', and 'D200'. Observations on fruit setting were made every two weeks until abscission of fully ripe fruit. Cross-pollination with 'D24', 'D168', 'D190', and 'D200' produced 7.14%, 13.04%, 8.70%, and 12.50% fruit set respectively. Preliminary data on harvested fruits showed that there was no significant difference on fruit weight, fruit circumference, length of stalk, thickness of stalk, spine length, rind thickness, number of locules per fruit, number of locules without pulp unit, seed weight per fruit, seed number per fruit, percentage of deflated seed, percentage of edible portion, flesh thickness, and total soluble solid content (TSS). Their flesh colour ranged from light yellow to yellow orange (RHS 11A, 11B, 12A, 12B, 12C). Pollen from different durian cultivars can affect percentage of fruit setting in 'Musang King'. There was no paternal effect from 'D24', D168, 'D190', and 'D200' on characteristics and morphology of 'Musang King' fruit. Preliminary sensory evaluation found that different pollen source could affect 'Musang King' pulp taste. Further study with more fruit samples and respondent could be done to further evaluate these findings.

Key words: Self-incompatibility, metaxenia, mix-planting, single cultivar, fruit set

INTRODUCTION

'Musang King' is one of the most valuable durian in Malaysia. It was registered by Department of Agriculture Malaysia with registration code D197 (Department of Agriculture, 2019). Other names for 'Musang King' are 'Raja Kunyit' and 'Mao Shan Wang'. It has yellow flesh colour with obovoid to oblong fruit shape, and mix of sweet, creamy and bitter taste. Musang King was produced naturally by natural pollination and being clonally propagated in large scale since its being registered in 1993. Nowadays, there are increasing of planting 'Musang King' planting area in Malaysia due to high demand for export to China.

'D24', 'D168', and 'D200' are among other popular durian cultivars being sold in Malaysia. Other names for 'D24' is 'Durian Sultan', for 'D168' are 'IOI' or 'Hajah Hasmah', while for 'D200' is 'Black Thorn'. Both 'D197' and 'D200' are commonly sold at premium price in Malaysia with price of more than RM 30 per kilogram, while 'D24' and 'D168' are sold at medium price of around RM 20 to RM30 per kilogram, following market demand and supply.

Durian has perfect flower with both male and female parts on the same flower. Durian flowers grow in cluster of 5 to 30 flowers on primary or secondary scaffold branches (Honsho *et al*. 2004b). The common pollinators for durian are fruit bats and honey bee (*Apis dorsata*) (Bumrungsri *et al*., 2008) because durian flowers have nocturnal character (Davis and Bhattacharya, 1974). In order to set fruit, durian pollination need to take place during night. According to Honsho *et al*. (2004b), anthesis of 'Mon Thong' durian started at 1600 hours and the flower will fully opened at around 1900 hours. The same characters observed on durian cultivars in Malaysia such as 'D24', 'D99', and 'Musang King' (personal observation).

Many popular durian cultivars were reported to be self-incompatible, including 'Musang King' (Muhammad Afiq *et al*., 2018; Kozai *et al*., 2014; Bumrungsri *et al*., 2009; Honsho *et al*., 2004, George *et al*., 1994). They need pollen from different cultivars to successfully set fruit. Indriyai *et al*. (2012) and Lim

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Table 1. Effect of male parent on mean period to fruit maturity from anthesis

Parent	Days
'D168'	110
'D200'	102
'D24'	104
'D190'	108

Table 2. Percentage of fruit set from different cross-pollination treatment on Musang King

Crossing combination	Number of cross-pollinated flowers	Number of fruit harvested	Fruit set percentage (%)
'Musang King' x 'D168'	23	3	13.04
'Musang King' x 'D200'	24	3	12.50
'Musang King' x 'D24'	28	2	7.14
'Musang King' x 'D190'	23	2	8.70

Table 3. Mean squares for characteristics of 'Musang King' fruit produced after cross-pollination with 'D24', 'D168', 'D190', and 'D200' pollen

Source	Df	Fruit character													
		Fruit weight [g]	Fruit circum ference [cm]	Length of stalk [mm]	Thickness of stalk [mm]	Spine length [cm]	No of locules per fruit	No. of locules without pulp unit	Rind thickness [mm]	Seed weight per fruit [g]	Seeds number per fruit	Percentage of deflated seed [%]	Flesh thickness (mm)	Edible portion [%]	TSS (%)
Treatment	3	678200.6ns	37.8ns	15.2ns	25.3ns	0.1ns	0.1ns	0.8ns	3.0ns	31791.1ns	51.5ns	1632.6ns	9.2ns	11.8ns	6.6ns
Error	6	892769.1	46.3	90.4	12.9	0.3	0.3	2.2	4.9	9314.4	30.6	577.6	6.4	40.0	15.2
Total	9														
CV		45.1	13.1	13.4	22.5	6.4	10.4	185.2	16.8	53.3	44.6	37.0	21.6	24.6	13.9
Mean		2095.3	52.0	70.9	16.0	8.5	5.3	0.8	13.1	181.2	12.4	64.9	11.8	25.7	28.1

ns: not significantly different at p<0.05

and Luders (2009) reported that pollen source can affect durian fruit characters such as fruit size and total number of seeds per fruit. The effect is commonly known as paternal-effect. There is still no information on paternal effect on 'Musang King'. This paper provides report on preliminary study of paternal effect on 'Musang King' fruit setting, fruit characters and taste. The information will be useful to identify the best pollen donor cultivarsfor 'Musang King' production.

MATERIALS AND METHODS

Plant Materials and Artificial Pollination

This study was conducted at Top Fruit Sdn Bhd durian farm in Parit Sulong, Batu Pahat, Johor (2°0'N, 102°52') during February to June, 2019, using a single 'Musang King' plant with age about 15 years old. Flowers at the 'white' stage (Kozai and Higuchi, 2011) were emasculated between 0900 hours to 1500 hours, and the flower clusters were covered with costume-made muslin bag to prevent natural pollination. Flower clusters were thinned to two to seven flowers per cluster. Seven to eight flower clusters were emasculated for each cross-pollination treatment. Fresh anthers were collected at 2000 hours from four different durian cultivars registered as 'D24', 'D168', 'D190', and 'D200'. The flowers were pollinated at anthesis around 2030 hours to 2300 hours. Flowers were covered after pollination and removed second weeks after anthesis. The number of flowers or fruits that remained attached to the plants was counted on 2, 4, 6, 8, 10, 12 weeks after anthesis and during mature fruit abscised. Fruits were tide with small rope at 12 weeks after anthesis to prevent fully ripe abscised fruit from drop to the ground.

Measurement of Fruit Characteristic and Morphology

Fully ripe abscised fruits were weighed and stored in cold room at 7°C within 7-14 weeks by following storage method by Nur Azlin *et al*. (2019), until further data measurement was made. The following characteristics and morphology were measured and recorded: fruit circumference, length of stalk, thickness of stalk, fruit colour, fruit shape spine length, spine shape, character of spine at fruit apex, character of spine at the base of the fruit pedicel, rind thickness, number of locules per fruit, number of locules without pulp unit, seed weight per fruit, seeds number per fruit, percentage of deflated seed,

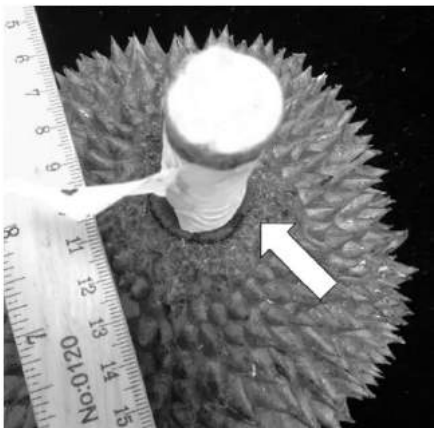

Figure 1. White arrow shows area with absent of spine around the base of the fruit pedicel which represent another common character for 'Musang King' fruit

Table 4. Fruit characteristics of 'Musang King' fruit produced after cross-pollination with 'D24', 'D168', 'D190', and 'D200' pollen

Pollen source	'D24'	'D168'	'D190'	'D200'
No. of fruits harvested / flowers pollinated	2/28 (0.07) *	3/23 (0.13)	2/23 (0.09)	3/24 (0.13)
Fruit weight [g]	2137.5±1037.5	1550.7±177.9	2850.5±650.5	2108.3±601.1
Fruit circumference [cm]	52.8±7.8	47.6±1.1	57.2±4.2	52.5±4.4
Length of stalk (mm)	70.8±0.3	72.9±4.1	72.6±3.8	67.9±8.3
Thickness of stalk (mm)	17.6±2.6	11.9±2.7	18.9±1.7	17.0±1.5
Spine length [mm]	8.7±0.3	8.5±0.2	8.4±0.8	8.4±0.2
Rind thickness [mm]	12.4±3.0	12.8±0.7	12.4±2.0	14.6±0.5
No. of locules per fruit	5.5±0.5	5.3±0.3	5±0	5.3±0.3
No. of locules without pulp unit	0.5±0.5	1.0±0.6	0±0	1.3±1.3
Seed weight per fruit [g]	151.1±96.1	90.0±25.1	365.9±103.9	169.4±44.8
Seeds number per fruit	12.5±4.5	7.7±1.5	19±3	12.7±4.3
Mean seed weight [g]	10.7±3.8	14.2±6.6	18.9±2.5	14.3±1.7
Percentage of deflated seed (%)	85.3±14.7	86.7±13.3	32.67±23.6	51.15±11.9
Thickness of flesh [mm]	14.0±2.6	12.6±0.8	11.7±3.0	9.5±0.9
Edible portion [%]	25.92±4.56	24.9±2.9	29.27±2.5	24.09±4.77
TSS (%)	29.95±1.05	27.71±2.02	29.34±3.6	26.33±2.55
Flesh colour	RHS 12B (yellow), RHS 12C (light yellow)	RHS 11A (yellow orange), RHS 12B (yellow)	RHS 11A (yellow orange), RHS 11B (light yellow)	RHS 12A, RHS 12B (yellow)

* Number in parenthesis indicated fruit set ratio

Table 5. Panel respond on similarity of tested sample to 'Musang King' character and taste (sample in form of pulp)

Sample	Respond on similarity of sample character and taste to 'Musang King' (%)	
	Agree	Not agree
OP1	85.7	14.3
OP2	100	0
OP3	71.4	28.6
OP4	33.3	66.7
OP5	75	25
'Musang King' x 'D24'	12.5	87.5
'Musang King' x 'D168'	37.5	62.5
'Musang King' x 'D190'	42.9	57.1

OP1: Fruit number 1 from natural pollination; OP2: Fruit number 2 from natural pollination; OP3: Fruit number 3 from natural pollination; OP4: Fruit number 4 from natural pollination; OP5: OP1: Fruit number 5 from natural pollination.

percentage of edible portion, flesh thickness, and total soluble solid content (TSS). The experimental design was a completely randomized design (CRD). One-way ANOVA was applied to evaluate the significant difference in selected fruit characteristics parameters, and the differences between treatment means were compared by the Least Significant Difference (LSD) method at ($P<0.05$).

Sensory Evaluation

Pulp from fruit for each cross-pollination treatment and from representable 'Musang King' fruits produced from natural pollination were stored in vacum container at -18^{o}C following storage method reported in Nur Azlin *et al*. (2016), within 14 days after fully ripe fruit abscission until survey on taste evaluation was conducted. Pulp were thawed at room temperature assisted with electrical fan for two and a half hours before survey. Blind sensory evaluation was made by eight well trained respondent who had experience of eating 'Musang King' more than 20 times and can recognize its characters and taste easily. The respondents were asked to evaluate each sample and compare their characters and taste with commonly present character on 'Musang King'. The need to evaluate base on flesh colour, aroma, texture, creaminess, and bitterness. After fully examine and taste each sample, they need to decide whether each sample is similar to 'Musang King' or not.

RESULTS AND DISCUSSION

Abscission of fully ripe fruits for all cross-pollination treatment occur within 102 to 110 days after anthesis (Table 1). During fully ripe fruit abscission, percentage of fruit harvested from cross-pollination treatment with 'D168', 'D200', 'D190' and 'D24' were 13.04%, 12.50%, 8.70%, and 7.14% respectively (Table 2). The result was consistent with Muhammad Afiq *et al*. (2018) on the ability 'D24' to be used as pollen source for 'Musang King'. In the report, fruit setting at 12 weeks after anthesis from cross-pollination with 'D24' produced 37.92% fruit setting. The reason of lower fruit retention in this study, probably due to different agronomic practice and environmental condition between both study area. Lim and Luders (2009) reported that fertilization failure, low level of nutrients, insufficient water, and adverse weather conditions during fruiting can cause low percentage of fruit set. Meanwhile, Masri (1999) reported that restricted soil moisture conditions could cause premature fruitlet drop.

There was no significant difference on fruit characteristics between different cross-pollination treatment including fruit weight, fruit circumference, spine length, number of locules per fruit, number of locules without pulp unit, rind thickness, seed weight per fruit, seeds number per fruit, percentage of deflated seed, percentage of edible portion, length of stalk, thickness of stalk, and TSS (Table 3). These findings consistent with Honsho *et al*. (2009) who found that fruit characteristics were not so affected by pollen. Paternal effect could only occur on cross-pollination between specific durian cultivars such as reported by Indriyani *et al*. (2012) and Lim and Luders (2009).

There was no difference on 'Musang King' fruit morphology produced from all cross-pollination treatment. They had green fruit colour, similar length of pyramid shape spine (Table 4), five number of locules, present of viable and deflated seeds in every fruit, present of "star-shaped" pattern at the fruit apex due to present of small spine, absent of some

spine and unique spine arrangement, absent of spine at the base of the fruit pedicel (Figure 1), and obovoid to oblong fruit shape which are common in 'Musang King'. These morphology characters could only be control by maternal effect. These morphology characters are valuable in identifying and verifying 'Musang King' fruit at market.

Table 5 shows taste evaluation on 'Musang King' fruits produced from different cross-pollination. The pulp was stored before evaluation, so that fruits from all treatment can be collected and chosen for taste evaluation at the same time. Comparison of samples between different cross-pollination treatment showed that sample from cross-pollination with 'D190' had highest agreement on its similarity to 'Musang King', followed by from cross-pollination with 'D168' and 'D24', with 42.9%, 37.5% and 12.5% respondent agree respectively. However, their similarity scores were lower than obtained by 'Musang King' fruits produced from natural pollination OP1, OP2, OP3, OP5 (71.4% – 100% agree), and almost similar to OP4 (33.3% agree). OP1, OP2, OP3, and OP5 could be produced from natural pollination of 'Musang King' flower with pollen form other durian cultivars available at the farm. Paternal effect could influence the volatile compound content in the fruit. Aziz et al. (2019) reported that durian unique flavour, taste and odour were determined by different type and amount of volatile compound available within the pulp. Further study could be done to further evaluate on the effect of paternal parent on volatile content of durian.

CONCLUSION

Pollen from different durian cultivars can affect percentage of fruit setting in 'Musang King'. There were no paternal effect from 'D24', 'D168', 'D190', and 'D200' on characteristics and morphology of 'Musang King' fruit. Preliminary taste evaluation found that different pollen source could affect 'Musang King' pulp taste. Further study with more fruit samples and respondent could be done to further evaluate these findings.

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TPGM

Preliminary study on encapsulation of *Aquilaria malaccensis* and *Endospermum malaccense* for *in vitro* germination and propagation

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Abstract

Aquilaria malaccensis and *Endospermum malaccense* are two of the potential timber species planted in Malaysia. Demand for conservation is increasing to restore their diversity in nature either for timber or non-timber products. The establishment of tree plantation programmes for reforestation has caused the high demand of planting materials. Synthetic seed technology could be employed for supplying *in vitro* planting materials via artificial/synthetic seed. The main objective in the present investigation is to study the *in vitro* germination and plantlet regeneration of *A. malaccensis* and *E. malaccense* germplasm as *in vitro* conservation method. Generally, these findings showed that the synthetic seeds of either species have equal probability to germinate on semi solid modified basal MS; vermiculite and garden soil under the same condition. Therefore, the formation of synthetic seed for *A. malaccensis* and *E. malaccense* was possible not only as an alternative planting materials but also for germplasm storage and conservation.

Key words: Biomass yield, forage maize, QTL, SSR markers, single marker regression

INTRODUCTION

Aquilaria malaccensis (Thymelaeaceae) or locally known as karas and *Endospermum malaccense* (sesendok) (Euphorbiaceae) are two examples of the potential timber species planted in Malaysia. *A.malaccensis* can be found growing in various habitats, from rocky, sandy or calcareous, well-drained slopes and ridges to swampy lands. *Aquilaria* produces agarwood or 'gaharu', as a result of infection in its heartwood by endophytic fungi. Gaharu, the valuable forest product that is sought for its uses in perfumery, incense and religious purposes. *Aquilaria* species are listed in the IUCN Red List and assessed as critically endangered (Harvey-Brown, 2018).

Sesendok is a useful indigenous and fast growing timber species (Hashim *et al*., 2015). It is suitable for a wide range of general utility purposes such as for making matchboxes, match splints, drawing boards, blackboards and toys. In medical, the bark is used to treat dropsy and the roots help to heal injuries.

The forest-based industries in the country have recognised that existing natural forests cannot continue to meet their raw material requirements. Demand for conservation is increasing to restore their diversity in nature either for timber or non-timber products. Such tree plantations could certainly help to increase forest cover in the country.

The establishment of tree plantation programmes for reforestation has caused the high demand of planting materials. Majority of tree species produces seeds only during particular seasons of the year. In the effort to ensure a continuous supply of planting materials, an interesting and growing field of seed biotechnology or encapsulation technology research, could be employed for supplying *in vitro* planting materials via artificial/ synthetic seed.

Synthetic seed technology provides an alternative system for propagation of transgenic plants, non-seed producing plants, polyploids with elite traits and plants with problems in seed propagation (Das *et al*., 2016). Encapsulation has been recently proposed as plant biotechnology important tool for mass propagation in fruit, forestry, horticultural, medicinal, and aromatic species (Rai *et al*., 2009; Reddy *et al*., 2012; Mandal *et al*., 2001; Murch *et al*., 2004).

The concept of synthetic seeds has evolved, resulting in a new definition: "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions" (Aitken-Christie, 1995). These encapsulated tissues or also known as synthetic seeds or artificial seeds or beads can be useful for the exchange of germplasm of elite genotypes and axenic plant material

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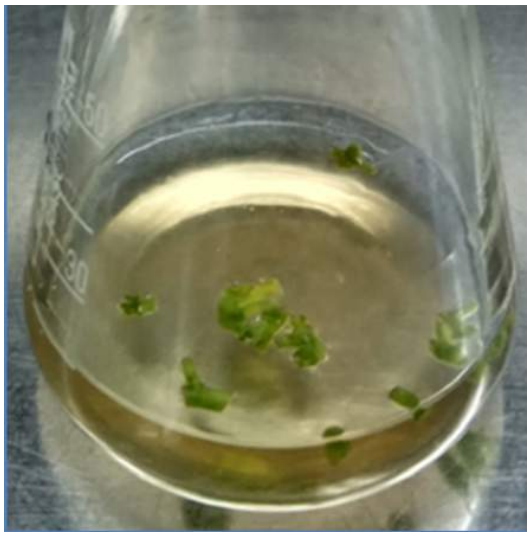

Figure 1. Explants in alginate solution

Figure 2. Synthetic seeds

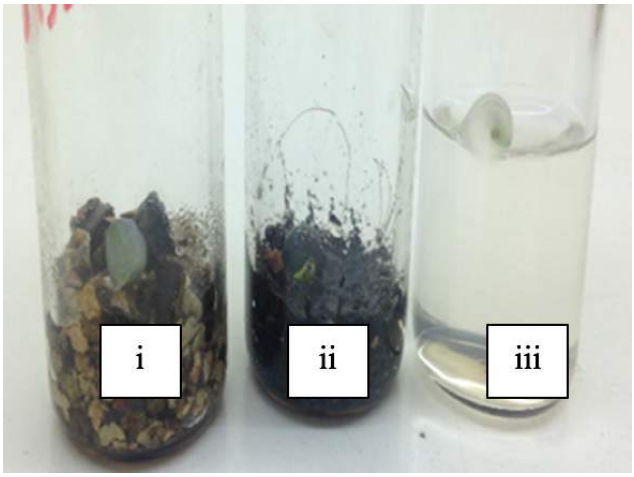

Figure 3. Synthetic seeds cultured on (i) vermiculite; (ii) garden soil; (iii) MS agar

between laboratories due to the small size and relative ease of handling (Naik and Chand, 2006). The synthetic seed can grow into a plant, either *in vitro* or *ex vitro*, through "conversion" or germination", like a conventional zygotic seed (Sharma *et al*., 2013). The main objective in the present investigation is to study the *in vitro* germination and plantlet regeneration of *A. malaccensis* and *E. malaccense* germplasm as *in vitro* conservation method.

