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About the Journal

Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. Pertanika Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Pertanika Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other Pertanika series include Pertanika Journal of Science and Technology (JST) and Pertanika Journal of Social Sciences and Humanities (JSSH).

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JTAS

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ABSTRACTING/INDEXING

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Foreword

Welcome to the **Third Issue 2014** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for the Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains **eight articles,** out of which **one** is a short communication and **seven** are regular research papers. The authors of these articles are from **Malaysia, Sierra Leone, Nigeria, India** and **Iran**.

The short communication paper discusses species diversity and external nest characteristics of stingless bees in meliponiculture (*Kelly, N., Farisya, M. S. N., Kumara, T. K.* and *Marcela, P.*). Their study of 161 stingless bee colonies at a bee farm in Kelantan, Malaysia, revealed five species of stingless bees and one unidentified species. The species included *Trigona itama*, *Trigona thoracica*, *Trigona terminata*, *Trigona laeviceps*, and *Hypotrigona scintillans* and one unidentified meliponid. From the list, only *T. itama* and *T. thoracica* were mostly used in meliponiculture

The seven research papers cover a wide range of topics. In the first research paper, researchers from Njala University, Sierra Leone, report on their study of the effects of different rates of palm kernel and barley residues as organic manures on growth and yield of maize on a degraded upland soil (*M. T. Lahai*, *J. P. C. Koroma* and *P. M. P. Mornya*). The next research paper from Nigeria discusses the influence of thermotolerance acquisition in broiler chickens through early feed restriction in response to acute heat stress (Abioja, M. O., Sodipe, O. G., *Abiona, J. A., Oladipo, K. A., Kasali, O. D., Akerele, Z., Ola, A., Oke, H., Ogundele, O.* and *Osinowo, O. A.*). The following research paper, from Universiti Putra Malaysia, briefly discusses the benefit of HYDRUS 2D Model simulation in silt pit efficiency towards conserving soil water (*Mohsen Bohluli, Christopher Teh Boon Sung, Ahmad Husni Mohd Hanif* and *Zaharah A. Rahman*).

In the next research paper, researchers from Sant Longowal Institute of Engineering and Technology, India, present a multivariate classification of promising paddy cultivars on the basis of physical properties (*Singh, Y.* and *Prasad, K.*) while the following research paper presents a multivariate and an ordination approach to classify the attractiveness of the plant species in pastoral lands (*Fazel Amiri* and *Abdul Rashid Mohamed Shriff*).

Collaboration work between Universiti Putra Malaysia and the Institute of Medicinal Plants-ACECR, Iran, examines the genetic diversity of *Fusarium solani* populations obtained from black pepper (*Piper nigrum* L.) fields in Malaysia and assess the phylogenic relationships of *F. solani* isolates using ISSR markers in order to manage yellowing disease

in black pepper (*Shahnazi, S., Meon, S., Vadamalai, G., Yazdani, D.* and *Shabanimofrad, M.*). The last research paper is from a group of researchers from Universiti Malaysia Sabah and Universiti Sains Malaysia collaborating on the demographics of horseshoe crab populations in Kota Kinabalu, Sabah, Malaysia, with an emphasis on *Carcinoscorpius rotundicauda* and some aspects of its mating behaviour (*Robert, R., Muhammad Ali, S. H.* and *Amelia-Ng, P. F.*).

I anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

I would also like to express my gratitude to all the contributors, namely, the authors, reviewers and editors, who have made this issue possible. Last but not least, the editorial assistance of the journal division staff is fully appreciated.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Short Communication

Species Diversity and External Nest Characteristics of Stingless Bees in Meliponiculture

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ABSTRACT

Stingless bee farming is a new and potential booming industry in Malaysia. A study was conducted on stingless bee species preferred by meliponiculturists in Malaysia. External nest characteristics of the stingless bee species were also described. Sampling of 161 stingless bee colonies at a bee farm in Kelantan, Malaysia (06°07'N, 102°19'E) revealed five species of stingless bees and one unidentified species. The species included *Trigona itama*, *Trigona thoracica*, *Trigona terminata*, *Trigona laeviceps*, and *Hypotrigona scintillans* and one unidentified meliponid that accounted for 83.2%, 11.2%, 2.5%, 1.9%, 0.6% and 0.6%, respectively. The external nest characteristics, including the height of tree trunk, the trunk circumference at the top and bottom, and the height of the entrance tube from the bottom, were measured together with the size and description of entrance tube for each species. It was apparent that of 17-32 known species of stingless bees in Malaysia, only *T. itama* and *T. thoracica* were mostly used in meliponiculture.

Keywords: Entrance, *Trigona*, honey, meliponiculture, nest, stingless bee

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INTRODUCTION

Stingless bee beekeeping is known as meliponiculture. This activity, which is generally undertaken by traditional communities, has local characteristics according to regional and traditional knowledge. In Australia, interest in production and marketing of Australian

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honey is expanding. Although the total annual honey production in Australia is currently small (i.e., with probably less than 100kg), there is potential for rapid growth. At present, the market wholesale price of honey is about AU $50 per kg. In Asia, stingless bee beekeeping for pollination purpose is only beginning to take root in southern Asia, India and in SE Asia including Malaysia and the Philippines (Cortopassi-Laurino *et al.,* 2006).

Stingless bee farmers in the state of Kelantan, Malaysia, commercialize three types of stingless bee products, namely honey, bee bread and propolis. The current market price for stingless bee honey is RM35 per 300g, RM30 for 200g of bee bread and RM25 for 10ml of propolis, depending upon consumers' demands. At the moment, however, the stingless bee species of economic value and its external nest characteristics in Malaysia remain unexplored. Hence, this paper explores the subject matter mentioned and provides measurements of the extracted tree trunks from the forest with stingless bee colony in it.

MATERIALS AND METHODS

Sampling was done at a bee farm located in the state of Kelantan, Malaysia (06°07'N, 102°19'E). Measurements of the height of tree trunks, height of the entrance tube from bottom, size and shape of the entrance tube were documented. In this study, five to ten specimens of stingless bees were caught in individual plastic bag [34.5cm (h) x 24.6cm (w)] for each colony and they were brought back to Biology Laboratory, Universiti Malaysia Kelantan, Malaysia. At the laboratory, the bee specimens were killed using chloroform, pinned and oven dried at 40°C overnight. Once preserved, the specimens were identified with the taxonomy keys provided by Schwatz (1939), Sakagami *et al*. (1990) and taxonomic revisions by Rasmussen (2008).

RESULTS AND DISCUSSION

A total of 161 colonies of stingless bees were measured and documented (Fig.1). Five species of stingless bee and an unidentified species were collected during the sampling. The five species were *Trigona* (*Geniotrigona) thoracica*, *Trigona (Heterotrigona) itama*, *Trigona* (*Lepidotrigona) terminata*, *Trigona* (*Lisotrigona) scintillans* and *Trigona* (*Tetragonula) laeviceps*. In particular, *T. itama* were highly preferred by bee farmers and this species contributed 83.2% of the total colonies in the farm, followed by *T. thoracica* (11.2%) (Table 1). The two species contributed 94.4% of the total stingless bee species in the farm. Stingless bees could be found throughout the world with more than 500 species estimated (Heard, 1988; Ruttner, 1988). In Malaysia, the number of stingless bee species varies between 17 to 32 species depending on the study areas (Norowi, 2010; Salim, *et al.,* 2012; Schwarz, 1939). However, within the diverse species in Malaysia, only two species were highly sought after by bee farmers for meliponiculture purposes.

Fig.1: Tree trunks with stingless bee colonies

TABLE 1
Diversity of stingless bees in the farm

Species	Colonies (n) (%)
Trigona (Geniotrigona) thoracica	18 (11.2)
Trigona (Heterotrigona) itama	134 (83.2)
Trigona (Lepidotrigona) terminata	4 (2.5)
Hyporigona (Lisotrigona) scintillans	1 (0.6)
Trigona (Tetragonula) laeviceps	3 (1.9)
Unidentified	1 (0.6)
Total	161 (100)

Bee farmers extracted wild colonies nesting in the tree trunks before they transferred the colony into a bee box and subsequently sold. Each species of the stingless bees has specific nest requirements according to their sizes, population and habitat quality (Fonseca, 2012). Thus, the tree trunks represent the preferences of the species towards specific nesting site. It was recorded that *T. thoracica* and *T. laeviceps* preferred tree trunk circumferences ranging from 82 cm to 129 cm, whereas *T. itama* and *T. terminata* preferred tree trunk circumferences ranging between 71 cm and to 164 cm (Table 2). Majority of the tree trunks are of rubbers trees with a few forest hardwood trees. Extraction of the wild colonies requires experience and a good estimation of the location of the brood within the tree trunks to avoid damages to the brood. To avoid damages to the brood during extraction, data on nest entrance and height of tree trunk from this study could be used as a reference point to cut or fell the tree trunk (Table 2). In this study, the nest

entrance of the stingless bee was found to be different according to the genus (Fig.2). The functions of the nest entrances are related to defense, foraging (Biesmeijer *et al.,* 2005) and physio-chemical regulation (Roubik, 2006). The narrow tube can be closed with resin or cerumen or externally coated with droplets of fresh resin where invaders like ants may be halted (Wittmann, 1989; Camargo, 1984). Among the five species, *T. thoracica* was found to form mount-shape entrance with the widest entrance compared to all other species. However, the structure of the nest entrance or the thickness of the resin enclosing the internal nest are influenced by the age of nest, bee genetics and micro environment including predators, parasites, symbionts, rain, wind and sun (Roubik, 2006).

TABLE 2
External nest characteristics of stingless bees by species

Species	Mean ± s.d (cm)			
	Height of tree trunk	Circumference of the tree trunk (top)	Circumference of the tree trunk (bottom)	Height of entrance from bottom
T. thoracica	108.7±43.7	105.3±23.7	111.6±17.5	54.7±29.8
T. itama	86.2±20.2	96.3±24.9	100.4±27.1	45.8±21.1
T. terminata	102.3±22.1	108.5±34.4	125±39.3	44.8±34.6
H. scintillans	45	69	110	45
T. laeviceps	70.3±42.2	84.3±1.52	101.7±18.9	37.7±37.8

TABLE 3
Size and descriptions of entrance tube of the sampled stingless bees

Species	Entrance tube (mean ± s.d) (cm)			Shape	Color	Rigidity
	Length	Width	Tube Length			
T. itama	1.53 ± 0.47	2.04± 0.69	7.84± 7.39	F, R	Br, Lb	S, H
T. throracica	3.97 ± 1.29	4± 0.92	7.38± 3.65	M	Br, Bl	H
T. terminata	1.84± 0.38	1.96± 0.1	7± 2.02	F	Lb	S
T. laeviceps	1.85± 0.35	2.75± 0.75	4.25± 1.75	F	Bl	S,H
H. scintillans	NA*			R	Br	S

Entrance shape: F= funnel, M= mount, R= round-ringed; Color: Br=Brown, Bl=Black, Lb=Light Brown ; Rigidity: S=soft, H=hard;
* NA: Not Available; entrance was accidentally destroyed

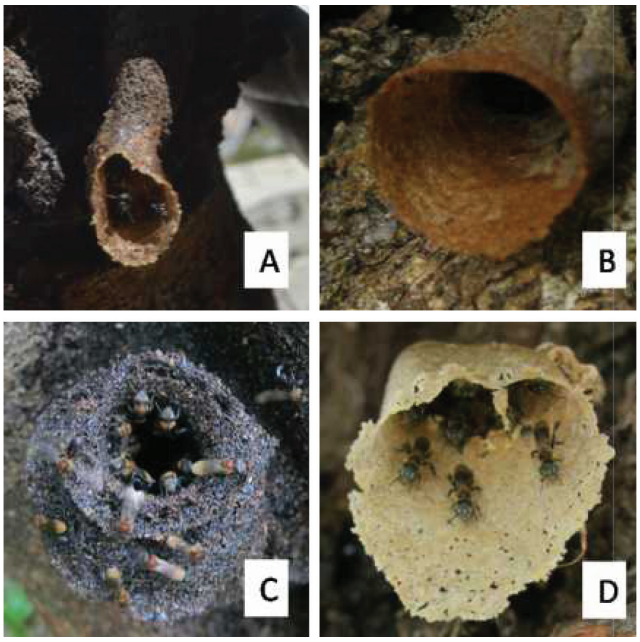

Fig.2: Shape of the entrance of the stingless bees' nest. A - Funnel shape entrance of *T. itama*; B - Round ringed entrance of *T. itama*; C - Entrance in shape of mount (*T. thoracica*), D - Funnel shape entrance of *T. terminata*

CONCLUSION

From this study, five species of stingless bees and one unidentified bee species were sampled. The species were *T. itama*, *T. thoracica*, *T. terminata*, *T. laeviceps*, and *H. scintillans*. Meliponiculture in Malaysia is limited to two species of stingless bees, namely *T. itama* and *T. thoracica*. Although the extraction of wild is not sustainable in the long term, it is the authors' hope that in the future, colony propagation will be a good alternative with the expansion of the knowledge and research on the biology of stingless bees.

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Effects of Different Rates of Palm Kernel and Barley Residues as Organic Manures on Growth and Yield of Maize on a Degraded Upland Soil

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ABSTRACT

Effect of organic manures and chemical fertilizers on maize (cv. DMR-ESR-Yellow) was studied in a degraded upland soil. Four levels (0, 1, 3, and 5 t ha^{-1}) of palm kernel and barley residues and a recommended rate of chemical fertilizers were tested in a randomized complete block design with three replications. Plant height, stem diameter, number of leaves, ear leaf area, 1000 grain weight, and grain yield increased with the increase rate of manures, but the increase was higher in palm kernel than the barley residue. The 3 and 5t ha^{-1} rates of palm kernel residue were the best treatments due largely to higher supply of N, P, K, Ca and Mg as. Under high rainfall, grain yield increased likely by reducing days to tasseling and silking and anthesis silking interval (ASI). Under low rainfall, both organic manures, which may have maintained more favourable soil moisture and temperature regimes, gave considerably higher yield than the recomended fertilizer rate (NPK 15:15:15). Organic manure treated plants, especially under palm kernel residue with higher N supply reached 50% tasseling and silking earlier and had shorter ASI and longer grain-filling period than inorganic fertilizer. The use of 3 to 5t ha^{-1} of palm kernel residue manure could substantially increase grain yield in Sierra Leone. However, the increase in yield with application rate showed that the highest rate used was not the optimum. Therefore, higher rates should be included in subsequent trials to obtain maize response curves for both manures.

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INTRODUCTION

Maize is one of the major cereal crops in West and Central Africa, particularly in

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areas with good access to fertilizer inputs and markets (Fakorede *et al*., 2003). In West Africa, Manyong *et al.* (1996) assessed maize as one of the five main crops of the farming systems in about 125 million hectares (about 72%) of the study area. The current on-farm average yield of maize in West Africa is about 2 t ha^{-1} compared with the average yield in the 1970s, which was below 1 t ha^{-1} (Fakorede *et al.,* 2003). Even though the magnitude of the recent gains has been remarkable, there is still a wide yield gap between the research stations and that grown under on-farm conditions. Nevertheless, individual farmers who grow the improved varieties under well-managed, high input conditions usually obtain much higher grain yields. While yield increases achieved by farmers have resulted from the impacts of both genetics and improved crop husbandry, the contribution of improved management to yield increases is likely to be smaller in the next decade due to high cost and non-availability of agro-chemicals, particularly chemical fertilizers and a reduction in soil fertility as a result of human activity (Kamara *et al*., 2004). Therefore, alternative nutrient sources to chemical fertilizers that are environmentally friendly, cheap and readily available need to be investigated for maize.

Organic manures made of natural materials that undergo little or no processing may be a good alternative to chemical fertilizers. Organic inputs play a key role in sustaining soil productivity as they contribute to fertility by adding nutrients such as N, P and K to the soil. They are also major sources of energy and nutrient for soil microbial communities. It improves physical properties of soil such as promoting soil aggregation and nutrient buffering capacity and reducing soil bulk density (Vanlanwe, 1996; Sanchez, 1987). In Sierra Leone, as in other parts of West Africa, where oil palm abounds and have established brewery industries, readily available organic materials in the forms of palm kernel and barley residues, by-products of palm kernel oil processing and brewery industries respectively are usually produced in large quantities annually, and these are normally left to waste. These materials may be used as alternatives to chemical fertilizers in the production of maize. However, little or no research has been reported in this direction for crops. Therefore, this study was initiated to determine the response of maize to different rates of palm kernel and barley residue organic manures on a degraded upland soil in Sierra Leone.

MATERIALS AND METHODS

Experimental Site Description

A field experiment was conducted at the east end of Freetown (latitude 8˚, 30'N, longitude 13˚, 17'W and 400 m above sea level) in the Western area of Sierra Leone in 2010 and 2011. Annual rainfall in the Western area ranges from 2000-3000 mm, with an average temperature of 26˚C. The experimental site was cultivated with okra, followed by groundnut before the trial was established. During the trial period, mean temperature and relative humidity were about the same in both years, but rainfall

was higher in 2010 than it was in 2011. The soil classified as ultisol (USDA, 1975) was sandy clay loam with a pH of about 4 in both years. The nutrient contents, particularly N, were low (Table 1).

TABLE 1
Weather and soil characteristics of the experimental site during the trial periods

Weather/Soil characteristics	2010	2011
Weather factor		
Rainfall (mm)	1194.3	991.5
Minimum temperature (°C)	23.3	23.6
Maximum temperature (°C)	30.6	30.8
Minimum relative humidity (%)	74.5	74.3
Maximum relative humidity (%)	86.8	84.5
Soil characteristics		
pH (H_2O)	4.1	4.0
Sand (%)	56	54
Silt (%)	20	22
Clay (%)	24	24
Organic Carbon (%)	0.36	0.29
N (%)	0.11	0.10
P (mg kg^{-1} Bray 1)	8.91	6.12
K (cmol kg^{-1})	0.54	0.38
Na (cmol kg^{-1})	0.26	0.17
Mg (cmol kg^{-1})	0.13	0.11
Ca (cmol kg^{-1})	0.32	0.24
CEC	6.00	4.70
Soil type	Sandy clay loam	Sandy clay loam

Planting Material

Zea mays L. (cv. DMR-ESR Yellow), obtained from the International Institute of Tropical Agriculture, Ibandon, Nigeria, was used as the planting material.

Experimental Design and Plot

The experiment was laid out in a randomized complete block design with three replications and four factors (palm kernel manure rate, berley manure rate, recommended chemical fertilizer rate and year) after manual land preparation. The plot size was 5 m x 3m with each plot consisting of 4 rows with 10 plant stands per row.

Manure and Fertilizer Rate and Method of Application

Four levels (0, 1, 3 and 5 t ha^{-1}) of palm kernel and barley residues and a recommended rate of chemical fertilizers (NPK 15-15-15) of 200 kg ha^{-1} were used. The organic manures were applied one week before planting using the pocket application method. Inorganic fertilizer was split applied to the appropriate plots, one-half at three weeks after planting and the other half at the onset of tasseling (anthesis). Two-hand weedings were carried out at 3 and 6 weeks after planting.

Seed Sowing and Harvesting

Planting was done on 30th May 2010 and 1st June 2011 at a spacing of 0.75 m x 0.50 m. Four seeds were planted and later thinned to two per stand one week after germination to give a population of 53, 333 plants ha^{-1}. The maize matured around 90 days after planting in both years. Harvesting was done on 29th August 2010 and 30th August 2011 for the first and second year, respectively. The 32 plants in the two middle rows of each plot (excluding the end plants) were harvested. The harvested ears were dried in the sun for two days, shelled and oven-dried at 60^{O}C for

4 days. Grain yield and 1000 grain weight were computed from the shelled dried grain adjusted to 12% moisture level.

Data Collection

Days from sowing to 50% pollen shed (anthesis date) and 50% silk extrusion (silking date) were determined on 40 plants in the two middle rows of each plot. Anthesis-Silking Interval (ASI) was calculated as the difference between days to silking and anthesis. Plant height and stem girth were measured on 10 plants per plot approximately 2 weeks after germination. The area of the leaf below the ear (cob) was measured *in situ* at mid-silking using a portable leaf area meter model LI-3000 A (head-scanner serial no. PAM 1684). The number of leaves per plant was determined at the same time as the ear leaf area.

Analysis of the Soil and Plant Samples

About 100g each of the palm kernel and barley residues were oven-dried at 60^{O}C for 4 days, crushed and passed through a 0.5 mm sieve. The organic manure materials were analyzed for N, P, K, Ca, Na and Mg concentrations. In addition, C:N ratio and organic carbon were determined for the manure materials. The Walkley-Black and Macro-Kjeldahl methods were used to analyze for organic C and total N, respectively. Meanwhile, the C:N ratio was calculated as the ratio of organic C to that of total N. Bray I method was used to extract P, while Ca, Mg, Na and K were analyzed after extraction with 1N ammonium acetate at pH 7.0. Ca and Mg were measured using atomic absorption spectrometry and Na and K by flame photometry (IITA, 1982).

The soil samples were collected in three positions in each replication at the depth of 0 – 20 cm with a 0.025 m-diameter Edelman auger. The soil samples were then bulked to make one composite sample. A subsample of 100 g was air dried, crushed and passed through a 0.5 mm and 2 mm sieved for the chemical and mechanical analyses, respectively. Soil pH was measured in 1:1 moisture of soil in deionized water using a glass electrode pH meter. The Walkley-Black and Macro-Kjeldahl methods were used to analyze soil organic C and total N, respectively. The C:N ratio was calculated as the ratio of organic C to that of total N. The Bray I method was used to extract available P. Exchangeable cations were analyzed after extraction with 1N HN_4OAc (ammonium acetate) at pH 7.0. Ca and Mg were measured using absorption spectrometry, and Na and K by flame photometry. The cation exchange capacity was determined by the summation of extractable cations by 1N NH_4OAc. The hydrometer method was used to determine the percentage of sand, silt and clay contents of the soil (IITA, 1982).

Statistical Analysis

The mixed model procedures of the Statistics Analysis System for Microsoft Windows Release 6.10 (SAS Institute, 1991) were used to detect differences between treatments for all the variables. Manure rate and year were the fixed effects and replication the random effect. The residual mean square was used to calculate approximate standard errors.

RESULTS

Nutrient Concentration in Manures

The palm kernel residue had about 3.1, 3.3, 4.1, 4.1, 1.2, 2.0% and 5.0% N, P, K, Na, C, Mg and Ca, respectively while barley residue contained 2.5, 2.9, 3.5, 5.6, 1.7, 1.8 and 3.9% N, P, K, Mg and Ca, respectively. This indicated that concentrations of N, P, K, Mg and Ca were 19, 11, 16, 11 and 21% higher in palm kernel than the barley residue, whereas, Na, C and C: N ratio were 27, 26 and 40% higher in barley than the palm kernel residue (Fig.1).

Grain Yield and Yield Components

Grain yield differed significantly between type ($p< 0.0001$) and rate ($p< 0.0001$) of manure, as well as year ($p< 0.0001$). Within each manure type, grain yield increased with the increase in the rate of manure in both years (Table 2). The palm kernel residue produced higher grain yield than the barley residue. Palm kernel residue treated plants gave about 19 and 28% higher grain yields than those treated with barley residue in 2010 and 2011, respectively. The plants treated with organic manures and NPK 15-15-15 fertilizer produced higher yield than the control. In 2010, only the 3 and 5 t ha^{-1} rates of palm kernel residue yielded higher than the 200kg ha^{-1} NPK 15-15-15 recommended fertilizer rate. In 2011, however, the 1, 3 and 5 t ha^{-1} rates of both manures gave higher grain yield than the NPK 15-15-15 fertilizer. Grain yield was about 39% higher in 2010 than that of 2011. Meanwhile, trends in 1000 grain weight are similar to those in grain yield (Table 2).

Phenology

Days to 50% anthesis and silking, as well as Anthesis-Silking Interval (ASI),

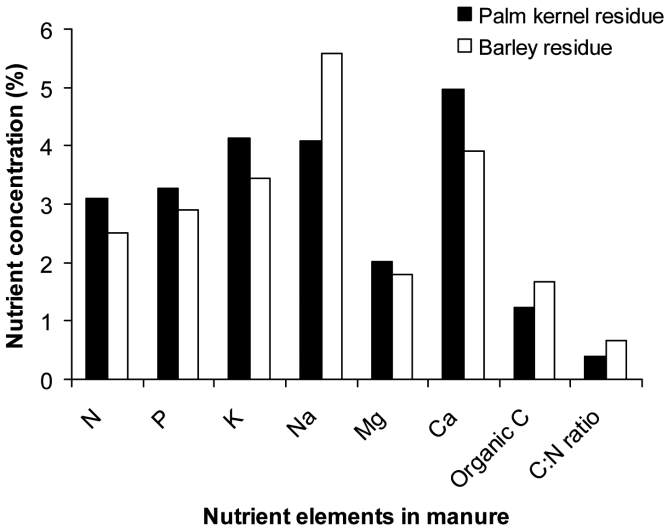

Fig.1: Concentration (%) of nutrient elements and C: N ratio in palm kernel and barley residue organic manures.

varied significantly ($p < 0.0001$) among rate and type of manure and year. In general, flowering (anthesis and silking) and ASI decreased as the rates of both manures increased in both years (Table 2). The plants treated with palm kernel residue reached 50% anthesis and silking about 2 and 3 days earlier than those plants treated with barley residue in 2010 and 2011, respectively. Anthesis-Silking Interval was the same for the plants treated with both manures in 2010. In 2011, ASI was 1 day shorter for the plants subjected to palm kernel manure than those of the plants treated with barley residue manure. Plants with no manure application had higher number of days to flowering and ASI than the manure and fertilizer treated plants in both the years. NPK 15-15-15 fertilizer treated plants flowered later and had higher ASI than the organic manure in both the years. The plants tasseled and silked were about 3 and 4 days, respectively, earlier in 2010 than in 2011. ASI was also lower in 2010 than in 2011 (Table 2).

