Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 36 (3) AUG. 2013

PERTANIKA
JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

Journal of Tropical Agricultural Science

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).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published four times a year, i.e. in **February**, **May**, **August** and **November**.

Goal of *Pertanika*

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Indexing of *Pertanika*

Pertanika is now over 33 years old; this accumulated knowledge has resulted in *Pertanika* JTAS being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge (BIOSIS & CAB Abstracts), EBSCO, DOAJ, CABI, AGRICOLA, ISC and MyAIS.

Future vision

We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

Aims and Scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: *agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance,* and *veterinary medicine*.

Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Editorial Board

2013-2015

Pertanika Editorial Office

Office of the Deputy Vice Chancellor (R&I), 1st Floor, IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8947 1622
E-mail: executive_editor.pertanika@upm.my
URL: http://www.pertanika.upm.edu.my/editorial_board.htm

Publisher

The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
Tel: +603 8946 8855, 8946 8854 • Fax: +603 8941 6172
E-mail: penerbit@putra.upm.edu.my
URL : http://penerbit.upm.edu.my

Pertanika Journal of Tropical Agricultural Science
Vol. 36 (3) Aug. 2013

Contents

Foreword

Welcome to the **Third Issue 2013** 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 **seven articles,** out of which **one** is short communication, while **five** are regular research papers and **one** is a case study. The authors of these articles are from **Malaysia, Nigeria and Thailand**.

The short communication reports an update made by the group of researchers in Malaysia. The group reports that the use of goat manure to fertilise grasses grown for animal feed may lead to parasitic diseases, specifically by strongyle infection (*Basripuzi, H. B., Sani, R. A., Ariff, O. M.* and *Chandrawathani, P.*). The study also suggests that the manure can still be applied as a safe fertiliser.

The five research papers cover a wide range of topics. A group of researchers from the National Fish Health Research Centre, Malaysia, reports an infestation of protozoan parasites in imported mangrove oysters. (*Kua, B. C., Mohd. Salleh, M. T.* and *Noraziah, M. R.*). The other research group from Universiti Malaysia Sarawak, Malaysia, describes in detail the genetic variations and population structure of the genus *Cynopterus*, a megabat in Malaysia (*Fong, P. H., Yuzine, E.* and *Abdullah, M. T.*), while another group from Malaysia describes the vitrification technique on *Lignosus rhinocerus* that can preserve dikaryotic mycelial cells with 100% regeneration and without trace of genotoxicity (*Lai Wei Hong, Ninie Noor Diana Enche Baharuddin, Shu San Loo, Azura Amid, Fauzi Daud, Abas Mazni Othman* and *Norihan Mohd Saleh*).

The researchers from Nigeria describe in detail the influence of feed restriction and realimentation on performance and carcass characteristics of growing rabbits in a humid environment (*Adeyemi, O.A, Ajeboriogbon, O.O.* and *Aderinoye, S.A.*), while another group from Nigeria reports that the root knot nematodes infection on cowpea can be managed with the leaf powder of *Azadirachta indica, Parkia biglobosa, Eucalyptus gigantea* and *Cassia siamea* (*Chimbekujwo, I. B., Bukar, A. M.* and *Channya, F. K.*).

The last manuscript in this issue is a case study by a group of researchers from Thailand. They report that electroejaculation was useful for semen collection in perineal urethrostomy goat. (*Sringam, S., Suwanathada, P., Sangkaew A., Thuangsanthia, A.* and *Leingchareon, N.)*.

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.

Chief Executive Editor
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

Pertanika J. Trop. Agric. Sci. 36 (3): 211 - 216 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Short Communication

Presence of Parasite Larvae in Goat Manure for Use as Fertiliser

Basripuzi, H. B.*[1], Sani, R. A.[2], Ariff, O. M.[3] and Chandrawathani, P.[4]

[1]*Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Locked Bag 36, 16100 Pengkalan Chepa, Kota Bharu, Kelantan, Malaysia*
[2]*Research Centre for Ruminant Diseases, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Veterinary Research Institute, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia*