MATERIAL & METHODS

Plant Material

Shoots and axillary buds from *in vitro A. malaccensis* and *E. malaccense* plantlets cultured on Murashige and Skoog (1962) (MS) medium served as explants. The plant materials were excised just above and below the nodes where only a small amount of expanded leaf primordial were retained, approximately 3 mm long. No distinction between the shoots and axillary buds was made as all explants were randomly mixed and used as experimental units.

Encapsulation and formation of beads

Preparation of encapsulation matrix was carried out according to Nor Asmah *et al.* (2011). The explants were transferred to the 3% sodium alginate solution. Explants in the alginate solutions were pipetted using a sterile Pasteur pipette with tip cut off individually dropwise into the 100mM calcium chloride solution ($CaCl_2.2H_2O$) and maintained for at least 30 min to polymerize the beads. When sodium alginate drops come in contact with calcium chloride solution, surface complexion begins and firm round beads are formed, each bead containing one explant. The beads were recovered by decanting the calcium chloride solution and blotted dry on filter paper.

Culture media

The encapsulated individual explants or the beads were directly sowed aseptically into three different following media: semi solid modified basal MS; vermiculite and garden soil in the 10 mm long test tubes. These media were sterilized by autoclaving at 121°C for 15 min prior to use.

Culture condition

The cultures were maintained in the culture room at 26 ± 1°C under 16 h photoperiod with a photon flux density of about 35 $\mu molm^{-2} s^{-1}$ provided by cool white fluorescent lamps to observe the germination ability of the beads. Each treatment (medium) consisted of six (*A. malaccensis*) and three (*E. malaccense*) individual explants as replicates and repeated three times. The germination responses of the encapsulated explants were scored weekly until the germination process ended. Vermiculite and garden soil were moistened with sterile distilled water as and when necessary throughout the study.

Table 1. Effect of media on the germination of synthetic seeds in both *Aquilaria malaccensis* and *Endospermum malaccense* after 6 weeks of inoculation (mean±SE).

Media	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Garden soil	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Vermiculite	0.00±0.00	22.22±22.22	33.33±22.22	33.33±22.22	27.78±16.67	25.00±19.44
MS agar	0.00±0.00	16.67±5.56	16.67±5.56	19.45±2.77	22.22±0.00	22.22±0.00

Table 2. Effect of species on the germination of synthetic seeds of *Aquilaria malaccensis* and *Endospermum malaccense* (mean±SE)

Sp	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Aquilaria malaccensis	0.00±0.00	3.70±3.70	7.41±3.70	9.26±4.90	11.11±6.41	9.26±6.68
Endospermum malaccens	0.00±0.00	22.22±12.83	25.92±16.14	25.92±16.14	22.22±12.83	22.22±12.83

Table 3. Effect of species and media interactions towards germination of synthetic seeds

Species	Media	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Aquilaria malaccensis	Soil	0.00	0.00	0.00	0.00	0.00	0.00
	Vermiculite	0.00	0.00	11.11	11.11	11.11	5.56
	MS agar	0.00	11.11	11.11	16.67	22.22	22.22
Endospermum malaccens	Soil	0.00	0.00	0.00	0.00	0.00	0.00
	Vermiculite	0.00	44.44	55.55	55.55	44.44	44.44
	MS agar	0.00	22.22	22.22	22.22	22.22	22.22

Statistical analysis

Data with three to six explants per replication with three repetitions were analysed using ANOVA CRD (completely randomized design). The means were compared using Duncan's Multiple Range Test (DMRT) when F-test was determined to be significant.

RESULTS & DISCUSSIONS

Production of synthetic seeds have been reported in many species (Chand & Singh 2004; Daud *et al.* 2008; Nor Asmah *et al.* 2011, 2012). The size of the synthetic seeds (beads) was controlled by varying the inner diameter of the Pasteur pipette depending on the explant sizes. Regrowth or conversion ability of encapsulated explants (shoots and axillary buds) was evaluated in terms of the percentage of explants able to break the calcium alginate matrix to develop shoots. The breaking of the calcium alginate matrix was determined as germination as when the expanded leaves of the explants appeared and break the gel (Machii, 1992).

It was observed that most of the encapsulated explants of both *A.malaccensis* and *E.malaccense* remained green after inoculation for at least one week. There were even some explants that maintained green until week 6. In general, no significant difference was recorded in the conversion of the synthetic seeds.

Effect of culture media on the germination of synthetic seed

The ability for the synthetic seeds in both *A.malaccensis* and *E.malaccens* to germinate after six weeks of inoculation was observed by the media used (Table 1).

Aquilaria malaccensis: The first shoot sprout from the encapsulated explants were observed on the semi solid modified basal MS at week 2. Later at week 3, beads on vermiculite medium were seen germinated and followed by more sprouts on MS medium. Some of the sprouting shoots and the explant of the not germinating bead, remained green and clean until week 6. However, some of them had fungal contamination at week 5. No germination was detected on soil medium despite being clean culture until week 4. Beyond week

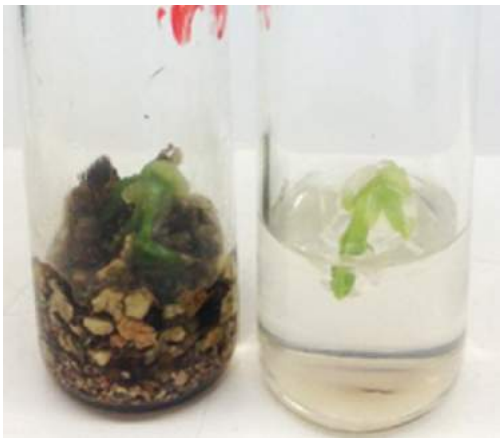

Figure 4. Synthetic seeds germination on vermiculite and MS agar

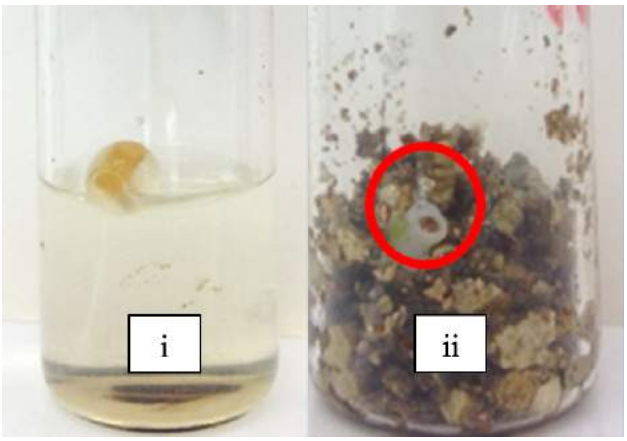

Figure 5. Synthetic seed (i) turned yellow; (ii) shrunken during culture.

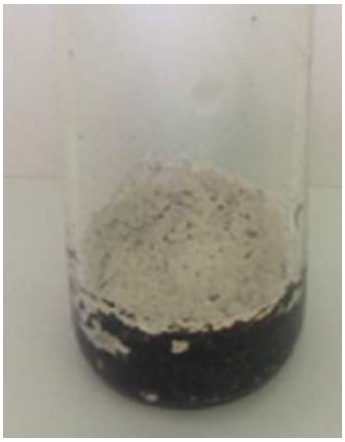

Figure 6. Fungal contamination

4, most of the explants of beads on soil had turned yellow and brown.

Endospermum malaccense: Germinations were seen on both semi solid MS and vermiculite media at week 2. At week 3, another bead germinated on vermiculite. The explants of the not germinating bead had turned from yellow to brown as early as week 2 and eventually shrunk. No germination was also detected on soil. Fungal contamination was only detected on vermiculite and soil media.

Generally, the highest cumulative germinations on vermiculite medium were 33.33% at week 3 while 22.22% on semi solid MS agar at week 5). There were no significant differences between the media used. However, some beads had fungal contaminations (5.6-11.1% data not shown).

Effect of species on the germination of synthetic seed

There was no significant difference statistically between both species with regards to germinations on the media used. Table 2 showed that *A. malaccensis* had the highest cumulative germination at week 5 (11.11%), while *E. malaccense* was at week 3 (25.92%). Generally, these findings showed that the synthetic seeds of either species have equal probability to germinate under the same condition.

Effect of species and media interactions on the germination of synthetic seed

There was no significant difference on the interactions of the species and media used. This showed neither the species nor the media have effect on the synthetic seed germination. Individual observations showed that the synthetic seed germinated better and faster on vermiculite (Table 3).

As there was no germination observed on soil medium, Malek (2009) suggested that the encapsulated explants could have dried completely within a week where the nutrients in the encapsulation matrix may not be sufficient for the explants to develop a balanced shoot and root system. It was also observed at week 6, some green explants of the synthetic seeds failed to germinate. This could be due to inhibition of respiration in the plant tissues because of alginate covers (Redenbaugh *et al*., 1987).

Due to the small number of samples available for this study, results shown were considered as preliminary findings for future studies. However, some improvements could be done to enhance germination or regeneration from the synthetic seeds. After the synthetic seed formation, the toxic residual of chloride and sodium ions could be washed a few times with distilled water (Gantait *et al*., 2015) as the free residual may lead to toxicity to the encapsulated explants. The beads will then be blotted dry for the subsequent processes.

With a limited number of explants, the beads were tested for their regeneration into shoots and roots (if any) only on basal MS semi-solid, garden soil and vermiculite media. More types of media could have been tried out such as any basal media in different concentrations (¼, ½, full-strength), at

semi-solid or liquid states and also in planting substrates like gravel, perlite, sand, soilrite, and vermicompost for complete regeneration into plantlets. There were some researchers who added MS nutrients at ¼- or ½- for watering the substrates as a substitute of distilled water which has improved the regeneration percentage (Chand and Singh, 2004; Sharma *et al*., 2009). Another problem encountered during the study was the contamination by microbes. Despite all necessary precautions being taken, fungal or bacterial contaminations was the main reason of losing the cultures. Thus, in the future, an extra precaution could be considered where Antonietta *et al*. (2007) has added fungicide of about 50–100 mgl^{-1} into the encapsulation matrix to minimize the contamination issue.

CONCLUSION

The present study has successfully developed a method of synthetic seed formation by encapsulating shoots and axillary buds from *in vitro* grown plantlets of fast-growing tropical tree species, *A.malaccensis* and *E.malaccens*. Although the number of plantlets regenerated was low, this study revealed that synthetic seeds are good alternative planting materials because they can be produced throughout the year through plant tissue culture techniques, integrating tissue culture manipulations with synthetic seed technology for germplasm storage and conservation.

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TPGM

Effect of water deficit on the agronomic characteristics and SDS-PAGE protein pattern of three Sabah dryland rice varieties

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Abstract

Drought is the major limiting factor to rice production worldwide. Thus, drought tolerance emerges as one of the most important aspects of rice breeding programs. A study on Sabah's local dryland rice varieties were carried out using Silabukan soil as planting medium at Faculty of Sustainable Agriculture, Universiti Malaysia Sabah. This study aimed to compare the agronomic characteristics between Beruang, Kelopak and Tiga Bulan dryland rice varieties under normal and drought stress conditions. Two cycles of drought treatments were applied at 50% heading stage to agronomic replicates. Completely randomized design was used in this study. Results showed that early maturing Kelopak variety performed the best under normal condition, exhibited the highest extrapolated yield (4.856 tonnes ha^{-1}). Drought stress at 50% heading stage led to a significant decrease in relative water content, percentage in filled grain and grain yield per plant. In this study, Tiga Bulan variety experienced the lowest percentage of yield reduction (28%) and gave the highest grain yield per plant (19.438 g plant^{-1}) under drought stress condition. Early flowering Tiga Bulan variety with slightly longer days to leaf rolling was selected for proteome analysis. Normal and completely rolled flag leaves of Tiga Bulan variety after first cycle of drought treatment at grain filling stage were subjected to Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis to compare their protein expressions. SDS-PAGE analysis revealed one and two bands were up- and downregulated significantly in response to drought respectively. These proteins may be involved in drought mechanism. Recent trend in climate change have predicted a further increase in drought intensity, therefore efforts to develop drought tolerant varieties are vital to sustain the rice production worldwide.

Key words: Drought, dryland rice, Silabukan soil, proteome, SDS-PAGE

INTRODUCTION

Rice is a staple food crop for nearly 3.5 billion people, with an annual consumption of more than 450 metric tons worldwide (Muthayya *et al*., 2014). Drought is a climate related natural disaster that continuously challenges global rice production, through its impact on limiting water supply (Wang *et al*. 2013). The frequency of drought event as a consequent to climate changes is expected to increase in tropical and subtropical regions (IPCC, 2014). Since rice production is closely associated with water irrigation, the occurrence of drought events will adversely impact food security and lead to vast economic cost on a global scale.

Taking Malaysia as an example, 1997/98 El Nino related drought event caused severe impacts to the environment, economic and social activities of the country (Simon, 2014). Water shortages experienced during this drought event had significantly reduced the rice production and affected the welfare of rice farming community. Given that there are about 674,000 hectares of rice fields, producing about 2.6 million tonnes of rice annually in Malaysia (DOA Peninsular Malaysia, 2014), the occurrence of drought events would have a threatening impact on the National Self Sufficiency Level (SSL) of domestic rice production.

In the East Malaysia particularly Sabah, dryland rice cultivation is mostly practiced by the rural communities as a self-sustaining agricultural activity. Beruang, Galigim, Hijau Manis, Kelopak, Kondinga and Tiga Bulan are among the Sabah's indigenous rain-fed dryland rice varieties. These varieties have been widely recognized to be more drought tolerant, as well as highly valued for their unique aroma, grain quality and palatability (Siambun, 2003; Ding *et al*., 2013). However, the grain yield of these dryland rice is much lower than that of wetlands (Hanafi *et al*., 2009). Due to its lower yield, research on rain-fed dryland rice varieties has often been neglected and thus resulting in the scanty agronomy information available for these varieties.

Proteins are the building blocks of all cells, where the biological processes of life maintenance, defense, replication and reproduction are exerted (Breda *et al*., 1999). Proteomics is the study of structure and function of proteins (Graves and Haystead, 2002). Although drought tolerance is among the

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important rice breeding goals, however, proteomic analysis for dryland rice varieties in response to drought stress has not been well investigated (Kumar *et al*., 2007). Alterations in cellular protein expression are responsible for drought tolerance in dryland rice (Salekdeh *et al*., 2002; Komatsu and Tanaka, 2005). The identification of drought responsive proteins using molecular approach able to assist rice breeding projects worldwide, by improving the drought tolerance of high-yielding but drought-susceptible rice varieties.

In this study, drought stress was applied during the 50 percent heading (agronomy analysis) and grain filling (proteomic analysis) stages since these two stages of rice are highly sensitive to drought stress (He and Serraj, 2012). For agronomy analysis, agronomic characteristics of all sample dryland rice varieties under normal and drought stress condition was compared.

Flag leaf was used for proteomic analysis because it is the major carbohydrate producer, and photosynthetic carbohydrates are the primary source of rice grain yield. Since leaf rolling is most noticeable on flag leaves, leaf rolling scale was used as an indicator for drought stress treatment (Pask *et al*., 2011). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used in this study to compare and analyze the effect of drought in the proteomic expressions of dryland rice variety. Two staining methods, namely Coomassie Brilliant Blue (CBB) and silver staining, were compared in this study to obtain an optimized method for proteomic analysis of dryland rice variety.

METHODOLOGY

This study was carried out in rain shelter no. 6 of Faculty of Sustainable Agriculture (FSA), Universiti Malaysia Sabah (5°55'N, 118°00'E) in year 2015. Three (3) rain-fed dryland rice varieties (Beruang, Kelopak and Tiga Bulan) were used in this study. The experimental soil was Silabukan soil (30 cm deep from the soil surface), which was collected from plot 16 and 17 of FSA. The collected soil was air-dried, grounded and sieved before use. Transparent plastic bags were placed inside all the nursery and planting pots prior to soil filling. Triplicate Silabukan soil samples were analyzed for total nitrogen (N) concentration in soil, soil available phosphorus (P), cation exchange capacity (CEC) and soil pH. This study was performed using completely randomized design (CRD) with 5 replicates (for agronomy analysis) and 3 replicates (for proteome analysis). The treatments used in this study were normal treatment and drought stress.

Planting of dryland rice

Hundred (100) seeds from each variety were soaked in distilled water for 24 hours and placed in germination boxes that filled with moist paper towels, until the formation of primary shoots and roots. Once germinated, dryland rice seedlings were transplanted into nursery clay pots. Fertilizer application with NPK ratio of 2:1:1 was split into two, which are basal dressing a week before transplanting and topdressing after a week. The transplanted seedlings were watered twice daily. Fourteen (14) days old dryland rice seedlings were then transplanted into planting pots. Fertilizer application with NPK ratio of 2:1:1 was split into three, which are basal dressing a week before transplanting and topdressing during active tillering and panicle initiation. The transplanted seedlings were watered twice daily until the drought treatment and resumed after treatment.

Application of drought treatment

At the 50% heading stage, watering was applied thoroughly into the soil and small holes were punched on the plastic bags to drain the water slowly. No watering was done since then until the flag leaf reached leaf rolling score of 3 (more than 67% of leaf rolled). Watering was then applied thoroughly again into the soil and the full water level was maintained for a day. Second drought stress was applied until the flag leaf reached leaf rolling score of 3. Watering was resumed until harvesting after the second cycle of drought treatment. Surrounding temperature and relative humidity (RH) were measured daily using thermohygrometer (Hanna Instrument HI93640) throughout the drought treatment. Soil moisture content was measured using soil moisture meter.

Methods for agronomic traits

Morphological characteristics, physiological characteristics and yield for dryland rice varieties under drought stress and control conditions were measured.

Morphological characteristics	Plant height, culm height and tiller number at harvest, panicle number and length, percentage of productive tiller
Physiological characteristics	Days to leaf rolling, days to 50% heading, relative water content
Yield	Spikelet number, percentage of filled and unfilled grain, thousand-grain weight, grain yield per plant, extrapolated yield

Table 1. Soil chemical properties of Silabukan soil

N	0.2084%
P	0.4218 ppm
CEC	18.3 $cmol_c\ kg^{-1}$
pH	5.37

SDS page analysis

Three (3) flag leaf samples were collected when the leaf rolling score reached 3 following first cycle of drought treatment during grain filling stage. Another 3 samples were collected from controls. One (1) dryland rice variety, namely Tiga Bulan which exhibited slightly longer days to leaf rolling were selected for proteome analysis. Collected flag leaf samples were immediately cut into pieces, wrapped with aluminium foil and immersed in liquid nitrogen. The frozen samples were then stored at -80 °C until further use.

Acetone dried powder (AcDP) for each flag leaf sample was prepared and stored at -80°C until further use. Pierce 660nm protein assay standard curve (R^2 = 0.99) was established. TCA-acetone precipitation method was used for protein extraction. Extracted proteins were then resolubilized and quantified using DeNovix DS-11 spectrophotometer. The

Table 2. Mean comparison of agronomic characteristics between selected dryland rice varieties under normal conditions.