TABLE 2
Yield and yield components and days to anthesis and silking of maize (cv. 'DMR-ESR-Yellow) as influenced by different rates of palm kernel and barley residue organic manures and NPK 15:15:15 fertilizer in 2010 and 2011 on the upland soil

Treatment		Grain yield (kg ha^{-1})		1000 grain weight (g)		Days to 50% silking		Days to 50% anthesis		Anthesis-silking internal (days)	
Manure type	Rate	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
No manure	0 t ha^{-1}	1826de	860f	189d	142f	57.7a	67.0a	51.7a	61.0a	6.0a	6.0a
NPK 15:15:15	200 kg ha^{-1}	2136bc	945ef	194cd	147de	54.7c	61.0b	49.7c	56.3b	5.0b	4.7d
Palm kernel residue	1 t ha^{-1}	1763e	1171cd	190cd	147de	54.0c	55.0de	49.7c	50.3de	4.3c	4.7d
Palm kernel residue	3 t ha^{-1}	2174b	1577b	202b	154c	53.0d	54.3e	49.3c	50.0e	3.7d	4.3e
Palm kernel residue	5 t ha^{-1}	2826a	1982a	222a	161a	52.7d	54.3e	49.0d	50.0e	3.7d	4.3e
Barley residue	1 t ha^{-1}	1683e	974def	190cd	146e	56.3b	58.7bc	51.7d	53.0c	4.6bc	5.7b
Barley residue	3 t ha^{-1}	1849de	1080de	195c	150d	54.7c	58.0c	51.0b	53.0c	3.7d	5.0c
Barley residue	5 t ha^{-1}	1969cd	1343c	195c	160a	54.3c	57.0cd	51.0b	52.3cd	3.3d	4.7d
Year mean		2028a	1241b	197a	151b	54.7b	58.2a	50.4b	53.2a	4.3b	4.9a

Treatment means in a column and year means in row for each variable followed by a common letter are not significantly different at 5% level determined by LSD test

Plant height, stem girth, leaf production and ear leaf area were significantly varied among manure type and rate, as well as year (Table 3). For both types of manure, plant height increased with the increase in rate of manure in both the years. Comparing the residues, palm kernel residue showed taller plants than the barley residue. The plants were also taller in the organic manure treated plots than the control. In 2010, both manures at the rate of 5 t ha^{-1} produced significantly taller plants than the 200 kg ha^{-1} NPK 15-15-15. In 2011, the plants treated with both types of organic manure at all application rates were taller than those treated with NPK-fertilizer. The plants were 17% taller in 2010 than those in 2011 (Table 3).

The stem girth increased with the increase in rate of organic manures, but at each rate the palm kernel residue treated plants produced bigger stems than those of plants treated with barley residue in both years (Table 3). In 2010, plants treated with organic manure had bigger stems than the control and the recommended fertilizer rate of 200 kg ha^{-1} NPK 15-15-15. On the contrary, in 2011 only plants treated with 5 t ha^{-1} palm kernel residue had bigger stems than those treated with NPK-fertilizer. In all cases the control (0 t ha^{-1}) produced the smallest stem size.

Leaf production increased as the rates of two manure types increased in both years (Table 3). At each manure rate, the palm kernel residue produced higher number of leaves than the barley residue. In 2010, NPK-fertilizer treatment plants produced more leaves than the control, but in 2011 both treatments had similar number of leaves. In 2010 and 2011, 200 kg ha^{-1} NPK 15-15-15 and the control treatments produced lower number of leaves than the manure treatments. On average, plants produced more leaves in 2010 than in 2011 (Table 3).

In each type of manure, ear leaf area increased with the increase in rate of manure. At each rate, plants treated with palm kernel residue produced larger ear leaf area than those plants treated with barley residue. Plants treated with palm kernel residue at all rates and at 5 t ha^{-1} for barley residue had significantly large ear leaf area than those of 200 kg ha^{-1} NPK 15-15-15 fertilizer, while the control had the least ear leaf area. In 2011, irrespective of the rate and type, the manure treated plants had larger ear leaf area than the 200 kg ha^{-1} NPK 15-15-15 ferilizer and the control. In both years, the chemical fertilizer treated plants produced larger ear leaf area than the control. The ear leaf area was about 12% larger in 2010 than in 2011 (Table 3).

DISCUSSION

Maize showed significant differences for grain yield and its components under different rates of palm kernel and barley residue manures. Grain yield and 1000 grain weight increased with the increase in the rate of both organic manures. The results are consistent with Kamara (1998) who reported that an addition of organic materials is important in maize production. Mugendi *et al*. (1994), and Sanginga and Swift (1992) reported that positive role is

TABLE 3
Effect of different rates of palm kernel and barley residue organic manures and NPK 15:15:15 fertilizer on morphological characters of maize (cv. 'DMR-ESR-Yellow) in 2010 and 2011 on a degraded upland soil

Treatment		Plant height (cm)		Stem girth (cm)		No. of leaves plant^{-1}		Ear leaf area (cm^2)	
Manure type	Rate	2010	2011	2010	2011	2010	2011	2010	2011
No manure	0 t ha^{-1}	162.4d	121.3e	1.8d	1.9c	15.3d	13.6d	223.8e	186.8d
NPK 15:15:15	200 kg ha^{-1}	175.5bc	130.1d	1.9cd	2.1b	16.0d	13.7d	286.4c	198.8d
Palm kernel residue	1 t ha^{-1}	172.2c	144.5c	2.2b	2.0bc	16.6cd	16.7b	290.2c	261.1c
Palm kernel residue	3 t ha^{-1}	176.1b	155.3b	2.4a	2.1b	19.7a	17.6b	326.4b	295.6b
Palm kernel residue	5 t ha^{-1}	185.4a	167.0a	2.5a	2.8a	19.8a	18.8a	385.3a	345.5a
Barley residue	1 t ha^{-1}	173.0bc	130.6d	2.0c	2.0bc	15.5d	15.4c	253.1d	258.5c
Barley residue	3 t ha^{-1}	173.5bc	149.2bc	2.2b	2.0bc	16.3cd	16.7b	280.7c	262.6c
Barley residue	5 t ha^{-1}	177.8b	153.4b	2.2b	2.1b	17.0c	17.1b	302.9bc	262.8c
Year mean		174.5a	143.9b	2.2a	2.1a	17.0a	16.2a	293.6a	259.0b

Treatment means in a column and year means in row for each variable followed by a common letter are not significantly different at 5% level determined by LSD test

played by N released from decomposing organic materials. A similar trend in the grain yield as that in 1000 grain weight suggests that the manures increased yield by partly increasing the individual grain weight. This is supported by Kamara *et al*. (2003) who reported that maize genotypes with higher individual kernel weight gave higher yields than those of lower kernel weight.

While the palm kernel residue at the rates of 3 and 5t ha^{-1} were the best treatments in both years, the 200 kg NPK 15-15-15 ha^{-1} yielded higher than barley residue at 1 and 3t ha^{-1} in 2010. In 2011, there was a 27-57% and 19-56% increases in yield with the application of 1-5t ha^{-1} palm kernel residue compared to the control and the recommended 200 kg ha^{-1} NPK 15-15-15. The corresponding figures for barley residue were 9-36% and 0.2-30%. This indicates that the use of both organic manures, particularly at 5 t ha^{-1}, has substantial yield advantage over the inorganic fertilizer. The results of this study corroborate with those of Kamara (1998) who observed that organic materials obtained from *G. sepium* and *L. leucecophala* gave higher maize yield than the inorganic N fertilizer at 90 kg ha^{-1}.

The barley and palm kernel residues contained about 2.5 and 3.1% N, respectively, which translated to 25 and 31 kg N for each ton of manure, respectively. The ability of plant residues to decompose and release nutrients is determined by their litter quality which is a function of the chemical composition of the plant materials. Plant residues with high N content show

high decomposition rates and nutrient release (Swift *et al*., 1979; Kamara, 1998). Therefore, the higher yield obtained from palm kernel residue application than that of barley residue could largely be due to higher N content, faster decomposition rate and nutrient release by the palm kernel than barley residue (Swift *et al*., 1979; Kamara, 1998). After oil extraction, the palm kernel residue is left in the form of fine particles, which might have contributed to its faster rate of decomposition and nutrient release. Thus, the use of barley residue as manure should be supplemented with inorganic nutrients to accelerate nutrient release as recommended by Mugendi *et al*. (1994) for pruning from tree leaves with low N content. Palm kernel residue could do without inorganic fertilizer.

Grain yield increased with the increase in rates of organic manures, due largely to increase in N supply, as was also observed by Mugendi *et al*. (1994) and Kamara (1998). The 200 kg of NPK 15-15-15 fertilizer had 30 kg N, indicating that at 3 and 5t ha^{-1}, the palm kernel residue contained about 3 and 5 times, while the barley residue had 2½ and 4 times more N than the chemical fertilizer treatment. Hence, the higher grain yields at these rates (3 and 5t ha^{-1}) for both organic manures than the 200 kg of NPK 15-15-15 fertilizer. The low availability and high cost of inorganic fertilizers put them out of the reach of many small farmers in West Africa (FAO, 1992). Also, leaching of nutrients in the soil reduces the efficiency of inorganic fertilizers in the tropics (Sanchez *et al*., 1997). Organic inputs have an important advantage over inorganic fertilizers with regard to fertility replenishment; they provide a carbon source for microbial utilization resulting in the formation of soil organic nitrogen. Inorganic fertilizers do not contain such carbon sources. Therefore, most of the fertilizer nitrogen not used by the crops is subject to leaching and de-nitrification losses, while much of the nitrogen released from organic inputs and is not utilized by the crops can build soil organic nitrogen capital (Sanchez & Palm, 1996). Hence, the use of 3-5t ha^{-1} of both manures, particularly palm kernel residue with higher nutrient content compared with barley residue, could substantially increase maize grain yield in Sierra Leone.

The 5t ha^{-1} rate contained about 125 and 155 kg N ha^{-1} for barley and palm kernel residues, respectively. Thus, the increase in grain yield and its components as the application rate of both manures increased showed that the highest rate used was not the optimum as maize has been reported to respond to up to 200 kg N ha^{-1} in Sierra Leone (George, 1986). Therefore, rates higher than 5t ha^{-1} should be included in subsequent trials in order to obtain maize response curves for both manures. Grain yield was about 39% higher in 2010 than in 2011, likely and partly due to higher rainfall (1615.5 mm) in the first than the second year (941.2 mm), which might have reduced drought stress in the first year (Table 1). During the period of lower rainfall, plants treated with both organic manures gave considerably higher grain yield than the chemical fertilizer compared with the yield

under high rainfall. The organic manures might have maintained more favourable soil moisture and temperature regimes during low rainfall thereby substantially increasing yield. Consistent with our results, Martinez *et al*. (2009) observed 50% increases in yield of quinoa with the addition of organic matter even under conditions of low irrigation.

Plants treated with both organic manures reached 50% pollen shed and silk emergence earlier and had shorter ASI than those of plants treated with inorganic fertilizer and no manure application, with plants treated with palm kernel residue having shorter days to reproductive development than those of plants treated with barley residue. However, all the treatments attained physiological maturity at about 90 days. The reduction in days to silking and ASI likely resulted in effective pollination and longer grain-filling period, which might have contributed to the increased in yield for especially plants treated with palm kernel residue. The results of this study are consistent with the observation that delayed silking is associated with barrenness (Herrero & Johnson, 1981) and appear to reflect reduced partitioning of assimilates to the developing ear at flowering (Edmeades *et al*., 1993; Kamara *et al*., 2003). Environmental stress such as N-deficiency delays reproductive development (Bolanos & Edmeades, 1993; Kamara *et al*., 2005) and reduce ear biomass at flowering (Lemcoff & Loomis, 1986). The increased supply of N to the maize plants with increasing manure rate might have contributed to the early tasseling and silking and also to shorter ASI as the rates of both organic manures increased. Delay in reproductive development under inorganic fertilization and barley residue as compared to palm kernel residue, respectively, could largely be attributed to lower N supplied by the former than the latter nutrient sources. The plants had shorter days to tasseling and silking and lower ASI in 2010 than in 2011. This could partly be attributed to the higher rainfall in 2010 than in 2011, which could have reduced drought stress in the first year, as drought stress has been reported to delay reproductive development in maize (Edmeades *et al.*, 1993).

Plant height, stem diameter, number of leaves and ear leaf area increased as the rate of both organic manures increased, but the increases in these growth parameters were higher under palm kernel than the barley residue. Kamara *et al*. (2005) showed that plant height, ear height and total dry matter increased with increasing rates of N application. The increase in nutrient availability, particularly N with increasing rate of manuring, may have contributed to the increase in growth parameters as the rate of both manures increased. This is supported by the fact that palm kernel residue with higher N, P, K, Ca and Mg produced taller plants that had bigger stems and higher number of leaves and ear leaf area than barley residue treated plants. Kamara *et al*. (2003) showed that maize genotypes with extended green leaf area duration have longer grain filling periods and higher kernel yields and also noted remobilization of assimilates from the stem to the ears. The manure treated plants with bigger stems and

higher number of leaves and ear leaf area, especially those under palm kernel residue likely had longer grain filling periods and more assimilates for remobilization to the developing ears and therefore higher grain yield than the recommended chemical fertilizer and the control.

CONCLUSION

Maize exhibited significant differences for growth parameters and yield under different rates of palm kernel and barley residue manures. These parameters increased with the increase in the rate of manures, but the increases were higher in palm kernel than the barley residue. Organic manure treated plants, especially under palm kernel residue, flowered earlier and had shorter anthesis silking interval (ASI) than those plants treated with inorganic fertilizer. High rainfall reduced days to tasseling and silking and ASI and increased yield. Under low rainfall, both organic manures yielded considerably higher than the inorganic fertilizer when compared with the yield under high rainfall. The use of 3 and 5t ha^{-1} of particularly, palm kernel residue could substantially increase grain yield without the use of inorganic fertilizer. However, the increase in yield with application rate showed that higher rates should be included in subsequent trials to obtain maize response curves for both manures.

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Thermotolerance Acquisition in Broiler Chickens through Early Feed Restriction: Response to Acute Heat Stress

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ABSTRACT

Broiler chicks were subjected to early feed restriction (EFR) on d3-5 post-hatch to determine the day that confers the best thermal tolerance during acute heat stress episode at market age. In Experiment I, 160 *Marshall* chicks were allotted to 4 treatments. One group received feed *ad libitum* (CONTROL), while others received no feed for a period of 24 hours on d3 (D3), d4 (D4) and d5 (D5), respectively. Each group had 4 replicates with a total of 10 birds per replicate. On d55 of age, rectal temperature (RT) was monitored and blood samples were taken from the birds before and 1h after exposure to temperature 37±2°C and 50% relative humidity. There were a total of 120 chicks in Experiment II and they were divided into 3 groups which were either fed *ad libitum* (CONTROL) or had feed withdrawn for 12h ($D5^{12}$) or 24h ($D5^{24}$) on d5 to determine the length of feed restriction that best conferred thermotolerance on the birds. RT and blood sampling were also done as described above. In Experiment I, haematological parameters were ($P>0.05$) similar among the treatments before and after exposure. Prior to the exposure to heat stress, RT was not affected ($P>0.05$) by feeding regimen but it was significantly ($P<0.05$) affected by the treatment after the exposure. D5 chickens had significantly ($P<0.05$) lower temperature than control. However, it was not different from D3 and D4 chickens. In Experiment II, initial RT did not differ among the 3 treatment groups although RT was ($P<0.05$) affected by the feeding regimen after 60 minutes of exposure. The control chickens had similar RT with that of $D5^{12}$. However, RT in control birds ($P<0.05$) was higher than RT in $D5^{24}$. Thus, applying EFR in broilers on D5 of post-hatch for 24

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hours may help reduce hyperthermia during heat spell at market age.

Keyword: rectal temperature, acute heat stress, broilers, H:L, early feed restriction

INTRODUCTION

Broiler production continues to suffer under the influence of heat stress in the tropics (Lin *et al.*, 2006; Abioja, 2010; Sunil Kumar *et al.*, 2011) where environmental temperature is often above thermal comfort zone of most poultry species. It results in hyperthermia (Altan *et al.*, 2000; Garriga *et al.*, 2006) which leads to a cascade of events that affect the well-being of the birds. Heat stress causes changes in leucocyte number and other haematological parameters (Maxwell *et al.*, 1992; Yalcin *et al.*, 2004).

Broilers are more susceptible at growing phase and at market age than starting phase. The envisaged increase in environmental temperature, as climate change becomes more evident, has created fear in broiler industry. Moreover, IPCC (2007) report pointed to Africa as a continent that is most likely to be affected by climate variations. Climate change, in terms of both climate means and variability, poses a great threat to farmers and the livestock in the region.

Stress is usually avoided because of its perceived repercussions on living tissues. However, evidences are emanating from various research works that exposure of broiler chicks to mild stress at early age help in acquiring thermotolerance at latter age. This was first reported in rats by Levine (1962). The author discovered that mild stress at early age affects the adrenocortical functioning during adulthood. It was later confirmed in broiler chickens (Yahav and Plavnik, 1999; Zulkifli *et al.*, 2000; Liew *et al.*, 2003). From this assertion, there are two strategies that have been explored: thermal conditioning (TC) and early feed restriction (EFR). TC involves exposing chicks at early age post-hatch to elevated ambient temperature (Arjona *et al.*, 1990; Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001) within the first six days while EFR subjected the chicks to varying degrees of hunger during the same period (Zulkifli *et al.*, 1994). TC has been demonstrated to be effective as a tool for thermotolerance acquisition in poultry but its application is not practicable in the traditional open-sided poultry houses common in most tropical and developing countries. The cost of heating poultry house to a desired temperature is high. Besides, the technicality associated with thermal conditioning such as length and degree of heat exposure may not be easily mastered by the local farmers. This leaves the only option for thermotolerance acquisition in the developing and under-developed world to application of early feed restriction.

Zulkifli *et al*. (2000) reported that acute heat stress resulted in increase in heterophil/lymphocyte ratio for all feed restricted groups and the *ad libitum* group. But broilers restricted from 60% of daily feed requirement on day 4, 5 and 6 had the least heterophil/lymphocyte ratio. The authors concluded it appeared 60% feed restriction is beneficial in improving growth

and survivability of female broiler chickens exposed to heat stress later in life. The authors restricted the feed by percentage on three consecutive days (day 4, 5 and 6). Exact feed intake of the birds may not be ascertained: feed intake depends on strain, genotype, environmental condition in the pen, body weight, *etc*. Farmer may have problem calculating the feed intake. Besides, percentage feed restriction may not give the exact day the EFR is most effective. Therefore it is important to determine exact day of life the chicks should be subjected to early feed restriction by farmer which ensures best acquisition of thermotolerance.

Therefore, the objective of this study is to determine the effectiveness of EFR in conferring thermotolerance on broilers at market age with respect to change in blood parameters and rectal temperature during acute heat stress. It also sought to establish the actual day and length of time chicks should be exposed to EFR which will ensure the least fluctuation in body temperature and blood picture.

MATERIALS AND METHODS

Meteorological observations: Minimum, maximum and mean temperature, and relative humidity at the level of the birds in the pen were monitored by means of digital thermo-hygrometer.

Animals and Management

Experiment I: This focused on appropriate day of exposure to EFR that confers the best thermotolerance on broiler chicks. One hundred and sixty *Marshall* broiler chicks obtained from a reputable hatchery were allotted to four treatments. One group received feed *ad libitum* (CONTROL) while others received no feed for a period of 24 hours on day 3 (D3), day 4 (D4) and day 5 (D5) respectively. Each group had 4 replicates and 10 birds per replicate. Starter (23.05% crude protein, 11.73 MJ/Kg, 3.93% ether extract and 3.67% crude fibre) and finisher mash (19.91% crude protein, 11.71 MJ/Kg, 3.89% ether extract and 3.79% crude fibre) were supplied *ad libitum* to all birds except on days of feed restriction while water was made available always.

Acute heat stress: Four birds per treatment were transferred from the pen into a pre-heated room (37±2^{o}C, 50% RH) to test heat tolerant response to 1-hour acute heat stress episode on d55. The temperature was 24^{o}C before the chickens were moved from the pen.

Blood analyses: Blood samples were collected from the birds via brachial vein before the transfer and 1 hour after transfer into the pre-heated room. The samples were centrifuged and the plasma stored at -20^{o}C for analyses. Wintrobes microhaematocrit and colorimetry methods (Lamb, 1991) were used to determine packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC) and white blood cell count (WBC). Blood collected into labeled EDTA bottles were placed in the microhaematocrit centrifuge and spun for 5 minutes at a revolution of 1988 x g. The PCV values were subsequently determined by measuring the height of

the red cell column and expressing this as a ratio of the height of the total blood column using microhaematocrit reader. Red blood cell count was done by diluting the blood sample with 0.9% NaCl and shaking well. The diluted blood was mounted on a haemocytometer and the number of erythrocytes counted microscopically. Four bBlood smears were stained using May-Grunwald and Giemsa stains approximately 4 h after preparation with methyl alcohol fixation. Leucocyte differentials (heterophils, lymphocytes, eosinophils, monocytes and basophils) were counted for each smear and heterophil:lymphocyte ratio was calculated according to Yalcin *et al.* (2005).

Blood glucose was determined using the enzymes glucose oxidase and peroxidase (Belo *et al.,* 1976). The former catalyses the oxidation of glucose to glucoronic acid and hydrogen peroxide. The hydrogen peroxide in the presence of the second enzyme and a chromogenic hydrogen donor, d-dinisidine forms a coloured substance. The following

TABLE 1
Haematological parameters and blood glucose (before and after acute heat stress episode) of broiler chickens exposed to 24-hour feed withdrawal during d3, d4 or d5 of age

Parameter		CONTROL	D3	D4	D5	Sem	P
Packed cell volume (%)	Before	32.3	33.5	32.5	34.0	1.33	0.97
	After	31.0	33.8	33.5	34.3	0.72	0.42
Haemoglobin concentration (g/dL)	Before	8.8	9.0	9.1	10.4	0.45	0.63
	After	9.0	10.0	8.8	9.0	0.37	0.70
Red blood cell ($x10^{12}$/L)	Before	2.3	2.2	2.1	2.0	0.13	0.79
	After	1.7	2.2	1.9	1.9	0.10	0.44
White blood cell ($x10^{9}$/L)	Before	11.9	14.7	15.2	13.7	0.69	0.35
	After	12.5	12.0	12.7	14.0	0.57	0.67
Heterophil (%)	Before	27.0	36.8	34.5	33.3	1.53	0.12
	After	32.3	35.0	33.3	34.3	1.62	0.95
Lymphocyte (%)	Before	72.0	62.5	64.8	65.8	1.59	0.18
	After	66.5	64.0	66.0	64.8	1.74	0.97
Eosinophil (%)	Before	0.25	0.25	0.50	0.00	0.144	0.73
	After	0.25	0.25	0.25	0.25	0.112	1.00
Basophil (%)	Before	0.00	0.25	0.25	0.75	0.151	0.38
	After	0.50	0.50	0.25	0.25	0.125	0.83
Monocyte (%)	Before	0.75	0.00	0.25	0.75	0.144	0.22
	After	0.50	0.50	0.25	0.25	0.125	0.83
Glucose (mg/dL)	Before	149.8	148.0	132.8	146.5	3.98	0.45
	After	144.5	150.8	148.5	148.5	3.10	0.93
Heterophil:Lymphocyte ratio	Before	0.39	059	0.54	0.51	0.33	0.14
	After	0.50	0.57	0.51	0.54	0.04	0.95

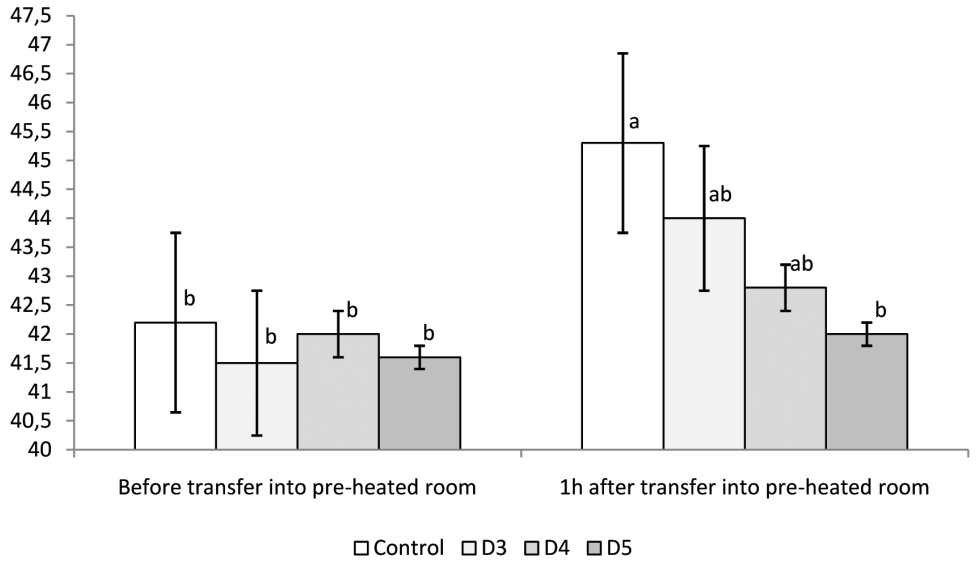
procedure was used: 1:10 somogyi-filtrate stock was prepared by adding 0.2 ml of blood to a test tube with 1 ml distilled water. After this, 0.4 ml of 0.3N barium hydroxide was added and stirred vigorously. After 5-10 minutes, 0.4 ml of 5% zinc sulphate and filtered. 0.1 ml of the somogyi-filtrate was put into a tube and mixed with 1 ml of the glucose oxidase enzyme. A blank with 0.1 ml water and standard with 0.1 ml of glucose solution was included with each set of unknowns. All the tubes were incubated in a water bath thermostatically maintained at 37^{o}C for 30-40 minutes. After incubation, 5 ml H_2SO_4 was added to each tube and mixed. The pink colour was read at 540mU against the blank tube (Spectronic® 20D model).