ABSTRACT

Some livestock farmers utilise goat manure to fertilise grasses grown for animal feed, which may lead to parasitic diseases caused by strongyle infection. Therefore, the presence of strongyle larvae in manure needs to be determined. In this study, goat faeces containing strongyle eggs were deposited into five replicates for daily sampling throughout 23 days and subjected to faecal egg count, larvae identification and enumeration. Absence of eggs was detected on Day 4 when the infective larvae of *Haemonchus contortus, Trichostrongylus* sp. and *Oesophagostomum* sp. were found. Larvae counts reached a maximum of 164 larvae on Day 8 and were negligible by Day 14, by which time the manure can be used as fertiliser to grow forage crops for animal feed.

Keywords: Strongyle larvae, goats, manure, fertiliser

ARTICLE INFO

Article history:
Received: 15 August 2012
Accepted: 2 November 2012

E-mail addresses:
basripuzi@yahoo.com (Basripuzi, H. B.)
rehana@vet.upm.edu.my (Sani, R. A.),
ariff@vet.upm.edu.my (Ariff, O. M.),
chandra1959@gmail.com (Chandrawathani, P.)
* Corresponding author

INTRODUCTION

One of the major infections affecting small ruminants throughout the tropics and sub-tropics is gastrointestinal strongylosis (Sani *et al.*, 2004a; Jackson & Miller, 2006; Krecek & Waller, 2006). The infected animal passes out strongyle eggs with its faeces and upon hatching, develop into free-

ISSN: 1511-3701

living stages, namely, first (L1), second (L2) and third (L3) stage larvae. The infective larvae L3 migrate to grass and are the source of infection to the grazing animal (Barger, 1999).

The survival of L3 in tropical or sub-tropical countries is much shorter than in temperate regions because of the limited feed reserves in the non-feeding larval stage (Waller, 2006). An investigation on the survival of L3 on open pasture and vegetation under tree crops in Malaysia revealed that L3 emerged within one week with a minimum time of 3.5 days after faecal deposition and survived up to eight weeks (Sani *et al.,* 2004b). Livestock farmers usually apply the available manure on their farms to fertilise grasses grown for animal feed. Knowing the survival period of strongyle L3 in animal manure is essential to reduce L3 ingestion by the animals.

Therefore, the objective of the present study was to determine the presence of strongyle larvae in goat manure to assess its safety for use as fertiliser.

MATERIALS AND METHODS

Site and Animals

A study was carried out at the Goat Unit, Livestock Section, University Agricultural Park, Universiti Putra Malaysia (UPM). Information on weather parameters during the study was obtained from the Department of Land Management, Faculty of Agriculture, UPM. The location was recorded as having a total rainfall of 273.5 mm in November 2011 when this study was conducted.

A goat house with raised wooden slatted floor and concrete ground floor was located at this unit. The grass growing in the fenced area around the goat house was maintained at a height of approximately 5 cm throughout the year. Twenty-two goats of both sexes comprising the breeds of Katjang, Saanen and Boer crosses which ranged from less than 1 year-old to more than 5 year-old were raised in this house. The goats were last dewormed with ivermectin six months prior to the study.

Faecal Egg Count

Out of the 22 goats, 18 were detected to be infected with gastrointestinal strongyles by the modified McMaster technique (Lyndall-Murphy, 1993) 24 hours before the start of the study. The faecal egg counts (FEC) of the 18 goats ranged from 100 to 8900 eggs per gram (epg) of faeces with a mean FEC of 2078 epg.

Deposition and Sampling of Faeces on Plots

Five plots separated by a distance of at least 1.5 m representing five replicates were identified in the area to deposit the faeces. The faeces deposited by animals over a 24-hour period were collected from the concrete ground floor and mixed thoroughly prior to deposition on plots. The bulked faeces were divided into five replicates weighing approximately 1 kg each and heaped on each plot to imitate a natural deposit in mound-form as usually practised by the farmers. Faecal samples were collected daily in the

morning from each mound of faeces until eggs and strongyle larvae were no longer detected. One gram of faeces was need for FEC, whereas approximately 5 grams of faeces was collected for larvae recovery from each mound of replicate.