Dryland Rice Variety	SGP (%)	50H (day)	PH (cm)	CH (cm)	TN	PN	PT (%)	PL (cm)	SN	%FG (%)	%UG (%)	1000-GW (g)	GY (g plant^{-1})	EY (tonnes ha^{-1})
Beruang	97.67a	106.8a	128.82	86.6	11.4b	9.2b	36.46b	20.17ab	929b	68.13	31.87	30.596a	20.634	3.309
Kelopak	84.67b	81.4b	127.8	84.81	18.6a	16.6a	59.71a	21.19a	1554.8a	76.31	23.69	25.355b	30.279	4.856
Tiga Bulan	93.33a	82b	118.52	82.42	22.4a	19.8a	59.41a	18.6b	1391.6ab	75.13	24.87	25.985b	27.092	4.345

SGP= Seed germination percentage; 50H= Days to 50% heading; PH= Plant height; CH= Culm height; TN= Tiller number; PN= Panicle number; PT= Percentage of productive tiller; PL= Panicle length; SN= Spikelet Number; %FG= Percentage of filled grain; %UG= Percentage of unfilled grain; 1000-GW= Thousand-grain weight; GY= Grain yield per plant; EY= Extrapolated yield; Means followed by the same letter in the same column are not significantly different according to Tukey's HSD test at P<5%

Table 3. Number of days to leaf rolling of Beruang, Kelopak and Tiga Bulan dryland rice varieties under two cycles of drought treatment

Dryland Rice Variety	Number of Days to Leaf Rolling (days)	
	First Drought Treatment	Second Drought Treatment
Beruang	4.4	3.4
Kelopak	2.2	2.2
Tiga Bulan	2.8	2.6

protein samples (15 uL for CBB staining; 5 uL for silver staining) were subjected to Bio-Rad Mini Protean Tetra Cell (#165-8000). The electrophoresis system was conducted at constant volt of 70 V for 15 minutes and continued at 160 V until the bromophenol blue line reached the bottom of the plate.

CBB and silver staining

Once the electrophoresis process had completed, respective gel samples were removed and subjected to CBB and silver staining. The stained gels were then subjected to Bio-Rad Gel Doc EZ Imager (#1708270) for analysis.

Data analysis

Statistical Analysis System (SAS) was used in this study for statistical analysis of all the collected data. For analysis of agronomic characteristics under normal condition, the collected data were subjected to one-way analysis of variance (ANOVA) and the differences within treatments were analyzed using Tukey's HSD test at p<5%. For comparison of agronomic characteristics under normal and drought conditions, the collected data were subjected to two-way analysis of variance (ANOVA) and the differences within treatments were analyzed using LSD test at p<5%. For proteome analysis, relative intensity of protein bands expressed under normal and drought stress conditions were compared. The results were subjected to paired sample t-test.

RESULTS AND DISCUSSION

Soil chemical properties of Silabukan soil

Low total nitrogen (< 0.25%), low available phosphorus (< 20 ppm) and moderately high acidity (pH 5.2 to 6.0) were observed in Silabukan soil (Marx *et al*., 1996), as indicated in Table 1. Similar results were obtained by Ligunjang (2010). Thus, it can be suggested that Silabukan soil is of low fertility and fertilizers inputs are necessary for planting activities. Acidic nature (pH 5.37) of Silabukan soil may reduce the solubility of nutrients in the soil, since most nutrients are in their most available state at pH between 6.0 and 6.5 (Brady, 1990).

CEC of Silabukan soil was found to be 18.3 $cmol_c$ kg^{-1}, thus it can be suggested that Silabukan soil is made up of clay loams. Triantafilis *et al*. (2007) reported that CEC for clay loams was between 15 to 30 $cmol_c$ kg^{-1}. Silabukan soil which composed mainly of Ultisol, is a member of low-activity clay (LAC) soils that possesses low CEC. Low CEC is correlated with composition of the clay mineral and low soil organic carbon content (Prasetyo et al., 2001).

Agronomic characteristics under normal condition

Kelopak and Tiga Bulan varieties are of medium flowering rice varieties, with average days to 50% heading of 81.4 and 82 days respectively, whereas Beruang variety, with 106.8 days to 50% heading is of late flowering species (Mitra and Biswas, 1977). Plant height at harvest, culm height at harvest, percentage of filled and unfilled grain, grain yield per plant for all three varieties were not significantly different.

Table 4. Mean comparison of agronomic characteristics between selected dryland rice varieties under normal and drought conditions

Treatment	Dryland Rice Variety	RWC (%)	%FG (%)	%UG (%)	1000-GW (g)	GY (g plant^{-1})	EY (tonnes ha^{-1})
Normal	Beruang	91.123	68.13	31.87	30.596	20.634	3.309
	Kelopak	93.918	76.31	23.69	25.355	30.279	4.856
	Tiga Bulan	93.724	75.13	24.87	25.985	27.092	4.345
Drought	Beruang	70.297	54.09	45.91	31.407	14.548	2.333
	Kelopak	90.169	42.1	57.9	25.658	15.531	2.491
	Tiga Bulan	80.638	57.95	42.05	25.426	19.438	3.117
SD (5%)		13.41^{ns}	35.17^{ns}	35.17^{ns}	70.13^{ns}	44.33^{ns}	44.33^{ns}

RWC= Relative water content; %FG= Percentage of filled grain; %UG= Percentage of unfilled grain; 1000-GW= 1000-grain weight; GY= Grain yield per plant; EY= Extrapolated yield; SD= Significant difference; ^{ns}= No significant difference

Tiller number at harvest differed significantly among the three varieties, in which Tiga Bulan recorded the highest number of tiller and the lowest belonged to Beruang variety. This result was in accordance to that of Yang et al. (2003), who reported that shorter rice varieties tend to have higher tillering capacity, since plant height at harvest for Tiga Bulan was the shortest, whereas the highest was achieved by Beruang variety. Each tiller holds the potential to bear panicle (Hardke, 2013), thus higher tiller numbers means higher probability to obtain higher number of panicles. This is in accordance with the results obtained, whereby Tiga Bulan variety with the greatest number of tillers, possessed the highest panicle number; and vice versa for Beruang variety. Percentage of productive tiller for Kelopak and Tiga Bulan variety were significantly higher than Beruang variety.

Panicle length determines the number of grains it holds (spikelet number), thus contributing to grain yield per plant (Huang *et al*., 2013). Based on the results, Kelopak variety exhibited the longest panicle length, the highest spikelet number and thus the highest grain yield. The panicle length of Tiga Bulan variety was significantly lower than the other two varieties. However, its number of spikelet and grain yield was found to be higher than Beruang variety. This suggests that this variety produced higher number of tillers to compensate for shorter length of panicle.

Comparison of agronomic characteristics under normal and drought conditions

Dryland rice varieties did not interact significantly with cycle of drought treatment for days to leaf rolling, and drought treatments for relative water content (RWC) of flag leaf, percentage of filled grain, percentage of unfilled grain, grain yield per plant and extrapolated yield.

Effects of drought on the agronomic characteristics of selected dryland rice

Morphological changes of dryland rice subjected to drought treatment include leaf rolling, reduction in spikelet fertility and percentage of filled grain. Leaf rolling symptom was developed by all dryland rice that subjected to drought stress, as a drought escape mechanism (Yue et al., 2005). This adaptive mechanism can be explained by the ability of rice plant to reduce the area of leaf exposed to the surrounding, thereby reduces the rate of transpiration on the upper surface of the leaf and light interception (Mackill *et al*., 1996).

Based on the results obtained, it can be seen that Kelopak variety possessed the shortest days to leaf rolling (average of 2.2 days), however, its relative decrease in RWC of flag leaf was the least (4%). This indicates that Kelopak variety began to roll at a higher leaf water potential than the other two varieties. According to Mackill *et al*. (1996), although leaves rolled at higher leaf water potential do improve the plant's water use efficiency, however, this reduces gas exchange and photosynthesis rates at an earlier time.

Since 50% heading is the critical stage in determining spikelet fertility, early leaf rolling resulted the plant to experience reduced photosynthesis earlier, and this led to greater decrease in fertile panicle number and filled grain percentage. Maisura *et al*. (2014) reported that decrease in grain yield during drought stress is due to reduced panicle formation and high spikelet sterility. Water shortage experienced during drought treatment caused dryland rice unable to absorb soil water, and thus their flowers failed to open (Bunnag and Pongthai, 2013). Closed flowers inhibited the pollens from reaching their surface and thus reduced the occurrence of pollination (Maisura *et al*., 2014). Liu *et al*. (2008) reported that drought stress can abort pollination for up to 67% of total grain per panicle.

As an evidence, Kelopak variety experienced the greatest reduction in percentage of filled grain (45%) following drought treatment. Thus, Kelopak variety experienced greatest reduction in grain yield per plant (49%). This result is similar to that reported by Dingkuhn *et al*. (1989), in which some drought-resistant upland rice where their leaves rolled at high leaf water potential, may possess greater yield reduction during periods of moderate drought stress.

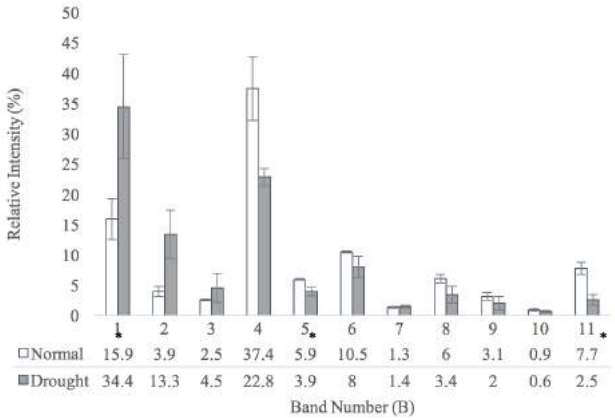

Figure 1. Relative intensity of protein expression of flag leaves under normal and drought conditions; the bars in the graphs represent an average intensity ± SD of three replicates
* indicates drought effect was significant according to t test at P<5%

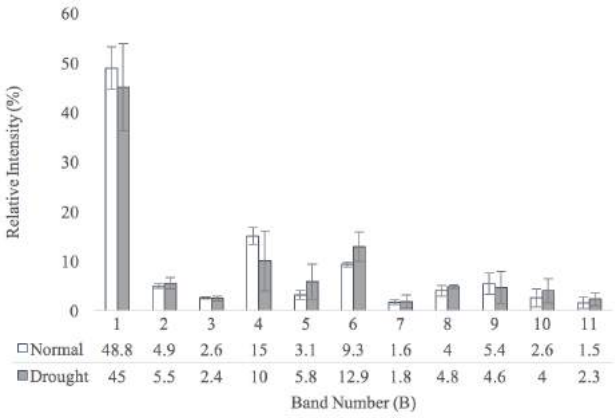

Figure 2. Relative intensity of protein expression of flag leaves under normal and drought conditions; the bars in the graphs represent an average intensity ± SD of three replicates
* indicates drought effect was significant according to t test at P<5%

Beruang variety, on the other hand, possessed the longest days to leaf rolling (average of 3.9 days). Delayed lead rolling is associated with drought resistance, in other words, the ability of Beruang variety to remain its leaves expanded and unrolled longer indicates that this variety is the best among the three in maintaining its leaf water potential (Singh and Mackill, 1990). This may be due to Beruang variety exhibited greatest amount of wax on the leaf surface which enhance its resistance to water loss (O'Toole *et al*., 1979).

However, its relative decrease in RWC of flag leaf was the highest (23%). This indicates that Beruang variety began to roll at a lower leaf water potential than the other two varieties. The ability of this variety to adjust its leaf water potential and delay the occurrence of leaf rolling, minimized the impact of drought towards the process of photosynthesis and spikelet fertility. As an evidence, Beruang variety experienced the lowest reduction in percentage of filled grain (21%) following drought treatment.

Days to leaf rolling of all the dryland rice varieties were found to be shorter (varied from 2.2 to 4.4 days) when compared to those reported in field experiment by Bunnag and Pongthai (2013), where drought tolerant rice started to show sign of leaf rolling after 7 days of drought treatment. This difference could be due to all the three varieties were grown in pots with confined rooting volume (Turner *et al*., 1986). This limited the ability of the rice plant to search for water sources during drought stress through root penetration deep into the soil profile, and eventually experienced early leaf rolling to avoid drought by limiting water loss.

Protein expression on SDS-page

Eleven (11) bands were detected at similar molecular weight in all the normal and stressed samples in both Coomassie Brilliant Blue (CBB)-stained and silver-stained gels. Three (3) bands (band 1, 5 and 11) in CBB-stained gel responded significantly towards drought stress. The intensity of band 1 were elevated, whereas the remaining two declined in intensity as a result of drought stress. None of the bands in silver-stained gel responded significantly towards drought stress.

(a) **Coomassie Brilliant Blue (CBB)-Stained Gel**

Band 1 (250 kDa) was upregulated in response to drought. According to Pessarakli (2011), many hydrolytic and oxidative enzymes show an increased activity in drought-stressed tissues of rice plants. These enzymes functions to degrade all types of all biological polymers including proteins via process of hydrolysis. Therefore, band 1 could be expressed by enzymes with hydrolytic and oxidative functions. Band 5 and 11 (43 and 12.5 kDa) were downregulated in response to drought. Reduced expression may be associated with the reduction in protein synthesis, increased activities of hydrolyzing enzymes, reduced availability of amino acids and denaturation of enzymes responsible for protein synthesis (Sharma and Dubey, 2005).

CBB is less sensitive than silver staining, as indicated by the less sharp bands produced. However, it has a wide dynamic range, thus it is more suitable for quantitating medium and high abundance proteins (Qin et al., 2006). Silver staining allows higher sensitivity (in the low ng range) to be obtained, as indicated by the sharp bands produced (Chevallet *et al*., 2006). However, it has a narrow linear dynamic range (the range at which the level of staining is linear to the concentration), thus it is less suitable for quantification.

(b) **Silver-Stained Gel**

CONCLUSION

All three varieties used in this study showed variations in terms of agronomic characteristics under normal and drought conditions. Beruang seeds exhibited the highest germination percentage (98%) and vigorousity, were of the best quality among all. Under normal cultivation using Silabukan soil with NPK rate of 120: 60: 60, early maturing Kelopak variety was

superior because this variety exhibited the longest panicle length (21.19 cm), the highest spikelet number (1554.8), the highest grain yield per plant (30.279 g), and contributed to the highest extrapolated yield of 4.856 tonnes ha^{-1}.

Leaf rolling symptoms were exhibited in all three varieties subjected to drought stress, as a drought escape mechanism. Drought stress at 50% heading stage reduced grain yield per plant significantly irrespective of rice varieties. Tiga Bulan variety experienced the lowest percentage of yield reduction (28%) and gave the highest grain yield per plant (19.438 g plant^{-1}) under drought stress condition.

Drought tolerance is a multigenic trait. Three mechanisms exhibited by Tiga Bulan variety were found to be directly involved in reducing yield loss during drought stress at 50% heading. These mechanisms include production of higher tiller number to compensate shorter panicle length, generation of greater amount of wax on the leaf surface to delay leaf rolling, as well as ability to maintain high RWC of flag leaf under drought stress.

Drought stress at grain filling stage induced changes in the expression of proteome patterns of Tiga Bulan's flag leaf. SDS-PAGE analysis revealed that one band was upregulated and two bands were downregulated significantly in response to drought. These proteins may be involved in drought mechanism. Recent trend in climate change have predicted a further increase in drought intensity, therefore efforts to develop drought tolerant varieties are vital to sustain the rice production worldwide.

Drought is the major limiting factor to rice production worldwide, thus rice breeding project on developing varieties capable of tolerating, escaping and or avoiding drought merits further attention. Sabah has a wide variety of dryland rice, thus similar study can be conducted in the future with varieties other than those selected in this study, to provide their agronomy information and to identify drought tolerant varieties. This information able to assist rice farmers in the selection of dryland rice varieties to be planted in drought-prone areas.

Although the changes in protein expressions of Tiga Bulan's flag leaf during drought stress can be identified using SDS-PAGE analysis, however, it is unable to prove the roles of these expressed proteins in drought tolerance without knowing their functions. Thus, this research can be continued using protein fractionation which improves the dynamic range of analysis. Further research can be conducted using 2DPAGE (Two-dimensional Polyacrylamide Gel Electrophoresis) analysis which allows for higher sensitivity and better resolution of protein visualization. Mass spectrometry can be used for the identification of drought responsive proteins.

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TPGM

Relationship between hybrid performance and genetic distance, specific combining ability and heterosis among parental inbred lines in forage corn

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Abstract

Selection of suitable parental lines for hybrid variety development using results of genetic distance analysis has been established and effectively used in many crops. Genetic distances among parental corn inbred lines based on simple sequence repeat (SSR) markers were used to estimate the magnitude of biomass yield traits in crosses among them. Six selected maize inbred lines were crossed in a half-diallel manner to produce 15 single cross F_1 hybrids. Biomass yield traits (fresh plant yield, fresh leaf yield, fresh stem yield and fresh ear yield) of the hybrids were evaluated along with their parents in a randomized complete block design (RCBD) with three replications, in two environments (Field 10 and Field 15, Universiti Putra Malaysia). Combining ability analysis revealed that specific combining ability (SCA) effects were significant for all biomass yield traits in both environments separately and when environments were pooled. Compared with the average value of the parents for fresh plant yield (25 388 kg/ha), the 15 hybrids showed a significant ($p<0.01$) yield advantage across environments, with an average of 151.9% mid-parent heterosis (MPH) when data from the environments were pooled. Highly significant positive correlations were observed between specific combining ability (SCA) and hybrid performance *per se* for fresh plant yield in Field 10, Field 15 and environments pooled (0.946, 0.900 and 0.796, respectively), while positive significant correlations ($p<0.05$) were observed between genetic distance and MPH in Field 10 and environments pooled (-0.546 and -0.537, respectively). Hence, the findings revealed a weak association between genetic distance and F_1 hybrid performance, although SCA remained to be an important factor in the determination of heterosis and hybrid performance *per se*.

Key words: Combining ability, heterosis, genetic distance, inbred lines, forage corn

INTRODUCTION

Genetic improvement for hybrid corn involves selection of potential inbred lines as parents, evaluation of the hybrid progenies and selection of the best hybrids for the traits involved in a breeding program. Selected inbred lines could be tested for their combining ability to estimate ability of the inbred lines to inherit good genes based on performance of their progenies. According to Guerrero *et al*. (2014), heterosis exhibited in the crosses also depends on the genetic diversity of the parents. The heterotic response of the hybrids also largely depends on the breeding value of the parents and the environmental conditions under which the hybrids are grown (Hallauer and Miranda, 1988; Young and Virmani, 1990; Abuali *et al.*, 2012). Based on a study by Guerrero *et al.* (2014), a positive mid parent heterosis (MPH) was exhibited for fresh forage yield, with estimates ranging from 8.0 to 21.5%, all exceeding the average parental yield in the 15 crosses utilized. Hybrid vigour can be predicted by the use of molecular tools to obtain maximum efficiency in hybrid breeding programs by producing promising hybrids. Many studies have utilized molecular markers to predict correlations between genetic distance of the parental inbred lines and the hybrid performance in tropical as well as temperate maize lines (Lanza *et al.*, 1997; Melchinger, 1999; Benchimol *et al.*, 2000). Although many investigators have reported heterosis and gene action for quantitative characters in corn, reports on genetic control for yield traits on forage corn are limited. Production and improvement of forage corn is needed in the country to utilize this potential crop as an alternative source of feed for ruminants. Thus, clear breeding goals are necessary to develop hybrid varieties for feed production. Hybrid varieties should acquire superior performance for the relevant forage traits, besides being vigorous, having synchronized and uniform flowering, and tolerant to pests and diseases.

MATERIALS AND METHODS

Six inbred lines from different heterotic groups as well as those that had contrasting characters for forage traits were selected

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Table 1. List of six parental inbred lines selected for the half-diallel cross scheme

Inbred line	Source population	Origin
CML152	P62	CIMMYT
CML331	REC	CIMMYT
CML383	P502	CIMMYT
CML428	P145	CIMMYT
CML491	REC	CIMMYT
CML498	POP21	CIMMYT

CIMMYT = International Maize and Wheat Improvement Center, Mexico.