Rectal temperature: The rectal temperature (RT) of the birds was measured using *Jorita* digital thermometer (±0.1^{o}C accuracy, model ECT-5) inserted into the rectum (colon) of the birds and held till the thermometer beeped as described by Abioja *et al.* (2012). The readings were taken before and 1 hour after being transferred into the pre-heated room.

Experiment II: Subsequently *Experiment II* focusing on the length of EFR on day 5 was designed. One hundred and twenty day-old chicks were divided into three treatment groups of 40 each. Group I was fed *ad libitum* (CONTROL) while the remaining 2 groups had feed withdrawn for 12 hours ($D5^{12}$) or 24 hours ($D5^{24}$). The methods employed and data collection are the same to those of Experiments I. Data were subjected to analysis of variance using SYSTAT (1992) computer statistical package. Ninety five percentage level of confidence ($P<0.05$) was taken as significant. Means that are statistically different were separated with Tukey test.

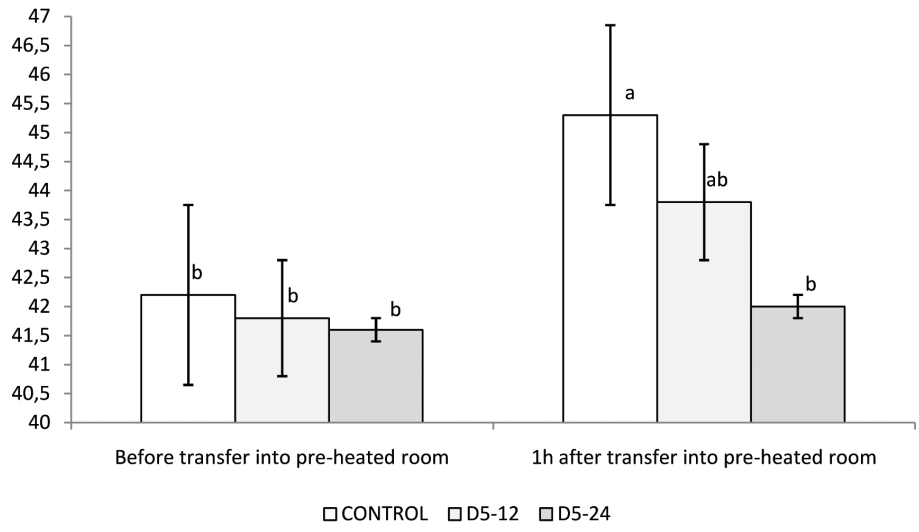
RESULTS AND DISCUSSION

Fig.1 shows the RT of the broiler chickens before and after the exposure to heat stress in Experiment I. There was no significant ($P>0.05$) difference in the RT of the birds in the four treatments before the exposure. The RT ranged between 41.5 and 42.2^{o}C. However, after 60 minutes of heat episode, RT revealed significantly ($P<0.05$) higher value in the control chicks than those exposed to EFR on D5. D3 and D4 birds had similar RT with that of the control. The change in the RT of birds in the four groups was not statistically ($P>0.05$) different (data not presented). The results of haematological parameters and blood glucose (before and after acute heat stress episode) in broiler chickens exposed to 24-hour feed withdrawal during D3, D4 and D5 of age are presented in Table 1. For packed cell volume, haemoglobin concentration, red blood cell count, white blood cell count, differential counts, H:L and blood glucose, there were no significant ($P>0.05$) differences among the four groups before and after the heat stress exposure. Fig.2 presents the results of the initial and final rectal temperature of broilers feed restricted on day 5 for either 12 or 24 hours. The initial RT was not ($P>0.05$) different from each other. Nevertheless, exposure to acute heat stress resulted in different ($P<0.05$)



[a,b] Means with different superscripts differ significantly (P<0.05)

Fig.1: Rectal temperature (before and after acute heat stress episode) of broiler chickens exposed to 24-hour feed withdrawal during d3, d4 or d5 of age



[a,b] Means with different superscripts differ significantly (P<0.05)

Fig.2: Rectal temperature (before and after acute heat stress episode) of broiler chickens exposed to 12 and 24-hour feed withdrawal during d5 of age

responses in the birds. In particular, $D5^{24}$ chickens had lower RT than that of the CONTROL chicks. The RT of the $D5^{12}$ broilers fell in between the two.

Rectal temperature is used as a major indicator of heat stress in poultry (Abioja *et al.*, 2012). Heat stress leads to elevated body temperature as the environmental temperature shoots ahead of the comfort zone of the birds (Kumar *et al.*, 2011). All the broiler chickens in this study experienced increased rectal temperature during acute heat stress. However, the increment was the highest in birds that had no prior treatment of EFR (Zulkifli *et al.*, 1994). EFR for 24 hours on day 5 gave the least increment in body temperature. This finding shows that they acquire heat tolerance that helps them in limiting the increased body temperature. Similarly, Yahav and Hurwitz (1996) reported that male broiler chickens exposed to thermal conditioning during d5 and d7 had lower body temperature when subjected to acute heat stress (35±1^{o}C; 20-30% relative humidity) than broiler that did not experience thermal conditioning. Yahav and Plavnik (1999) corroborated the report of Yahav and Hurwitz (1996). Birds on restriction on day 3 and day 4 could also tolerate the elevated ambient temperature to some extent but not as much as those on day 5. Most authors who had worked on EFR did not report on its effects on body temperature. Comparing the duration of EFR, the results showed that a whole day restriction conferred a better heat tolerance on the broiler chickens than half day restriction.

Although early feed restricted broiler chickens could resist the effects of excess environmental temperature by limiting the increase in body temperature, there was no influence of EFR in any day compared with the control in haematological parameters and blood glucose. This finding is similar to that of Zulkifli *et al*. (2000) who stated that although exposing the birds led to an increment in blood glucose, the concentrations of glucose were not affected by feeding regimes. In other words, the stress imposed by feed restriction may not be sufficient enough to elicit the thermotolerant response anticipated. Moreover, feed withdrawal was only done for a period of 24 hours. In contrast, Zulkifli *et al.* (2000) reported that 60% EFR was found to have lowered H/L ratio compared to the results of the present study where there was no difference detected in the H/L ratio regardless of whether the restriction was on day 3, day 4 or day 5.

It can be concluded that blood parameters of acutely heat-stressed broilers were not affected by EFR. However, applying EFR to broiler chicks of d5 post-hatch for 24 hours may help reduce hyperthermia that is common during heat spell in pen at market age.

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Silt Pit Efficiency in Conserving Soil Water as Simulated by HYDRUS 2D Model

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ABSTRACT

Silt pit is one of the recommended soil water conservation practices in oil palm plantations. It is commonly regarded that the larger and deeper the silt pit, the more effective the pit would be to conserve soil water. This hypothesis was tested in this paper, where the effectiveness of four silt pit dimensions on conserving soil water in the oil palm active rooting zone was simulated using the HYDRUS 2D model. These silt pits had different sizes and total wet wall-to-floor area ratio (W:F): H1 silt pit (1x1x1 m of width, length, and depth, respectively, and W:F ratio of 4.0), H2 (1.5x1x1 and W:F of 2.5), H3 (2x1x0.5 and W:F of 1.5) and H4 (2×1×2 and W:F of 1.5). Simulations showed that silt pits with larger W:F ratios could store water for longer periods and feed water to a farther horizontal distance within the soil compared to silt pits with smaller W:F ratios. H1 took the longest to dry out, whereby it took 14 to 19 hours longer to dry out compared to than H2, H3 and H4. H1 and H3 could feed water as far as 80 cm away from the pit more than H2 and H4 (60 and 50 cm, respectively). This is because silt pits with larger W:F ratios had larger horizontal water flow than the vertical water flow. Meanwhile, the depth of a silt pit should not be below the oil palm active rooting depth, which water would flow out of reach by the roots. This study is a preliminary work to a field experiment where simulations from this paper would be validated against measurements obtained in the field before recommending the use of silt pits and their size to be constructed in oil palm plantations.

Keywords: HYDRUS 2D, oil palm, silt pit, soil water conservation

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INTRODUCTION

Further expansion of new oil palm plantations in Malaysia is currently limited to marginal lands which include steep lands. Accelerated

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soil erosion on steep slopes causes soil fertility reduction, fresh and ground water pollution and other environmental problems. Hartemink (2006) reported that the erosion under natural forests is less than 1 Mg ha^{-1} yr^{-1}, while the maximum soil erosion under oil palm plantations is 78 and 28 Mg ha^{-1} yr^{-1} for Oxisols and Ultisols, respectively. Chew *et al*. (1999) also determined 7 to 21 Mg ha^{-1} yr^{-1} of soil erosion in matured oil palm plantations. Soil erosion reduces oil palm production not only by decreasing soil fertility and organic matter but also by reducing soil water infiltration, soil water content and soil water holding capacity that would consequently cause lesser available soil water content.

Although Malaysia experiences high total annual rainfall (2000-3000 mm), studies have shown that proper water management could further increase oil palm yield. Annual oil palm production, for example, could increase by a mean of 73% when all of the oil palm's daily water demands were fulfilled through irrigation (Foong, 1999). Roslan (2006) observed that irrigation could increase fresh fruit bunches yield of oil palm by 10% compared with rain-fed oil palms in the first harvesting year. Gawankar *et al*. (2003) showed that water stress reduced leaf production by 30 and 12.5% at the early and later growth periods, respectively, as well as a decrease by 91% in the number of fresh fruit bunches due to the reduction of female inflorescences by 86%.

Hartemink (2003) has shown that soil erosion in oil palm plantations is highly related to slope steepness and soil water management practices. Silt pit is one of the recommended soil-water conservation methods in Malaysia (Teh *et al*., 2011). Goh *et al*. (1994) mentioned that maximum oil palm yield production in Malaysia could be increased by yield intensification through land management practices such as silt pits. Silt pits are close-ended trenches dug between oil palm plantation rows, particularly on steep slopes, to break the slope length to smaller catchments, collect run-off and sediments (which contain nutrients) and to redistribute water and nutrients into the oil palm root zone area after rainfall. Silt pits function by reducing soil erosion, controlling run-off and sedimentation, increasing oil palm yield through supplying more water, especially during dry weather, as well as protecting and increasing soil fertility through reduction of nutrient loss and redistribution of eroded nutrients back into the soil. Although silt pits have been practiced for several decades, there have been only a few studies done to determine the effectiveness of silt pits, particularly in high rainfall areas like in Malaysia.

One of the few studies was by Soon and Hoong (2001) who had recorded the highest runoff for plots with pruned fronds stacked down the slope (30.83%), followed by plots with contour stacked fronds (17.88%), and plots with the least runoff were those with contour stacked fronds + silt pit (10.68%). Hidayat and Sutarta (2008) reported that bund terraces were effective to reduce overland flow and soil erosion by 50.8% and 67.5%, respectively. Compared with the

control, however, silt pit reduced overland flow and soil erosion by 94.9% and 98.1%, respectively. Murtilaksono *et al*. (2008) observed that the number of fronds, number of bunches, average bunch weight and fresh fruit bunches (FFB) were significantly increased by silt pit. Nonetheless, bund terrace increased FFB (25.2 t ha^{-1}) more than silt pit (23.6 t ha^{-1}) and both these methods yielded more than that of the control (20.8 t ha^{-1}). Although bench terraces were able to improve oil palm production more than silt pits, silt pits were more effective in reducing erosion and increasing soil water conservation compared with bench terraces. For example, the combination of bund terraces and silt with mulches reduced overland flow to 26.17 and 1.99 mm, respectively, and total soil erosion to 238.68 and 13.90 kg ha^{-1}, respectively, compared with the control (60.66 mm overland flow and 729.28 kg ha^{-1} total soil erosion) (Hidayat & Sutarta, 2008). Murtilaksono *et al*. (2011) also found that bench terraces and silt pits increased soil water contents from 133.80 to 141.25 mm and 165.11 to 200.98 mm, respectively, compared to the control.

Silt pits have been used in the "Ngoro" system in Tanzania for several hundred years. This indigenous soil and water conservation method enables plantation to be done in areas with 10 to 60% slopes (Kayombo *et al*., 1999). Mally *et al*. (2004) studied the effects of different pit sizes in the Ngoro system for maize cultivation. They reported that application of pits reduced soil erosion significantly and that the larger the pit, the higher the yield of maize would be because of the higher water infiltration and soil water content.

It is commonly believed that the larger and deeper the silt pit, the higher the amount of stored water would be returned to the soil. A silt pit must be able to capture maximum run-off and also to redistribute the collected water into the oil palm active root zone to avoid it from being lost through deep percolation. The water must also be stored for long periods of time for it to be used by oil palm during the dry periods. Hence, the main question in this study was: how does the size of silt pit (dimensions) affect the effectiveness of pits to conserve water into oil palm root zone? In other words, is it better to have larger and deeper silt pit? The objective of this study was to simulate the effectiveness of various silt pit dimensions in conserving soil water content by using the HYDRUS 2D model. This simulation is a preliminary work to a field experiment in which the effectiveness of silt pit dimensions, as reported in this paper, would be compared with one another in field conditions.

The HYDRUS 2D/3D model (Simunek *et al*., 2006; Sejna & Simunek, 2007) is a software package used for the simulation of two- and three-dimensional movement of water (as well as heat and multiple solutes) in a variably saturated media. The HYDRUS model assumes the following: an insignificant role of air phase in liquid flow process, zero pressure head along a drain located in saturated zone, water leaves the saturated zone by overland flow, and the mass transfer rate is proportional to the

differences in water contents rather than pressure head.

The most important input parameters required by HYDRUS are the water flow parameters including residual water content (Q_r), saturated water content (Q_s), saturated hydraulic conductivity (K_s), empirical coefficients of the hydraulic functions (α and n) and pore-connectivity (I), which affect total soil water capacity and soil water movement (Brooks & Corey, 1964). Water flow parameters can be estimated from measured soil water retention curve, measured soil texture or soil textural classes, which are represented as defaults in the soil hydraulic catalogue of HYDRUS.

There are almost one thousand peer-reviewed journal references where the HYDRUS model has been used. For example, Tadaomi *et al*. (2012) simulated the distribution of water content and water fluxes in a water harvesting system (ditches filled with sand) by using HYDRUS 2D. Comparisons between simulated and measured results showed an acceptable agreement. The validity of HYDRUS 2D in terms of water distribution and infiltration in terraced slopes had been tested by Haishen *et al*. (2008), who reported a good agreement between simulated and measured data. Meanwhile, Raoof and Pilpayeh (2011) examined the accuracy of numerical inversion solution of HYDRUS 2D in simulating soil wetting profile (1m depth) under saturated and unsaturated conditions in different degrees of slope steepness. They reported an estimation average error of 3.22% and root mean square error of 0.032 between measured and simulated data. Zhang *et al*. (2013) investigated the distribution of infiltrated water in the cross section of furrow irrigation system through laboratory experiments and HYDRUS 2D model. They remarked that the observed and simulated data were highly in agreement based on the mean square error and coefficient of determination (R^2).

MATERIALS AND METHODS

Four treatments of silt pit dimensions were selected and shown in Table 1.

The data on soil hydraulic and physical properties were collected from Tuan Mee oil palm plantation (03° 16' N and 101° 28' E) at Sg. Buloh in Malaysia. The field site

TABLE 1
Silt pit sizes used in the simulations

Treatment	Silt pit size (m) Width × Length × Depth	Volume (m^3)	Opening or floor area (m^2)	Wet wall to floor area ratio (W:F)	Head of water* (m)
H_1	1.0×1.0×1.0	1.0	1.0	4.0	1.00
H_2	1.5×1.0×1.0	1.5	1.5	2.5	0.75
H_3	2.0×1.0×0.5	1.0	2.0	1.5	0.50
H_4	2.0×1.0×1.0	2.0	2.0	1.5	0.50

* Indicates the height of stored water from the floor of silt pit. A fixed volume of $1m^3$ of water was placed in all silt pits at the beginning of simulation.

has a slope steepness of 11°. The soil of this area is classified as Bungor Series (Typic Paleudult), which comprises low grade schists or in-durated shales. The mean total rainfall in Tuan Mee estate is 2440 mm, with an average of 160 mm monthly rain. The data used in the model were measured from the field: bulk density (1.35 Mg m^{-3}, core ring method, Blake & Hartage, 1986); sandy clay loam texture (USDA) with 24.92, 7.34 and 67.74 % clay, silt and sand, respectively; and the soil hydraulic properties (pressure plate and membrane technique. Richards (1974) were: permanent wilting point (0.13 m^3 m^{-3}), saturated point (0.44 m^3 m^{-3}), parameter α and n in the soil water retention function (2.00 cm^{-1} and 1.30, respectively), saturated hydraulic conductivity (0.02 m day^{-1}) and tortuosity parameter in the conductivity function (0.50).

The HYDRUS model numerically solves Richards' equation for water flow:

$$\frac{\partial \theta}{\partial t} = \frac{\partial}{\partial x_i}\left[K\left(K_{ij}^{A}\frac{\partial h}{\partial x_j} + K_{iz}^{A}\right)\right] - S \quad [1]$$

where θ is the volumetric water content (L^3L^{-3}), h is the pressure head (L), S is the sink term (T^{-1}), x_i (i = 1, 2) is the spatial coordinates (L), t is the time (T), K_{ij}^{A} is the components of dimensionless anisotropy tensor and K is the unsaturated hydraulic conductivity (LT^{-1}).

Van Genochten (1980) function was used to characterize the shape of the soil water retention curve:

$$\theta(h) = \theta_r + \frac{\theta_s - \theta_r}{[1+(\alpha|h|)^n]^{1-1/n}} \quad [2]$$

where θ(h) is the water content (L^3L^{-3}), $|h|$ is the pressure (L^{-1} or cm of water), θ_s is the saturated water content (L^3L^{-3}) and θ_r is the residual water content (L^3L^{-3}) taken to be the permanent wilting point in this study. Note that θ_s, θ_r, α and n are the four independent parameters that must be estimated from the measured soil water retention data. Meanwhile, α can be determined by solving the following:

$$\alpha = \frac{1}{h}\left(\Theta^{-1/m} - 1\right)^{1/n} \quad [3]$$

where Θ is the dimensionless normalized volumetric soil water content and m is given by:

$$m = 1 - 1/n \quad [4]$$

The following empirical formula was used to estimate m:

$$m = \begin{cases} 1 - \exp\left(-0.8\, S_p\right) & (0 < S_p \leq 1) \\ 1 - \frac{0.5755}{S_p} + \frac{0.1}{S_{p^2}} + \frac{0.025}{S_{p^3}} & (S_p > 1) \end{cases} \quad [5]$$

where S_p is the slope of the soil water retention curve at a point halfway between θ_r and θ_s.

The soil geometrical and environmental conditions were set as constant across all silt pit treatments (dimensions). The topography was flat. Simulations assumed no evaporation and no plant water uptake and free drainage was further assumed in order to make the simulation simple.

A 50-cm soil depth was selected for the determination of soil water content because oil palm has a shallow active root system within the first 50 cm soil depth (Gray & Hew, 1968).

RESULTS AND DISCUSSION

Simulations showed that the smaller the silt pit floor area (or opening area), the longer it took for the pits to dry out (see Fig.1). In particular, H1, H2, H3 and H4 dried out in 72, 57.6, 52.8 and 52.8 hours, respectively. The simulations also showed that a larger horizontal distance (80 cm) of wetted front for H1 and H3, compared with 60 and 50 cm for H2 and H4, respectively (Fig.2). This means H1 and H3 could feed water horizontally farther into the top soil than H2 and H4.

Water can infiltrate vertically and laterally into a soil (Lal & Shukla, 2004). There would be a larger horizontal movement of water for silt pits with larger total wet wall-to-floor area (W:F) ratio. The narrow opening area of silt pits (H1 and H2) have larger W:F ratios (4 and 3.3, respectively) than H3 and H4 (1.5 for both). When a pit has a larger W:F ratio, the height of stored water in the silt pit would be nearer to the soil surface. Therefore, a larger wall area would be wetted, and this in turn would lead to a larger total water flux through the walls and smaller total flux through the floor compared with a silt pit with smaller W:F. Meanwhile, Sawhney and Parlange (1974) reported that the vertical infiltration is about twice as fast as horizontal infiltration. Therefore, the horizontal water infiltration is always slower than the vertical water infiltration into the soil. This is because vertical soil water movement is driven by both gravity and water potential differences compared with horizontal water movement which is driven only by the water potential

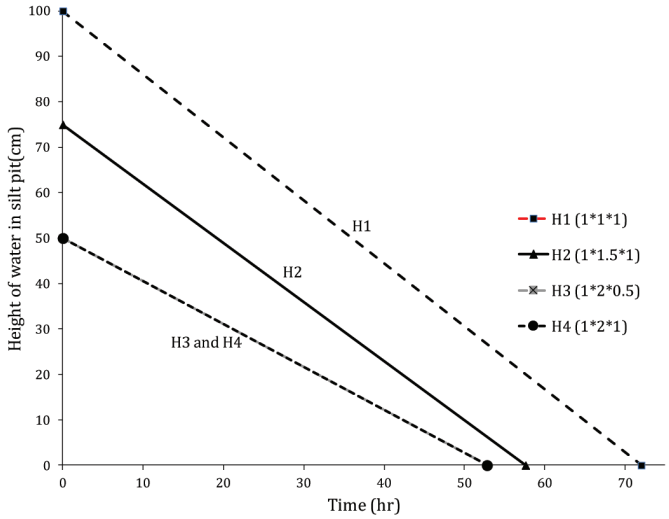

Fig.1: Temporal changes to the height of stored water from the floor of silt pit (Note: lines for H3 and H4 overlap each other)

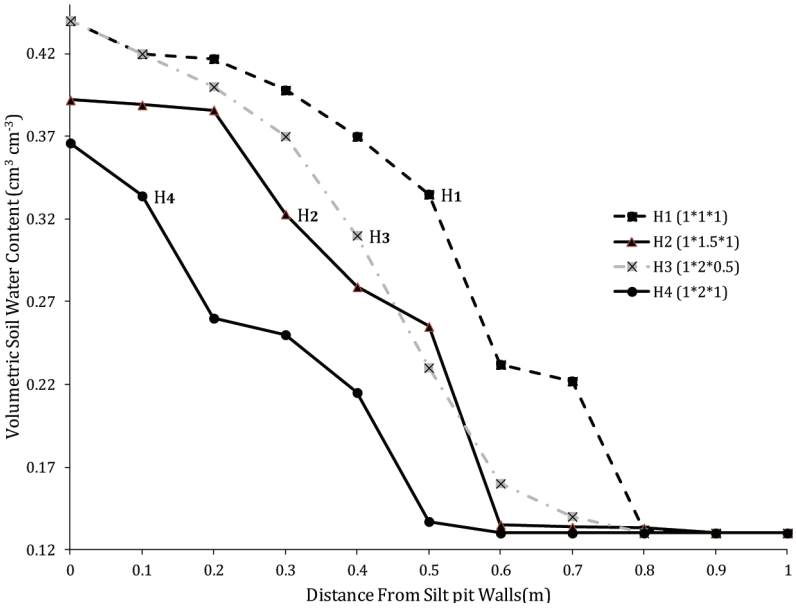

Fig.2: Volumetric soil water content at various horizontal distances from the silt pit walls. Soil water content at 0.50 m soil depth and at 72 hours.

gradient. Hence, silt pits with larger floor area lose water more through vertical than horizontal infiltration.

There is a slowing effect of horizontal infiltration on vertical infiltration (Talsma, 1969; Talsma & Parlange, 1972). In other words, an increasing horizontal infiltration causes a decreasing vertical infiltration. Slowing effect is another reason why silt pits with larger W:F ratios could release water slower (hence, store water longer). When larger wall areas in H1 become in contact with water, the quantity of horizontal infiltration will increase. This will then reduce the vertical infiltration.