Parasitological Techniques

Each faecal sample was subjected to FEC by the modified McMaster technique until zero epg was detected to confirm the absence of eggs in all replicates. The samples were also subjected for larvae recovery by the modified Baermann method (van Bezooijen, 2006) that was carried out from Day 4 of zero epg until no free-living larva was recovered. The larvae were collected for identification and enumeration of pre-infective and infective stages. Identification of L3 was done to the genus level.

RESULTS AND DISCUSSION

FEC of 2000 epg indicated a worm burden of 1800 adult *Haemonchus contortus* and 1500 adult *Trichostrongylus* sp. (Israf *et al*., 1996). The results of the present study indicated that the goat farm with a mean FEC of 2078 epg revealed a parasitic problem. Mean FEC of manure replicates declined from 660 epg on Day 1 to 0 epg on Day 4. The absence of strongyle eggs starting from Day 4 indicated that by then all eggs had hatched. This led to the detection of both pre-infective and infective larvae on Day 4 until Day 22 (Fig.1). The pre-infective strongyle larvae consisted of L1 and L2, which were observed on Days 4 and 5. Their absence thereafter indicated that the pre-infective larvae had moulted into infective larvae by Day 6. The findings were consistent with those of Sam-Mohan (1995), who observed that L3 emerged from the faeces on Day 4 and migrated to herbage on Day 6.

Fig.1 shows that the number of *Haemonchus contortus* larvae peaked on Day 6 with 127 larvae and decreased steadily thereafter until no larva was detected on Day 21. *Trichostrongylus* sp. infective larvae existed longer until Day 22 when only one L3 was found. It peaked with 85 larvae on Day 8. *Oesophagostomum* sp. larvae were recovered in low numbers throughout the study with the highest count of 10 on Days 8 and 9 and absent by Day 16. Overall, the highest L3 count on the manure was observed on Day 8 with 164 larvae, which consisted of *Haemonchus contortus, Trichostrongylus* sp. and *Oesophagostomum* sp. In a study by Dobson *et al.* (1990a), geometric mean of total worm burden observed in lambs infected with 200 *Trichostrongylus colubriformis* L3 per day, 5 days per week for 7 weeks was 3167. However, the estimated threshold worm burden of 3532 must be exceeded before any substantial resistance to infection begins to develop (Dobson *et al.,* 1990b). Therefore, small numbers of L3 ingested continuously contributed to a build up of total adult worm burden which led to devastating effects on the goats.

Following the pattern of larvae presence in the faeces, the goats might be predisposed to parasitism if the manure containing L3 was used as fertiliser on