Table 2. Mean squares in ANOVA for biomass yield traits measured on 15 corn hybrids and their parental lines evaluated at Field 10 and Field 15, Universiti Putra Malaysia

Source of variation	d.f.	Mean squares							
		At Field 10, UPM				At Field 15, UPM			
		Fresh plant yield	Fresh leaf yield	Fresh stem yield	Fresh ear yield	Fresh plant yield	Fresh leaf yield	Fresh stem yield	Fresh ear yield
Genotypes	20	1111428225**	86563072**	85800580**	250669287**	1115387532**	106979110**	85939424**	214295669**
Blocks	2	202307006*	8178850^{ns}	14269781^{ns}	59492860*	506996981*	19996474^{ns}	27494106^{ns}	192534610**
Error	40	53596487	6182112	6501819	12767979	138279555	13859581	12502633	31967865
C.V. (%)		13.45	15.38	17.92	14.87	22.91	23.42	27.9	24.84

d.f. = degrees of freedom, and C.V. = coefficient of variation

as parents for a half-diallel cross. The selected inbred lines were expected to reveal high heterosis in their hybrids. The list of the parental inbred lines selected is shown in Table 1. Fifteen hybrid progenies, six parental inbred lines and two control open-pollinated varieties, Pool26 and BTL1 were evaluated at Field 15, UPM, Serdang, from August to November 2014, and at Field 10, UPM, Serdang from February to June 2015. All genotypes were planted and evaluated in a randomized complete block design (RCBD) with three replications, where each plot consisted of either an inbred line or a hybrid. At three weeks after silking, the whole plants were harvested by cutting at 20 cm above soil level and separated into ears, leaves and stems. All fresh parts were weighed and recorded as fresh plant yield (kg/ha), fresh leaf yield (kg/ha), fresh stem yield (kg/ha) and fresh ear yield (kg/ha). The Duncan's New Multiple Range Test (DNMRT) was applied to compare mean performances among the hybrids, parental inbred lines and the check varieties. Estimates of heterosis was calculated as the percentage of superiority of the hybrids over their mid-parent (MP) values. Mid-parent heterosis (MPH) was estimated using the mean values of the hybrids (F_1) and their mid-parental inbred line values (MP) as follows:

$$\text{Mid-Parent Heterosis (\%)} = \left(\frac{F_1 - MP}{MP}\right) \times 100$$

The diallel analysis was conducted following Griffing's Method 2 (one set of F_1 progenies and their parental lines) (Griffing, 1956). Genetic distances were determined among the six corn inbred lines used as parents in the diallel cross, based on microsatellite DNA markers using 1 - Nei's genetic similarity coefficients (Nei and Li, 1979). Pearson's correlation coefficients were used to determine the relationships among genetic distances revealed by the molecular characteristics of the parental lines, hybrid performance, mid-parent heterosis and specific combining ability for biomass yield traits, using the Statistical Analysis System (SAS) computer package version 9.3 (SAS Institute Inc., 2011).

RESULTS AND DISCUSSION

Results of the analysis of variance (ANOVA) revealed that genotype effects were highly significant for all biomass yield traits (significant at $p \leq 0.01$), evaluated in Field 10 and Field 15, as presented in Table 2. Results on performance of single cross hybrids evaluated in Field 10 and Field 15 are presented in Table 3. In Field 10, the highest fresh plant yields were produced with the range of 60 444 kg/ha (Hybrid HF15) to 75 333 kg/ha (Hybrid HF5), while in Field 15, the highest fresh plant yield were produced with the range of 48 111 kg/ha (Hybrid HF12) to 70 889 kg/ha (Hybrid HF6), which were comparable to the performance of the check variety, BTL1 (65 889 kg/ha). Among the single cross hybrids evaluated, HF10, HF3, HF15, and HF1 showed high biomass yield performances in Field 10 and Field 15, Universiti Putra Malaysia.

All hybrids showed superiority over their parental inbred lines for biomass yield traits studied, indicating the existence of substantial amount of heterosis in the hybrids. Compared with the average value of the parents for fresh plant yield (25 388 kg/ha), the 15 hybrids showed a significant ($p<0.01$) yield advantage across environments, with an average of 151.9% mid-parent heterosis (MPH) when data from the environments were pooled. Mid-parent heterosis estimates revealed from the evaluation were positive for biomass yield

Table 3. Mean performance of single-cross hybrids, their parental inbred lines and two check varieties, for biomass yield traits, evaluated at Field 10 and Field 15, Universiti Putra Malaysia

Genotype	At Field 10, UPM				At Field 15, UPM			
	FPY (kg/ha)	FLY (kg/ha)	FSY (kg/ha)	FEY (kg/ha)	FPY (kg/ha)	FLY (kg/ha)	FSY (kg/ha)	FEY (kg/ha)
Hybrid:								
HF1	63778 abcde	17220 bcde	15822 abcd	30736 abcde	61556 a	17663 abc	14329 abcd	29564 abc
HF2	66222 abcde	19429 abcd	15996 abcd	30796 abcd	58555 ab	18940 abc	14820 abc	24795 abc
HF3	61833 abcdef	18652 abcd	19853 a	23329 ef	69556 a	20636 ab	21217 a	27703 abc
HF4	64778 abcde	18269 abcd	19740 a	26768 bcdef	64522 a	17558 abc	16943 abc	30020 abc
HF5	75333 a	20000 abcd	18508 ab	36825 a	30652 cd	9554 de	7784 defg	13314 def
HF6	66000 abcde	19028 abcd	15368 abcd	31604 abc	70889 a	20726 ab	15462 abc	34701 a
HF7	65111 abcde	19994 abcd	17441 abc	27676 bcdef	63000 a	21517 ab	15712 abc	25771 abc
HF8	74445 ab	21944 ab	18725 ab	33775 ab	61444 a	18427 abc	13771 bcd	29247 abc
HF9	66333 abcde	21356 abc	17603 abc	27375 bcdef	65667 a	20401 ab	14893 abc	30373 abc
HF10	73333 abc	22870 a	19867 a	30597 abcde	68296 a	21245 ab	16337 abc	30714 abc
HF11	68667 abcd	19719 abcd	15792 abcd	33155 abc	68889 a	23212 a	18045 ab	27632 abc
HF12	59167 cdef	16105 def	13995 bcd	29066 bcdef	48111 abc	14765 bcd	10656 cdef	22691 bcd
HF13	57889 def	16979 cde	14654 abcd	26256 cdef	68556 a	20399 ab	18353 ab	29803 abc
HF14	59500 bcdef	18419 abcd	18792 ab	22289 f	57333 ab	19277 abc	15605 abc	22451 bcd
HF15	60444 abcdef	18061 abcd	15530 abcd	26853 bcdef	68667 a	21699 ab	17078 abc	29890 abc
Parent:								
CML152	19282 ij	6291 hi	4737 e	8254 hi	27519 cd	8340 de	6403 efg	12776 def
CML331	12516 j	4654 i	3032 e	4830 i	20067 d	5669 e	4478 fg	9920 f
CML383	26556 i	7781 hi	5753 e	13021 gh	22037 d	7044 e	3388 g	11605 ef
CML428	42111 gh	13127 efg	13100 cd	15884 g	38482 bcd	12498 cde	11465 bcde	14518 def
CML491	31519 hi	9894 gh	7472 e	14152 gh	23511 d	7161 e	4953 efg	11398 ef
CML498	28126 i	9758 gh	7046 e	11322 ghi	20711 d	7120 e	4491 fg	9100 f
Check:								
BTL1	48222 fg	12357 fg	12166 d	23699 def	65889 a	16993 abc	15798 abc	33097 ab
Pool26	51933 efg	15659 def	12879 cd	23397 def	52630 ab	19182 abc	14709 abc	21109 cde
S.E.	2324.5	664.2	665.0	1106.9	2524.3	774.1	711.5	1164.6

Mean values followed by the same letter in the same column are not significantly different at p<0.05, based on DNMRT
FPY = fresh plant yield, FSY = fresh stem yield, FLY = fresh leaf yield, FEY = fresh ear yield, and S.E. = standard error.

traits (Table 4). In Field 10, mid-parent heterosis estimates for fresh plant yield ranged from 57.2% to 301.1%, while in Field 15, they ranged from 27.1% to 236.7%. These results indicate the preponderance of dominant gene action in the genetic control of fresh plant yield. The data from the environments pooled showed that 13 out of 15 hybrids exhibited more than 100% heterosis for fresh plant yield, indicating the presence of favorable dominant genes in the parents of the hybrids. The high values of heterosis found from this study for biomass yield traits were the consequence of prior selection of parental inbred lines from different heterotic groups. Similarly, an investigation on heterosis among eight maize landraces conducted by Gabriel *et al.* (2009) has revealed significant mean heterosis obtained from crosses with diverse parents from different heterotic groups. Hybrids HF3 (CML152 x CML428), HF7 (CML331 x CML428) and HF10 (CML383 x CML428) were the top-yielding, expressing high heterosis for the traits measured and sharing a common parental inbred line, CML428. The parental inbred lines of each from these three crosses have been selected from different heterotic groups, indicating that they were genetically distant. Most of the top yielding hybrids were not necessarily those that revealed the top heterosis estimates, but all the top yielding hybrids exhibited high heterosis for the traits concerned.

Significant specific combining ability (SCA) effects were found for all biomass yield traits in Field 10, Field 15 and when data from the environments were pooled, as presented in Tables 5 and 6. This result indicates the presence of significant additive and non-additive gene actions controlling the traits measured. According to Kearsey and Pooni (1998), the magnitudes of GCA and SCA estimates show the importance of additive and non-additive gene effects in the inheritance of quantitative traits and offer vital genetic information about potential genetic progress in breeding programs.

Results from analysis of association were correlated (at p≤0.05) between data from genetic distances using 100 microsatellite markers among the parental inbred lines and the estimates of mid-parent heterosis for fresh plant yield in Field 10 and when data from the environments were pooled (Table 7). There was a significant negative relationship between genetic distances of the parental inbred lines and mid-parent heterosis for fresh plant yield (r = -0.537) when the data from the environments were pooled, revealing the effectiveness of the markers used in this study for prediction of hybrid performance for forage traits. Low correlation values were found between genetic distances among parental inbred lines and hybrid performance *per se* in both environments separately. The associations between the corresponding SCA estimate and hybrid performance *per se* were found to be highly significant for fresh plant yield (0.946 in Field 10, 0.900 in Field 15 and 0.796 in environments pooled), fresh leaf yield (0.663 in Field 10, 0.894 in Field 15 and 0.673 in environments pooled), fresh stem yield (0.833 in Field 15 and 0.673 in environments pooled) and fresh ear yield (0.951 in Field 10, 0.935 in Field 15 and 0.681 in environments pooled). Highly significant correlations were also found between corresponding

Table 4. Estimates of mid-parent heterosis revealed by 15 corn hybrids for biomass yield traits measured at Field 10 and Field 15, Universiti Putra Malaysia

Hybrid	At Field 10, UPM				At Field 15, UPM				Environments Pooled			
	FPY (%)	FLY (%)	FSY (%)	FEY (%)	FPY (%)	FLY (%)	FSY (%)	FEY (%)	FPY (%)	FLY (%)	FSY (%)	FEY (%)
HF1	301.1	307.4	214.6	369.8	158.7	152.2	163.4	160.5	215.8	189.1	207.5	237.1
HF2	188.9	205.0	176.1	189.5	136.3	146.2	202.7	103.4	161.6	187.0	170.0	143.5
HF3	101.4	122.6	92.1	93.3	110.8	98.1	137.5	103.0	106.3	113.9	109.4	98.4
HF4	155.0	223.4	125.7	138.9	152.9	126.5	198.4	148.4	154.0	155.7	169.2	143.8
HF5	217.8	214.2	149.2	276.2	27.1	23.6	42.9	21.7	121.6	106.2	106.0	141.9
HF6	237.8	249.9	206.0	254.1	236.7	226.1	293.1	222.4	237.3	239.8	235.8	236.8
HF7	138.4	116.2	124.9	167.2	115.2	136.9	97.1	110.9	126.4	111.8	127.2	136.7
HF8	238.1	256.5	201.7	255.9	182.0	187.3	192.0	174.4	210.2	197.9	218.4	212.8
HF9	226.4	249.3	196.4	239.0	222.1	219.1	232.1	219.4	224.2	210.1	232.4	228.4
HF10	113.6	110.8	118.8	111.7	125.7	117.4	120.0	135.2	119.3	119.3	114.1	122.8
HF11	136.5	138.8	123.1	144.0	202.5	226.8	332.7	140.3	165.5	190.3	184.4	142.3
HF12	116.4	118.7	83.6	138.8	125.1	108.5	170.5	119.2	120.2	110.6	113.3	129.8
HF13	57.2	42.5	47.5	74.8	121.2	107.5	123.6	130.0	86.5	79.2	74.3	100.4
HF14	69.4	86.6	61.0	63.9	93.7	96.5	95.6	90.1	80.5	75.2	91.5	76.1
HF15	102.7	113.9	83.8	110.8	210.6	203.9	261.7	191.6	148.6	141.5	158.5	146.9

FPY = fresh plant yield, FSY = fresh stem yield, FLY = fresh leaf yield and FEY = fresh ear yield.

Table 5. Mean squares in ANOVA for biomass yield traits measured on 15 forage corn hybrids from a 6 x 6 diallel cross (Griffing's Method 2), evaluated at Field 10 and Field 15, Universiti Putra Malaysia

Source of variation	d.f.	Mean squares							
		At Field 10, UPM				At Field 15, UPM			
		Fresh plant yield	Fresh leaf yield	Fresh stem yield	Fresh ear yield	Fresh plant yield	Fresh leaf yield	Fresh stem yield	Fresh ear yield
Blocks	2	202307006	14269227	8178553	59491617*	506996981*	19996474	27494106	192534610**
Genotypes	20	1111428225	85799904**	86563358**	250669217**	1115387532**	106979110**	85939424**	214295669**
GCA	(5)	80461355	31567480**	12802054	24947436	375296315	40414145*	56436649**	62571350
SCA	(14)	1455083848**	103877379**	111150459**	325909811**	1362084605**	129167432**	95773683**	264870442**
Error	40	53596487	6501757	6182078	12768034	138279555	13859581	12502633	31967865
C.V. (%)		13.45	17.92	15.38	14.87	22.91	23.42	27.90	24.84

Table 6. Mean squares in ANOVA for biomass yield traits measured on 15 forage corn hybrids from a 6 x 6 diallel cross (Griffing's Method 2), from pooled data of the two environments

Source of variation	d.f.	Mean squares			
		Fresh plant yield	Fresh leaf yield	Fresh stem yield	Fresh ear yield
Environments	1	301058772	2320171	76116481**	50464029
Reps/Env.	4	354651994**	14087513	20881667	126013114**
Genotypes	20	2022201481**	179004376**	157235276**	406114964**
GCA	(5)	333762290*	45201577**	79811391**	52102144*
SCA	(15)	2585014544**	223605309**	183043238**	524119237**
Genotypes x Env.	20	204614276**	14538092	14504052	58849922**
GCA x Env.	(5)	121995380	8014621	8192737	35416641
SCA x Env.	(15)	232153908**	16712582	16607824	66661015**
Error	80	95938021	10180669	9342356	22367949
C.V. (%)		18.52	19.74	22.91	20.22

Table 7. Pearson's correlation coefficients between genetic distances revealed by 100 microsatellites (GD) among six parental inbred lines and hybrid performance for biomass yield traits, mid-parent heterosis (MPH) and specific combining ability (SCA) for fresh plant, leaf, stem and ear yields from 15 corn hybrids, evaluated at Field 10, Field 15 and when data of the two environments were pooled.

Environment	Parameter	Pearson's correlation coefficient											
		Hybrid performance				Mid-parent heterosis (MPH)				Specific combining ability (SCA)			
		FPY	FLY	FSY	FEY	FPY	FLY	FSY	FEY	FPY	FLY	FSY	FEY
Field 10	GD	0.133	0.386	0.309	-0.154	-0.546*	-0.553*	-0.409	-0.576*	-0.104	-0.028	0.088	-0.217
	MPH	0.512	0.216	0.029	0.654**	-	-	-	-	-	-	-	-
	SCA	0.946**	0.663**	0.491	0.951**	0.747**	0.631*	0.722**	0.776**	-	-	-	-
Field 15	GD	-0.019	0.147	0.124	-0.366	-0.366	-0.288	-0.307	-0.389	-0.251	-0.129	-0.120	-0.334
	MPH	0.686**	0.666**	0.316	0.871**	-	-	-	-	-	-	-	-
	SCA	0.900**	0.894**	0.833**	0.935**	0.846**	0.834**	0.719**	0.900**	-	-	-	-
Pooled environments	GD	0.051	0.305	0.293	-0.300	-0.537	-0.424	-0.487	-0.592*	-0.307	-0.125	-0.078	-0.507
	MPH	0.420	0.140	0.121	0.718**	-	-	-	-	-	-	-	-
	SCA	0.796**	0.673**	0.681**	0.910**	0.809**	0.662**	0.681**	0.886**	-	-	-	-

FPY = fresh plant yield, FLY = fresh leaf yield, FSY = fresh stem yield and FEY = fresh ear yield.

SCA estimate and mid-parent heterosis for fresh plant yield (0.747 in Field 10, 0.846 in Field 15 and 0.809 in environments pooled), fresh leaf yield (0.663 in Field 10, 0.894 in Field 15 and 0.673 in environments pooled), fresh stem yield (0.722 in Field 10, 0.719 in Field 15 and 0.681 in environments pooled) and fresh ear yield (0.776 in Field 10, 0.900 in Field 15 and 0.886 in environments pooled) among hybrids. This emphasises the importance of SCA in the determination of heterosis and hybrid performance *per se*.

CONCLUSION

Positive heterosis estimates were obtained for the corn biomass yield traits including fresh plant yield, revealing the significant contribution of dominance in the manifestation of the forage traits measured. These results indicate that selection of parental lines based on molecular characteristics during the evaluation of inbred lines have proven effective in accumulating dominant genes for the traits, resulting in high heterosis in the hybrids produced. The findings also revealed a weak association between genetic distance and F_1 hybrid performance, although SCA remained to be an important factor contributing to high heterosis and high hybrid performance *per se*.

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TPGM

Genetic parameters estimation and selection of *Pisum sativum* accessions in lowland

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Abstract

Peas are classified in the Fabaceae family which has high economic value. In Indonesia, peas are only developed in the highlands. Efforts to expand the cultivation area need to be done to increase production capacity. Breeding needs to be done to get the pea genotype that adapts to the lowlands. Successful selection requires information about genetic parameters such as genetic variability and heritability. This research was conducted to estimate the value of some genetic parameters and to obtain genotypes that have high yield potential in the lowlands. 82 accessions of peas originating from several areas in Indonesia were planted in experiments that followed augmented design together with three comparative genotypes. Based on the character of dry pod weight per plant and dry seed weight per plant, 3 accessions were selected namely 03 (16) (2) -1, Batu-1-1 and Batu-2.

Key words: Genetic variability, heritability, lowland, adaptation

INTRODUCTION

Pea (*P. sativum* L.) is a leguminous plant that has quite high economic value and good as a food source because it has high nutritional value (Dahl *et al*., 2012). The seeds contain 22-25% protein, complex carbohydrates, and fiber content, a variety of vitamins, minerals, and phytochemicals which valuable for human food and animal feed. The dried pods rank third for the most developed bean species in the world (Poblaciones *et al*., 2013; Smýkal *et al*., 2012).

Pea is a winter plant (cool-season), so it will grow optimally in low-temperature areas. In Indonesia, a pea can only be grown in the highlands, so it's increasing demand cannot be accompanied by the availability of peas because of the limited area.

Pea breeding is directed to get high yielding and adaptive varieties in lowland areas through selection activities. The effectiveness of selection for character improvement is determined by genetic diversity and heritability value (Handayani and Hidayat, 2012; Jameela *et al*., 2014). Selection activities will be effective if the value of genetic progress is high which is supported by a high value of genetic diversity and heritability (Herawati *et al*., 2009). The purpose of this study is to estimate the diversity and heritability of character of 82 accessions of pea in lowland areas and determine the potential of pea genotypes developed in the lowlands.

MATERIAL AND METHODS

Eighty-two pea accessions were planted in the Agro Techno Park greenhouse, Universitas Brawijaya, in the rainy season of 2019. The research location was at an altitude of ± 400 m asl. with a minimum temperature of 24°C and a maximum temperature of 31°C. Genetic material is pea accessions obtained from various places in Indonesia. The plants were planted with standard cultivation procedures following an augmented design with three comparative genotypes. Observations were made on 32 quantitative and 22 qualitative characters based on the National Draft Descriptor Guidelines for the Conduct of Test for Distinctness, Uniformity, and Stability of Pea (*Pisum sativum* L.) (UPOV, 2006).