Fig.3 displays a relationship between total water flux from walls and W:F values. When W:F ratio increases, the proportion of total horizontal water flux to total vertical flux increases. There is an inverse relationship between vertical flux and W:F. Therefore, H3 and H4 with the same W:F had similar horizontal and vertical total flux. The similar performance of H3 and H1 in terms of the wetted front distance (80 cm for both; Fig 2) was because both these pits had equal height of water inside the pit (50 cm) at 50 cm soil depth where the infiltration water was measured.

Increasing the W:F ratio resulted in increasingly more water flux out of the silt pit through its walls than through its floor. This is an important implication because the oil palm roots are located around the walls and not below the floor of the silt pit. Therefore, a silt pit with a large W:F ratio is able to redistribute the stored water to the surrounding root zone and avoid it from being lost through percolation via the silt pit floor and away from the root zone. This

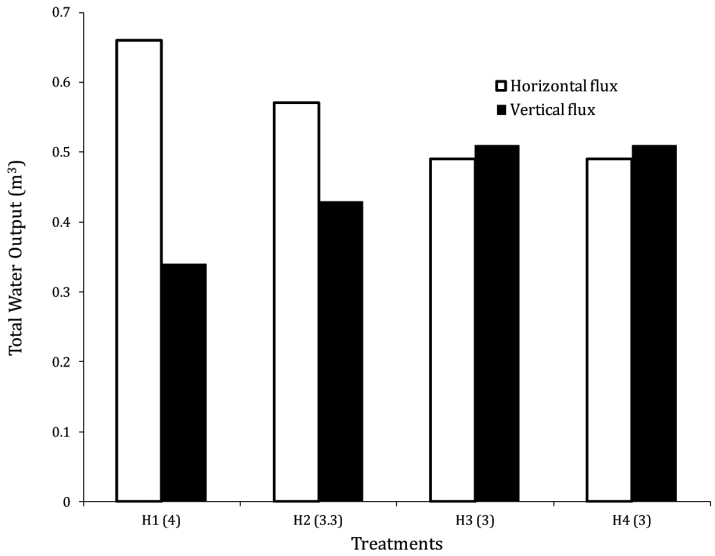

Fig.3: The amount of water output from the walls and floors of the silt pit treatments. Values in parentheses indicate total wall-to-floor area ratio (W:F) for the silt pit dimension. Width, length and depth for H1, H2, H3 and H4 are 1×1×1, 1.5×1×1, 2×1×0.5 and 2×1×1 m, respectively).

also means that the floor depth of a silt pit cannot be lower than the depth of the active oil palm roots, which will cause water loss to be particularly large for silt pits with very small W:F ratios.

As stated previously, water evaporation was not considered in this simulation. It was however expected that evaporation from a larger opening area would be higher from that of a smaller opening area. Therefore, water loss via evaporation would be more in a silt pit with a bigger opening area than that from a silt pit with smaller opening area. The opening area of H4 is twice of H1, which also means evaporation from H4 is double of that from H1. So, if evaporation was to be included in the simulations, H3 and H4 would dry out even quicker (see Fig.1) and have a smaller wetting front distance (Fig.2) than that reported here.

CONCLUSION

The simulations carried out in this study have shown that the water conservation ability of silt pits is dependent upon their W:F ratios. The larger the W:F ratio, the longer the silt pits could store water and the farther the water could be fed back to the surrounding soil. For the same volume of water, silt pits with a larger W:F ratio would have higher water head and are more horizontal than the vertical movement of water out of the silt pit. This made silt pit, H1 (1x1x1), to store the water the longest and had the farthest horizontal wetting distance than the other silt pit sizes (H2, H3 and H4). Hence, H1 was the best silt pit dimension to conserve soil water. The depth of the silt pit floor could not be lower than oil palm active rooting depth.

The next step of this study would be to carry out a field experiment using the four silt pit dimensions so as to determine the validity of the simulation carried out in this earlier work. Hence, the researchers hope to be able to recommend not only using silt pits as a soil water conservation method in oil palm plantations, but also the silt pit sizes to be constructed.

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Multivariate Classification of Promising Paddy Cultivars on the Basis of Physical Properties

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ABSTRACT

Properties of materials play important role in the development of processing, storing and handling equipment. Genetic diversity is one of the important tools to qualify genetic variability in both cross and self-pollinated crops. Through this study, efforts were made to find out variability and relationship between paddy cultivars of various economically important traits. Paddy of twenty two selected genetically diverse strains was evaluated for physical and optical characteristics. The moisture content of kernels of selected paddy cultivars ranged from 7.89 to 14.15% (DWB). Multivariate classification techniques were applied to determine the coherent physical and optical characteristics in order to reduce dimensionality problems. The properties heavily loaded on different components are dimensional with frictional in first principal component, dimensional and optical in the second component and optical with gravimetric in the third components found responsive for major variations (79.36%) in the Eigen analysis of data. The observed physical and optical characteristics differed significantly and found to be cultivar dependent, which has paved the way for the classification of selected paddy cultivars on the basis of principal component and cluster analysis. Accordingly, the dominating similar prominent physical and optical characters formed at least three verifiable distinct separate and coherent clusters of paddy cultivars, indicating the usefulness multivariate analysis in identifying promising cultivars with the appropriateness in design and development of agricultural and processing equipment based on opto-physical characteristics.

Keywords: Paddy, oryza sativa, physical properties, principal component analysis, cluster analysis

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INTRODUCTION

Paddy (*Oryza sativa* L) is a widely cultivated cereal crop of the family Gramineae. The paddy production in India has witnessed

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more than three folds increase in the past 50 years and contributed to more than one fifth of the total paddy production in world. The present production figure of paddy in India is 152.6 MMT (FAO, 2013). Among 23 recognized species of *Oryza* in the world, *sativa* is widely cultivated mainly due to economic reasons with limited cultivation of *glaberrima* on ritual context specifically grown in West Africa. Also, the desired attributes such as disease and insect resistances with higher climatic adaptability of other wild species have been explored to transfer the desired characteristics to cultivated species through inter breeding for the development of diverse improved paddy variants. The diversities among the cultivars also existed for kernel size, shape, cooking, colour, flavour, milling, nutritive value and their ability to make different rice products. Apart from calorie and protein, rice being staple food, also supplies to some extent vitamins and minerals in the poor-man's diet.

Paddy undergoes different levels of processing prior to its conversion into usable form (Singh & Prasad, 2012) for various product preparations (Prasad *et al*., 2013). The knowledge of physical properties comprised of dimensional, gravimetric and frictional characteristics are thus necessary in the design of equipment for their effective implementation in various operations (Mohsenin, 1980; Sahay & Singh, 1994; Ghadge & Prasad, 2012) starting from harvesting till their conversion into edible form mainly as white rice or rice powder (Prasad *et. al.,* 2012). The obtained data on opto-physical properties may be utilized in the development of equipment related to direct seeded rice (DSR) technology in order to have associated potential benefits of labour, water (20-25%) and cost of cultivation (Kamboj *et al*., 2012).

Multivariate statistical methods have been applied for the classification of paddy genotypes based on coherent physical and optical characteristics to reduce dimensionality problems. Principal component analysis (PCA) and cluster analysis (CA) as multivariate statistics are often employed purposely (Kara, 2009) to get the relationships among parameters of the original data matrix (Kosa *et al*., 2001). The multivariate analysis has successfully been applied by the author in characterization of okra genotypes (Sharma & Prasad, 2010a; Prasad & Sharma, 2012).

The comparative characteristics of Indian promising paddy varieties for their classification based on the physical and optical properties are not available yet. Hence, the present study was conducted to determine the physical and optical properties of paddy in order to classify it based on the chemo-metric approach supported by the clustering technique of multivariate analysis.

MATERIALS AND METHODS

Twenty two cultivars of paddy kernels were procured from different agro climatic regions of India (Table 1). The acquired cultivars of paddy were cleaned in an air classifier to remove the foreign matter, broken and immature kernels. The initial moisture

TABLE 1
Paddy cultivars and source

Paddy cultivar	Source	Symbol
BR 4-10	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	1
CSR 10	Indian Agricultural Research Institute, New Delhi	2
Haryana Mahak 11	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	3
HBC 19	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	4
HKR 95-157	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	5
HKR 95-407	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	6
HKR 99-66	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	7
HKR H7	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	8
IR 64	Indian Agricultural Research Institute, New Delhi	9
Jehlum	Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu & Kashmir	10
Muchhal	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	11
Palman 579	Punjab Agricultural University, Ludhiana, Punjab	12
PAU 201	Punjab Agricultural University, Ludhiana, Punjab	13
Pusa 1121	Punjab Agricultural University, Ludhiana, Punjab	14
Pusa Basmati	Indian Agricultural Research Institute, New Delhi	15
Pusa Sugandh	Indian Agricultural Research Institute, New Delhi	16
Shabnam	Seed Collection Centre, Sangrur, Punjab	17
Shalimar 1	Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu & Kashmir	18
Shalimar 2	Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu & Kashmir	19
Shalimar 3	Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu & Kashmir	20
Sugandha	Seed Collection Centre, Sangrur, Punjab	21
Usha	Seed Collection Centre, Sangrur, Punjab	22

content of paddy cultivars was determined as per standard method (USDA, 1990) and it was found to vary between 7.89±0.37 to 14.15±0.28% (dry weight basis). The physical properties of paddy cultivars were determined for the dimensional, gravimetric and frictional characteristics (Ghadge & Prasad 2012).

Physical Properties

The dimensional characteristics were evaluated for three major perpendicular dimensions: length (L), breadth (B) and thickness (T). The dimensions were measured manually using a dial type vernier caliper (Mitutoyo Corporation, Japan) with the least count of 0.02 mm.

The geometric mean dimension (*GMD*) and aspect ratio (*AR*) of the sample were assessed using the relationship given by Mohsenin (1980) as:

$$GMD = (LBT)^{1/3} \quad (1)$$

$$AR = \frac{B}{L} \times 100 \quad (2)$$

The criteria used to describe the shape of paddy kernel are sphericity and aspect ratio. Thus, the sphericity (SPH) was accordingly computed (Mohsenin, 1980) as:

$$SPH = \frac{(LBT)^{1/3}}{L} \times 100 \quad (3)$$

The surface area (*SA*) of paddy kernel was evaluated using the relationship given by McCabe *et al.* (1986) as:

$$SA = \pi(GMD)^2 \quad (4)$$

The weight of the paddy samples was recorded using electronic balance (Ishida Co. Ltd., Japan) to an accuracy of 0.001 g. The bulk density (*BD*) of the seed sample was evaluated using the methods suggested by Williams *et al*. (1983). True density (*TD*) was determined using liquid displacement technique (Shepherd & Bhardwaj, 1986). Toluene as liquid was used in spite of water to prevent absorption and also to get the benefit of low surface tension (Ogut, 1998). The porosity (POR) of seeds was computed from the values of true density and bulk density by using the following relationship by Mohsenin (1980):

$$POR = \frac{TD - BD}{TD} \times 100 \quad (5)$$

The angle of repose (AOR) was determined using the relationship:

$$AOR = tan^{-1} \frac{(2H)}{D} \quad (6)$$

Where, H and D are the height and diameter of the heap in mm.

The static coefficient of friction (COF) was determined for four frictional surfaces namely glass (COFG), galvanized iron sheet (COFGI), plywood parallel (COFPAR) and plywood perpendicular (COFPER). A plastic cylinder of 50 mm diameter and 60 mm height was placed on an adjustable tilting flat plate faced with the test surface and filled with nearly 100 g sample. The cylinder was raised slightly to avoid touching the surface. The structural surface with material filled cylinder on it was inclined gradually until the cylinder just started to slide.

Optical Properties

The optical characteristics of the paddy samples were evaluated using the Hunter Colorimeter (Gretag Macbeth, Model No. i5, USA) in terms of l, a, b and ΔE values, where, l corresponds to the luminance or brightness and a, b to the chromaticity. The 'a' value particularly represents the red-green component from positive to negative values; the 'b' value represents the yellow-blue component in similar ways (Prasad *et al*., 2010a; Prasad *et al*., 2010b). Total colour difference (ΔE) is the measure of modulus of the distance vector between the reference colour values and the actual colour coordinates. The total colour difference indicates the colour difference from the standard plate or the reference sample colour (Rhim *et al*., 1999), which was evaluated as:

Where, l_0, a_0 and b_0 represented the least observed colour values among the selected paddy cultivars. The total colour difference can be used for the analytical classification on optical basis as small , distinct (and very distinct (colour difference (Adekunte *et al*., 2010).

Statistical Analysis

In order to determine any statistically significant effects prevailed due to paddy cultivars, analysis of variance (ANOVA) was carried out and critical difference (CD) at $P \leq 0.05$ was determined. Descriptive statistics including the means of each attributes were determined and represented graphically for assessing the varietal performance and dependent separations. Principal components analysis (PCA) and hierarchical cluster analysis (CA) for multivariate data were statistically analysed using MINITAB v 13.2 and Microsoft Excel v 2000. The principal component and hierarchical cluster analyses provided the characteristic patterns to classify the selected 22 paddy cultivars. Clustering of the samples was done according to Ward (1963) based on minimizing the loss of information from joining two clusters (Sharma & Prasad, 2010a; Sharma & Prasad, 2010b).

RESULTS AND DISCUSSION

Physical Properties

The comparative characteristic variability of twenty two paddy cultivars (Fig.1) with their overall individual response with respect to their mean is presented in Figures 2 and 3. The analysis of variance (ANOVA) revealed that the differences in selected cultivars were significant for all the attributes indicating the presence of associated variability among them ($p \leq 0.05$). The length (L), breadth (B) and thickness (T) of the paddy cultivars varied from 8.10±0.40 to 11.83±0.40 mm, 2.19±0.08 to 3.06±0.19 mm and 1.77±0.11 to 2.20±0.06 mm, respectively. The comparison of the dimensional data with the existing work on paddy can play a vital role in machine design and thus be sufficient in making symmetrical projections towards process equipment adaptation (Mohsenin, 1980). The geometric mean diameter (GMD), which integrates three major dimensional parameters, varies from 3.46±0.01 to 3.85±0.14 mm. Aspect ratio (AR) and sphericity (SPH) of the samples varied in the range of 18.92±0.00 to 36.154±0.68% and 31.16±0.58 to 45.48±0.92%, respectively. Lower values of sphericity with intermediate value of aspect ratio suggest that the paddy tend towards a cylindrical shape rather spherical indicating a likely difficulty in getting the kernel to roll than sliding on surface. This tendency of sliding is necessary in the design of hoppers or sowing equipment. As evident, the surface area (SA) of the paddy kernels ranging from 37.68±0.28 to 46.67±3.43 mm^2 is a relevant tool in determining the material shape. The dimensional characteristics of the selected paddy cultivars significantly differ ($p \leq 0.05$) to each other, support the results reported by Reddy and Chakraverty (2004).

The significant ($p \leq 0.05$) difference in bulk density (BD) 323.863±2.281 to

612.126±11.713 kg/m^3 and true density (TD) 756.533±3.101 to 1521.737±3.069 kg/m^3 have been found evident from the works of Correa *et al*. (2007), Muramatsu *et al*. (2007), and Singh *et al*., (2005). Higher level of porosity (POR) ranging from 46.42±1.68 to 75.17±0.08% has indicated the dependency on bulk and true densities of the material.

The frictional characteristics for paddy cultivars were found to have significant difference. The experimental values of the angle of repose (AOR) ranged from 30.555±0.288 to 43.807±2.788° (Fig.3). Similar trends have been reported by Ghasemi *et al*. (2008) for emptying angle of repose for rough rice. The coefficient of friction (COF) was found to be minimum for glass surface (COFG) and maximum for plywood surface vertically aligned (Fig.3). The differences in the values may be due to the fact that the roughness of the material used for determining the coefficient of friction (Correa *et al*., 2007). Thus, the frictional characteristics have imperative roles in the food grain processing, particularly in the designing of the hopper and sowing equipment applied for the purpose of direct seeded rice technology.

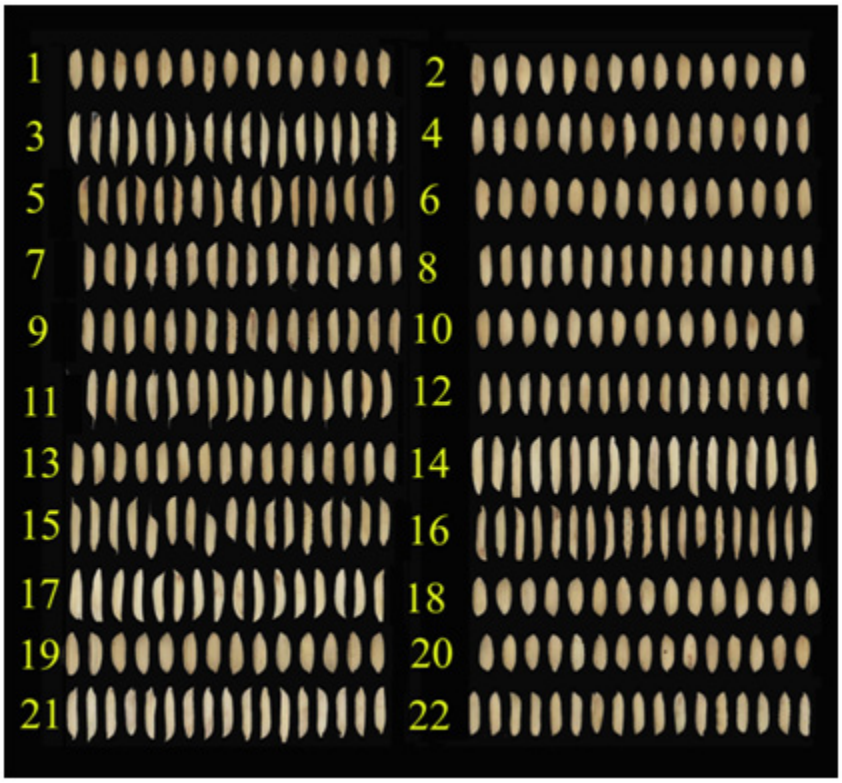

Fig.1: Charged coupled device snapshot showing selected paddy cultivars

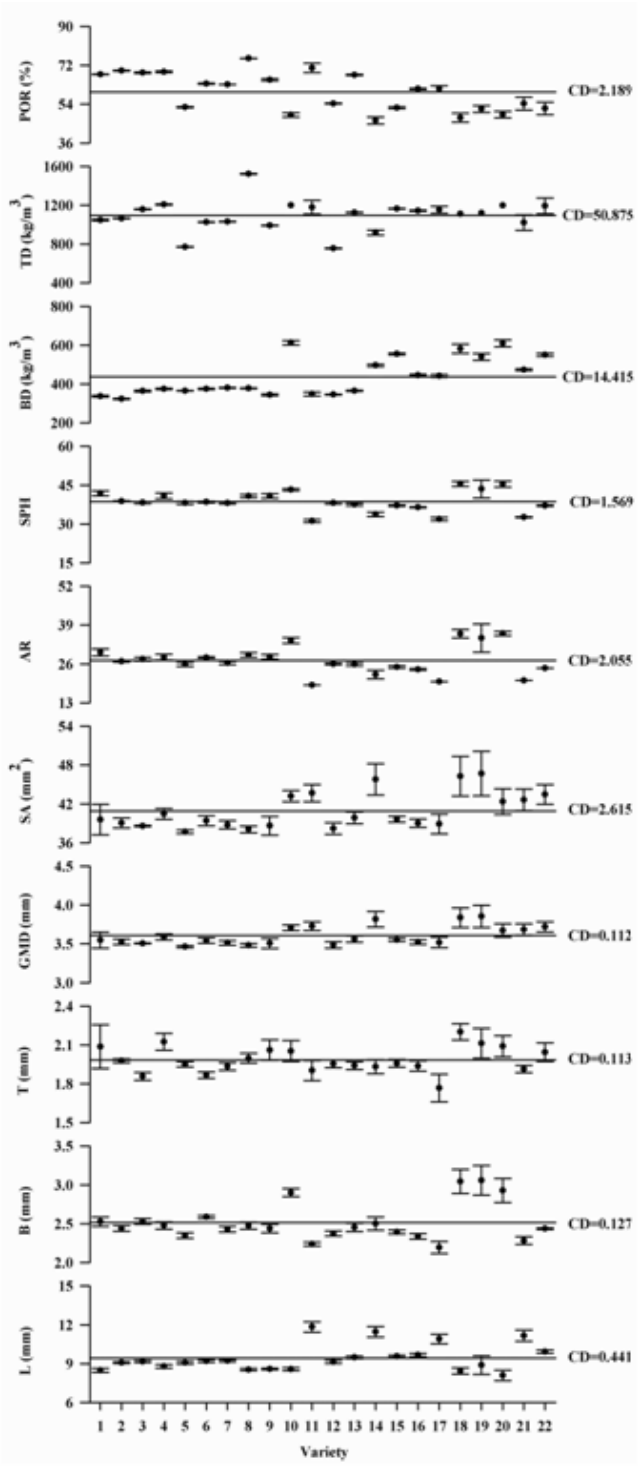

Fig.2: Dimensional and gravimetric properties of selected paddy cultivars

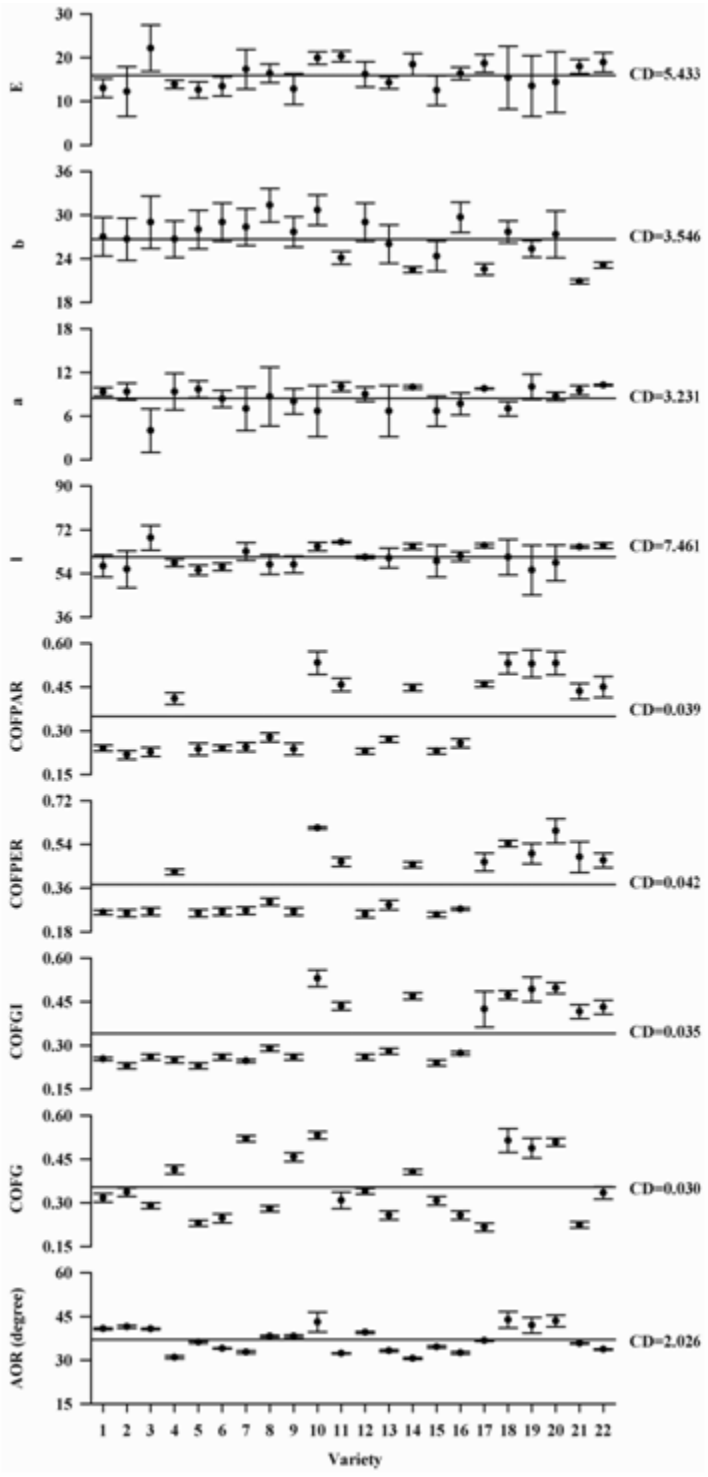

Fig.3: Frictional and optical properties of selected paddy cultivars

TABLE 2
Correlation matrix of variable properties of the paddy cultivars

Variables	B	T	GMD	SA	AR	SPH	BD	TD	POR	COFG	COFGI	COFPER	COFPAR	AOR	l	a	b	DE
L	-0.59	-0.62	0.24	0.24	-0.86	-0.92	-0.06	-0.14	-0.04	-0.44	0.25	0.15	0.19	-0.61	0.62	0.36	-0.73	0.50
B		0.70	0.59	0.59	0.92	0.84	0.61	0.18	-0.45	0.73	0.52	0.52	0.52	0.65	-0.26	-0.20	0.30	-0.15
T			0.45	0.45	0.76	0.81	0.38	0.16	-0.26	0.69	0.24	0.35	0.36	0.43	-0.43	0.08	0.21	-0.40
GMD				1.00	0.26	0.15	0.68	0.10	-0.57	0.49	0.85	0.80	0.84	0.13	0.24	0.25	-0.43	0.23
SA					0.26	0.15	0.67	0.10	-0.57	0.49	0.84	0.79	0.84	0.13	0.23	0.25	-0.43	0.22
AR						0.98	0.42	0.19	-0.26	0.69	0.23	0.28	0.26	0.72	-0.46	-0.28	0.55	-0.34
SPH							0.32	0.18	-0.18	0.66	0.09	0.17	0.15	0.68	-0.54	-0.26	0.59	-0.42
BD								0.25	-0.80	0.47	0.76	0.75	0.73	0.29	0.18	-0.05	-0.20	0.16
TD									0.37	0.06	0.21	0.28	0.27	0.07	0.12	-0.18	0.14	0.22
POR										-0.38	-0.6	-0.54	-0.53	-0.20	-0.10	-0.11	0.27	-0.03
COFG											0.43	0.47	0.46	0.39	-0.07	-0.14	0.22	-0.04
COFGI												0.94	0.95	0.28	0.39	0.25	-0.35	0.43
COFPER													0.99	0.25	0.34	0.25	-0.33	0.37
COFPAR														0.21	0.31	0.31	-0.38	0.34
AOR															-0.23	-0.21	0.36	-0.11
l																-0.23	-0.33	0.94
a																	-0.49	-0.20
b																		-0.13

Optical Properties

The optical properties, l, a and b values of the samples lie in the range of 55.333±2.082 to 68.667±5.132, 4.00±2.92 to 10.243±0.110 and 20.865±0.292 to 31.333±2.309, respectively. The value of total colour difference (ΔE) which varied from 12.230±4.861 to 22.134±4.641 further confirms the existence of very distinct colour difference (Adekunte *et al*., 2010) with a variety of specific associated characteristic variability ($p \leq 0.05$) in distinguishing on the basis of considered parameter (Fig.3).