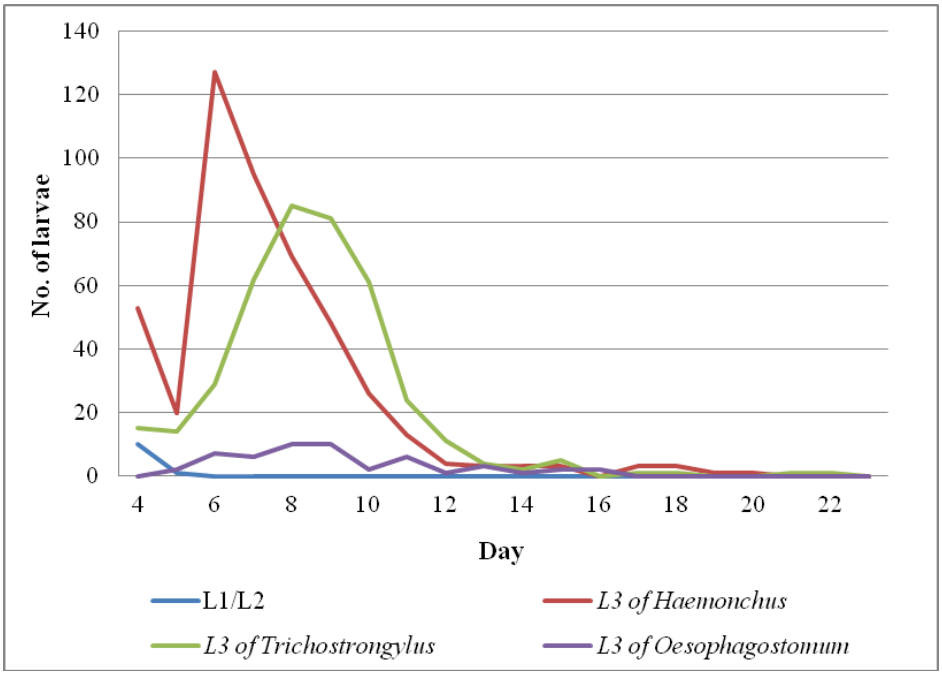


Fig.1: Presence of strongyle L1, L2 and L3 in goat manure.

grasses between Days 4 to 14 of faecal deposition. However, all the larvae were undetected on Day 23 due to their ability to migrate to a more favourable micro-environment, which also attributed to their longevity (O'Connor, 2006). Consequently, goat manure purposely accumulated in the farm to be used as fertiliser should be kept for at least 14 days before being applied on grasses meant for animal feed as the larvae count was found to be negligible by that time. Throughout the study, *Haemonchus contortus* (51%) was the most predominant species followed by *Trichostrongylus* sp. (43%) and *Oesophagostomum* sp. (6%).

CONCLUSION

The most hazardous time to use goat manure as fertiliser was on the eighth day after manure deposition as it contained the highest number of infective larvae. The recovery of infective larvae of gastrointestinal strongyles in goat manure until Day 22 indicated that an original manure mound made without continuous addition of fresh faeces was free of the larvae from Day 23. However, the negligible number of L3 in the mound by Day 14 suggests that the manure when applied as fertiliser to grasses and other forage crops did not harbour many infective larvae.

REFERENCES

Barger, I. A. (1999). The role of epidemiological knowledge and grazing management for helminth control in small ruminants. *International Journal for Parasitology, 29*(1), 41-47.

Dobson, R. J., Waller, P. J., & Donald, A. D. (1990a). Population dynamics of *Trichostrongylus columriformis* in sheep : The effect of infection rate on loss of adult parasites. *International Journal of Parasitology, 20*(3), 359-363.

Dobson, R. J., Donald, A. D., Barnes, E. H., & Waller, P. J. (1990b). Population dynamics of *Trichostrongylus columriformis* in sheep: Model to predict the worm population over time as a function of infection rate and host age. *International Journal of Parasitology, 20*(3), 365-373.

Israf, D. A., Sani, R. A. and Halim, R. A. (1996). Caprine helminthiasis : Relationship between faecal egg count and worm burden. *Jurnal Veterinar Malaysia, 8*(1), 33-35.

Jackson, F., & Miller, J. (2006). Alternative approaches to control—Quo vadit?. *Veterinary Parasitology, 139*, 371-384.

Lyndall-Murphy, M. (1993). Anthelmintic resistance in sheep. In L. A. Corner & Bagust, T. J., *Australian standard diagnostic techniques for animal diseases* (pp. 8-9). CSIRO Publications.

Krecek, R. C., & Waller. P. J. (2006). Towards the implementation of the ''basket of options'' approach to helminth parasite control of livestock: Emphasis on the tropics/subtropics. *Veterinary Parasitology*, *139*, 270–282.

O'Connor, L. J., Walkden-Brown, S. W., & Kahn, L.P. (2006). Ecology of the free-living stages of major trichostrongylid parasites of sheep. *Veterinary Parasitology, 142*, 1–15.

Sam-Mohan. (1995). Epidemiology of Trichostrongylus of sheep grazing under oil palm. (MSc. Thesis dissertation). Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Sani, R. A., Adnan, M., Cheah, T. S., & Chandrawathani, P. (2004a). Helminth control for small ruminants in Malaysia. *Jurnal Veterinar Malaysia, 16* (1 & 2), 1-8.

Sani, R. A., Adnan, M., Cheah, T. S., & Chandrawathani, P. (2004b) Worm control for small ruminants in Malaysia. In Sani, R. A., G. D. Gray, & R. L. Baker, *Worm control for small ruminants in Tropical Asia* (pp. 189-199.). ACIAR Monograph 113.

van Bezooijen, J. (2006). *Methods and techniques for nematology*. p. 20. Wageningen University.