Quantitative data were analyzed using analysis of variance (ANOVA) for augmented design (Sharma, 2006). If there are real differences, the least significant increase (LSI) test is performed to determine which genotypes have better results

$$LSI = t_{\alpha}\sqrt{\frac{(b+1)(c+1)\,MSe}{b.c}}$$

where:

t_{α} = Value t one-way table of degrees free of MSe
MS_e = Mean Square of Error
c = number of check
b = number of block

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Estimation of genetic and phenotype variance components is calculated by the formula:

$$\text{Environment Variance } (\sigma^2_e) = MS_e$$
$$\text{Phenotype Variance } (\sigma^2_f) = MS_e$$
$$\text{Genetic Variance } (\sigma^2_g) = \sigma^2_p - \sigma^2_e = MS_g - MS_e$$

Genetic Coefficient Variance (GCV) and Genetic Coefficient Variance (PCV) of each character are determined based on the equation of Singh and Chaudhary (1979), as follows:

$$GCV = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100\%$$

$$PCV = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100\%$$

Where:
σ^2_g = genotype variance
σ^2_p = phenotype variance
$\bar{x}$ = mean of each character

Criteria of GCV and PCV based on Singh dan Chaudhary (1979) are: low (GCV and PCV < 10%), moderate (10% ≤ GCV and PCV ≤ 25%) and high (GCV and PCV > 25%).

Estimation of broad-sense heritability value was conducted based on the separation of variance components using equations by Acquaah, (2012):

$$h^2 = \frac{\sigma^2_g}{\sigma^2_p} \qquad h^2 = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_e}$$

where:
σ^2_g = genetic variance
σ^2_e = environment variance

Criteria of heritability value:

high ($h^2 > 0.50$), moderate ($0.20 \leq h^2 \leq 0.50$) and low ($h^2 < 0.20$) (Stansfield, 1991).

RESULT AND DISCUSSION

The result of the ANOVA showed differences among pea accessions in some characters. These show that variability occurs in each character is caused by the effect of the genotypic factor.

Genetic and phenotype variability

Variability of 82 pea genotype characters observed from the variability of quantitative characters and qualitative characters. The variability of quantitative characters is determined through the GCV and PCV values. Phenotypic variability and estimated heritability value are some genetic parameters that can be used as a basis for consideration so that selection activity runs effectively and efficiently (Hapsari, 2014).

Table 2 shows that the GCV and PCV values of 82 pea accession planted in lowland have varying values. The characters with GCV and PCV values with high criteria were found on the characters of the stem length to the first pod, the number of brachtea, leaf stover weight, dry pod weight per plant, and dry seed weight per plant. Jhanavi *et al.* (2018) explain that high GCV and PCV values indicate sufficient variability in the genetic resources used and the trait is regulated by additive genes. This shows that there are many opportunities to improve the character through direct selection.

The results show that the PCV value tends to be greater than the GCV value, this indicates that the variability appears is more influenced by environmental factors. Similar result found by Ton *et al.* (2018); Jaiswal *et al.* (2013); Saxesena *et al.* (2014); dan Katoch *et al.* (2016) which shows the results of PCV values are generally higher than GCV values because of variations by environmental factors are more dominant than variations in genetic factors.

Qualitative characters observed in the study indicate that there are 6 characters that have uniformity between genotypes and 16 other characters that vary between genotypes (Table 5). Syukur *et al.* (2012) explain that qualitative characters are controlled more by simple genes (one or two genes) and are not or less influenced by the environment. The appearance of qualitative characters 82 genotypes of peas are more influenced by genetic factors and can be inherited in offspring.

The estimated value of heritability

The estimated value heritability values of 82 pea genotypes in the lowlands are shown in Table 4. The results showed that there are several characters that have high criteria for heritability. Heritability is a quantity that shows the proportion of genetic factors to environmental factors displayed by a character. Heritability is a genetic parameter that describes the ability of a genotype in a population to pass on the characters possessed to their offspring. The diversity of a character caused by genetic factors can be known through heritability calculations (Sa'diyah *et al.,* 2013). Characters that have estimated value heritability with high criteria include plant height, distance between nodes, age of flowering, dry harvest age, length of stipule, width of stipule, distance of axile to tip of stipule, length of axillary to first leaflets, width of leaflets, leaf stover weight, stem stover weight, dry pod weight per plant, dry seed weight per plant, dry seed weight per pod, dry pod length, dry pod width, dry seed length and dry seed width.

High heritability value indicates that the appearance of a character is more influenced by genetic factors than environmental factors (Syukur *et al.,* 2011). Barcchiya *et al.* (2018)) explained that the heritability value in a broad sense would assist in the identification of characters suitable for selection and help breeders to choose superior genotypes based on the phenotypic appearance of quantitative traits. Genotype selection for selection purposes is better if it is based

Table 1. Analysis of variance of observed characters of 85 accessions using augmented design planted in lowland

No.	Characters	Mean	Range		MS Genotype	Error
			Min	Max		
1	Plant height (cm)	164.26	96.20	244.35	1064.73**	141.23
2	Length of internode (cm)	10.20	7.00	14.63	2.47**	0.50
3	Number of Branch	1.75	0.00	4.00	0.79^{ns}	1. 90
4	Max number of leaflets	34.61	17.50	83.25	134.37^{ns}	152.82
5	Number of fertile node	17.37	13.00	24.50	5.26^{ns}	3.45
6	Days of flowering	35.77	29.00	48.00	26.40*	7.19
7	Days of harvesting	63.43	56.00	79.00	29.61*	7.39
8	Stipulate length (cm)	4.95	3.25	6.85	0.53*	0.16
9	Stipulate width (cm)	2.55	1.50	4.00	0.23*	0.07
10	Axil-stipulate tip distance (cm)	3.73	2.35	4.87	0.28*	0.11
11	Axil-first leaflets distance (cm)	5.00	3.60	7.20	0.49*	0.16
12	Leaflets lenght (cm)	3.84	2.75	4.87	0.22^{ns}	0.32
13	Leaflets width (cm)	2.20	1.50	3.25	0.14^{ns}	0.06
14	Stalk-first pod lenght (cm)	6.15	1.28	14.58	8.75^{ns}	5.40
15	Distance of 1st pod – 2nd pod (cm)	0.14	0.00	2.85	0.23^{ns}	0.26
16	Number of brachtea	4.72	0.00	24.25	32.80^{ns}	46.45
17	Leaf stover weight (g)	1.66	0.39	5.37	1.07**	0.20
18	Root stover weight (g)	0.03	0.00	0.12	0.00^{ns}	0.00
19	Stem stover weight (g)	2.22	0.54	7.16	2.12**	0.19
20	Number of dry pod	8.26	2.25	17.25	9.88^{ns}	7.48
21	Pod dry weight (g)	6.16	1.94	14.69	5.51*	2.04
22	Seed dry weight (g)	5.39	1.68	13.43	4.29*	1.55
23	Number of dry seed	30.33	8.50	73.50	133.53^{ns}	82.59
24	100 seed weight (g)	17.86	13.43	24.55	4.43^{ns}	3.54
25	Seed dry weight per pod (g)	0.81	0.50	1.44	0.03**	0.00
26	Number of dry seed per pod	4.36	3.00	6.00	0.44^{ns}	0.33
27	Dry pod lenght (cm)	5.93	4.45	9.42	0.53*	0.18
28	Dry pod width (mm)	10.38	7.70	16.87	3.06**	0.45
29	Thickness of dry pod (mm)	6.46	5.76	8.21	0.19^{ns}	0.13
30	Dry seed lenght (mm)	7.48	6.60	8.13	0.10^{ns}	0.04
31	Dry seed width (mm)	6.89	5.95	7.71	0.13^{ns}	0.05
32	Dry seed thickness (mm)	5.54	4.83	7.43	0.11^{ns}	0.09

Note: * = significantly different at 5%; ** = significantly different at 1%; ns = non-significant

on high GCV values together with high heritability and genetic progress (Selvaraj *et al.,* 2011).

The results showed that some characters have negative heritability. The heritability value is obtained from the comparison of the value of genetic variance with phenotype variance, while the value of genetic variance is obtained from the operation of reducing phenotype variance with environmental variance. In theory, the value of genetic variance cannot be negative, but it can be negatively fielded due to the addition and subtraction of the components of the variance so that a negative value is obtained. The value of environmental variance is greater than the phenotype variance results in negative genetic variance. The results of the analysis of variance on characters that have a negative heritability value have results that are not significantly different (non-significant), so it is considered to have no variability or a value of 0 (zero).

Table 2. The value of GCV and PCV of pea characters planted in lowland

No.	Characters	GCV	Criteria	PCV	Criteria
1	Plant height (cm)	21.15	Moderate	22.71	Moderate
2	Length of internode (cm)	15.87	Moderate	17.76	Moderate
3	Number of Branch	23.94	Moderate	20.40	Moderate
4	Max number of leaflets	12.03	Moderate	32.47	High
5	Number of fertile node	7.56	Low	12.90	Moderate
6	Days of flowering	12.17	Moderate	14.26	Moderate
7	Days of harvesting	7.35	Low	8.48	Low
8	Stipulate length (cm)	13.53	Moderate	16.14	Moderate
9	Stipulate width (cm)	17.49	Moderate	20.94	Moderate
10	Axil-stipulate tip distance (cm)	12.37	Moderate	15.72	Moderate
11	Axil-first leaflets distance (cm)	12.31	Moderate	15.12	Moderate
12	Leaflets lenght (cm)	8.81	Low	12.72	Moderate
13	Leaflets width (cm)	14.85	Moderate	19.44	Moderate
14	Stalk-first pod lenght (cm)	30.00	High	48.51	High
15	Distance of 1st pod – 2nd pod (cm)	12.39	Moderate	23.28	Moderate
16	Number of brachtea	25.33	High	51.64	High
17	Leaf stover weight (g)	60.65	High	67.40	High
18	Root stover weight (g)	1.57	Low	2.50	Low
19	Stem stover weight (g)	71.33	High	74.76	High
20	Number of dry pod	18.26	Moderate	37.06	High
21	Pod dry weight (g)	33.07	High	41.64	High
22	Seed dry weight (g)	33.76	High	42.25	High
23	Number of dry seed	23.53	Moderate	38.10	High
24	100 seed weight (g)	5.92	Rendah	13.21	Moderate
25	Seed dry weight per pod (g)	22.17	Moderate	24.18	Moderate
26	Number of dry seed per pod	8.04	Moderate	16.10	Moderate
27	Dry pod lenght (cm)	10.44	Moderate	12.78	Moderate
28	Dry pod width (mm)	16.41	Moderate	17.78	Moderate
29	Thickness of dry pod (mm)	4.00	Low	7.10	Low
30	Dry seed lenght (mm)	3.38	Low	4.42	Low
31	Dry seed width (mm)	4.11	Low	5.30	Low
32	Dry seed thickness (mm)	2.93	Low	6.16	Low

Selected genotypes

The selection of pea genotypes is carried out to look for genotypes that have the potential to be developed in the lowlands. The selection was performed on characters that had significantly different variance analysis results and affected yield characters, namely the characters of flowering age, dry harvest age, dry pod weight per plant, dry seed weight per plant and dry seed weight per pod. Selected genotypes are genotypes that have an average value greater than the average comparator + LSI value, except for the character of flowering and dry harvest age. For both of these characters, the selected genotype is a genotype that has a smaller average than the comparison average - the LSI value. The selection is then based on the scoring of each character. The character that has the most significant difference compared to the comparison is the genotype chosen in the highlands. The notation "a" indicates a better genotype than cv. Calibra, notation "b" is better than SMG(H)(03), notation "c" is better than 05-(16)-1 (Table 5).

Table 3. Performance of qualitative pea characters planted in lowland

No	Characters	Catagory	% Genotype	No	Characters	Catagory	% Genotype
1	PAC	Not exist	13.41	12	LC	Yellowish green	6.10
		Exist	86.59			Green	93.90
2	AAC	Not exist	13.41	13	LCI	Bright	9.09
		Single ring	64.63			Moderate	67.53
		Double ring	21.95			Dark	23.38
3	LE	Exist	100	14	LS	Exist	100
4	WC	Redish purple	100	15	FBC	White	100
5	FBS	Very convex	4.88	16	AS	Acumulated	43.90
		Moderate concex	28.05			Acute	45.12
		Flat	67.07			Round	10.98
6	LD	Not exist /very weak	9.76	17	PP	Not exist/partly	81.71
		Weak	24.39			Entire	18.29
		Moderate	41.46	1.	PWT	Not Exist	89.02
		Strong	24.39			Exist	10.98
7	LSD	Rare	46.55	19	PC	Not Exist /very weak	41.46
		Moderate	53.45			Weak	46.34
		Dense	41.38			Moderate	12.20
8	PTS	Pointed	56.10	20	SS	Ellipse	26.83
		Blunt	43.90			Cylincrical	73.17
9	CC	Yellow	92.68	21	TC	Reddish brown	28.05
		Orange	7.32			Brown	50.00
10	PCL	Green	100			Brownish green	21.95
11	WH	Identic to testa	75.61	22	ST	Exist	100
		Darker than testa	24.39				

Note: PAC = plant anthocyanin color; AAC = Axile anthocyanin color; LE = leaflets existance; WC = wing color; FBC = flower based color; LC = leaf colorwarna daun; LCI = leaf color intencity; LS = leaf spot; FBS = flower base shape; AS = apex shape; LD: leaf dentation; PP: pod parchment; PWT = pod wall thickness; LSD = leaf spot density; PC = pod curve; PTS = pot tip shape; PCL = pod color; CC = cotyledon color; SS = seed shape; TC = testa color: HC = hylum color; ST = sed texture.

It is important to increase the genetic resources of peas to produce new high yielding varieties that can increase production, management, profits and increase marketing of pea seeds (Saxesena *et al.,* 2014). Selection is carried out to search for pea genotypes that have the potential to be developed in the lowlands. Selection is based on the LSI test by comparing the average of the tested genotype with the comparison (check) used. On the character of flowering age and harvest age, it can be seen that there are 16 genotypes that have flowering age and dry harvest age better than the two cv comparison. Calibra and SMG(H)(03), i.e. genotypes with the notation "ab". While the results of economic value are dry weight per plant and pod weight per plant, there are 3 genotypes that have an average yield better than the three comparisons, namely genotype followed by the "abc" notation.

CONCLUSION

There are several pea characters that have a high diversity and heritability in the lowlands so that selection is still very possible. Based on the character of dry pod weight per plant and dry seed weight per plant, 3 accessions were selected namely 03 (16) (2) -1, Batu-1-1 and Batu-2.

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Tabel 4. The estimated value of heritability (h^2) of pea characters planted in lowland

No.	Characters	σ^2_g	σ^2_g	σ^2_p	h^2	Criteria
1	Plant height (cm)	141.23	923.51	1064.73	0.87	High
2	Length of internode (cm)	0.50	1.97	2.47	0.80	High
3	Number of Branch	0.23	-0.13	0.10	-1.38	Low
4	Max number of leaflets	152.82	-18.45	134.37	-0.14	Low
5	Number of fertile node	3.45	1.81	5.26	0.34	Moderate
6	Days of flowering	7.19	19.21	26.40	0.73	High
7	Days of harvesting	7.39	22.22	29.61	0.75	High
8	Stipulate length (cm)	0.16	0.38	0.53	0.70	High
9	Stipulate width (cm)	0.07	0.16	0.23	0.70	High
10	Axil-stipulate tip distance (cm)	0.11	0.18	0.28	0.62	High
11	Axil-first leaflets distance (cm)	0.16	0.32	0.49	0.66	High
12	Leaflets lenght (cm)	0.32	-0.10	0.22	-0.48	Low
13	Leaflets width (cm)	0.06	0.08	0.14	0.58	High
14	Stalk-first pod lenght (cm)	5.40	3.35	8.75	0.38	Moderate
15	Distance of 1st pod – 2nd pod (cm)	0.06	-0.01	0.04	-0.28	Low
16	Number of brachtea	1.00	0.32	1.33	0.24	Moderate
17	Leaf stover weight (g)	0.20	0.86	1.07	0.81	High
18	Root stover weight (g)	0.00	0.00	0.00	0.40	Moderate
19	Stem stover weight (g)	0.19	1.93	2.12	0.91	High
20	Number of dry pod	7.48	2.40	9.88	0.24	Moderate
21	Pod dry weight (g)	2.04	3.48	5.51	0.63	High
22	Seed dry weight (g)	1.55	2.74	4.29	0.64	High
23	Number of dry seed	82.59	50.94	133.53	0.38	Moderate
24	100 seed weight (g)	3.54	0.89	4.43	0.20	Moderate
25	Seed dry weight per pod (g)	0.00	0.02	0.03	0.84	High
26	Number of dry seed per pod	0.33	0.11	0.44	0.25	Moderate
27	Dry pod lenght (cm)	0.18	0.35	0.53	0.67	High
28	Dry pod width (mm)	0.45	2.61	3.06	0.85	High
29	Thickness of dry pod (mm)	0.13	0.06	0.19	0.32	Moderate
30	Dry seed lenght (mm)	0.04	0.06	0.10	0.58	High
31	Dry seed width (mm)	0.05	0.08	0.13	0.60	High
32	Dry seed thickness (mm)	0.09	0.02	0.11	0.23	Moderate