Multivariate Analysis

The correlation matrix of physical and optical characteristics spread over dimensional (seven), gravimetric (three), frictional (five) and optical (four) attributes are shown in Table 2. Nearly, 80% of the correlation coefficients in the matrix are over 0.20 (Table 2). Kernel length was observed having positive significant correlation with optical parameters (l, a, ΔE) whereas negative correlation with dimensional (breadth, sphericity, aspect ratio, thickness), optical (b value) and frictional (AOR) attributes. The strong correlation is indicative for the cultivar associative character towards longer kernel length and the feeble correlation vice versa. The results are in agreement with the physical properties of white rice reported elsewhere (Bhatia *et al*., 2009).

The data set of the observed measurements was subjected to principal component analysis (PCA), which has eliminated the prevailed variations of highly inter-correlated nature. The initial statistics of the Eigen analysis is given in Table 3. It can be seen that three principal components (PCs) appeared to account for 79.36% of the total variance in the data. According to Kaiser Criterion (Kaiser, 1960), only the first four PCs could be retained because the Eigen values of more than one reduced dimensionality descriptor space to four. The descriptors were represented graphically in the form of loading plot for three components (Fig.4).

TABLE 3
Eigen analysis of principal components

No.	Eigen value	Individual Percent	Cumulative Percent
1	7.524	39.60	39.60
2	5.480	28.84	68.44
3	2.075	10.92	79.36
4	1.252	6.59	85.96
5	0.722	3.80	89.75
6	0.663	3.49	93.25
7	0.447	2.35	95.60
8	0.330	1.74	97.33
9	0.228	1.20	98.53
10	0.152	0.80	99.33
11	0.083	0.44	99.77
12	0.021	0.11	99.88
13	0.010	0.05	99.93
14	0.006	0.03	99.97
15	0.004	0.02	99.99
16	0.002	0.01	100.00
17	0.000	0.00	100.00
18	0.000	0.00	100.00

The loading of components on the principal axes indicated the presence of variability among the selected paddy

cultivars (Fig.4). In order to study the prevailed pattern for the measured attributes and to categorize factors that are substantively meaningful, the chemo-metric approach was applied. It can be seen that the first component explaining 39.60% of variance is highly correlated both positively (porosity) and negatively (dimensional properties, coefficient of friction and other gravimetric properties). Thus, it classifies and distinguishes the score of paddy cultivars on the basis of these components. The second principal component explaining 28.84% of total variance is highly correlated with most of the observed parameters leaving the gravimetric attributes, while the third component has explained only 10.92 % of the total variance and is loaded with mainly the optical attributes and the mass per unit volume occupied by different paddy cultivars as represented in terms of true density (Fig.2).

Fig.4 illustrates the biplot of scores and loadings on the three principal components for the observed parameters of the paddy cultivars. The biplot of principal component 1 (PC1) and principal component 2 (PC2) shows the separation of the paddy cultivars according to their respective scores. The first quadrant of the plot contains samples having positive PC1 and PC2 scores. The cultivars forming a distinct cluster with five cultivars, namely Muchhal (11), PUSA 1121 (14), Shabnam (17), Sugandha (21) and Usha (22), are found loaded with the length and colour difference (ΔE) attributes (Fig.4). Another distinct cluster formed by cultivars Jehlum (10), Shalimar 1 (18), Shalimar 2 (19) and Shalimar 3 (20), the short bold rice kernel characteristics and occupied their position in third quadrant in principal space (Fig.4). The remaining 13 cultivars have formed a separate cluster and occupied their place in the fourth quadrant in principal space (Fig.4).

A graphical depiction of classification based on measured characteristics of selected paddy cultivars was obtained by means of cluster analysis (CA) of standardized compositions using Ward's method (Ward, 1963) as an amalgamation rule and squared Euclidean distances as the measure of proximity between samples. A dendogram is shown in Fig.5. As a result of applying CA to the principal component score matrix, the paddy cultivars were grouped into three different clusters (Table 4) supporting the findings obtained using principal component analysis. It was revealed that clusters I, II and III have 5, 4 and 13 cultivars, respectively. The formed first cluster represents mainly the long grain rice varieties (Singh & Prasad, 2013) and was found susceptible to breakage during milling (Singh & Prasad, 2012). The clustering pattern of paddy cultivars reveals considerable characteristic diversity among themselves pertaining basically on the attributes of dimensional and total colour difference characteristics by occupying three clusters. Thus, the above characterizations of paddy cultivars on the basis of dissimilarity in scores with respect to extracted principal components justify the existence of variability and may be used varietal improvement programme with the development of equipment pertaining to the processing and agricultural purposes.

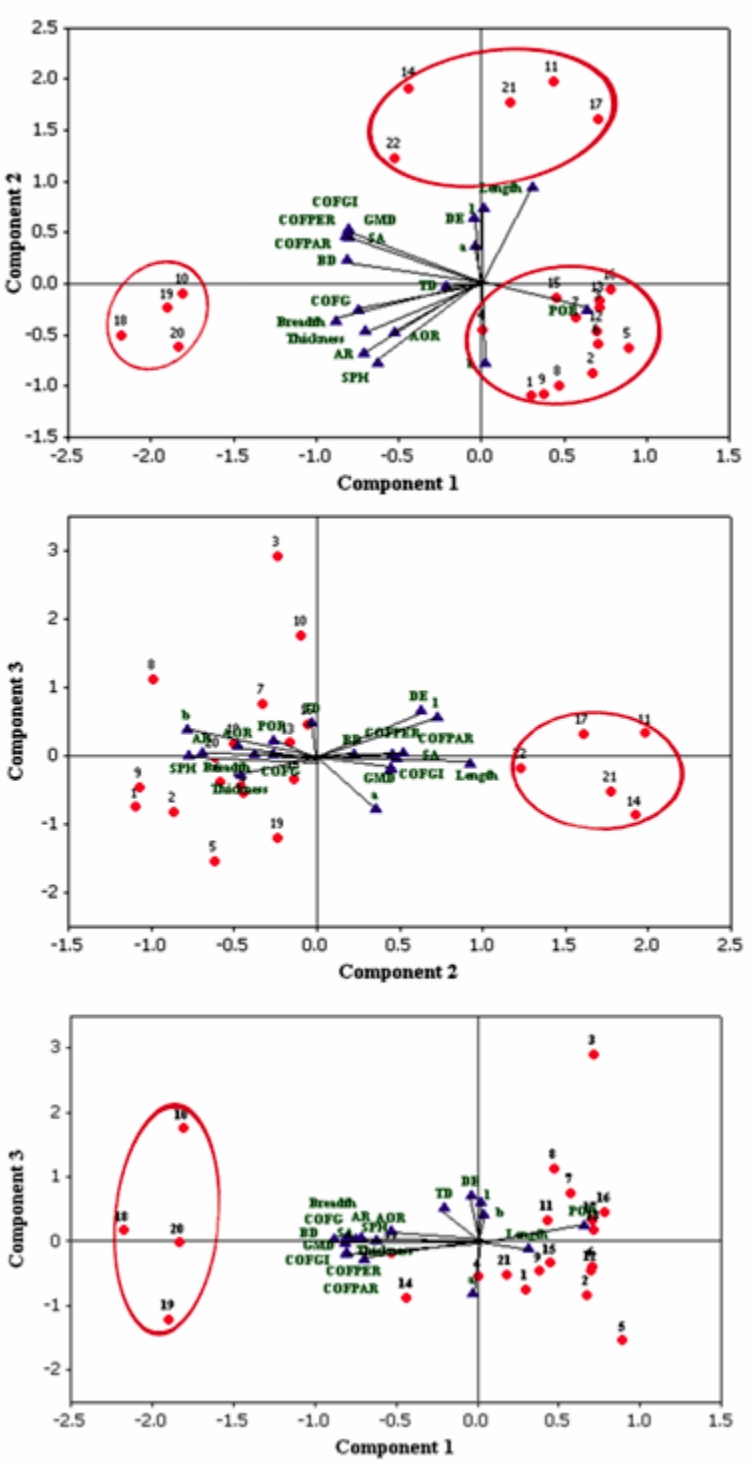

Fig.4: Biplot of loadings and scores for paddy cultivars on principal axes 1, 2 and 3

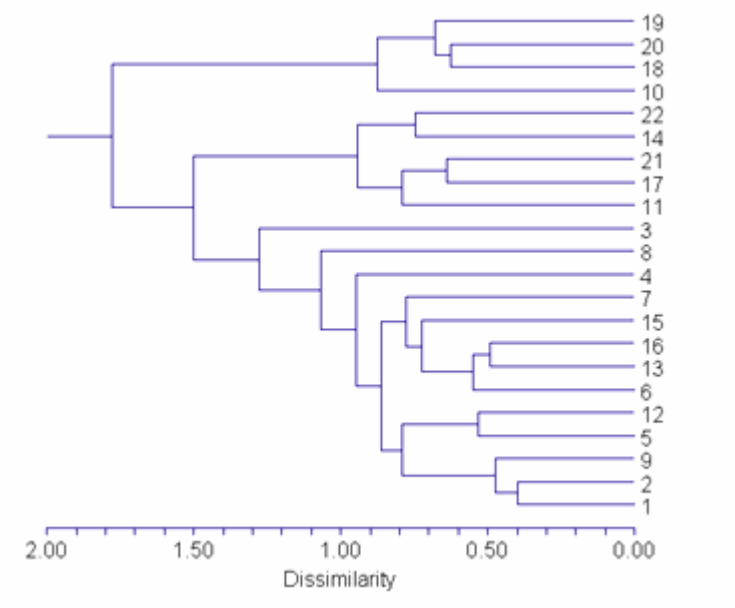

Fig.5: Dendogram of Cluster Analysis for selected paddy cultivars

TABLE 4
Distribution of 22 paddy cultivars into three clusters

Cluster No.	Paddy cultivars in different cluster
I	Muchhal (11), PUSA 1121 (14), Shabnam (17), Sugandha (21) and Usha (22)
II	Jehlum (10), Shalimar 1 (18), Shalimar 2 (19) and Shalimar 3 (20)
III	BR 4-10 (1), CSR 10 (2), Haryana Mahak 11 (3), HBC 19 (4), HKR 95-157 (5), HKR 95-407 (6), HKR 99-66 (7), HKR H7 (8), IR 64 (9), Palman 579 (12), PAU 201 (13), PUSA Basmati (15) and PUSA Sugandh (16)

CONCLUSION

Characteristic variations among physical and optical properties existed among the paddy cultivars. Cultivars Muchhal, PUSA 1121, Shabnam, Sugandha and Usha showed greater potentials in terms of handling attributes as it may outperform processing as compared the other cultivars due to significant dimensional variations. This indicates the usefulness multivariate analysis in identifying promising cultivars for the likely possibility to produce new recombinants with desired characters or appropriateness in the development of agricultural and processing equipments based on the opto-physical characteristics.

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Multivariate and an Ordination Approach to Classify the Attractiveness of the Plant Species in Pastoral Lands

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ABSTRACT

To identify the foraging plants by honeybees and determine apicultural potential, a sample collection of species was carried out with the commencement of rangeland plants at 6 different stations in the Freidan summer rangelands during 2010. Attractive index (AI) of each plant was determined by direct observations based on the average number of bees visiting and the length of time that each bee spents on the flowers. By using ordinate method, the AI data were classified into four main groups. The most import vegetation families utilized by the bees in the area were Compositae Labiatae, Umbeliferae, Cruciferae, Gramineae, Liliaceae and Caryophyllaceae. The results of the assessment carried out on the plant diversity showed that between 88 plant species, 70 species were utilized by the bees. These species were unpalatable for grazing animals and were dominant in degraded rangelands. The Class I group comprised of *Papaver dubium, Onobrychys sativa, Astragalus gossipianus, Thymus kotschyanus, Eryngium billardieri, Echinops cephalotes* and *Alhagi camelorum*. The Class II group comprised of *Eremurus persicus, Peganum harmala, Astragalus sp., Centaurea sp., Scariola orientalis, Medicago lupulina, Ferula gummosa* and *Mentah longifolia*. The Classes III and IV comprised of the species, *Euphorbia, Tragopagon caricifolius, Centaurea, Salvia sp, Acantholimon erinaceum, Convolvulus arvensis, Achillea falcate* and *Cynodon dactylon*. The results indicated that the more extensive uses of the pollen of these plants by the honeybees were associated with the higher attractiveness of the flowers and the presence of more nutritive materials in the pollen and nectar.

Keywords: Multivariate, Cluster analysis, Attractive index (AI), beekeeping, *Apis mellifera persica*

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INTRODUCTION

To utilize the natural resource capabilities for apicultural purposes, there is a need for precise and comprehensive information regarding the attractiveness of plant species in order to raise and keep honeybees. The existence of plants with high pollen and nectar producing potential in semi-arid rangeland will enable employment of these species for bee keeping as one of the multiple aspects of rangeland use. Thus, their development based on native knowledge is an important strategy to create new employment and compensate for the income decline among ranchers due to the recommended decrease in livestock pressure to be applied to rangelands.

Recognition of favorite bee plants, their dispersion areas, determination of the attractiveness of plant species, as well as phenology (especially during flowering period) is important planning tools for the protection and prevention of rangeland destruction and beekeeping development (Amiri & Mohamed Shariff, 2012). Since there is close interaction between bees and flowering plants, it is necessary to identify and study plants used by honeybees and be aware of their biological needs in order to raise them (Abou-Shaara, 2013). Therefore, detailed knowledge is necessary to achieve maximum production in bee keeping (Abou-Shaara *et al*., 2013).

Lack of attractiveness of plants would indicate lack of apicultural capacity of the rangelands. Hence, the study on the nectar and pollen producing potential of plants in an area is important to determine bee keeping effectiveness. In this respect, Ghalechnia (2006) studied the summer rangelands of Mazandaran province and noted that the most nectar bearing species were from the Leguminosae, Labiatae, Compositae and Rosaceae families. In the Markazi province, Asadi *et al*. (2004) reported 139 plant species from among 32 plant families, while Nazarian *et al*. (2006) identified 186 genera with 301 plant species used by honeybees among 54 plant families and specified their attractiveness. In the summer rangelands to the east of Mazandaran, Razaghi Kamrodi (2009) introduced plant species from the Labiatae, Compositae, Leguminosae, Rosaceae, and Cruciferae families. In the Tarobar basin area, Sabaghi *et al*. (2006) named the more important families as Compositae, Labiatae, Rosaceae, Leguminosae, Cruciferae, Umbelliferae, Scrophulariaceae and Plumbaginaceae. In Esfahan province, however, Faghih *et al*. (2005) attached greater importance to Papilionaceae, Compositae, Labiatae and Umbelliferae families. This was due to the existence of flowers bearing a great deal of pollen and the special characteristics of compounds in the pollen and nectar of the flowers. In Far province, Karimi and Jafari (2009) also noted that Compositae, Papilionaceae, Labiatae, Rosaceae, Umbelliferae and Cruciferae families are important families. In Mazandaran province, Akbarzadh and Razaghikamrodi (2006) identified 123 species from 22 families and 68 genera based on nectar and pollen bearing potential of the plant species. Coffey and Breen (1997) identified 76 nectar and

pollen bearing plant species in Ireland. In Ghana, Amoako (1997) studied 399 plant species from 59 families and introduced 255 pollen and nectar bearing species. Freitas (1994) identified 62 plant species used by honeybees by analyzing pollen samples in the Caatinga area of Brazil. Shahid (1992) studied the attractiveness of 178 plant species from 45 plant families and reported that the most important plant families in the frontier province of northwest of Pakistan are Compositae, Rosaceae, Leguminosae and Labiatae. Maskey (1992) reported that the most important plant families in Kathmandu are Rosaceae and Cruciferae. In Nepal, Verma and Attri (2008) identified 31 plant species and specified their attractiveness to honeybees. There is a wealth of literature on the apicultural capacity of many species of plants. However, little is known on the nature and apicultural capacity of plant species in the rangelands of Freidan, which is located in the north-west of Isfahan Province. Hence, the aim of this study was to identify the diversity of plant species of the area and suggest suitable strategies to enhance apicultural capacity of the rangelands. The study also aimed to identify the diversity of the plant species in terms of their pollen and nectar bearing potential and determine the attractiveness of the species to *Apis mellifera persica* bees.

MATERIALS AND METHODS

Study Area

The study area included 6 different stations in the Freidan rangelands in the north-west of Isfahan Province (50º, 00´-50º, 12´E and 32º, 56´-33º, 48´N) covering a 25,221 ha

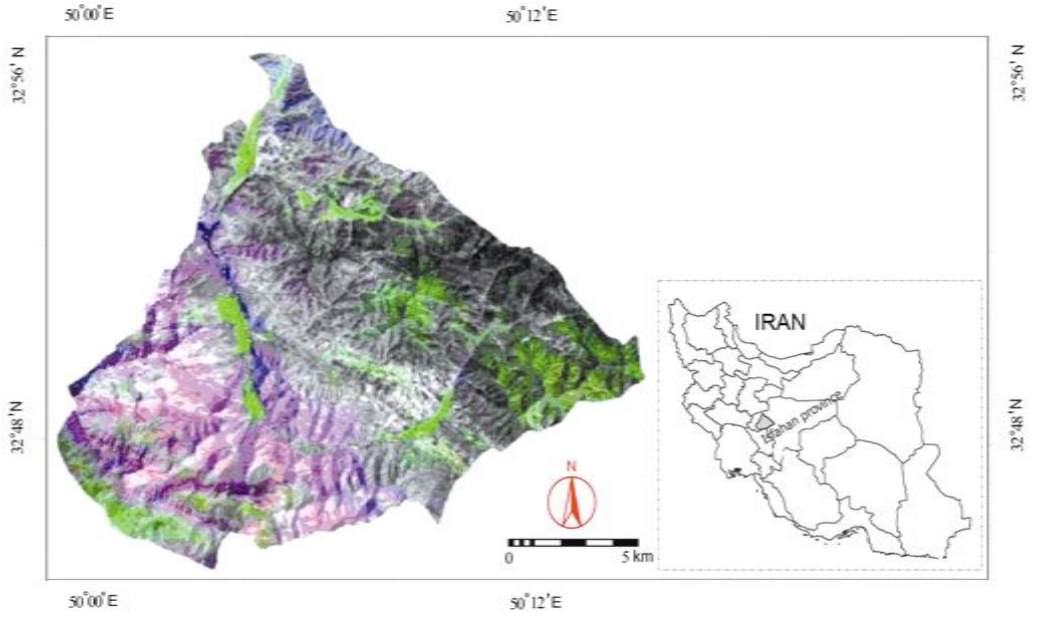

Fig.1: Location of the study area

plain. The average altitude of this region is 2828 meters above sea level (see Fig.1). It has semi-arid climate with an average annual rainfall of 452 mm yr^{-1}. Rainfall mainly occurs in the autumn and winter. The mean annual temperature is about 10 degrees Celsius. About 86.62% (21666 hectares) of the study area are rangelands, which include 10 vegetation types.

Sampling Methods

A quantitative survey of the vegetation was carried out during May and June 2010. The survey included the concentration of honeybee colonies. The survey was carried out with the aid of 1:20,000 and 1:50,000 scale maps.

Vegetation Types and Identification of Diversity of the Plant Species

A random-systematic sampling method was used to sample the diversity of plant species in each vegetation type (Potts *et al*., 2009). In each vegetation type, two 200 meter transects were made along and perpendicular to the slope (Fig.2). Along each transect, one square meter (1 m^2) of the sampling areas was marked at 20 meter intervals and plant characteristics within the sampling areas were determined. The

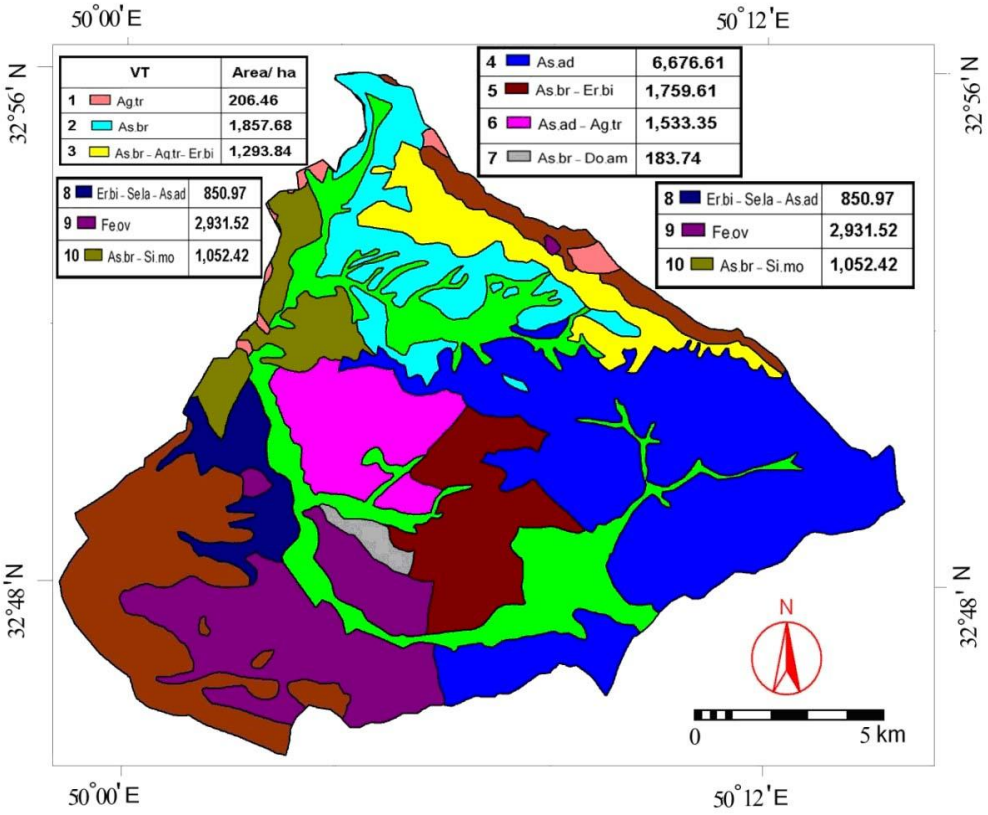

Fig.2: Vegetation type mapping of Freidan summer rangelands

presence or absence and cover percentages of nectar and pollen producing plants within the sampled plots were also determined (Ebeling *et al*., 2008). The plants were identified by comparing them with available herbarium samples, published information and were based on interviews with local bee keepers (Asadi *et al*., 2004; Afzali, 2006; Faghih *et al*., 2005; Nazarian *et al*., 2006; Amoako, 1997; Coffey & Breen, 1997).

Flowering Period

The flowering periods of plants were recorded from start to finish during weekly field visits to the area. Plant communities with at least 10 to 20% of dominant flowering pattern were determined.

Attractiveness Index of Plant Species

The attractiveness of plants for honeybees were determined in field observations by recording the number of visiting bees, and the time bees settle on each species during the flowering period (Rastgar *et al*., 2007). At 100 to 1000 meter intervals, honeybee colonies were counted and recorded in the relevant forms. The number of visitor bees on each species was counted within a square meter area during 10 minutes. The honeybee settlement period of each species was measured using a stopwatch. The attractiveness of the species was determined as follows:

The average time and number of honeybees settling on each plant was divided by the total time and number of bees settling on all plants, and multiplied by 100, respectively. The two indices were added up and the mean was considered as the attractiveness index (AI) of each plant species.

$$AI = \frac{R_n + R_t}{2} \quad (1)$$

where AI is the attractiveness index, R_n and R_t are the average time and number of honeybees settling on each plant, respectively.