Waller, P. J. (2006). Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. *Animal Feed Science and Technology*, *126*, 277–289.

Pertanika J. Trop. Agric. Sci. 36 (3): 217 - 224 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

A Case Study of Protozoan Parasite Gregarine *Nematopsis* spp. (Apicomplexa: Sporozoa) Infestation in Mangrove Oyster *Crassostrea belcheri* Imported from Thailand

Kua, B. C.[1]*, Mohd. Salleh, M. T.[2] and Noraziah, M. R.[1]

[1]National Fish Health Research Centre, NaFisH FRI Batu Maung, 11960 Batu Maung, Penang, Malaysia
[2]National Fish and Prawn Production and Research Centre, FRI Pulau Sayak, 08500 Kota Kuala Muda, Kedah, Malaysia

ABSTRACT

One hundred mangrove oysters (weight: 248.41 ± 28.41g and length: 10.69 ± 0.73cm) from Thailand meant for human consumption were examined for parasite infestation. Gross observation showed that the infestation comprised mostly cliona (43%) followed by mud (1%) and water blister (1%). Histopathological study revealed high prevalence of gregarine *Nematopsis* sp oocysts (99%), metaplasia (92%), inclusion cells (15%), brown cells (14%), oedema (5%), ceriod (8%) and abscess (1%). A large number of phagocytes with gregarine oocysts inside the gill and connective tissues were observed. Most of the oocysts were located within parasitophorous vacuoles and phagocytes. The oocysts appeared ellipsoidal at 73.55µm wide and 140.56µm long with a thick outer surface. The clusters of oocysts consisted of a single vermiform sporozoite engulfed by phagocytes. The number of oocysts in each phagocyte varied ranging from 1 to 9 with an average of 4.9. The findings revealed that gregarine oocysts did not cause much pathological damage as there was no irregular arrangement and disruption of gill filament or loss of cilia. Similar histopathology was also observed in four molluscs (i.e. *Anadara granosa, Perna viridis* and *Arcuatula arcuatula* and *Paphia undulate*) as reported in Thailand, and three cultivated molluscs (*A. granosa, P. viridis* and *C. iredalei)* as reported in Malaysia. The present study provides significant baseline information on the health profile of the important molluscan species, particularly on *Nematopsis* spp. infestation in oysters.

ARTICLE INFO

Article history:
Received: 5 July 2010
Accepted: 26 January 2013

E-mail addresses:
kuabeng@fri.gov.my (Kua, B. C.)
salehtaha93@yahoo.com (Mohd. Salleh, M. T.),
norazianr@gmail.com (Noraziah, M. R.)
* Corresponding author

ISSN: 1511-3701

Keywords: Mangrove oyster, *Crassostrea belcheri*, protozoa, *Nematopsis* sp., oocysts

INTRODUCTION

Crassostrea belcheri, known locally as mangrove oyster or white-scar oyster in Thailand, is mainly cultured in Asia, particularly in Thailand and Malaysia. Four species of oysters are found in Malaysia, namely, *Crassostrea belcheri, C. rivularis, Saccostrea cucullata* and *Ostrea folium*. Among the four species, *C. belcheri* is mainly cultured with an average production of 1000 tones per year. The production of oysters in Malaysia is low compared to the production of other bivalve species (Table 1) (Annual Fisheries Statistics 2006, 2007, 2008, 2009 & 2010). *Crassostrea belcheri* is in high demand from the local Malaysian seafood market, and generally, the demand is higher than the local supply. The importation of oysters from Thailand is an alternative measure by local suppliers to fulfill the high demands. Generally, the oysters are sold in shell-on forms which are consumed fresh.

In Malaysia, a monitoring programme on the molluscan health focusing on the Office International des Epizooties (OIE) revealed that gregarine was the most frequently listed parasite since 1999 until 2009, which showed that gymnospores of gregarine (*Nematopsis* sp.) were the parasites most frequently encountered in tropical oysters (*Crassostrea iredalei*) and blood cockles (*Anadara granosa*). Although they can be observed in most of the specimens in both cultured species, no significant health effects have been found. Bower and McGladdery (2001) reported that gymnospores and oocysts of gregarines are usually associated with a focal, benign inflammatory response and there is no serious effect on or damage to the host. Gymnospores need marine arthropods such as crabs or shrimps in order to complete their life cycle. Hence, multiplication of gregarines is limited to bivalves.