Table 5. List of selected accession based on yield character

No.	Genotype		Characters				
			DF (dap)	DH (dap)	PDW (g)	SDW (g)	SDP (g)
1	01(16)|2|-1		32.28a	59.94a	6.28a	5.54a	0.76a
2	01(16)|3|-1		40.61	65.28a	6.67a	5.67a	0.75a
3	02(16)|2|		41.61	78.94	3.23	2.79	0.75a
4	03(16)|2|-1		45.28	68.94	14.99abc	13.64abc	1.13abc
5	03(16)|2|-2		38.61	68.61	5.1a	4.47a	0.76a
6	03(16)|3|-1		37.61a	62.61a	6.17a	5.55a	0.78a
7	03-(16)-|3|-2		32.61a	58.61a	7.03a	7.14a	0.92ab
8	03(16)|3|-3		32.61a	58.94a	2.72	2.48	0.61a
9	03-(16)-1		36.61a	66.61a	8.46a	7.65a	0.93ab
10	04(16)|1|U1		43.28	75.94	5.88a	4.97a	0.62a
11	04(16)|1|U2		42.28	67.94	7.57a	6.63a	0.83a
12	04(16)-5		47.28	68.61	2.63	2.31	0.74a
13	05(16)|2|-1		33.28a	62.94a	8.33a	7.5a	0.92ab
14	05-(16)-1		32.28a	60.61a	2.04	2.05	0.66a
15	06-(16)-(11)-1		34.28a	66.61a	7.42a	6.6a	0.9ab
16	06(16)1-1		29.28ab	59.61a	5.3a	4.97a	0.78a
17	06(16)2-1		29.61ab	57.94a	0.15	0.18	0.56a
18	10-(16)-(1)		36.28a	59.61a	5.5a	5.01a	0.61a
19	3(16)1-2		36.61a	63.61a	9.41ab	8.42ab	0.99abc
20	Batu 1		43.61	69.94	2.04	1.8	0.68a
21	Batu 1-1		45.28	69.61	11.46abc	9.9abc	1.02abc
22	Batu 1-2		43.28	68.61	9.04a	7.58a	0.91ab
23	Batu 1-4		45.61	78.94	5.95a	4.94a	0.64a
24	Batu 2		46.28	69.94	11.07abc	9.6abc	1.08abc
25	Batu 3		47.28	69.94	4.63	3.95	0.81a
26	Bromo 1		39.28	69.61	5.76a	4.66a	0.75a
27	Bromo 2		42.28	66.94a	5.3a	3.04	0.59a
28	Bromo 3		40.28	67.94	6.8a	5.48a	0.9ab
29	Bromo 4		43.61	68.61	8.55a	6.89a	1.3abc
30	Bromo 5		43.61	68.94	4.3	3.56	1.36abc
31	Bromo 6		38.61	62.94a	6.15a	4.82a	0.81a
32	BTG-1		37.61a	65.94a	2.56	2.07	0.53a
33	BTG-2		32.28a	62.94a	7.86a	6.77a	0.76a
34	BTG-3		38.28	68.61	4.9	4.25a	0.64a
35	BTG-4		37.28a	68.61	5.94a	5.25a	0.76a
36	BTG-5		34.61a	63.94a	9.43ab	7.9a	1.13abc
37	GRT-(03)		30.61a	62.61a	6.4a	5.53a	0.85ab
38	GRT(04)|1|		30.61a	58.61a	7.67a	6.49a	0.84a
39	GRT(PSO-1-1)		32.61a	60.61a	8.48a	7.17a	0.72a
40	GRT(PSO-1-2)		33.61a	63.61a	6.7a	5.76a	0.71a
41	GRT(PSO-2-1)		36.28a	62.61a	5.73a	5a	0.74a
42	GRT(PSO-2-2)		35.61a	64.28a	9.18a	8.23ab	0.88ab
43	GRT(PSO-3-1)		33.28a	57.94a	2.61	2.28	1.46abc
44	GRT-02(1)-1		28.61ab	55.94ab	4.83	4.05	0.87ab
45	GRT-02(2)-1		31.61a	55.94ab	2.21	1.73	0.74a
46	GRT-02(2)-2		28.61ab	55.94ab	4.51	3.66	0.86ab
47	GRT04(1)-1		32.28a	61.94a	4.4	3.66	0.9ab
48	GRT04(1)-2		30.61a	56.28ab	6.51a	5.47a	0.78a
49	GRT04(3)-1		30.28a	56.94ab	3.66	3.04	0.92ab
50	GRT04(3)-2		35.28a	64.61a	7.01a	6.23a	1.01abc
51	SMG(C)(1)		32.28a	59.94a	7.21a	6.59a	0.73a
52	SMG(C)(2)		35.28a	64.61a	4.75	4.35a	0.87ab
53	SMG(C)(3)-1		33.28a	57.94a	4.19	3.79	0.71a
54	SMG(D)(3)		34.28a	59.61a	6.12a	5.62a	0.87ab
55	SMG(E)(3)1		31.28a	57.94a	4.24	3.68	0.6a
56	SMG(H)(03)		33.61a	62.94a	6.76a	6.06a	0.77a
57	SMG(H)(05)		34.61a	58.94a	1.53	1.46	0.65a
58	SMG(H)(05)-1		31.61a	56.94ab	2.33	2.19	0.7a
59	Taichung (C)		44.61	69.94	5.66a	5.04a	0.92ab
60	Taichung (H)		43.61	67.94	8.86a	7.82a	0.81a
61	TMG 1-1		33.61a	63.94a	0.59	0.65	0.45a
62	TMG 1-2		35.28a	68.94	7.56a	6.87a	0.81a
63	TMG 1-3		33.28a	57.94a	8.84a	8.03a	0.86a
64	TMG 1-4		29.28ab	56.94ab	4.79	4.34a	0.78a
65	TMG 2-1		33.28a	56.94ab	8.17a	7.48a	0.81a
66	TMG 2-2		36.61a	70.61	3.36	2.98	0.64a
67	TMG 2-3		35.61a	60.28a	8.77a	7.87a	0.82a
68	TMG 2-4		39.61	63.28a	5.28a	4.46a	0.55a
69	TMG 3-1		36.28a	59.61a	3.56	3.31	0.79a
70	TMG 3-2		30.61a	54.61ab	4.98	4.41a	0.61a
71	TMG 4-1		31.61a	56.61ab	8.01a	7.28a	0.81a
72	TMG 4-2		34.61a	60.28a	5.77a	5.17a	0.7a
73	TMG 4-3		29.28ab	59.61a	5.47a	5.01a	0.85a
74	TMG 5-1		29.28ab	56.94ab	3.17	2.76	0.72a
75	TMG 5-2		30.61a	56.28ab	3.05	2.65	0.74a
76	TMG 6-1		32.28a	59.61a	5.6a	5.06a	0.87ab
77	TMG 6-2		34.61a	57.94a	2.49	2.23	0.79a
78	TMG 6-3		35.61a	60.28a	7.46a	6.62a	0.87ab
79	TMG 7-1		33.61a	58.61a	6.09a	6.29a	0.77a
80	TMG 7-2		33.28a	59.61a	5.14a	4.72a	0.78a
81	TMG 8-1		29.61ab	56.61ab	4.47	3.97	0.83a
82	TMG 8-2		32.28a	59.61a	4.41	3.88	0.7a
cv. Calibra		a	44.00	73.83	1.82	1.42	0.29
SMG(H)(03) [CEK]		b	36.33	63.33	6.14	5.29	0.71
05-(16)-1 [CEK]		c	28.50	57.50	7.40	6.56	0.78
	LSI		6.06	6.14	3.22	2.82	0.15

Note: DF (days of flowering); DH (days of harvesting); PDW (pod dry weight); SDW (Seed dry weight); SDP (Seed dry weight per pod). Notasi "a" (lebih baik dibandingkan cv. Calibra); "b" (lebih baik dibandingkan SMG(H)(03) [CEK]); "c" (lebih baik dibandingkan 05-(16)-1 [CEK]); Tanpa notasi (tidak lebih baik dibandingkan ketiga pembanding).

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TPGM

Telomere length diversity under the influence of heat and feed restriction stresses in broiler chicken

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Abstract

Telomere is nucleoprotein and noncoding sequence of DNA which protects end of chromosome from degradation. It becomes shortened with exposure to various types of stress and knowledge on how it is affected by heat and feed restriction stresses still remains uncleared. This work is designed to evaluate telomere length as a well-being biomarkers in chickens exposed to both conditions of stress. The experiment was carried out at Institute of Tropical Agriculture and Food Security, UPM livestock unit. Forty broiler birds of equal weight (1700 g ±100 g) were selected from 300 Cobb broilers at 36 days of age and subjected to treatments namely heat stress, heat stress negative control, feed restriction and feed restriction negative control. Ten birds were placed in each treatment group. The heat stress group were allotted into climatic chamber and exposed to 34˚C for 6hrs while the feed restriction group were exposed to 60% feed reduction of the feed given to the control groups in previous day. It was observed that there were significant changes (P<0.05) in the body weight and body conformation traits of the birds exposed to the stresses when compared to the control groups. It was observed that the telomere length was reduced in feed restricted birds in week 1 and 2. Heat stress also led to telomere length attrition in both weeks. There is inverse correlation between telomere length and body weight. Telomere length had a strong and negative correlation with body length in week 1(-0.98, P<0.05). This study revealed that feed restriction and heat stress reduced the growth performance of the birds and the telomere length of the birds under stressed conditions. The study also revealed that shorter telomere is associated to higher body mass index. It is therefore concluded that telomere length could be used as an important physiological stress bio-markers in chickens.

Key words: Black pepper, transcriptomic, sequencing, gene ontology

INTRODUCTION

In general, chickens are exposed to a variety of external and internal stressors, including stocking density, temperature, transportation, feed restriction, feed contamination, fear, and disease (Keles *et al*., 2010; Zulkifli *et al.*, 2009). These stressors have adverse effects on the growth and production performances of chickens. Daghir (1995a) revealed depressed growth rate and decreased feed consumption of broilers raised in higher environmental temperature that have been reported in many studies over a number of years. Studies conducted under artificially regulated housing have consistently shown that feed restriction at early age stimulated the ability of chickens to withstand high ambient temperatures at younger age than those fed ad libitum throughout the experiment (Zulkifli *et al*., 1994a, 2000a). Many studies have been conducted to identify biological markers for assessing the physiological and immunological responses of birds under stress conditions. Some of the immune parameters, such as the hematological values, and plasma corticosterone levels are well known as physiological indicators (de Jong *et al.*, 2002). The use of well-being markers as a tool to determine survivability, adaptability and as a measure of fitness traits is becoming promising in the future of poultry sector. One of the well-being markers is Telomere.

Telomeres are DNA and nucleoprotein complexes which consist of tandem repeat of TTAGGG sequences. Telomere is located at the ends of eukaryotic chromosomes shielding them from fusion and degradation and are subject to shortening or attrition due to cell division or DNA replication (Monaghan, 2014; Boonekamp *et al*., 2014). The aforementioned transcription factors are the upstream signaling pathways sensing the free radical generation and trigger a wide array of response to stress. Telomeres shorten with the age of the organism in most of the somatic cells of vertebrates (Kim *et al.*, 2011). However, telomere attrition is usually aggravated by exposure to stress conditions (Von Zglinicki, 2002; Richter and Proctor, 2007). Damage to DNA naturally occurs under normal physiological conditions, but the extent of the damage to DNA is accelerated under various stressors (Chen *et al.*, 2007).

Telomere has been documented as biomarkers for measuring the level of stresses to which animals are exposed. Richter and Proctor (2007) reported a worsened situation of telomere attrition when exposed to stressors. Sohn *et al.* (2011) observed significant lymphocyte telomere shortening in the broiler birds exposed to feed restriction stress higher than the control. Therefore, the telomere length may effectively represent the individual animal wellbeing status. Many works had been done on how ill-health situations affect telomere

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Table 1. Oligomers used for absolute telomere assay in chicken

	Oligomers names	Oligomers sequences	Amplicon sizes
PCR Primers	**Telomere**	ACACTAAGGTTTGGGTTTGGGTTTG GGTTTGGGTTAGTGT- **F** TGTTAGGTATCC CTATCCCTATCCCTATCCCTATCCCTAACA-**R**	**79bp**
	GAPDH	ACTATGCGGTTCCCAGTGTC-**F** TGCCACCATCAGAAAAATGA-**R**	**215bp**

F=Forward primer, R=Reverse primer

Table 2. Representation of master mix preparation

Reagents	Volumes for one sample (µl)	Final Concentration
Power SYBR Green master mix (2x)	10	1x
Primer telomere-F (2µM)	1	0.1 µM
Primer telomere-R (2µM)	1	0.1 µM
H_2O	4	
DNA template (5ng/µl)	4	20ng

length in various species including human beings. However, studies on how stresses affect telomere length in broiler is still not cleared and this necessitated this work. This study is therefore designed to determine the effect of stress factors of both heat and feed restriction on telomere length and how the variation in telomere length affect traits of economic important in broiler chicken.

MATERIALS AND METHODS

The Fourty (40) birds of 1700 ±100g which include 10 each of heat, heat control, feed restriction and feed restriction control were randomly selected from 300 cobb broilers at 36 days of age. They were tagged accordingly for proper identification and adapted for 3 days after which they were allotted into their various cages. The heat stress group (H) was allotted into climatic chambers (environmentally controlled house) and exposed to 34˚C for 6hrs daily while their control group (HC) was allotted to climatic chamber with 25 ˚C. Their cohort, the feed restriction group (FR) was exposed to 60% feed reduction of the feed given to the control groups daily while their control group (FRC) was given feed and water ad-libitum. The Feed restricted group and their control were raised in an open sided house under cyclic temperature.

Body weight, body conformation traits and blood sampling were taken right from the first day of the experiment and at week interval in order of day 1, week 1 and week 2. The body weight was measured with electronic weighing scale while the body conformation traits were taken with measuring tape.

Blood sampling and DNA extraction

Blood samples were collected from the wing veins of five randomly picked birds from each group into EDTA vacutainer and stored at -20˚C before the DNA extraction. DNA samples were extracted from 150µl blood using innuPREP Blood DNA Mini Kit following the recommendation of the manufacturer (Analytikjena). The qualities of the DNA were tested using nanodrop (Spectrophotometer, MultiskanGo) and DNAs with 1.8-2.0 (260/280) values were stored for telomeric length determination.

For the discovery of the telomere length, the primers were adopted from Cawthon (2009).The GAPDH (glyceraldehyde-3-phosphatase) primer sequences were designed and sequenced using information in gene bank (NCBI) (Table 1) with reference number NC_006088.5 (region between 77619440---77623355) with Tm 60˚C and exon count of 12 and it was specific to chicken. The two genes were optimized using normal PCR before the real-time PCR analysis.

The same master mix preparation was used for the reference gene (GAPDH) as shown in Table 2. The SYBR green mix (SensifAST™ SYBR No-ROX One-Step) was obtained from Bioline chemical company and stored at -20˚C. Primer sequences were synthesized by Apical chemical company. Water was added to obtain concentration of 100µM and working solution of 2µM was prepared. The DNA concentration was diluted to obtain a working solution of 5ng/µl.

Running the qPCR (Timing: 2 hrs)

Samples were arranged accordingly in the qPCR machine (Biorad-CFX96) with identifier including the NTC and the standards.

Cycling conditions (for both telomere and GAPDH amplicons) are: 10 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 1 min, followed by a dissociation (or melt) curve.

Absolute telomere length using Ct values was calculated using method described by Callaghan and Fenech (2011) using kilobase per diploid genome. The samples were run in duplicate and standard deviation (SD) was used to choose the best Ct. Any Ct with SD above 1 was not picked.

Table 3. Means (±SE) of Body weight and conformation traits of broiler birds exposed to feed and heat stressors

	Opened sided House		Environmentally controlled House		
	Feed Restriction	Feed Restriction control	Heat Stress	Heat control	P>F
Body weight (g)					
Day 1	1753.98^{ab}±27.96	1712.00^{b}±21.07	1758.10^{ab}±18.36	1787.40^{a}±25.37	<0.1748
Week 1	2006.00^{c}±33.18	2324.3^{b}±54.44	2264.56^{b}±48.05	2604.00^{a}±54.00	<0.0001
Week 2	2223.30^{c}±39.07	2696.90^{b}±72.30	2578.40^{b}±56.94	3052.7^{a}±69.77	<0.0001
Body length (cm)					
Day 1	20.70±0.37	20.00±0.49	20.30±0.42	19.80±0.44	<0.4945
Week 1	20.90^{b}±0.41	21.50^{ab}±0.40	20.78^{b}±0.40	22.50^{a}±0.29	<0.0157
Week 2	22.80^{a}±0.33	23.30^{a}±0.31	21.20^{b}±0.20	23.70^{a}±0.42	<0.0019
Body girth (cm)					
Day 1	28.00±1.68	29.60±0.34	30.20±0.48	29.00±1.82	<0.6776
Week 1	32.15^{d}±0.31	34.96^{b}±0.49	33.53^{c}±0.42	36.55^{a}±0.44	<0.0001
Week 2	33.70^{d}±0.33	37.10^{b}±0.69	35.40^{c}±0.68	38.80^{a}±0.47	<0.0001

^{a,b,c} = Means with the same superscript within the same row for a particular parameter are not significantly (P>0.05) different.

Table 4. Means (±SD) of telomere length (Kb/diploid genome) of the stressed and control birds

Days	Feed restriction control	Feed restriction	Heat stress Control	Heat stress
Week 1	384.90 ± 110.08	329.5 ± 123.29	344.78 ± 66.17	219.98 ± 44.02
Week 2	363.38 ± 49.70	328.70 ± 57.70	395.15 ± 61.48	329.36 ± 80.40

^{a,b,c} = Means with the same superscript within the same row for a particular parameter are not significantly (P>0.01) different.

Table 5. Correlations between telomere length and body conformation traits of broiler birds in week 1

Traits	FRC TL	FR TL	HC TL	H TL
Body weight	0.21	-0.69	-0.03	-0.31
Body length	0.45	-0.98*	-0.06	-0.36
Body girth	-0.06	0.26	-0.04	-0.29

FRC=Feed restriction control, FR=Feed restriction, HC= Heat control, H= Heat stress, TL= Telomere length
P<0.05= (*)

Statistical analysis

Data on body weight, body conformation traits and Kb/diploid chicken genome of telomere were subjected to general linear model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, US, 2002-2012). The data were subjected to test of normality and satisfied the conditions for normal distribution curves. Means were separated using Duncan New multiple range test.

RESULTS

Results of the means of body weight and body conformation traits (BCT) of the broiler birds exposed to heat and feed restriction stresses at various weeks were shown in Table 3. It was observed that there were significant changes (P<0.05) in the parameters estimated of the birds exposed to the stresses and the changes follow the same pattern across the ages. The body weight and BCT were significantly (P<0.05) reduced by the two stressors in both the weeks. The body conformation traits are indices of body weight of animals as both are highly correlated.

The results of the telomere length (T/S) were presented in Table 4. The results revealed lower telomere length in FR than FRC. The heat stress revealed slightly lower telomere length than their control in week 1. The feed restriction and the heat stress also caused telomere erosion in week 1 and week 2. The results were depicted in Figures 1 and 2.

The results of correlations between telomere length, body weight, body length and body girth were presented in tables 5 and 6. The results revealed low and negative correlations (-0.06 to -0.35) between telomere length, body weight, body length and body girth. Strong and positive correlation were observed between telomere length and body length in week 1 for the feed restriction group control. The correlation between telomere length and body length in feed restricted group was strong and negative (-0.98, P<0.05) in week 1. Low and negative correlations were also obtained in week 2 between the telomere length and body weight, body length and body girth (ranging between -0.03 and -0.55) among the treatment groups. However, correlation between

Table 6. Correlations between telomere length and body conformation traits of broiler birds in week 2

Traits	FRC TL	FR TL	HC TL	H TL
Body weight	-0.29	-0.23	-0.03	0.64
Body length	-0.33	-0.55	0.43	-0.12
Body girth	0.15	-0.53	-0.19	-0.12

FRC=Feed restriction control, FR=Feed restriction, HC= Heat control, H= Heat stress, TL= Telomere length

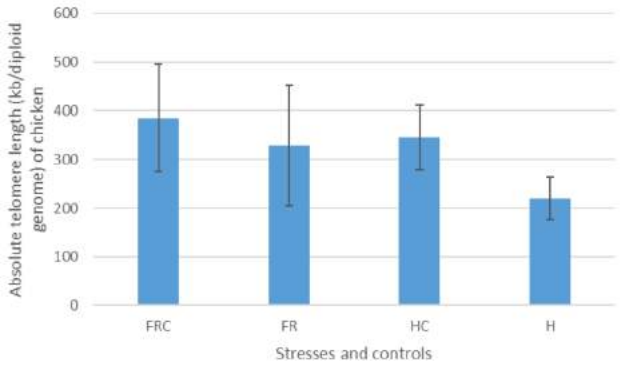

Figure 1. Distribution of the telomere length as affected by both stresses in week 1
FRC=Feed restriction control, FR=Feed restriction, HC= Heat control, H= Heat stress, TL= Telomere length

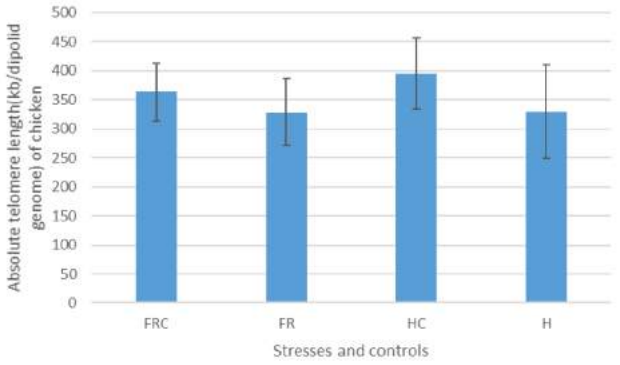

Figure 2. Distribution of the telomere length as affected by both stresses in week 2
FRC=Feed restriction control, FR=Feed restriction, HC= Heat control, H= Heat stress, TL= Telomere length

telomere length and body length in heat stressed group was strong and positive.

DISCUSSION

Body performance

The results of this studies as depicted above revealed that feed deprivation and exposure to heat caused a profound reduction in the body weight, body conformation. Daghir (1995) in his study had revealed the depression in body weight of broiler birds raised under high environmental temperature. Zuprizal *et al*. (1993) and Siegel (1995) associated the cutback in body weight gain during heat stress regime to the reduction in both feed consumption and true digestibility of protein and amino acids. Body weight and feed utilization obtained in ECH birds were higher and well optimized under 25˚C. Al-Aqil *et al.* (2009) stated that in order to have feed utilization and body weight optimized in broiler chicken, the environmental temperature should be maintained at about 23˚C.

Telomere length

Our results revealed attrition in telomere length of the birds fed 60% of ad libitum feed (FR) in the week 1. This indicated that telomere length could be used as sensitive physiological stress markers in chickens. Sohn *et al.* (2012) reported significant difference in telomere length shortening and DNA damage in birds exposed to feed restriction (70% of the ad libitum) and high stocking density for 30 days. In our findings, the heat stress reduced telomere length in week 1 and in week 2. This study therefore showed that telomere length was associated with stressful status. As stressful condition can increase the rate of telomere attrition (Epel *et al*., 2004; Boonekamp *et al.*, 2014; Trusina, 2014), we expected loss in telomere length in the feed restricted and heat stressed group for both one and two weeks durations. Previous studies had equally shown a profound evidence of telomere loss in both short (Meillere *et al.*, 2015; Salomon *et al.*, 2016) and long periods (Kotschar *et al.*, 2017; Boonekamp *et al.*, 2014).