Data Analysis

Multivariate analysis was performed using SPSS® software, with XlStat an add-in package of Microsoft Excel 2010. Agglomerative hierarchical clustering (AHC) and Gap statistic (Gs) were used to identify differences between the classes and to cluster the samples with similar coefficients. AHC was undertaken according to the Ward-algorithmic method. Results are presented in a dendrogram where steps in the hierarchical clustering solution and values of distances between clusters (squared Euclidean distance) are represented. Thus, species having excellent attractiveness (Group, I), species with good attractiveness (Group II), species having average attractiveness (Group III), and species with weak attractiveness (Group IV) were separately classified into the distinct groups. The attractiveness index (AI) of each plant species was classified into four levels as in Table 1.

TABLE 1
The Score of each species based on AI index

Range	Class
5	I
4	II
3	III
1	IV

RESULTS

The results of assessment of plant diversity showed that out of 88 plant species belonging to 29 families existing in the area, 70 species were of interest to honeybees. Among the identified plants, 14 species belonging to the Leguminosae and 11 and 13 species of Compositae and Labiatae families, respectively, were preferred by the honeybees, while Gramineae and Umbelliferae had 5 and 7 species that were preferred by honeybees, followed by Caryophyllaceae with 4 species and Cruciferae and Liliaceae families with 3 species each. Among the identified plant species preferred by honeybees, 16 species (22.8%) belonged to the "excellent" attractiveness group, 23 species (32.8%) were of "good" attractiveness, 22 species (31.4%) with "average" attractiveness, and 8 species (11.4%) had "weak" attractiveness. Floristic listing of species used by honeybees, including the flowering period and the plant attractiveness for bees is presented in Table 2. The results of the cluster analysis showed 65% similarity level. Based on the two characteristics, the honeybee settling time and the number of visits by bees, all the plant species were classified into the four general groups (see Fig.3A and 3B).

Species with Excellent Attractiveness (Group I)

In this group, *Papaver dubium* species had the most number of visitor bees, and the longest bee settling time, with an average of 26 visitors lasting about 234 seconds on average, with a DA_{Index} equal to 130. The main reason for the high index value is the flowering period of this species, which is from late April to the middle of June. The lowest attractiveness was observed for *Alyssum linifolium* and *Alhagi camelorum* species with attractiveness indices of 69.7 and 72.2, respectively. The average number of bees visiting *Astragalus camelorum* species was 14 and the settlement time was 125.2 seconds. *Astragalus camelorum*, *Astragalus linifolium*, Bellevalia sp., *Astragalus lycioides*, *Onobrychys melanotricum*, *Echinops cephalotes*, *Stachys acerosa*, *Phlomis persica*, *Astragalus adsendence*, *Astragalus parroaianus*, *Eryngium billardieri*, *Thymus kotschyanus*, *Stachys inflate*, *Astragalus gossipianus*, *Onobrychys sativa* and *Papaver dubium* had a similarity level of 65% and were placed in one class (Fig.2; Fig.3a). The common aspects between these species are the number of visitor honeybees and the high rate of settlement time for each plant species. Based on the similarity characteristics of the species with each other (80%), four subgroups are distinguishable (Fig.3b).

TABLE 2
Floristic list of species used by honeybees, flowering period and the plant attractiveness rate for bees in the study area

No.	Scientific name	Family	Growth form	Flowering period		Bee activity			Bee activity of the plant			
				Start	End	nectar	pollen	nectar and pollen	Exellent (I)	Good (II)	Moderate (III)	Weak (IV)
1	Acantholimon erinaceum	Plumbaginaceae	SH.	late June	Late July	*						*
2	Acanthophyllum bracteatum	Caryophyllaceae	SH.	June	Early July			*			*	
3	Achillea falcate	Compositeae	PF.				*					*
4	Aegilops sp	Gramineae	AF.	-	-	-	-	-	-	-	-	-
5	Agropyron trichophoum	Gramineae	PG.	-	-	-	-	-	-	-	-	-
6	Ajuga chamasistus	Labiatae	SH.	Early June	Early July	-	-	-	-	-	-	-
7	Alhagi camelorum Fisch.	Legominosae	PF.	late June	Early August			*	*			
8	Alyssum linifolium	Cruciferae	PF.	Mid May	late June			*	*			
9	Anthemis	Compositae	AF.	-	-	-	-	-	-	-	-	-
10	Artemisia aucheri	Compositae	SH.	-	-	-	-	-	-	-	-	-
11	Astragalus adsendence	Legominosae	BT.	Early July	Mid August	*			*			
12	Astragalus brachycalyx	Legominosae	SH.	Late May	Mid June	*				*		
13	Astragalus canesens	Legominosae	SH.	Late May	late June	*				*		
14	Astragalus cyclophylus	Legominosae	PF.	Late May	late June	*				*		
15	Astragalus gossipianus	Legominosae	SH.	Mid July	Early August	*			*			
16	Astragalus lycioides	Legominosae	SH.	Late May	Mid June	*			*			
17	Astragalus parroaianus	Legominosae	SH.	Mid June	Mid July	*			*			
18	Astragalus. sp	Legominosae	PF.	Mid July	Early August	*					*	

cont'd Table 2

19	Bellevalia sp	Liliaceae	PF.	May	Early June	*			*				
20	Bieberstenia multifida	Geraniaceae	PF.	May	June		*				*		
21	Bromus tomentellus	Gramineae	PG.	Early June	late June	-	-	-	-	-	-	-	
22	Caradaria draba	Cruciferae	PF.	Late May	Early July			*			*		
23	Carex stenophylla	Cyperaceae	PF.	-	-	-	-	-	-	-	-	-	
24	Centaurea behen	Compositae	PF.	late June	Mid July			*		*			
25	Centaurea virgata	Compositae	PF.	late June	Mid September	*				*			
26	Convolvulus arvensis L.	Convollvulaceae	PF.	Early June	Late August			*				*	
27	Cousinia bachtiarica	Compositae	PF.	Late June	Early August	-	-	-				*	
28	Cousinia cylanderica	Compositae	PF.	Late June	Early August			*		*			
29	Cynodon dactylon (L.)	Gramineae	PG.	June	July		*					*	
30	Daphne macronata	Thymelaeaceae	BT.	Early July	MidJuly		*				*		
31	Dianthus crinitus Sm.	Caryophyllaceae	AF.	Late June	Mid July	-	-	-	-	-	-	-	
32	Echinophora platyloba	Umbelliferae	PF.	Mid July	Late August		*				*		
33	Echinops cephalotes DC.	Compositae	PF.	June	July	*			*				
34	Eremostachys mocrophylla	Labiatae	PF.	Late May	Late June		*					*	
35	Eremurus persicus	Liliaceae	PF.	Late May	Mid June		*			*			
36	Eryngium billardieri	Umbelliferae	PF.	Mid July	late June			*	*				
37	Euphorbia decipiens Boiss.	Euphorbiaceae	PF.	Mid May	Mid June			*			*		
38	Euphorbia virgata	Euphorbiaceae	PF.	June	July		*				*		
39	Euphorbia macroclada	Euphorbiaceae	PF.	Mid May	Mid June	*					*		
40	Ferula gummosa Boiss.	Umbelliferae	PF.	Late May	Mid June			*		*			
41	Ferula ovina	Umbelliferae	PF.	Early July	Late July			*			*		
42	Festuca ovina	Gramineae	PG.	Early June	late June	-	-	-	-	-	-	-	

cont'd Table 2

43	Gallium Verum L.	Rubiaceae	PF.	Early June	Mid August		*			*		
44	Geramium tuberosum	Geraniacae	PF.	Mid May	Mid June			*			*	
45	Gundelia tournefortii	Compositae	PF.	Late May	late June			*		*		
46	Iris s p	Iridaceae	PF.	June	July		*					*
47	Isatis capadosica	Cruciferae	AF.	Early June	Early July			*			*	
48	Ixilirion tataricum	Amaryllidaceae	PF.	Mid May	Early June		*				*	
49	lactuca scarioloides	Compositae	PF.	Mid June	Early July			*			*	
50	Mathiola ovatifolia	Cruciferae	BF.	-	-		*				*	
51	Medicago lupulina L.	Leguminosae	PF.	Mid June	Early August			*		*		
52	Medicago sativa	Leguminosae	PF.	Mid June	Early August			*				
53	Melica persica	Gramineae	PG.	Early June	Late June	-	-	-	-	-	-	-
54	Mentah longifolia	Labiatae	PF.	Mid June	Mid August			*		*		
55	Nepeta micranatha	Labiatae	PF.	June	July			*		*		
56	Noea mucronata	Chenopodiaceae	SH.	Early August	Late August	-	-	-	-	-	-	-
57	Onobrychys melanotricum	Legominosae	PF.	Late May	Mid June			*	*			
58	Onobrychys sativa	Legominosae	PF.	Late May	Early August			*	*			
59	Papaver dubium	Papaveraceae	AF.	Late May	Mid June		*		*			
60	Peganum harmala	Zygophyllaceae	PF.	May	July		*			*		
61	Phlomis olivieri	labiatae	PF.	June	July			*			*	
62	Phlomis persica	labiatae	PF.	Mid June	Late July	*			*			
63	Plantago major L.	Plantaginaceae	PF.	June	July		*			*		
64	Poa bulbosa	Gramineae	PG.	Mid May	Mid June	-	-	-	-	-	-	-
65	Ploygonum sp	Polygonaceae	AF.	Early July	Late July	-	-	-	-	-	-	-
66	Prangus ferulacea	Umbelliferae	PF.	Late May	Mid June		*			*		

cont'd Table 2

67	Pterocephalus canus	Dipsaceae	PF.	May	June	*				*			
68	Ranunculus sp	Ranunculaceae	PF.	May	June			*			*		
69	Rheum ribes	Polygonaceae	PF.	-	-	-	-	-	-	-	-	-	
70	Rosa sp	Rosaceae	SH.	May	June			*			*		
71	Salvia sp	Labiatae	PF.	May	June			*			*		
72	Sanguisorba minor	Rosaceae	PF.	Late May	Mid June			*			*		
73	Scariola orientalis	Compositae	PF.	Early September	Early July		*			*			
74	Scrophullaria sp	Scrophullariaceae	PF.	Late May	late June	-	-	-	-	-	-	-	
75	Silene arbescula	Caryophyllaceae	PF.	May	June	-	-	-	-	-	-	-	
76	Silene sp	Caryophyllaceae	PF.	Mid May	June	-	-	-	-	-	-	-	
77	Stachys aserosa	Labiatae	SH.	Early June	Early July	*			*				
78	Stachys inflata	Labiatae	PF.	late June	Late July	*			*				
79	Stachys pilifera	Labiatae	PF.	June	July	*				*			
80	Taraxacum polycphalum	Compositae	PF.	Early May	late June			*		*			
81	Thalictrum isopyroides	Ranunculacea	PF.	Early May	Early June		*						*
82	Thymus kotschyanus	Labiatae	PF.	late June	Early August	*			*				
83	Tragopagon caricifolius	Compositae	PF.	Late May	late June			*		*			
84	Trigonella sp	Legominosae	AF.	Late May	Mid June			*		*			
85	Trifolium repens L.	Legominosae	PF.	Mid June	Mid August			*		*			
86	Tulipa sp	Liliaceae	PF.	Late April	Mid May		*				*		
87	Valeriana officinalis	Valerianaceae	PF.	Early June	late June	-	-	-	-	-	-	-	
88	Veronica orientalis	Scrophulariaceae	PF.	Mid May	late June			*			*		

This symbol (*) demonstrates bees' activities on nectar, pollen, nectar and pollen, and each plant group: (-) Dash symbol shows inactivity of honybee.

AF. = Annual Forb PF. = Perennial Forb SH. = Shrub

BF. = Biennial Forb PG. = Perennial Grass BT. = Bush Tree

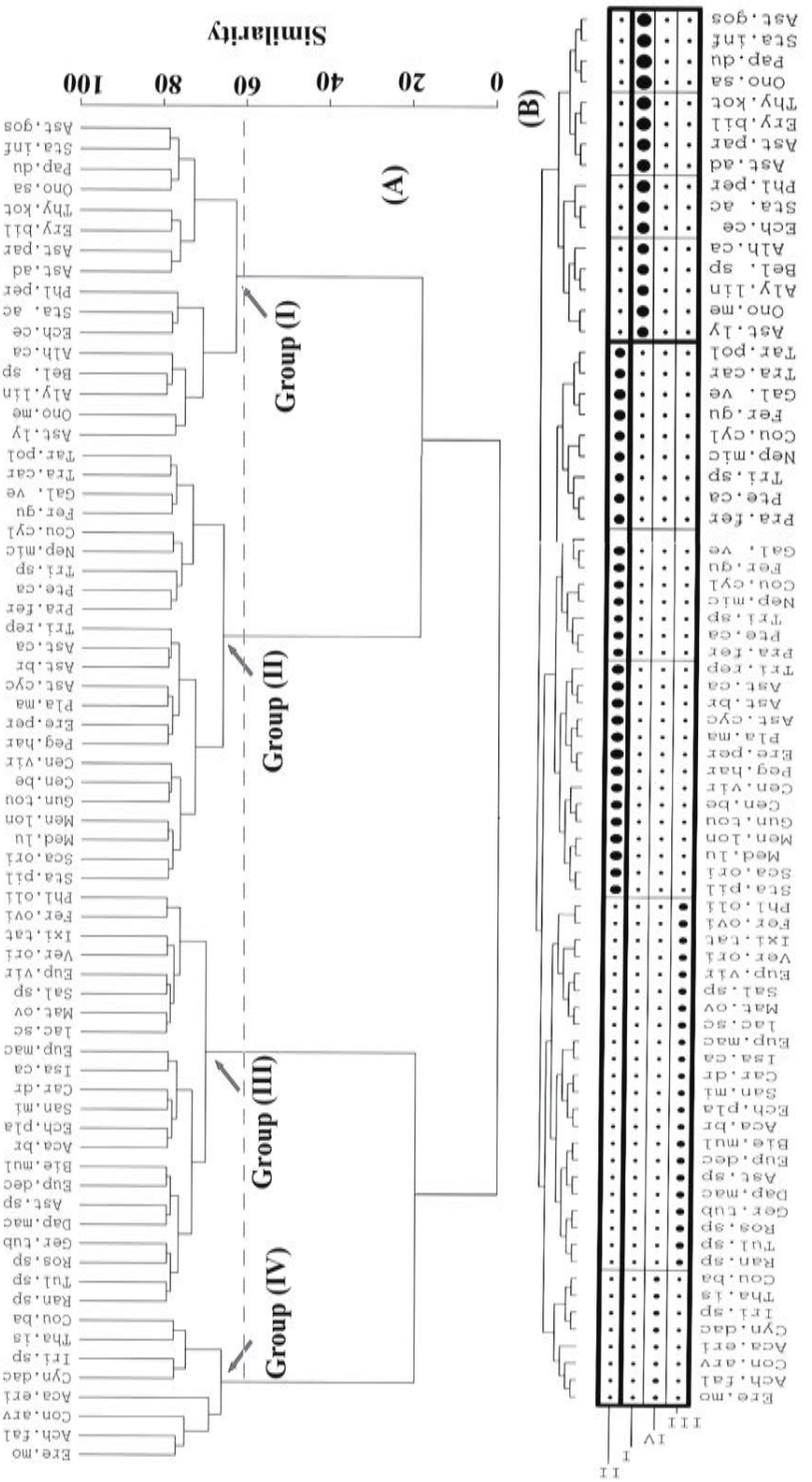

Fig.3: The cluster analysis of plants used by honeybees in the study area

Species with Good Attractiveness (Group II)

The results of the cluster analysis showed that at the similarity level of 75%, 3 subgroups were classified separately based on the number of visitor honeybees and the fairly high rate of bee settlement time on each plant species (Fig.3 a, b). Species located in subgroups based on the similarity percentage are presented separately (Fig.2 b). The number of visitor honeybees and the settlement time in this group are associated with *Eremurus persicus,* with an average of 12.7 visiting bees and a time of 172 seconds, yielding an average index of attractiveness of 92.35, while the lowest rate was associated with *Trigonella sp.*, with an average of 7.32 visiting bees and a time of 98.8 seconds yielding an average index of attractiveness of 53 (Table 2).

Species with Average and Weak Attractiveness (Groups III and IV)

In Group III, *Phlomis olivieri* species with an average of 6.5 visiting bees for a period of 85.3 seconds and *Isatis capadosica* with an average of 6.5 visiting bees for the same period of time resulting in attractiveness indices of 45.9 and 30.3 respectively, were the most and the least attractive species of this group. The most attractive index (48.1) in Group IV was associated with *Cousinia bachtiarica* with 4.1 visitor bees for a period of 26.1 seconds, while the least A_{Index} (27.6) was associated with *Acantholimon erinaceum* with an average of 2.3 visiting bee numbers for a period of 14.9 seconds (Table 2). The results of the cluster analysis indicated that in Group III with 75% similarity level, there were 3 subgroups, while in the group IV with a 70% level there were 2 subgroups (see Fig.3a,b). The differences between the species of the two groups were the average low numbers of visitor honeybees and the average weak period of bee settlement of each species.

DISCUSSION

Due to the differences in elevation and topography of the study area, there was high species diversity, while the presence of various species showed adaptations to local conditions. The most important plant families used by honeybees in the study area were Leguminosae, Compositae, Labiatae, Gramineae, Umbelliferae, Caryophyllaceae, Cruciferae and Liliaceae (Table 2).

Nazarian *et al*. (2006) reported the most important plant families as: Compositae, Leguminosae, Labiatae, Rosaceae, and Cruciferae, while Maskey (1992) in Kathmandu stated Rosaceae and Cruciferae as important families. The results of the present study when compared to previous studies showed that the plant families including Leguminosae, Compositae, Labiatae, Umbelliferae, Rosaceae and Cruciferae are very important families for bee keeping, especially in terms of the number of species, accumulation, dispersion, and attractiveness in addition to the production of nectar and pollen. These families have attractive and nutritious materials useful to honeybee nutrition are of significant importance (Nation & Robinson, 1968; Ebadi & Ahmadi, 2006). The results

obtained in this research correspond with similar studies carried out in Iran and elsewhere in the world. A comparison of the species in the area showed that most plants used by honeybees are species producing nectar and pollen. The results of the study showed that the number of plants producing nectar and pollen is more than the number of plants producing nectar or pollen.

The results of the classification analysis showed different levels of attractiveness (excellent, good, average and weak). The differences among the plant species mainly result from the density of flowers on each plant, density and dispersion of species in the area, physical characteristics of flowers, flowering date, climatic factors and plant distances from honeybee colonies. Hegland and Boeke (2006) found the diversity of floral resources and the diversity and abundance of pollinators in a temperate grassland community resulted in differences in attractiveness of different species to the bees.

The results of the classification analysis indicated that the species located in the low palatable class had the highest level of attractiveness in terms of apicultural applications and formed the species of Group I. Further, the flowering dates of these species may have been favourable and attracted the honeybees. In other words, the absence of competing plants can cause greater attraction of bees to one special species. Moreover, the results of Rabinowitch *et al*. (1993) showed that the distance of the colonies from flowers influences the attraction of bees to one effective species.

Lack of knowledge of the existing environmental resources in the area resulted in the utilization of these resources more for livestock production. However, the presence of plant species with potential for other usage, including recreation and bee keeping, can result in increased income. Behan (1984) stated that multiple usage of environmental resources should be based on scientific planning in agreement with politics, law, economy and sociology under the supervision of an authorized committee for decision making.

An increase in the number of livestock to increase income and fulfil the economic needs caused a change in the species composition and a decline in the diversity of plant species. An increase in the livestock caused extinction of palatable species and a decrease in livestock performance. According to the food communion of livestock and honeybees, early grazing before the flowering period of plant species will cause a decrease in forage produced in the following years and a decrease in the use of flowers during the flowering period by honeybees and soil degradation (Vulliamy *et al*., 2006). The entry date of livestock in the area should be after the flowering period so that the use of that particular bee species could be made possible. Among the invasive and secondary plant species unpalatable species not preferred by livestock are attractive and of interest for honeybees (Wilke & Irwin, 2010). Ralphs (2002) studied the ecological interaction among unpalatable plants in the west pastures of America and found that some species are useful for beekeeping.

CONCLUSION

Knowledge on plants, their dispersion areas and phonology (especially flowering period) is an important planning tool for the protection of the natural environment and development of beekeeping. Awareness of honeybee biology, knowing the plants preferred by honeybees and studying plant cover types are necessary to raise honeybees. In the study area, the floristic list of available plants and plant types in terms of nectar and pollen producing were identified and classified. Considering the geographical width of the area and the climate, soil, and topography, the interacting characteristics provide a good natural environment to raise honeybees. The results of the study on the floristic composition, the climatic conditions and the presence of nectar and pollen producing species indicate good potential for rehabilitation of the area. Based on the diversity of the plant species available in the area, it can be concluded that the environmental resources indicate a strong potential for bee keeping.

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Genetic Diversity of *Fusarium solani* Isolates from Black Pepper (*Piper nigrum* L.) in Malaysia by ISSR marker

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ABSTRACT

For effective management and control of yellowing disease, inter-simple sequence repeat (ISSR) markers were employed to investigate genetic diversity among 34 isolates of *Fusarium solani*, which had been isolated from major growing areas of black pepper in Malaysia. Over 15 primers, with fragment sizes ranging from 200 bp to 3600 bp, and a total of 253 amplified loci were amplified in all 34 isolates, with an average of 17 bands per primer; among which 248 (98.02 %) were polymorphic. High genetic diversity at species level was revealed (Nei's gene diversity h = 0.2671 and Shannon information index I = 0.4134, respectively). Sarikei population was found to have a higher degree of polymorphism compared to other populations. The dendrogram generated from UPGMA cluster analysis categorized the 34 *F. solani* isolates into two major clusters based on Jaccard's similarity coefficients. Cluster I contained a unique isolate. Cluster II contained 33 isolates which were split into two different subclusters. Results showed that the clusters were not related to geographic origins. Meanwhile, ISSR fingerprinting generated highly polymorphic markers that could be used to study genetic characterization of *F. solani* and to clarify phylogenic relationships, as well as useful for efficient management of yellowing disease and finding breeding programmes to find cultivars of black pepper that are resistant to *F. solani.*

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INTRODUCTION

One of the most recognized spices in the world is black pepper (*Piper nigrum* L.; Family: Piperaceae). Black pepper is a perennial climbing vine grown for its berries and it is extensively used as spice in traditional medicine. Malaysia is one of the major producers and exporters of pepper (Ravindran, 2000). The productivity of black pepper is slowly declining (Krishnamoorthy & Parthasarathy, 2010), while crop losses caused by diseases are a major production limitation in Malaysia and other pepper-producing countries (Kueh & Sim, 1992; Sarma *et al.,* 1992).

The most serious diseases of black pepper are ones caused by fungal pathogens and plant parasitic nematodes (Bong & Saad, 1986; Ramana & Eapen, 2000; Thankamani *et al*., 2008). One of the most important diseases of black pepper is yellowing disease, which is also known as slow decline (Kueh *et al*., 1993; Sitepu & Mustika, 2000). *Fusarium solani* was identified as the causal agent of this particular disease (Hamada *et al.,* 1988). It is important to note that *Fusarium* infection in black pepper plantation has been reported to reduce the economic life of the plantation from 20 to 6-8 years and the productivity per plant from 3.0 to 1.5 kg (Anandaraj, 2000).

Genetic characterization of plant pathogens variants established in an area is required for effective management and to increase crop yield. The *Fusarium* wilt disease can be managed by the use of resistant cultivars (Jalali & Chand, 1992). Plant pathogens are continuously mutating and changing, resulting in new strains and new challenges to growers (Koike *et al*., 2000). Development of a molecular marker technique to differentiate highly virulent and nonpathogenic isolates is important in managing the *Fusarium* disease in a variety of crops (Belabid *et al*., 2004). Disease management is difficult due to the presence of several pathogen types. For any one crop, the grower must deal with a variety of fungi, bacteria, viruses and nematodes. Hence, an increase in the genetic diversity of the crop host rotation is an important management step that incorporates ecological considerations (Koike *et al*., 2000).

The choice of a molecular marker technique for genetic diversity assay is dependent upon its reproducibility and simplicity (Bornet & Branchard, 2001). The most common used PCR-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs) (Pradeep Reddy *et al*., 2002). The main limitations of these techniques include low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop specific SSR primers. ISSR is a technique that overcomes most of these limitations (Zietkiewicz *et al.,* 1994; Pradeep Reddy *et al*., 2002).

A new molecular marker method called ISSR has been available since 1994 (Zietkiewicz *et al*., 1994). ISSR is a technique which involves the use of microsatellite sequences as primers in a PCR to generate multilocus markers (Pradeep

Reddy *et al*., 2002). ISSRs are known to be abundant, very reproducible and highly polymorphic tandem repeat motifs composed of 1 to 7 nucleotides (Bornet *et al*., 2004).