Mass mortality caused by gregarine *Nematopsis* sp. has not been reported in Malaysia although mass mortality of cockles and clams in Portugal had been associated with it (Azevedo & Cachola, 1992). Infection of Apicomplexan protozoan *Nematopsis* has been regularly reported in molluscs, which acts as the intermediate host with crustaceans as the definitive host (Spraque, 1970; Lee *et al*., 2000; Tuntiwaranuruk *et al*., 2004 and 2008). In Thailand, infestation of gregarine *Nematopsis* spp. has been widely reported in bivalve species, such as cockles (*A. granosa*), mytilid mussels (*P. viridis* and *A. arcuatula*) and venerid

TABLE 1
Total production of cultured molluscan (tonnes) in Malaysia from 2006 to 2010.

Species	2006	2007	2008	2009	2010
Blood cockle	45.674	49.620	61,138	64,938	78,024
Green mussels	6.904	4.034	8,993	10,596	10,529
Oysters	915	869	275	2,128	812

clams (*P. undulate*) except rock oysters (*Saccostrea cucullata*) during a survey of infestation of *Nematopsis spp* sporozoites in Thailand (Tuntiwaranuruk *et al*., 2004). *Nematopsis* sp., *Tylocephalum* sp., *digenetic trematodes* and *ciliates* were also reported from *C. iredalei* in the Philippines (Erazo-Pagador, 2010). In Malaysia, our survey of OIE listed parasites in tropical oysters (*C. iredalei*) in 2000 and 2003 found a prevalence of gregarine *Nematopsis* sp. oocyst, ranging from 33.33% to 73.33% (Kua & Taha, 2004).

Despite the commercial value and gradual increase in the production of *C. belcheri* in Malaysia, there has been no report or any specific study on problematic diseases of the oysters. Several reports on the diseases, particularly those caused by parasites, have been described from various shellfish in other countries (Sindermann, 1990). Itoh (2002) reported the paramysean ovarian parasite in pacific oysters and the economic loss caused by the disease, which was estimated to be a few hundred million Japanese yen in Okayama Prefecture. Imanaka *et al*. (2001) reported that the ovarian parasite may kill wild oysters. Taveekijakarn *et al*. (2008) also highlighted on the occurrence of *Marteilia* sp., *Perkinsus* sp., and trematodes in oyster samples from the Gulf of Thailand. In Malaysia, a molluscan health monitoring programme has been initiated focusing on the OIE listed parasites in three main species (*C. iredalei*, *P. viridis* and *A. granosa)*. In 2007, we had an opportunity to investigate 100 specimens of mangrove oysters (*C. belcheri*) imported from Thailand as provided by the Fish Quarantine Centre, Bukit Kayu Hitam, Kedah. Thus, the main objective of this study was to examine the presence of parasites in these imported mangrove oysters. The findings were significant and contributed new knowledge to the research community, especially on *Nematopsis* infestation in the commercially important mangrove oysters, *C. belcheri*.

MATERIALS AND METHODS

All the 100 specimens of *C. belcheri* were measured, opened and examined for colour, conditions (fat, medium or watery), macroparasites, as well as shell and tissue abnormalities. The body was removed and fixed in 10% buffered formalin for 4 hours. The fixed specimens were then cut in cross-section at the gills, stomach, digestive gland and intestine with 2-3 cm of thickness. The specimens were fixed again with the same fixative for another 24 hours before being processed by an automatic tissue processor (Leica ASP 300, Germany) and embedded in paraffin wax. The embedded specimens were sectioned at 5 µm thick, stained with haematoxylin and eosin (H&E), and finally mounted with DPX before being examined under a compound microscope (Leica DM5000B, Leica Microsystems, Germany) connected to a digital camera (Leica DFC 320, Leica Microsystems, Germany) and equipped with a computer software (Leica QWin, Leica Microsystems, Germany). The histological techniques were based on the method suggested by Humason (1979).