Correlations

Our results revealed that short telomere is associated with body weight, body length and body girth. This observation suggests that birds with higher body weight, body length and body girth risks shorter lifespan in the production cycle. This could be as a result of high fat deposition in birds with high body mass index (BMI) which in turn causing oxidative damages to the telomeric DNA. Many studies in human had revealed that higher BMI and hip circumference were inversely correlated with telomere length (Sangmi *et al.*, 2009). Strong and positive correlation obtained in week 2 in heat stress group between telomere length and body weight suggest fat depletion in this group of birds. Sangmi and colleagues, 2009 revealed that most marked decrease in telomere length was found in the women who had overweight or obese BMI.

CONCLUSION

It is concluded that feed restriction and heat stress had reduced the growth performance of the birds and caused telomere length attrition of birds. The study also revealed that shorter telomere is associated to higher body mass index. This therefore implies that telomere could be used as physiological stress biomarkers in chickens and can be used to evaluate lifespan of broiler birds.

Ethics Statement

The level of feed restriction and heat stress to which birds were exposed to including blood sampling had been approved by institutional animal care and use committee of Universiti Putra Malaysia.

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TPGM

Phenotypic characterization and genetic variation of Nile tilapia (*Oreochromis niloticus*) from wild and culture populations

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Abstract

Rice kernel

The phenotype and gene diversity studies provide basic information of the fish species as it gives the level of diversity and population expansion within them. This study was designed to describe the phenotypic (morphological and meristic) characterization and genetic variations of wild and culture *Oreochromis niloticus* from two different populations (Oyan Lake and a private farm) in Ogun State, Nigeria. One hundred fish samples were collected for the study comprising 50 samples from each population. Twenty samples from each population were used for genomic studies. The data on morphometric and meristic characters were subjected to T-test and cluster analyses at $\alpha = 5\%$ using SPSS. There were significant differences ($P < 0.05$) in the morphometric traits (except the Post Orbital Length) and meristic parameters (except Dorsal fin spines, Pectoral fin ray right and number of vertebrae) between the two populations. Both populations had high genetic variation among population and low genetic variation within species based on the distinctive classification from the cluster analysis. All sampled populations were polymorphic, but Nile tilapia from the wild were more polymorphic than the culture. The wild population could be a viable reservoir for high variability stock useful in fish breeding and future conservative programmes.

Key words: Cluster analysis, phenotype, polymorphism, tilapia, variation

.INTRODUCTION

The phenotype and gene diversity provide basic information of fish species as it gives the level of diversity and population expansion within species. *Oreochromis niloticus* is an indigenous species of Africa, found in freshwater and feed on different kinds of food such as aquatic plants, invertebrates, benthic fauna, detritus and bacterial films (Abd El-Kader *et al*., 2013). The Nile tilapia (*Oreochromis niloticus*) is the most commonly farmed tilapia species in Nigeria after *Clarias gariepinus* with good aquaculture qualities such as ability to withstand poor water quality and wide range of feed that support its cultivation. The Nile tilapia is a teleost fish, a member of the cichlid family and notably one of the most important species in aquaculture worldwide (McCune, 1981).

However, Tilapias has become one of the most important groups of fish, with increasing worldwide production (FAO, 1999). Tilapia has fast growth rate that can be further improved in a more favourable environment (FAO, 2012). About 75% of this production is from aquaculture. The most important species is the Nile tilapia, *Oreochromis niloticus* (Bo-Young *et al*., 2005), because they are fast growing, easily fed, are resistance to poor water quality and disease and are easily reproduced (Argue and Phelps, 1995).

Nile tilapia has special phenotypic characteristics such as cycloid scales, grey/black body bars, pointed mouth part that eases its classification. The Nile tilapia has its native range in Africa and is widely known for its importance in aquaculture. This species has been recognized for its ability to tolerate a wide range of salinity, dissolved oxygen and temperature (Farmer and Beamish, 1969; Avella *et al.,* 1993), high fecundity and rapid growth rate (Welcomme, 1967) and an omnivorous mode of feeding (El-Sayed, 1999). The species can adapt rapidly to new environments, possesses a wide range of biological responses to varying environmental conditions both in culture and in nature (Schofield *et al*., 2011; Grammer *et al*., 2012). The level of plasticity and/or the high degree of physiological tolerance of this species has enabled self-establishment in extreme environmental conditions such as temperate winter conditions (Peterson *et al,*. 2005; Grammer *et al*., 2012), hot springs (Trewavas 1983; Nyingi *et al.,* 2009; Ndiwa *et al*., 2014) and saline waters (Schofield *et al.*, 2011).

The study provides an insight into morphological responses of these fish populations based on their adaptability to the wild and culture environment. Occurrence of phenotypic differences between the populations studied will confirm that the environment and/or the genetic background play an important role in evolution of cichlids, while lack of phenotypic

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Table 1. Primer design used in the study

Primer pair	Primer sequence (5′ - 3′)
Pair 1	CRb forward5′-GGATTYTAACCCYTRCCCC-3′
	Reverse 3′-AGTAAAG TCAGGACCAAGCC-5′
Pair 2	MtD-loop forward 5′-GGATTTTAACCCTTACCCC-3′
	Reverse 3′-AGTAAAGTCAGGACCAAGCC-5′

Table 2. The probabilities and means of morphometric traits of Nile Tilapia (*Oreochromis niloticus*) from wild and culture populations

Trait	P	Wild	Culture
Weight (w) (g)	0.0000	129.42±11.55[a]	65.18±2.93[b]
Total length (TL) (cm)	0.0000	18.50±0.59[a]	15.72±0.24[b]
Standard length (SL) (cm)	0.0000	16.02±0.56[a]	12.65±0.19[b]
Head length (HL) (cm)	0.0430	4.50±0.14[a]	4.18±0.07[b]
Pre-orbital length (POL) (cm)	0.5160	1.25±0.72	1.30±0.03
Postorbital length (POSL) (cm)	0.0000	4.22±0.22[a]	1.95±0.04[b]
Eye diameter left (ED) (cm)	0.0070	1.04±0.04[a]	0.93±0.02[b]
Upper jaw length (UJL) (cm)	0.0390	1.11±0.06[b]	1.24±0.03[a]
Pre-dorsal fin length (PDFL) (cm)	0.0020	5.15±0.16[a]	4.56±0.09[b]
Pre-pectoral fin length (PPFL) (cm)	0.0000	3.80±0.19[a]	1.95±0.04[b]
Pre-anal fin length (PFL) (cm)	0.0000	12.15±0.58[a]	9.31±0.19[b]
Post-anal fin length (PSAFL) (cm)	0.0000	14.17±0.65[a]	11.50±0.18[b]
Body depth (BD) (cm)	0.0000	6.35±0.28[a]	4.80±0.09[b]
Caudal peduncle depth (CPD) (cm)	0.0000	2.39±0.14[a]	1.84±0.05[b]

[a b] Means with different superscripts across the same row are significantly different ($p < 0.05$), (±SE) = Standard error

differences will imply that the high degree of tolerance in Nile Tilapia is related to its propensity to invade new environment, and in keeping a very canalized phenotype. Therefore, this study was designed to describe the phenotypic (morphological and meristic) characterization and genetic variations of wild and culture *Oreochromis niloticus* from different populations (Oyan Lake and a private farm) in Ogun State, Nigeria.

METHODOLOGY

Study Area And Experimental Fish Sample

The experimental fish used in this study were collected from two different locations in Ogun State consisting Oyan Lake and a private Fish Farm in Ilaro, Ogun State Nigeria. The Oyan Lake (latitude 7° 15′ 30`` N and longitude 3° 15′ 20`` E) is located in Abeokuta North local government area of Ogun State,South west Nigeria. The lake crosses the Oyan River, a tributary of the Ogun River. Oyan Lake covers 4,000 hectares and has acatchment area of 9,000 km^2. The crest length of the lake is 1044 m while its height is 30.4 m and gross storage capacity of 270 million m^3. A total of 100 fish samples were collected for phenotypic (morphological and meristic) characterization comprising 50 samples each from Oyan lake being the wild population (consisting of 27 males (44.3%) and 23 females (59%)) and the private fish farm population situated at Ilaro, Ogun State (comprising 34 males (55.7%) and 16 females (41%). Twenty samples from each population were used for genomic studies

Morphometric And Meristic Measurements

Thirteen (13) morphometric measurements were recorded according to the method described by Teugels (1986). These were: Total length (TL), Standard length (SL), Head length (HL), Pre-orbital length (POL), Postorbital length (POSL), Eye diameter left (ED), Upper jaw length (UJL), Pre-dorsal fin length (PDFL),

Table 3. The probabilities and means of meristic traits of Nile Tilapia (*Oreochromis niloticus*) from wild and culture populations

Trait	P	Wild	Culture
Dorsal fin spines (DFS)	0.5320	17.02±0.12	16.94±0.05
Dorsal fin ray (DFR)	0.0000	9.60±0.35[b]	11.20±0.09[a]
Pectoral fin ray right (PFR)	0.4810	13.58±0.28	12.48±1.53
Pectoral fin ray (PELFR)		12.00±0.00	12.00±0.00
Anal fin ray (AFR)	0.0000	9.94±0.09[a]	8.96±0.99[b]
Number of the lateral line scales (LLS)	0.0230	20.54±0.25[b]	21.74±0.46[a]
Number of ventral tail ray (VTR)	0.8430	30.50±0.07	30.52±0.07
Number of gill ray (GIR)	0.047	29.16±0.12[b]	29.68±0.23[a]

[a b] Means with different superscripts across the same row are significantly different ($p < 0.05$), (±SE) = Standard error

Table 4. Genetic diversity indices estimated from two tilapia populations

Location/Sample ID	Hob	H	K	π
PFF	3	0.464	13.107	0.07085
OLP	8	1.000	65.821	0.27199

PFF = Samples from the culture population; OLP = Samples from the wild population; *Hob*=number of haplotypes; *H*= haplotype diversity; π= nucleotide diversity.

Pre-pectoral fin length (PPFL), Pre-anal fin length (PFL), Post-anal fin length (PSAFL), Body depth (BD) and Caudal peduncle depth (CPD).

Eight (8) meristic variables were determined by counting the number of dorsal spines, soft rays and fins on the body of the fish. These were: Dorsal fin spines (DFS), Dorsal fin ray (DFR), Pectoral fin ray right (PFR), Pectoral fin ray (PELFR), Anal fin ray (AFR), Number of the lateral line scales (LLS), Number of vertebrae (VTR) and Number of gill rakers on the first gill arch (GIR).

Blood Sample Collection, DNA Extraction, PCR And Electrophoresis

Blood samples were collected from the fish through cardio-puncture (puncture at the peduncle) and FTA paper was used in collecting the blood directly from the fish immediately after each puncture was done. The DNA extraction and Electrophoresis profiling was carried out. Agarose gel electrophoresis was used to quantify the amplified DNA. Two pairs of primers were designed (Table 1) for the mitochondrion DNA cytochrome b and displacement loop. The PCR reaction was performed in a mini cycler (Bio-Rad, USA) within a reaction mixture of 25µl containing 12.5ul enzyme (2 mM $MgCl_2$, 200µl M of dNTP, 2.5 l of 10 × PCR buffer), 2µl template DNA, 0.25µl primers and 12.30µl nuclease free water (NFW) with the cycling condition of initial denaturation step at 95˚C for 5 minutes, followed by 35cycles of denaturation at 94˚C for 40 seconds, annealing at 53˚C for 45 seconds, extension at 72˚C for 1 minute and final extension at 72˚C for 10 minutes for the cytochrome b region. However, for the second primer forward and reverse the cycling condition of initial denaturation step was at 60˚C for 5 minutes, followed by 35 cycles of denaturation at 59˚C for 40 seconds, annealing at 57˚C for 45 seconds, extension at 55˚C for 53 minutes and final extension at 52˚C for 10 minutes.

Sequence Data Analysis

Sequences were edited using BioEdit version 5.0.9 software, and then blasted in GenBank (http://www.ncbi.nlm.nih.gov/) to confirm that the DNA fragment was partial mtDNA D-loop sequence of tilapia. Alignment was carried out using DNASP version 5.10.01 (Rozas *et al.* 2003). MEGA X software was used to determine nucleotide compositions, the structure of the sequence and genetic distance (D) of the different populations with genetic diversity indices.

Statistical Analysis

The data on morphometric and meristic parameters were analyzed using T-test and cluster analyses. Significant differences were tested at α = 5% using SPSS version 20

RESULTS AND DISCUSSION

Significantly differences ($P < 0.05$) were observed in the means values of morphometric traits between wild and culture populations, except in the values of preorbital length (POL). The values of all the morphometric trait determined were higher in the in the wild than the those measured in the cultured population, except in the values of preorbital length. The mean preorbital length observed in the culture population was 1.30±0.03 while 1.25±0.72 was recorded in the wild population. Ola-Oladimeji *et al.*, (2016) reported significant differences in morphometric traits of wild and culture *Clarias gariepinus*. The

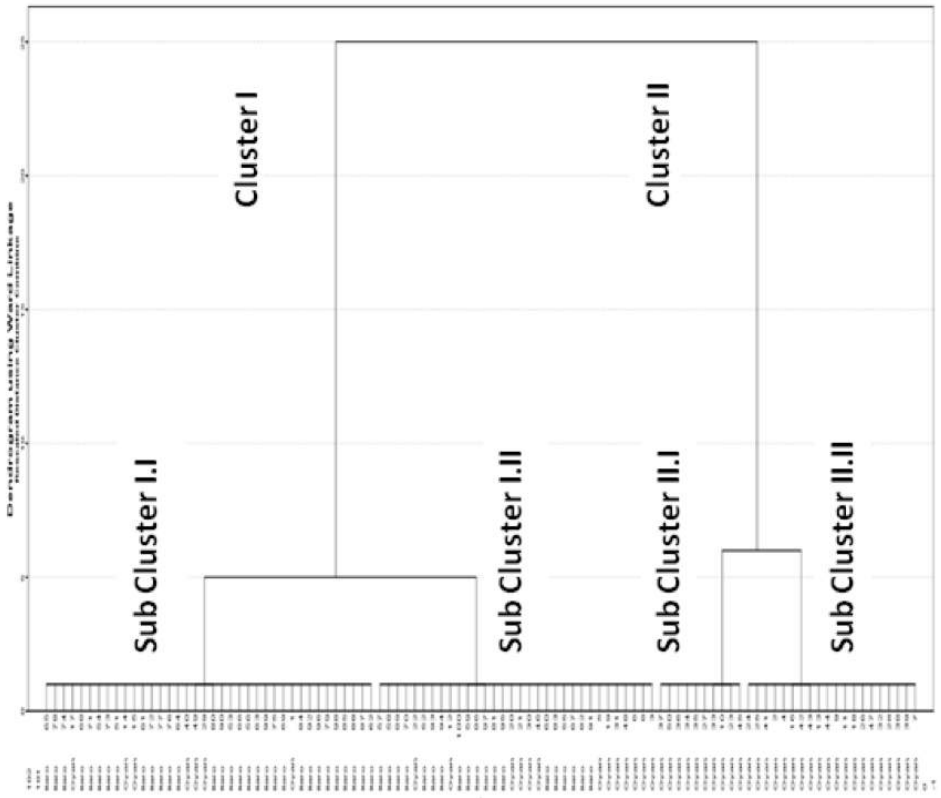

Figure 1. Dendrogram showing the morphological traits of *Oreochromis niloticus* from wild and culture

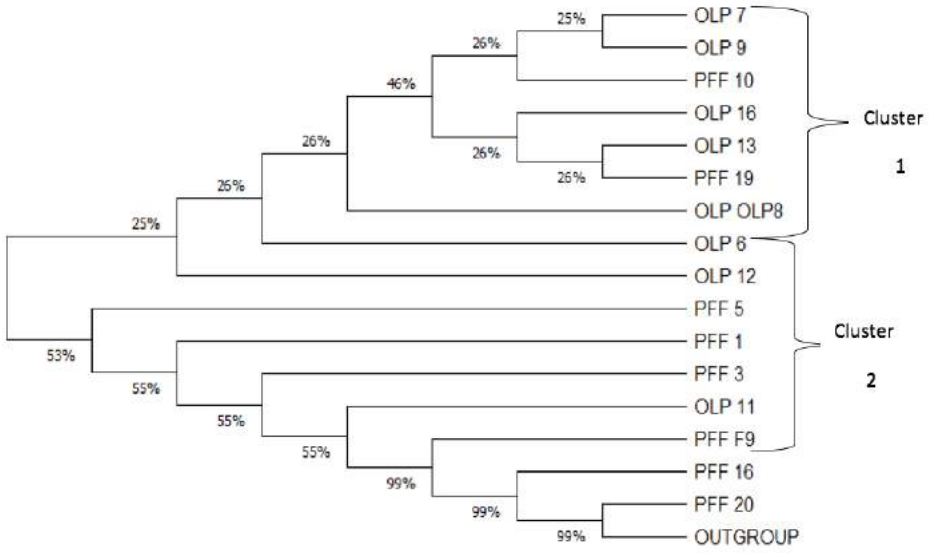

Figure 2. Neighbor-joining phylogenetic tree showing the relationship between tilapia fish from the wild and culture populations based on mtDNA D-loop region
(PFF = Samples from the culture population; OLP = Samples from the wild population)

values of meristic traits determined show no significant difference (P >0.05) in the values of DFS, PFR, PELFR, and VTR between the wild and culture *O. niloticus*. But there were significant differences (p<0.05) in the values of DFR, AFR, LLS and GIR.this shows that there are variances in the two population. Solomon *et al.,* (2015) stated that fish is more susceptible to environmental induced morphological variation.The cause of variation in the morphometric and meristic characters may range from variability to the intraspecific which is under the influence of environmental parameters (Akinrotimi *et al.*, 2018).

The cluster analysis based on the dendrogram of the morphological trait of *O. niloticus* from the two populations is divided into two main clusters, each with two sub-clusters (Figure 2). Cluster I comprised more of *O. niloticus* from culture population (Ilaro) with some from the wild (Oyan lake). Sub-cluster I.I had 31 samples from the culture with 7 samples from the wild. Sub-cluster I.II has 19 samples from the culture with 13 samples from the wild. Cluster II had all samples of Sub-cluster II.I (10 samples) and Sub-cluster II.II (20 samples) all from the wild. Both populations had high genetic variationamong population and low genetic variation within species based on the distinctive classification from the cluster analysis.

Genomic DNA was successfully extracted from the 20 fish samples of *O. niloticus* and gel electrophoresis confirmed the presence of DNA in the extracted samples.Gel electrophoresisis showed that the primers were stable in spite of different annealing temperature. Hence, 53°C was chosen as annealing temperature for the reaction procedure. The length of the amplified segment ranged from 250-260bp. All sampledpopulations were polymorphic, but Nile tilapias from the wild were more polymorphic than the culture based on the result of the DNA polymorphism.They were 121 polymorphic sites in both populations, 193 mutation sites, 60 singleton variable and 61 parsimony sites. The genetic diversity study of the two populations (Table 4) showed nucleotide diversity of 1.000 for Nile tilapia from the wild and 0.464 for the culture population. Other results for average number of nucleotide difference and nucleotide diversity were very much separated within the populations.

The Neighbor-Joining phylogenetic relationship of tilapia fish from wild and culture populations based on mitochondrial D-loop is presented in Figure 3. The numbers at the node represent the percentage bootstrap values for branches with 500 bootstrap replications. Two major clusters were revealed, which were based on the two populations. Cluster I consisted almost all the samples from the wild with various sub-cluster groups (with culture population inclusive). Similarly, cluster II consisted of all the samples from the culture population D-loop with only one sub-cluster from the wild. So samples from wild and culture D-loop were grouped together under the same major sub-cluster in cluster 1 (might be from same lineage). This could suggest that the two populations are genetically distinct although they may share a common ancestor. The findings here corroborate the earlier report by Agbebi *et al*., (2016) that populations of tilapia species in Nigeria showed no evidence of sub-structuring in their phylogeny but may be traced to a single ancestral lineage.