PCR analysis using ISSR primers has been acknowledged as a valuable technique in studies concerning genetic diversity, phylogenies and evolutionary biology (Pradeep Reddy *et al*., 2002). The information about genetic diversity within a species is helpful to come up with proper management programmes (Frankham, 1995). Distinct genetic differentiation within the same races can be assumed as an environmental factor affecting pathogen properties (Weller, 1988).

The aim of the present study was to examine the genetic diversity of *F. solani* populations obtained from black pepper fields in Malaysia and to assess the phylogenic relationships of *F. solani* isolates using ISSR markers to managing yellowing disease in black pepper in future studies.

MATERIALS AND METHODS

Fungal Isolates

For the purpose of this study, the roots of black pepper plants with yellowing disease symptoms and rhizosphere soils were collected from major growing areas in Sarawak (Sibu and Sarikei) and Johor (Kulai). Based on their morphological characteristics and molecular techniques (DNA sequencing of the ITS regions), 34 *Fusarium solani* isolates were obtained. The isolates of this species were used for ISSR fingerprinting.

DNA Extraction

Genomic DNA of *F. solani* isolates was extracted using CTAB method (Doyle & Doyle, 1990) and DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., USA) by computing UV absorbance at 260/280-nm ratio. The isolated genomic DNA was diluted to 50 ng/mL and stored at -20ºC for ISSR amplification.

PCR Amplification

A total of 20 primers were initially screened for PCR amplification and 15 primers that produced clear and reproducible banding patterns were chosen for final analysis. The ISSR markers were amplified using 15 primers (First Base Laboratories Sdn. Bhd., Malaysia), unanchored and anchored on 5' or 3'- ends with di- or tri-nucleotide repeats in the ISSR analysis (Table 1). The PCR amplification was carried out in 20 µl reaction mixtures containing 2 µl of 10x PCR buffer (100 mM Tris-Hcl [pH 8.3], 500 mM KCl, 20 mM $MgCl_2$), 2 µl of dNTP mixture (2.5 mM each), 0.4 µl of Taq (5 U/µl) (iNtRON Biotechnology, Inc., Seoul, Korea), 1 µl of ISSR primer (10 µM) and 1 µl of diluted genomic DNA.

The PCR amplifications were performed in Biometra Tpersonal Thermocycler. This involved an initial step at 94ºC for 5 min, followed by 40 cycles of denaturing at 94ºC for 1 min, annealing at primer specific temperature (Table 1) for 45 seconds and extension at 72 ºC for 2 min, followed by a final extension step for 7 min. The

amplification products were separated via electrophoresis on 1.4% agarose gel with 1x TBE buffer at 110 Vcm^{-1} for 2.5 hours by electrophoresis. The 1000 plus DNA ladder (iNtRON Biotechnology, Inc., Seoul, Korea) was used as a DNA marker for electrophoresis. To prepare ethidium bromide solution, 0.01 g ethidium bromide was dissolved in 1 ml distilled water and 50 µl of this stock was added to 500 ml distilled water. The gels were stained with ethidium bromide and the bands were visualized and photographed under UV light using a gel documentation imaging system (Bio-Rad, USA).

Data Analysis

Amplified bands from each primer were scored as present (1) or absent (0). Only the bands which had been amplified consistently were considered. Fragments of the same molecular weight were considered as the

TABLE 1
Primers, amplification conditions and polymorphism of ISSR markers

ISSR primer type	Sequence (5'-3')	Annealing temperature (ºC)	G + C content (%)	NAB[a]	NPB[b]	PPB[c] (%)	PCR product size range (bp[d])
UBC 807	$(AG)_8T$	51	47.1	10	10	100	300-1200
UBC 808	$(AG)_8C$	49	52.9	19	19	100	250-1700
UBC 809	$(AG)_8G$	52	52.9	12	11	91.67	250-1500
UBC 810	$(GA)_8T$	48	47.1	20	20	100	300-3200
UBC 811	$(GA)_8C$	51	52.9	22	22	100	250-2400
UBC 818	$(CA)_8G$	49	52.9	16	15	93.75	250-2200
UBC 825	$(AC)_8T$	50	47.1	8	7	87.50	550-2400
UBC 835	$(AG)_8YC$	55	52.8	20	19	95	200-2200
UBC 841	$(GA)_8YC$	49	52.8	24	23	95.83	200-2100
UBC 855	$(AC)_8YT$	50	47.2	22	22	100	250-2500
UBC 856	$(AC)_8YA$	54	47.2	19	19	100	250-3200
UBC 858	$(TG)_8RT$	53	47.2	8	8	100	400-3000
UBC 864	$(ATG)_6$	52	33.3	12	12	100	400-1800
UBC 885	$BHB(GA)_7$	53	51	27	27	100	200-3600
UBC 886	$VDV(CT)_7$	49	51	14	14	100	250-2200

[a] NAB: number of amplified bands; [b] NPB: number of polymorphic bands; [c] PPB: percentage of polymorphic bands; [d] bp: base pair. *Note:* R= (A,G)= Purine, Y= (C,T)= Pyrimidine, B= (C,G,T) (i.e. not A), D= (A,G,T) (i.e. not C), H= (A,C,T) (i.e. not G), V= (A,C,G) (i.e. not T)

same locus. The data were used to calculate the number of bands produced by each primer and the percentage of polymorphic loci.

To study the genetic relationships among the isolates, a pair-wise similarity matrix was generated using Jaccard's similarity coefficient. Cluster analysis was performed to develop a dendrogram. The dendrogram was constructed using an unweighted paired group method of cluster analysis using arithmetic averages algorithm (UPGMA) of NTSYS-pc Version 2.1, a numerical taxonomy and multivariate analysis software package (Rohlf, 2000).

The binary data matrix was input into POPGENE 32 version 1.31 (Yeh *et al*., 1999), assuming HardyeWeinberg equilibrium. Meanwhile, the POPGENE software was used to calculate genetic similarity coefficient and Nei's unbiased genetic distance among the populations. The percentage of polymorphism band (PPB), Nei's gene diversity (h), Shannon's information index (I), observed number of alleles per locus (n_a) and effective number of alleles per locus (n_e) were calculated to estimate the genetic variation.

RESULTS

ISSR polymorphism

Three populations of *F. solani* were studied for genetic diversity based on locations and geographical distributions (Table 2). Over the 15 primers, the fragment size obtained from *F. solani* isolates ranged from 200 bp to 3600 bp and a total of 253 amplified bands (loci) were generated, with an average of 17 bands per primer. The number of amplified bands per primer ranged from a maximum of 27 discrete bands in BHB$(GA)_7$ to a minimum of 8 in $(AC)_8$T and $(TG)_8$RT (Table 1). The number of polymorphic bands was 248 while the percentage of polymorphism was 98.02% among 34 individual isolates (Table 3). The average number of polymorphic bands per primer was 16.5. It is important to note that BHB$(GA)_7$ produced the greatest number of polymorphism (Table 1).

Genetic Diversity

Among the populations of *F. solani*, the percentage of the polymorphic bands for populations I (Sibu), II (Sarikei) and III (Kulai) were 68.77, 92.09 and 55.34%, respectively (Table 3). The observed average

TABLE 2
Population distribution of *F. solani*

Populations	Sample size	Location	Latitude	Longitude
I	10	Sarawak-Sibu	2° 34' N	111° 55' E
II	18	Sarawak-Sarikei	2° 07' N	111° 31' E
III	6	Johor-Kulai	1° 39' N	103° 36' E

TABLE 3
Genetic diversity of *F. solani* populations

Populations	Sample size	No. of PB[a]	PPB[b] (%)	[c] n_a ([g] S.D.)	[d] n_e (S.D.)	[e] h (S.D.)	[f] I (S.D.)
Overall populations	34	248	98.02	1.9802 (0.1395)	1.4446 (0.3505)	0.2671 (0.1707)	0.4134 (0.2199)
Within populations							
I (Sibu)	10	174	68.77	1.6877 (0.4643)	1.3531 (0.3523)	0.2135 (0.1858)	0.3276 (0.2627)
II (Sarikei)	18	233	92.09	1.9209 (0.2704)	1.4523 (0.3601)	0.2680 (0.1779)	0.4107 (0.2339)
III (Kulai)	6	140	55.34	1.5534 (0.4981)	1.3536 (0.3985)	0.2010 (0.2077)	0.2982 (0.2936)

[a] PB: Polymorphic bands; [b] PPB: Percentage of polymorphic bands; [c] n_a: Observed average number of alleles; [d] n_e: Average effective number of alleles; [e] h: Nei's (1973) gene diversity;
[f] I: Shannon's information index; [g] S.D.: Standard deviation

number of alleles (n_a) for populations I (Sibu), II (Sarikei) and III (Kulai) were 1.6877, 1.9209 and 1.5534, respectively, while the average effective numbers of alleles (n_e) were 1.3531, 1.4523 and 1.3536, respectively (Table 3).

In the total populations of *F. solani*, the values of Nei's gene diversity (h) for populations I (Sibu), II (Sarikei) and III (Kulai) were 0.2135, 0.2680 and 0.2010, respectively, while the values of Shannon's information index (I) for populations I (Sibu), II (Sarikei) and III (Kulai) were 0.3276, 0.4107 and 0.2982, respectively (Table 3).

The genetic structure was further investigated using Nei's gene diversity statistics, including total genetic diversity (H_t), genetic diversity within populations (H_S) and the mean coefficient of genetic diversity among the populations (G_{ST}). An estimate of gene flow among populations (N_m) was computed using the formula of McDermott and McDonald [$N_m = (1 \_ G_{ST})/2G_{ST}$] (McDermott & McDonald, 1993). Among the three populations of *F. solani*, total genetic diversity (H_t) was 0.2635 and genetic diversity within populations (H_S) was 0.2275. The proportion of genetic diversity among populations (G_{ST}) was 0.1365 and gene flow (N_m) was 3.1623.

In order to study the relationships among populations, genetic similarity coefficients (Nei's coefficient) of different populations were analyzed. The highest genetic similarity coefficient was 0.9764 and the lowest genetic coefficient was 0.9140 (Table 4).

TABLE 4
Genetic similarity coefficient and genetic distance among *F. solani* populations based on the ISSR markers

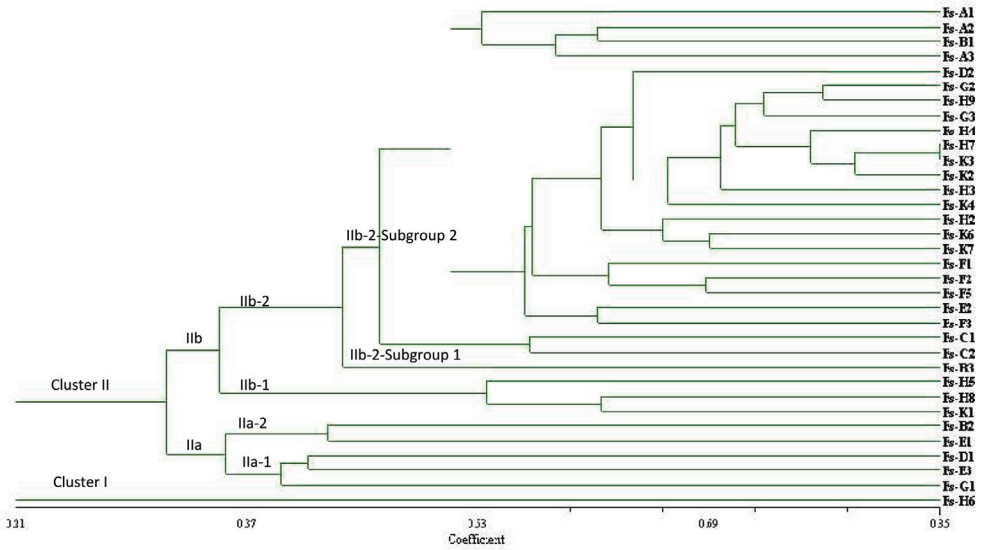
Populations	I	II	III
I	****	0.9764	0.9140
II	0.0239	****	0.9479
III	0.0899	0.0535	****

Nei's unbiased genetic identity is listed above diagonal and genetic distance is listed below diagonal.

Cluster Analysis

To examine the genetic similarity among the isolates, a pair-wise similarity matrix was generated using Jaccard's similarity coefficient. The values of Jaccard's genetic similarity coefficients among 34 tested isolates of *F. solani* were found to range from 0.15 to 0.85.

The UPGMA method of clustering was used to estimate the phylogenetic relationships among isolates. The dendrogram generated using the UPGMA cluster analysis based on Jaccard's similarity coefficients categorized the 34 *F. solani* isolates into two major clusters (see Fig.1). Cluster I contained a unique isolate Fs-H6. Cluster II was split into two subclusters that were designated as subclusters IIa and IIb. Subcluster IIa was further divided into two small groups. Group 1 consisted of three isolates (Fs-D1, Fs-E3, Fs-G1) and Group 2 consisted of two isolates (Fs-B2, Fs-E1). Similarly, subcluster IIb was also divided into two groups. Group 1 included three isolates (Fs-H5, Fs-H8, Fs-K1) and group 2 was further split into two subgroups. Subgroup 1 included Fs-



The scale is based on Jaccard's similarity coefficient

Fig.1: The dendrogram based on ISSR polymorphism and UPGMA clustering method showing genetic relationships of 34 *F. solani* isolates.

B3 isolate and subgroup 2 included the remaining 24 *F. solani* isolates. Similarity index was calculated at 21% between the two major clusters. The isolates in cluster II showed 31.6% similarity. The isolates in subcluster IIa showed 35.6% similarity and the isolates in subcluster IIb exhibited 34.9% of similarity (Fig.1).

DISCUSSION

Fusarium disease is difficult to control because the pathogen has survived in infected soil for many years (Haware *et al*., 1996). Due to the variability of genetic structure, one of the useful methods to control *Fusarium* wilt is the development of resistant cultivars and the races of pathogen (Nene & Haware, 1980). Successful management is largely dependent upon a complete knowledge of the species, including levels and structure of genetic variation (Jin & Li, 2007).

In this study, the ISSR technique has been demonstrated as applicable to evaluation of genetic diversity among the *F. solani* isolates. First, a great number of primers (20 primers) were assayed with (AG), (GA), (AC), (CA), (TG), (CT), (ATG) repeats in order to select suitable polymorphic ISSR markers for the study. Five primers [$(GC)_9YR$, $(AT)_9YR$, $(CT)_8RG$, $(CA)_8RT$ and $(GA)_8YT$] failed to produce any amplification patterns. In this study, the optimum annealing temperature for the ISSR markers varied from 48º to 55ºC, and the ISSR markers gave constant and reproducible bands across separate PCR runs. Regardless of the relatively small sample sizes, 248 polymorphic ISSR loci were sufficient enough to detect the variation and to differentiate *F. solani* populations with different geographical origins.

The UPGMA cluster analysis of Jaccard's similarity coefficients generated a dendrogram which categorized the 34 *F. solani* isolates into two major clusters. Cluster I contained a unique isolate that showed a unique banding pattern and was distinct from other isolates. Cluster II contained 33 isolates that were further split into two subclusters. The low similarity index value (21%) between the two clusters showed the high genetic variability among the isolates. The relatively low value of the similarity index was mainly due to isolate Fs-H6. This isolate was clearly distinct from the rest of *F. solani* isolates based on morphological characteristics. The dendrogram indicated that the clusters were not related to geographic origins.

Many studies revealed that there is a clear association between population characteristics and the environments in which they occur (Zhao *et al*., 2007). The genetic relationships between the populations in a species do not often accord with their geographical distance, especially for the species with large distribution area. Other factors including mutation, reproduction mode, gene flow, geographic range of populations, number of populations studied, sample size of populations and type of markers used can influence the patterns of genetic variability among and within the populations (Ma *et al*., 2008). Lack of genetic diversity decreases the ability of a

particular species to survive environmental changes (Cao *et al*., 2006).

The genetic diversity of *F. solani* was high at both the species and population levels. It is similar to the genetic diversity of some other *Fusarium* species revealed by the ISSR markers as reported by Nagarajan *et al.* (2004), Mishra *et al*. (2006) and Dinolfo *et al*. (2010). Among the three populations of *F. solani*, Population II (Sarikei) showed the maximum variation (92.09%), while population III (Kulai) showed the minimum variation (55.34%). The present study found the PPB value of 92.09% in population II, indicating that the population tested had a higher degree of polymorphism compared to other populations, while population III had the lowest genetic diversity levels. Based on Nei's coefficient, the highest genetic similarity coefficient was between Sibu population and Sarikei population, whereas the lowest genetic coefficient was revealed between Sibu population and Kulai population. Based on Jaccard's coefficients, the lowest similarity coefficient (0.15) was found between Fs-H6 and Fs-F2 isolates, whereas the greatest extent of similarity coefficient (0.85) was shown between Fs-K3 and Fs-H7 isolates.

Among the overall populations of *F. solani*, the observed average number of alleles (n_a) and the average effective number of alleles (n_e) were 1.9802 and 1.4446, respectively. The analysis of the ISSR markers using different approaches (Nei's gene diversity and Shannon's information measure) demonstrated similar interpretations of the genetic structure of the populations of *F. solani*. Similarly, Nei's gene diversity and Shannon's information index also revealed high genetic variation at species level of *F. solani* (0.2671 and 0.4134, respectively), with an average of 0.2275 and 0.3452, respectively. Genetic variation at species level was found to be generally higher than at the population level.

Meanwhile, gene flow affects genetic diversity level among and within populations. In population genetics, a value of gene flow (N_m) < 1.0 and a value of gene differentiation (G_{st}) > 0.25 are regarded as significant population differentiation (Slatkin, 1987).

The low coefficient of genetic diversity (G_{ST}) and the high gene flow (N_m) among the overall populations influenced the emergence and evolutionary development of *F. solani* (G_{ST} =0.1365, N_m =3.1623). This result is supported by Mishra *et al*. (2006). The result suggests that frequent gene flow and low coefficient of genetic diversity are predominant evolutionary forces determining the evolution and development of this particular fungus in Malaysia.

The distribution of genetic diversity among the *F. solani* isolates taken from major growing areas of black pepper in Malaysia was examined and a high level of variability was observed among the isolates by ISSR. Genetic characterization of *F. solani* is essential for an efficient management of yellowing disease through the use of resistant cultivars in black pepper growing areas.

CONCLUSION

The results of this study have proven the suitability and reliability of ISSR-PCR fingerprinting as a useful tool for population structure studies and discrimination among individual fungal isolates. ISSR is a reliable technique for genetic analysis of *Fusarium solani* and clarify phylogenic relationships in order to establish breeding programmes. In more specific, the ISSR fingerprinting generated highly polymorphic markers for *F. solani* and it is useful molecular markers for further genetic diversity studies. This method could be helpful for breeders to find cultivars of black pepper that are resistant to *F. solani.*

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Demographics of Horseshoe Crab Populations in Kota Kinabalu, Sabah, Malaysia with Emphasis on *Carcinoscorpius rotundicauda* and Some Aspects of its Mating Behaviour

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ABSTRACT

Survey results of two populations of *Carcinoscorpius rotundicauda* at a fishing ground and within a protected area were compared so as to establish effects of human activities on the species. *Tachypleus tridentatus* and *Tachypleus gigas* were also found in both sites but in substantially less abundance. The operational sex ratios were male-biased at both sites; 5.50 (n = 52) at the fishing ground (Site 1) and 2.58 (n = 68) at the protected area (Site 2). Size distributions at both sites were similar of which females were approximately 16% larger than males. A captive experiment was conducted where pair-forming behaviour of *C. rotundicauda* was observed for 30 days. Amplexus were most frequently formed when the sex ratio was balanced, lasting for 2.44 ± 2.03 days, and eight days maximum. Female body size and amplex-forming frequency were positively correlated (r = 0.678, n = 7, p = 0.10), attributable to fitness projected by the phenotype. Solitary males did not exhibit aggression towards paired males, allowing the latter to retain mate exclusivity for considerable periods. Anthropogenic pressures caused an unnatural shift in the population structure of local horseshoe crabs that could lower their reproductive success, making eventual extirpation possible.

Keywords: Carcinoscorpius rotundicauda, horseshoe crab, mating behaviour, population structure, *Tachypleus gigas*, *Tachypleus tridentatus*

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INTRODUCTION

All three Asian species of horseshoe crab are present in the waters of Sabah, namely, the tri-spine horseshoe crab, *Tachypleus tridentatus*, the Indian horseshoe crab,

Tachypleus gigas, and the mangrove horseshoe crab, *Carcinoscorpius rotundicauda* (Waterman, 1958; Sekiguchi, 1988a). A survey conducted from 2004 through 2006 along the coastal fishing villages in Peninsular Malaysia and Sabah indicated that the population of horseshoe crabs in Malaysia is undergoing a rapid decline (Christianus *et al.*, 2008). Horseshoe crabs exhibit site-specificity especially in areas where eggs and juveniles have developed successfully, making them vulnerable to overexploitation. In addition to being philopatric, the populations of horseshoe crabs have low genetic connectivity and high genotypic variations based on molecular genetics data (Obst *et al.*, 2012; Rozihan *et al.*, 2013) attributed to low migratory ability and hence demographic exchange between them. Unlike two other Asian species, *C. rotundicauda* does not travel out to deeper water after spawning since adults can be found buried 2 - 3 cm deep in the mud, while juveniles remain near the surface (Cartwright-Taylor *et al.*, 2011). Habitat loss is particularly hazardous to the genetically and ecologically constrained *C. rotundicauda* in Kota Kinabalu city where rapid coastal developments and reclamation over the past few decades have greatly decimated its mangrove cover.

All Asian horseshoe crab species share a similar habit of travelling upstream following incoming tides during full moon and new moon to spawn (Sekiguchi *et al.*, 1988; Chatterji *et al.*, 1992). A five-year survey on *C. rotundicauda* in Mandai Mudflats at Kranji in Singapore found that spawning occurred year-round and the sex ratio was balanced, showing consistently little change (Cartwright-Taylor *et al.*, 2009; Cartwright-Taylor & Hsu, 2012). Male dominant populations are regarded as a rarity in Asian horseshoe crabs, while male-biased populations are a norm in the American horseshoe crab, *Limulus polyphemus* (Sekiguchi *et al.*, 1988; Brockmann & Smith, 2009). A mating pair of horseshoe crab consists of a male mounting the back of a female and clasping the latter at the margin of its opisthosoma, forming an amplexus.

Limulus polyphemus is the most intensely-studied marine invertebrate in the world and its mating ecology has been well documented relative to the Asian species. According to Botton *et al.* (1996), although the Asian horseshoe crabs and *L. polyphemus* have over 100 million years of isolation in their lineages, their mating system are fundamentally the same. Studies showed that the species' behaviour in selecting mates is governed by a number of sensory cues which include visual, chemical and tactile cues (Hassler & Brockmann, 2001; Barlow, 2009; Saunders *et al.*, 2010). Size was suspected to play a role in mating selection as males tended to form amplexus with larger females (Suggs *et al.*, 2002). The same could be said of the Asian species since all the four extant species of horseshoe crabs share a lot of common features in terms of their biology and life history.

In Sabah, there have been very few studies done to determine the population status of horseshoe crabs. Demographic

data on vulnerable species are vital in understanding the mechanisms that are driving their decline, especially factors that perturb the balance between fecundity and longevity (see Owens & Bennett, 2004). Thus, this study aimed to compare the ecological status of *C. rotundicauda* populations at a fishing ground and a protected area in the vicinity of Kota Kinabalu to demonstrate the effects of anthropogenic pressure on a population and to provide information on some aspects of its mating behaviour. A brief discussion on *T. tridentatus* and *T. gigas* in Sabah is also included here so that the results of this study can be used as baseline data to be adopted in future appraisals of horseshoe crabs at other localities.

MATERIALS AND METHODS

Study Sites

Surveys were conducted at two sites, Menggatal River (Site 1) (6° 3' 52.9"E, 116° 7' 21.1"N) and Kota Kinabalu Wetland Centre (Site 2) (5° 59' 6.07"N, 116° 5' 14.48"E) (Fig.1). Site 1 is a river that is approximately 60 m wide and lined with narrow (5 to 10 m) strips of mangroves that, at most parts, have been cleared by fishermen living along the banks. Fishing activities are intense in the area for subsistence and commercial harvest of a myriad of species including bivalves and gastropods that form the major dietary components of horseshoe crabs (see Akbar John *et al.*, 2012). Site 2 is a 2.4 km^2 patch of mangrove which was once part of an extensive mangrove forest lining the coastline of Kota Kinabalu. Site 2 was accorded Bird Sanctuary status in 1996 and declared a State Cultural Heritage Site in 1998. A small stream that is approximately 10 m in width connects the mangrove patch to Likas Bay, where horseshoe crabs have been frequently sighted. Samples collected were brought to Borneo Marine Research Institute (BMRI) of Universiti Malaysia Sabah, Kota Kinabalu, for further analyses and captive experiment.

Sampling Technique

Periodicity of breeding activities of *C. rotundicauda* at both sites coincided with lunar phases, as reported by Sekiguchi (1988b). In order to ensure sampling efficiency, horseshoe crabs were intercepted when they were swimming upstream into the mangrove to spawn, usually in the evening following the rising tide. Sampling was conducted twice per month, i.e. during spring tides of full moon and new moon for two consecutive days in the span of four months (a total of 16 nights) from October 2008 through January 2009 at Site 1 and from January through April 2013 at Site 2.