The similarity in Post Orbital Length (morphometric trait), Dorsal fin spines, Pectoral fin ray right and Number of ventral tail ray (meristic parameter) in the study species is an indication of relatedness between the two populations from different population and/or location. The differences encountered between populations are attributable to the existence of heterogeneity between the two populations. Usually, meristic traits remain the same in the same species except otherwise conditioned by environmental pollution changing the phenotype. Fish can show high modifications in morphological characters both within and between populations of species than any other vertebrates (Wimberger, 1992). Modification of this sort is an adaptive mechanism to survival in its habitat (Meyer, 1987; Gulliet *et al.,* 2003). *Oreochromis niloticus*, among the members of the Cichlidae family is among the fish species that is categorized in showing wide range of variations in body shapes and conformity (McCune, 1981).

CONCLUSION

This study revealed variability in culture population as compared to wild population of Oreochromis niloticus. Although, the wild population could be a viable reservoir for high variability stock useful in fish breeding and future conservative programmes. The information obtained in this study could be useful in establishing phenotypic characterization and estimating genetic variability among other components of fish population dynamics.

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TPGM

Morphometric variations of *Oreochromis niloticus* from three wild populations in South-west (Eleyele, Owalla and Owena) Nigeria

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Abstract

One hundred and fifty (150) fish specimens of *Oreochromis niloticus* representing fifty (50) from each of the population were sampled from the fishermen landings to study their phenotypic variations. Fish samples were collected from Eleyele, Owalla and Owena (Oyo, Osun and Ondo States of South-West, Nigeria respectively). Data on morphometric and meristic were subjected to One Way ANOVA at 5% level of significance and Principal Component Analysis. The Kaiser-Meyer-Olkin Measure of Sampling Adequacy for Eleyele (0.9), Owalla (0.8) and Owena (0.9) were adequate. Two principal components (PC) were extracted in two populations (Eleyele and Owalla) and one principal component in Owena. With Owena populations showing the highest percentage variability for PC1 (83.2%), followed by Owalla and Eleyele populations (78.1% and 71.4%) respectively. The existence of morphological variations in wild populations showed that Owena population is the biggest, followed by Owalla and the smallest, Eleyele in terms of size. This signifies that all morphometric parameters were important for the classification of *Oreochromis niloticus*. From this study, it was concluded that body depth, total length, standard length and head length had the highest and direct positive contribution to the enhancement of body size of the Oreochromis niloticus across the three rivers.

Key words: Fish, meristic, morphometric, *Oreochromis niloticus*, population

INTRODUCTION

Tilapia is a large genus in cichlid family *Cichlidae*, which according to Nelson (2006) is the third largest fish in the world and one of the most diverse fish species. Tilapia is the generic name of a group of cichlids endemic to Africa. The group consists of three aquaculture important genera *Oreochromis, Sarotherodon* and *Tilapia*. Several characteristics distinguish these three genera, but possibly the most critical relates to reproductive behaviour. All tilapia species are nest builders; fertilized eggs are guarded in the nest by a brood parent.

The species is chosen because of its relatively short generation time of about six months and its suitability for investigation of the application of genetics in aquaculture from conservation of genetic resources to breeding programmes Eknath and Hulata (2009) and breed in captivity and they are enjoying wide acceptance as food fish because of their high palatability and history of use from inland fisheries.

The species can be discriminated physiologically; it is very difficult to identify them with meristic index for they have many overlapping characters especially at the juvenile stage (Bailey, 1997). However, morphological parameters and biometrical characteristics which involve morphometric measurement and meristic count have over the years been employed to identify fish stocks (Turan *et al*., 2004). To this day it still remains the simplest and most direct way among methods of species identification. The study of differences and variability in morphometric and meristic characters of fish stocks is important in phylogenetics and providing information for subsequent studies on the genetic improvement of stocks. Morphometric and meristic characters are generally used for fish identification. Morphometric characters are measurable characters of a fish while the meristic counts are countable characters. Examples of meristic counts are the number of dorsal fin spines, the number of dorsal fin rays, the number of pectoral fin rays to mention but a few while morphometric characters are total length, standard length, head length and so on (Solomon *et al.*, 2015).

Identification of species is a primary step towards any research work and plays a key role for the behavioural study. Fish species identification is traditionally based on external morphological features, including body shape, pattern of colours, scale size and count, number and relative position of fins, number and type of fin rays, or various relative measurements of body parts (Strauss and Bond, 1990). Gill rakers are sometimes counted to differentiate very similar species (Iffat, 2002). However when none of the aforementioned characters are not available otoliths can be occasionally used to identify fossils or stomach contents (Pierce and Boyle, 1991; Granadeiro and Silva, 2000).

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Table 1. The probabilities and means of morphometric parameters of *O. niloticus* from three lakes (Eleyele, Owalla and Owena)

Trait	P	Eleyele	Owalla	Owena
WT(gram)	0.0000	44.04±5.09 ^{b}	143.62±12.39 ^{a}	171.34±11.79 ^{a}
TL	0.0000	12.56±0.45 ^{b}	20.36±0.52 ^{a}	21.68±0.59 ^{a}
SL	0.0000	10.23±0.38 ^{b}	17.39±0.47 ^{a}	18.46±0.51 ^{a}
HL	0.0000	3.34±0.10 ^{b}	5.37±0.16 ^{a}	5.51±0.19 ^{a}
POL	0.0000	1.17±0.08 ^{b}	1.25±0.05 ^{b}	1.68±0.08 ^{a}
POSL	0.0000	1.89±0.08 ^{c}	2.17±0.13 ^{b}	2.69±0.09 ^{a}
ED	0.0000	0.89±0.03 ^{b}	1.14±0.03 ^{a}	1.24±0.06 ^{a}
UJL	0.0000	0.74±0.05 ^{c}	1.38±0.06 ^{b}	1.72±0.08 ^{a}
PDFL	0.0000	3.50±0.13 ^{b}	5.97±0.16 ^{a}	6.39±0.21 ^{a}
PPFL	0.0000	3.41±0.17 ^{a}	2.14±0.11 ^{b}	2.49±0.10 ^{b}
PAFL	0.0000	7.69±0.29 ^{b}	12.21±0.43 ^{a}	12.96±0.35 ^{a}
PSAFL	0.0000	8.97±0.33 ^{b}	14.15±0.50 ^{a}	15.20±0.38 ^{a}
BD	0.0000	4.61±0.17 ^{c}	6.77±0.23 ^{b}	7.52±0.18 ^{a}
CPD	0.0000	1.55±0.06 ^{c}	2.29±0.10 ^{b}	2.56±0.12 ^{a}

$^{a\,b\,c}$ Means with different superscripts across the same row are significantly different ($p < 0.05$), (±SE) = Standard error

Morphometric measurements and meristic counts are considered as easiest and authentic methods for the identification of specimen which is termed as morphological systematic (Mamuris *et al.,* 1998). Morphological measurements, meristic counts, provide data useful for taxonomic status. In general, fish demonstrate greater variances in morphological parameters both within and between populations than other vertebrates and are more susceptible to environmentally induced morphological variations. The cause of variation in the morphometric and meristic characters may range from variability to the intraspecific which is under the influence of environmental parameters (Akinrotimi *et al.*, 2018). Therefore, this study examined morphometric variations of *Oreochromis niloticus* from three wild populations in south-west (Eleyele, Owalla and Owena) Nigeria.

METHODOLOGY

Description of Study Area

The fish samples were collected from Eleyele, Owalla and Owenna rivers in Oyo, Osun and Ondo state, Nigeria respectively. Eleyele lake is situated in North-west of Ibadan, Oyo State, Nigeria at an altitude of 125m above sea-level and between7°25'00' and 7°26'30'N latitudes and 3°51'00' and 3°52'30'E longitudes. The lake is man-made and was formed in 1939 by damming of the Ona River (part of a dense network of inland waterways that flow southward into the Lagos Lagoon), and the Otaru, Awba, Yemoja and Alapo streams in Oyo State also empty into the lake.

Owalla lake is located within latitudes 07° 53.5’ and 07° 59.0’N and longitudes 004° 31.5’ and 004° 35.0’E with an average elevation of 336±8m above the mean sea level. The lake is situated in the North of Osogbo, Osun State capital, about 35km North of Ile-Ife. The lake wall is about 677m long and 27.5m high. The total length of the reservoir is about 12km from the dam wall to the upstream with a maximum width of 3.5km. It has an area of about 14.5km^{2} and capacity of about 94×106m^{3}.

Owenna is situated in Ile-oluji Okeigbo, Ondo state, Nigeria; its geographical coordinates are 7° 12' 0"N, 5° 1' 0"E.

Sample Collection

One hundred and fifty (150) fish specimens of *O. niloticus* representing fifty (50) fishes from each of the location were sampled from the fishermen landing site.

Morphometric and Meristic Measurement

The determination of body weight, total length and standard length were carried out onsite. Lineal morphometric measurements (M) were taken on the left side of the fish, so as to minimize artificial error. The greater number of the morphometric characters were measured following the conventional method described by Morales *et al.* (1998) and Diodatti *et al.* (2008). The fish were measured using measuring board and transparent ruler, and then weighed with an electronic OHAUS weighing balance. Meristic characteristics (m) were examined according to method by Froese and Pauly (2007). A total of twenty-two (22) body measurements were taken, including fourteen (14) morphometric variables and eight (8) meristic variables.

Table 2. The probabilities and means of meristic parameters of *O. niloticus* from the three lakes (Eleyele, Owalla and Owena)

Trait	P	Eleyele	Owalla	Owena
DFS	0.000	16.80±0.06 [b]	17.12±0.05 [a]	17.16±0.06 [a]
DFR	0.0210	12.44±0.07 [a]	12.10±0.07 [b]	12.18±0.12 [b]
PFR	0.000	10.06±0.23 [c]	12.52±0.08 [b]	13.48±0.14 [a]
PELFR		12.00±0.00	12.00±0.00	12.00±0.00
AFR	0.0000	9.28±0.22 [b]	10.2±0.15 [a]	9.76±0.08 [a]
LLS	0.0000	24.00±0.24 [a]	21.88±0.19 [b]	21.16±0.31 [c]
VTR	0.9010	30.48±0.07	30.52±0.07	30.48±0.07
GIR	0.0140	29.04±0.12 [b]	29.64±0.23 [a]	29.04±0.12 [b]

[a b c] Means with different superscripts across the same row are significantly different ($p < 0.05$), (±SE) = Standard error
Legend:
*Dorsal finspines (DFS), the number of dorsal fin rays (DFR), the number of pectoral fin rays(PFR), the number of pelvic fin rays(PELFR), the number of anal fin rays(AFR), number of the lateral line scales (LLS), number of vertebral (VTR) and number of gill rack on the first arch (GIR).

Statistical Analysis

The results obtained from the morphometric and meristic studies were determined and subjected to Analysis of Variance and correlation statistical analysis using SPSS version20. Duncan Multiple Range Test was used to separate the means at $p<0.05$.Morphometric data obtained were subjected to factor analysis using principal component analysis (PCA).

RESULTS

The results (Table 1) showed that morphometric parameters of *O. niloticus* in the three lakes were significantly ($p<0.05$) different. The average weight of *O. niloticus* in Owena (171.34±11.79g)>Owalla (143.62±12.39g)>Eleyele (44.04±5.09g). Thus, majority of the morphometric parameters such as total length, standard length, head length, pre-orbital length, eye diameter, pre-pectoral fin length, pre-anal fin length and post-anal fin length in Owena > Owalla > Eleyele.

Analysis of Meristic Parameters of *O. niloticus* From the Three Lakes (Eleyele, Owalla And Owena)

In Table 2, the mean values of O. niloticus meristic count in the lakes were significantlydifferent ($P<0.05$) in the numbers of dorsal fin spines (DFS), dorsal fin rays (DFR), pectoral fin rays(PFR), pelvic fin rays(PELFR), anal fin rays(AFR), the lateral line scales (LLS) and the number of gill rakes on the first arch. The number of dorsal fin spine of O. niloticus from Owalla (17.12±0.05) and Owena (17.16±0.06) where higher than those of Eleyele (16.80±0.06). The number of anal fin ray between samples from Owalla and Owena had no significant difference ($P<0.05$) but had a significant difference when compared to samples from Eleyele ($P<0.05$). The number of dorsal fin ray was not significantly higher ($p>0.05$) in Eleyele (12.44±0.07) than in Owalla (12.10±0.07) and Owena (12.18±0.12). The number of pectoral fin rays of O. niloticus from Owena (13.48±0.14) was the highest while those from Eleyele had the lowest (10.06±0.23).The number of lateral line scales had a significant difference across all three locations ($P<0.05$). There were no variations in the number of pelvic fin ray and vertebral across all three locations.

The chi-square distribution analysis in Table 3 shows that there was no significant difference ($P>0.05$) between the sexes. This means that the number of males and females were well represented across the locations. The numbers of males in Eleyele, Owala and Owena lakes were 21, 20 and 29 while the numbers of females were 29, 30 and 21, respectively.

Analysis of Morphometric Parameters of O. niloticus From the Three Lakes (Eleyele, Owalla And Owena)

Principal Component Analysis

From Table 4, the principal component analysis of all the morphometric parameters across the three locations extracted two principal components (PC1 and PC2) in Eleyele and Owalla while in Owena only one principle component (PC1) was extracted. The highest variance was contributed by PC1 (83.172%) in Owena, all parameters contributed high to PC1 with highest contributions from TL(0.977), HL(0.976), SL(0.967), PDFL(0.954), PAFL (0.949), BD (0.938), PSAFL(0.933),PPFL(0.926) and POL(0.916). The highest communality was from TL(0.954) while the lowest was from CPD(0.386). Owalla samples contributed a variance of 78.096% and 9.225% in PC1 and PC2 respectively. All parameters contributed high to PC1 with highest contributions from UJL(0.951), PAFL(0.948), TL(0.927), BD and CPD both contributed 0.921. Variation in PC2 was contributed mostly from PDFL(0.452). The highest communality was from POSL (0.941) while the lowest was from ED(0.645). Samples from Eleyele contribute a percentage variance of 71.353% and 7.852% in PC1 and PC2 respectively. All parameters contributed high to PC1 with highest contributions from SL(0.988), HL(0.969),CPD(0.968), PSAFL(0.968), BD(0.965) and PDFL(0.955). Variation in PC2 was contributed mostly from POL(0.864). The highest communality was from SL(0.977) and the lowest was from UJL(0.498).

Table 3. Chi-Square distribution of *O. niloticus* from the different lakes (Eleyele, Owalla and Owena)

Sex	Frequency	Eleyele	Owalla	Owena	Total
Male	Count	21	20	29	70
	Expected Count	23.3	23.3	23.3	70
Female	Count	29	30	21	80
	Expected Count	26.7	26.7	26.7	80
Total	Count	50	50	50	150
	Expected Count	50	50	50	150
Chi-Square value	3.911				
Df	2				
P	0.142				

Legend:
*Df = degree of freedom, P = probability

DISCUSSION

According to Turan *et al*. (2006), the introduction and domestication of a fish species especially those from the wild leads to high adaptation to a wide range of geographical locations, which leads to phenotypic variations. In order to determine morphological differences between wild and cultured fish of the same species, different authors have used morphometric and meristic variables (Narváez *et al*., 2005; Solomon *et al*., 2015) to quantify biological variation and identify adaptive processes of different populations of the same species.

In this study, O. niloticus meristic variations revealed a significant difference in the DRS, DFR, PFR, PELFR, LLS and AFR across all three the locations. Owena and Owalla samples had slightly higher meristic mean values of 17.16±0.06 and 17.12±0.05 respectively than samples from Eleyele with 16.08±0.06. It was observed that significant differences existed among the mean values of meristic variations measured. The meristic variations that were higher in Owena and Owalla sampled populations than Eleyele sampled populations may be due to the age differences of the sampled population and as a result of environmental fluctuations. Since the age and ecological effect of samples were not determined, it is possible that sampled population were influenced by age and environment. Ikpeme *et al*., (2017) reported that age and environmental fluctuations have direct effect on meristic counts. Kashefi *et al*. (2010) also noted that meristic characteristics are affected by considerable changes in environmental factors such as temperature, radiation, salinity and dissolved oxygen. Considerable changes in environmental conditions generally occur as a result of differences in geographical region.

The morphometric parameters using PCA showed that the sample size using the Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO test) for Eleyele (0.9), Owalla (0.8) and Owena (0.9) were adequate. The minimum KMO standard is 0.5 which shows that the data for PCA were adequate. This also supports the findings of Paknejad et al (2013) where KMO matrics for sampled data was appropriate to precede with a factor analysis procedure. Two (2) principal components were extracted in Eleyele and Owalla while one was extracted in Owena. The result indicated that the highest percentage composition (83.17%) by variance was recorded for Owena when compared with Eleyele and Owalla variability in PC1 (71.353%) and (78.096%) while PC2 for both Eleyele and Owalla (7.852%) and (9.225%) respectively which means all morphometric parameters where important for the classification of O. niloticus. All the measured parameters were found within this component which further shows their relatedness and contribution to genetic variability. Amarasinghe and Fernando (2011) reported three principal components in morphological parameters of two tilapia species with the highest variability in PC1 (20.6%).The high communality values for Eleyele (0.977), Owalla (0.941) and Owena (0.954) obtained for the measured parameters in thisstudy may be an indication of their contribution to the observed variability between the populations. These variations are important for their survivability in the advent of environmental changes as reported by Ikpeme *et al*. (2017).

CONCLUSION

This study revealed the existence of morphological variations in wild populations of *O. niloticus* across three lakes in south-western states of Nigeria. The study showed that Owena is bigger when compared with Owalla and Eleyele in terms of fish size. It is important to note that fish is marketed by size. Thus, the bigger the size of fish, the higher market price it commands. Therefore, any morphological feature of fish that contributes to size gain will be of paramount interest to a fish farmer. From this study, it was noted that body depth, total length, standard length and head length had the highest and direct positive contribution to the enhancement of body size of the *O. niloticus* and with increased interest in this fish, to meet market demands, this study recommends that the wild populations should be protected against overfishing and deliberate/ indeliberate release of exotic species into this populations should avoided.

Table 4. Eigen values and share of total variance along with factor loadings and communalities of the morphometric parameters of *O. niloticus* from the three lakes (Eleyele, Owalla and Owena)

Location	Parameters	PC 1	PC 2	Communalities
Eleyele	TL	0.654	-0.355	0.554
	SL	0.988	0.005	0.977
	HL	0.969	-0.006	0.938
	POL	0.353	0.846	0.839
	POSL	0.759	0.018	0.576
	ED	0.699	-0.389	0.64
	UJL	0.695	0.122	0.498
	PDFL	0.955	0.063	0.915
	PPFL	0.788	0.049	0.623
	PAFL	0.96	0.06	0.925
	PSAFL	0.968	- 0.026	0.938
	BD	0.965	0.025	0.933
	CPD	0.968	-0.048	0.94
	Eigen values	**9.276**	**1.021**	
	Percentage of Variance	**71.353**	**7.852**	
Owalla	TL	0.927	0.238	0.916
	SL	0.883	0.388	0.931
	HL	0.908	0.274	0.9
	POL	0.873	0.13	0.778
	POSL	0.902	- 0.356	0.941
	ED	0.734	0.326	0.645
	UJL	0.951	0.043	0.907
	PDFL	0.79	0.452	0.828
	PPFL	0.865	-0.426	0.93
	PAFL	0.948	-0.144	0.919
	PSAFL	0.921	-0.294	0.934
	BD	0.921	-0.156	0.872
	CPD	0.838	-0.385	0.85
	Eigen values	**10.153**	**1.199**	
	Percentage of Variance	**78.096**	**9.225**	
Owena	TL	0.977		0.954
	SL	0.967		0.936
	HL	0.976		0.952
	POL	0.916		0.84
	POSL	0.893		0.798
	ED	0.887		0.787
	UJL	0.86		0.739
	PDFL	0.954		0.91
	PPFL	0.926		0.857
	PAFL	0.949		0.901
	PSAFL	0.933		0.871
	BD	0.938		0.88
	CPD	0.621		0.386
	Eigen values	**10.812**		
	Percentage of Variance	**83.172**		

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