Horseshoe crabs at Site 1 were caught using three-layer trammel nets that had been deployed along the banks of the river with the help of a local fisherman. The nets were left overnight and checked for horseshoe crabs in the morning. Meanwhile, samples from Site 2 were collected through direct capture because accumulation of debris in the nets deployed made them difficult to be hauled. The conditions in which the individuals from Site 2 were found (solitary or amplexed) were also recorded to

provide supplementary data for the mating behaviour study. Amplexus in Site 1 were often dissociated upon entanglement in the net, and thus, it could not be ascertained whether the individuals caught were solitary or amplexed. Captures were brought to BMRI for species identification, sexing, and to be measured.

Specimens were placed in culture tanks with aerated seawater that was partially exchanged once every day. In captivity, they were fed chopped fish and squid, as recommended by Lee and Morton (2005). The samples were then identified and sexed using key morphological characteristics as described by Yamasaki *et al.* (1988). The operational sex ratios (OSRs) defined as the ratio of adult individuals (Emlen & Oring, 1977) was computed as the number of males per female. Size was determined by measuring the maximum prosomal carapace width to the nearest 0.02 mm using a Vernier calliper. To prevent duplications in the results, the captures were released at

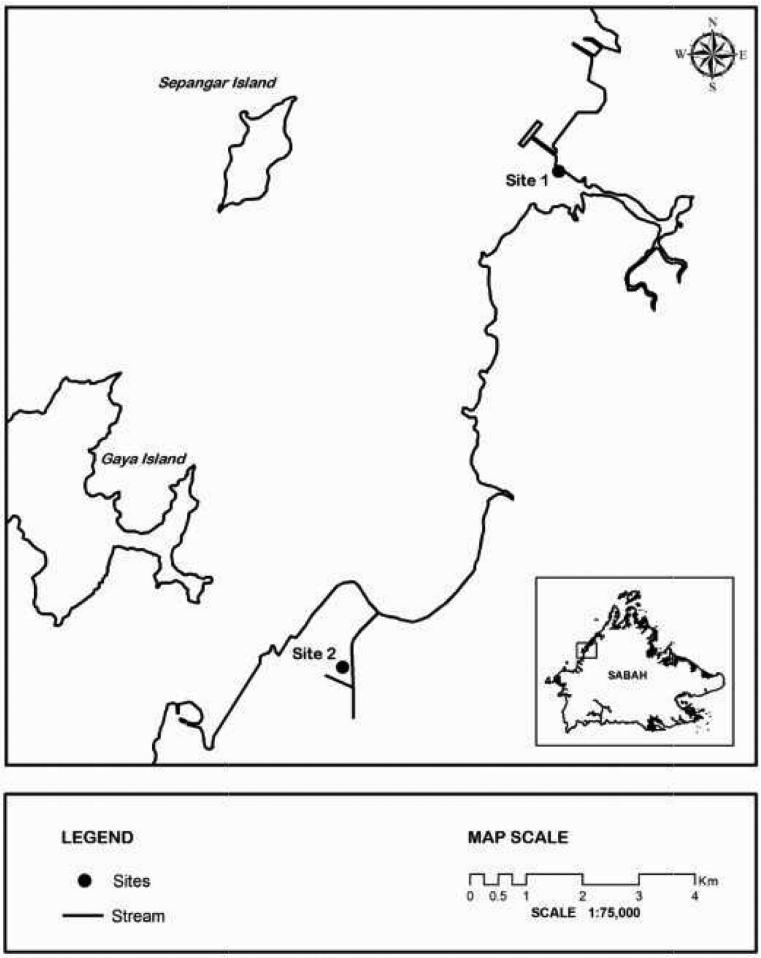

Fig.1: Map showing the sampling sites for horseshoe crabs in Kota Kinabalu

the original sites where they were collected only after sampling ceased.

Experiment of Mating Behaviour

A captive experiment to investigate the mating behaviour of *C. rotundicauda* was carried out using randomly selected individuals from Site 2. They were marked using oil paint with serial numbers and their sizes were measured and recorded, as shown in Table 1. Three circular HDPE tanks of 2m diameter, filled with 0.5 m deep seawater, were used (Tanks A, B, and C). Four males were put in each tank before four females were placed in Tank A, two in Tank B, and one in Tank C to give a male:female ratio of 1:1, 2:1 and 4:1, respectively. The experiment commenced when the females were added into the tanks. Observation was made for the first two hours to determine the duration for the formation of the first amplexus and subsequent observations for pair formations were made once every day at 1100 hours for 30 consecutive days.

RESULTS AND DISCUSSION

Adult *C. rotundicauda*, *T. gigas*, and *T. tridentatus* were found in Site 1 during

TABLE 1
Serial number, size (in mm) and maturity conditions of the male and female individuals used in the captive experiment on mating behaviour

Tank (OSR)	Horseshoe crab no.	Prosomal width (mm)	Maturity condition
Tank A (1:1)	CRM1	137.00	Mature
	CRF1	151.38	Ovigerous
	CRM2	123.62	Mature
	CRF2	152.00	Ovigerous
	CRM3	138.00	Mature
	CRF3	162.26	Ovigerous
	CRM4	128.40	Mature
	CRF4	159.10	Ovigerous
Tank B (2:1)	CRM1	133.34	Mature
	CRF1	136.70	Ovigerous
	CRM2	124.82	Mature
	CRF2	159.30	Ovigerous
	CRM3	124.34	Mature
	CRM4	135.40	Mature
Tank C (4:1)	CRM1	126.28	Mature
	CRF1	150.26	Ovigerous
	CRM2	138.52	Mature
	CRM3	127.50	Mature
	CRM4	126.88	Mature

surveys in October 2008 through January 2009. However, only adult *C. rotundicauda* and juvenile *T. tridentatus* were found during the January through April 2013 surveys in Site 2. Nonetheless, this finding confirmed the previous report by Sekiguchi (1988a) on the overlapping habitat of all three species in Sabah. A female *T. tridentatus* from Site 1 was 421.0 mm, larger than the 393.0 mm reported by Yamasaki (1988) who noted that *T. tridentatus* from Kota Kinabalu were the largest compared to its conspecifics in Southeast Asia. Of the 61 individuals from Site 1, 53 were *C. rotundicauda* (52 adults and a small juvenile). The OSR of *C. rotundicauda* in Site 1 was male-biased, with only eight (15%) adult females and 44 (85%) adult males to give an OSR of 5.50. The size of the males was averaged at 130.0 ± 5.3 mm (± *S.D*; *n* = 44), ranging from 120.4 mm through 145.3 mm, while the females were averaged at 150.8 ± 7.7 mm (± *S.D*; *n* = 8) and ranged from 139.6 mm through 160.9 mm (Figures 2 and 3). This indicates that the females are 16% larger than the average males.

Slightly more *C. rotundicauda* were caught in Site 2. The captures comprised of 68 adult *C. rotundicauda* and four juvenile *T. tridentatus*. There were 49 (72%) males and 19 (28%) females, giving a less-skewed OSR of 2.58. The average female size (± *S.D*) was 148.7 ± 10.7 mm, which is approximately 16% larger than the average male (± *S.*D; 128.2 ± 5.9 mm) (see Fig.2). The largest male was 143.9 mm, while the smallest was 115.4 mm, and the sizes of the females ranged between 129.0 mm through 171.2 mm (the largest *C. rotundicauda* in this study) (Fig.3). Adults from Site 2 had a wider size range but they were on average smaller than the adults in Site 1 (Fig.2); this is attributable to inter-population variations. Eighteen amplexed pairs were collected at Site 1. The mean (± *S.D*) size of the females in amplexus was 149.4 ± 10.6 mm (*n* = 18). The mean size (± *S.D*) of the amplexed males was 129.3 ± 6.8 mm (*n* = 18), which was slightly larger than the size of solitary males (± *S.D*; 127.6 ± 5.4 mm, *n* = 31). Only one pair was size-matched as the amplexed females were always bigger than their mate, with the mean percentage difference (± *S.D*) of 15.8 ± 10.5% (*n* = 18) ranging from as little as 3% to 37%.

In the captive experiment, the first amplexus was formed 55 minutes after the experiment had commenced in Tank B. The amplexus were more frequently formed in Tank A, observed in 18 days out of 30. The longest lasted for eight days between CRF3 and CRM4 in Tank A. They were a recurrent pair, forming amplexus on three separate occasions for a total 11 days. The biggest female in Tank A (CRF3) formed amplexus with the highest frequency and it was the only female which was courted by every male. The biggest female in Tank B (CRF2) was the only one paired, while the other female was ignored. Some individuals did not form amplexus throughout the experiment. The average number of days (± *S.D*) for every pairing episode was 2.44 ± 2.03 days.

In captivity, the females in amplexus were always bigger than the males attached

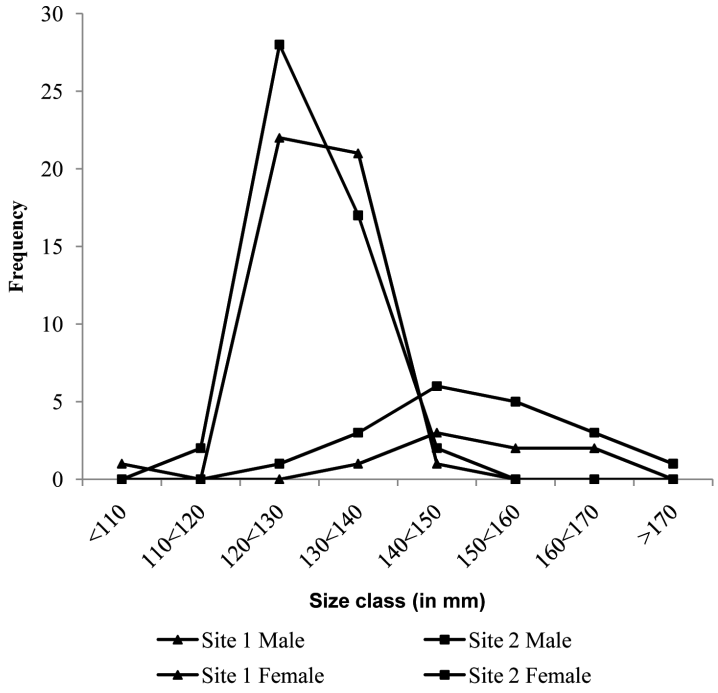

Fig.2: Frequency of size class intervals of the male and female *Carcinoscorpius rotundicauda* from Site 1 and Site 2

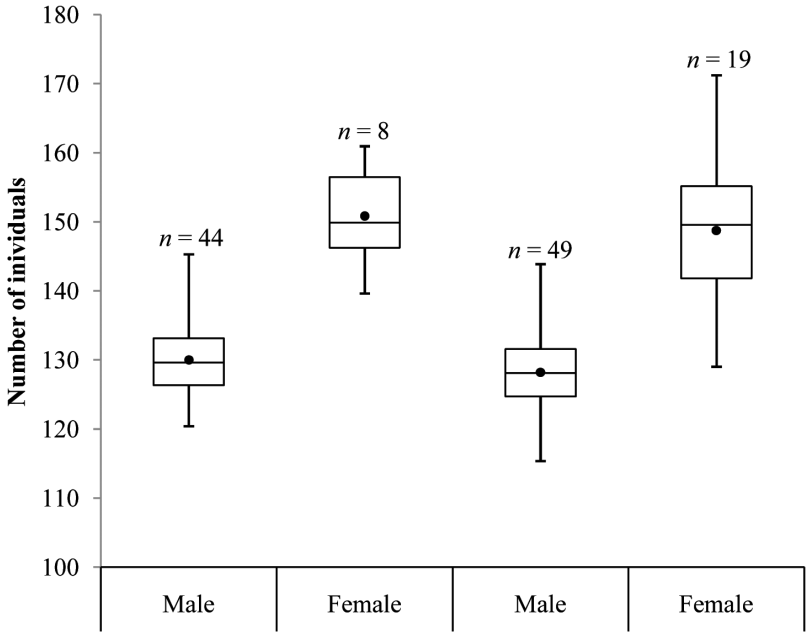

Fig.3: Size distribution of male and female individuals caught from Site 1 and Site 2

to them, reflecting the conditions in the wild. Correlation analyses with levels of significance of 0.10 were employed to illustrate the relationship between size of individuals and their propensity to form amplexus. Although the correlation was weak, the negative coefficient (r) value (r = -0.184; p = 0.10, $d.f$ = 10) suggested that smaller males formed amplexus more frequently as compared to larger ones (Fig.4).

On the contrary, the frequency of a female to form amplexus was positively correlated with size (r = 0.678; n = 0.10, $d.f$ = 5), and this indicated the preference towards large-sized females in the mating system (Fig.5). The largest female in Tank A was the only one which had formed a tandem when an additional male grasped on a paired male, which was never observed in the wild, supporting our theory that mating is size-assortative.

According to Cartwright-Taylor and Hsu (2012), breeding of *C. rotundicauda* in Singapore was year-round with no apparent seasonal pattern. However, the breeding period of *T. tridentatus* and *T. gigas* in Malaysia is thought to be seasonal (personal communication, Christianus, 2013), with marked increase in spawning activities towards the end of the year starting from September in Sabah (Muhammad Ali S. H.; personal observation). The presence of juvenile *T. tridentatus* at Site 2 from January

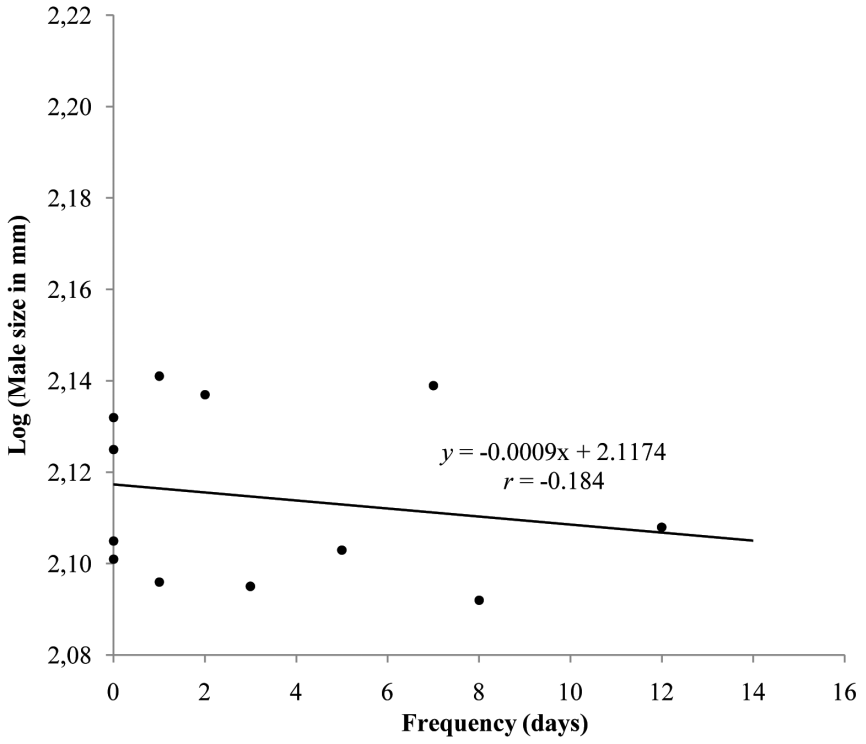

Fig.4**:** Correlation analysis of male body size and the frequency of forming amplexus

through April may be the product of recent spawning activities at the latter half of the previous year that resulted in recruitment of new individuals in the following months.

The OSRs of *C. rotundicauda* at both Sites 1 and 2 were male-biased but it was even more extreme at the former. Skews as high as 16 males per female (see Mattei *et al.*, 2010) in *L. polyphemus* were caused by selective harvest of larger and ovigerous females for the biomedical and bait industry (Carmichael *et al.*, 2003). Unlike *L. polyphemus*, large-scale commercial harvest of horseshoe crabs in Sabah is hitherto non-existent. However, during a survey carried out late November 2012 or before the survey at Site 2 was commenced, female tachypleans were seen being sold at a local market in Kota Kinabalu, purportedly caught opportunistically. In Southeast Asia, horseshoe crab eggs are highly sought-after despite documented cases of lethal poisoning after consuming toxic *C. rotundicauda* (Ngy *et al.*, 2007; Kanchanapongkul, 2008). The fishing communities in Kota Kinabalu are aware of this, and this explains why *C. rotundicauda* are not sold to the public. Nonetheless, they claimed to know the organs that need to be removed when preparing a dish of *C. rotundicauda* admitting that the females have been captured occasionally for their own consumption. Perhaps, the extremely low number of females in Site 1 was due to

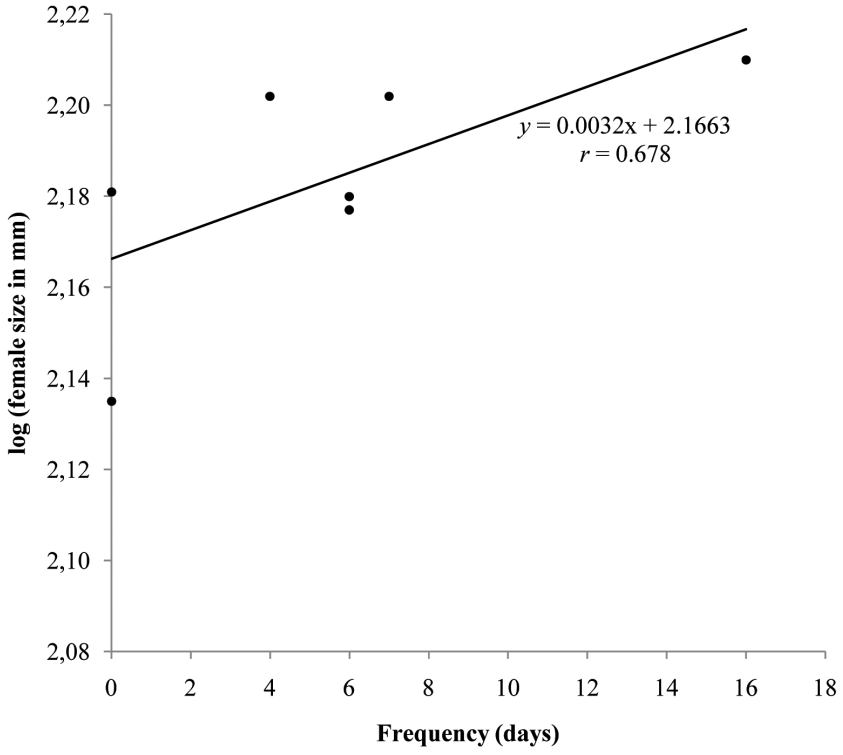

Fig.5: Correlation analysis of female body size and the frequency of forming amplexus

the selective removal of females trapped in fishing nets set up around Site 1.

Even though the adults in Site 2 were not subjected to the same anthropogenic influences as that of Site 1, there was still a slight imbalance in OSR that might be caused by sex-specific spatio-temporal segregation as previously reported in *L. polyphemus*. During spawning periods, unpaired females remained offshore while amplexed ones would travel to spawning grounds above the high tide mark accompanied by solitary males which clustered around spawning pairs (Brockmann, 1990). Of the 19 adult females caught in Site 2, only one was solitary, while others were in amplexus that lent credence to the theory that solitary females did not travel upstream. Female *L. polyphemus* would leave to deeper water after egg-laying, while solitary males would frequent the spawning beaches (Cohen & Brockmann, 1983; Brockmann, 1990; Smith *et al.*, 2013), indicating that over a spawning cycle, the number of males at a spawning beach would be consistent while the number of females would decline.

Despite the differences in the average sizes of individuals at both sites attributable to population variations, the proportion of sexual size dimorphism was similar at both sites. The females were 16% larger than the males, whereas ranges previously reported by Yamasaki *et al*. (1988) and Srijaya *et al.* (2010) were from 4% through 15%. Coincidentally, the mean size of the amplexed females was also 16% larger than the amplexed males. Adult males in all species of horseshoe crabs are smaller than females and tend to be within a narrower range of sizes. Male horseshoe crabs reach terminal moulting upon attaining sexual maturity where their pedipalps (in all extant horseshoe crabs) and the first ambulating legs (in Asian horseshoe crabs) are modified to form claspers. The reproductive benefit of having smaller body size is that males have better mobility for mate searching ergo increasing their chances of finding a mate.

Female horseshoe crabs may grow larger than males because of the continuous growth spurred by juvenile hormones (Levin, 2003). Females may moult continuously throughout their lives, enabling them to attain significantly larger sizes than males. In a mating system where females have to carry males, maintenance of a female-biased size dimorphism is necessary due to loading constraints exerted on them (see Otronen, 1997). In captivity, male *C. rotundicauda* in an amplexus may be very persistent, clinging onto the same female usually for more than one day. Extensive copulation time coupled with resistance of aquatic locomotion in the wild would exacerbate loading constraints on female horseshoe crabs. Consequently, they possess larger body sizes to compensate for the higher bioenergetics requirements of mating (Crespi, 1989). In addition, female size was found to be positively correlated with fecundity in horseshoe crabs (Chatterji & Parulekar, 1992; Leschen *et al.*, 2006). The two factors combined were suspected to be key factors why males showed preference for the largest females in captivity, resulting

in size-assortative mating, and in turn, female-biased size dimorphism in the extant horseshoe crabs.

Females exhibited polyandry as they acquiesced to any courting male evident through the lack of resistance to grasping males or attempts to dismount them. Horseshoe crabs have highly-sensitive mechanoreceptors throughout their bodies that enable them to detect important physical cues such as hydraulic pressure and to locate mates (reviewed in Saunders *et al.*, 2010). Females in amplexus grasped by hand during sampling displayed an exaggerated response to this stimulus, ostensibly to forcefully dissociate themselves to ease escape. In captivity, a courting male manoeuvred itself deliberately around a female before grasping the latter's opisthosoma. Grasped females were rarely startled by the males' advances as indicated by their calm demeanour.

Results of the captive experiment showed that smaller males had formed amplexus more frequently than the larger males (Fig.4; r = -0.184, n = 12, P = 0.10), although in the wild amplexed males were of similar size to solitary males, showing size-independent mating. Despite having smaller body size, there was a lack of size constraint for males to form amplexus, as demonstrated by a pair of *C. rotundicauda* from Site 2 that consisted of a female which was 37% larger than the male, twice the average size difference. Younger males that have recently moulted are smaller in size and thus lighter in terms of weight, which is more favourable for the females. Older individuals can be visually discerned from younger individuals by having larger body sizes and their carapace appears darker due to degrading actions of chitinase bacteria and more heavily encrusted due to attachment of symbiotic epibionts (mainly barnacles and limpets) that contribute to an increase in their weight.

Asian horseshoe crabs have undergone further modifications in their morphology to remain amplexed for longer periods than *L. polyphemus* (Botton *et al.*, 1996). Prolonged precopulatory amplexus ensures that the male retains access to the female when breeding periods commence, and thus, ensuring its reproductive success (Rudloe, 1980). An alternative mating tactic for solitary males, which is common in older *L. polyphemus*, is to engage in sperm competition with amplexed males and amongst themselves without investing in primary access to a female (Brockmann, 1990; Smith *et al.*, 2013). Hordes of solitary males (n = 31) were captured swimming along with amplexed pairs in Site 2, and excess males from Site 1 were most probably solitary as well, indicative of an alternative mating tactic in *C. rotundicauda*. Solitary males may still contribute to the genetic diversity of offspring despite being deprived of an exclusive mate in addition to ensuring a higher rate of fertilisation of the eggs.

CONCLUSION

The two populations studied in this work can be ecologically distinguished from each other mainly from the discrepancies in term of their OSRs. Lack of protection on horseshoe crabs would result in a further

decline of its already low numbers in the vicinity of Kota Kinabalu. Since it takes almost a decade for an individual to attain maturity, loss of adults due to natural and anthropogenic factors may compromise the long-term health of a population and affected populations may require longer time to rebound. Baseline data of horseshoe crabs at other locations in Sabah and studies on their breeding periodicity are therefore necessary to instigate conservation and protection measures of the valuable species.

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Journal of Tropical Agricultural Science

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(Manuscript Preparation & Submission Guidelines)

Revised: June 2014

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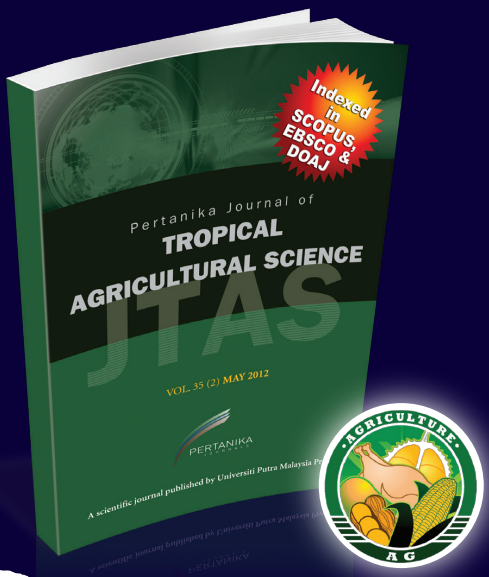

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