RESULTS AND DISCUSSION

Gross observation on the mangrove oysters showed mostly cliona (43%) followed by mud (1%) and water blister (1%). Fouling organisms, such as barnacles, ascidians and algae, were not observed during the study. The post-harvest cleaning process should have largely reduced the fouling organisms on the oysters before being exported to Malaysia, therefore, only a few fouling organisms were observed in the present study. Apart from the fouling organisms, small crabs and polychaete worms were also found on the soft tissues of the oysters. The cleaning process after the mangrove oysters were harvested from the culture sites before exporting to Malaysia resulted in the observation of a few fouling organisms.

Histopathological sections revealed high infestation of gregarine *Nematopsis sp*. oocysts (99%), metaplasia (92%), inclusion cells (15%), brown cells (14%), oedema (5%), ceriod (8%) and abscess (1%). Large numbers of phagocytes with gregarine oocyts were seen in the gills and connective tissues (Fig.1). Most of the gregarine oocysts were located within parasitophorous vacuoles and phagocytes (Fig.2). The clusters of gregarine oocysts showed individual gregarine oocysts, which consisted of a single vermiform sporozoite engulfed by phagocytes. The number of gregarine oocysts in each phagocyte varied from 1 to 9 with an average number of 4.9. The gregarine oocysts appeared ellipsoidal (73.55 ± 24.69 µm wide and 140.56 ± 27.77 µm long) with a thick outer surface. The comparison of the diameter of gregarine oocysts showed that there was a slight difference in size. The diameter of the single ellipsoidal oocyts in the study was bigger compared to *Nematopsis mytella* (12.9 – 13 µm long with 8.0 – 8.6 µm wide) as reported for *C. rizophorae* from Brazil (Padovan *et al*., 2003) and *Nematopsis spp.* (10 - 15 µm long with 6.4 - 12.7 µm wide) from Thailand (Tuntiwaranuruk *et al*., 2004).

Infestation of gregarine *Nematopsis sp* oocysts in other bivalve species has been documented (Table 2). However, gregarine *Nematopsis sp.* oocyst infestation of cultured or wild oyster *C. belcheri* from Thailand and Malaysia has never been reported. This study reports a prevalence of 99% of gregarine oocysts in *C. belcheri* from Thailand, which provides baseline information on health profile of the important molluscan species, particularly on *Nematopsis sp.* infestation in oysters. If high prevalence occurs under the condition of food shortage or reduced water flow, the abundant presence of parasites in the gills of oysters could further reduce gas exchanges and food intake, thus weakening the infected oyster.

However, there are several reports on high prevalence of gregarine oocyst infestation in mollusc, which do not show significant health effects. Carballal *et al*. (2001) mentioned the prevalence (76%) of *N. veneris*, *N. ostrearum*, and *N. schneideri*, *Nematopsis spp.* with low intensity occurring in clam, *Cerastoderma edule,* from 34 locations along the coasts of Galicia, Spain, which showed no impact on the health of the clam. Desser and Bower (1997) highlighted little evidence of pathological

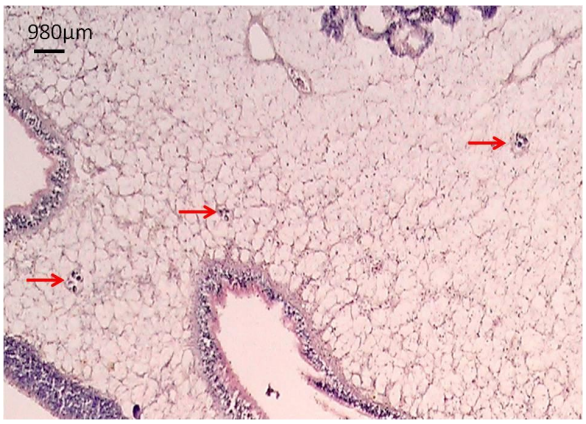


Fig.1: Phagocytes with gregarine oocyts (arrow) in connective tissues of mangrove oyster infested with *Nematopsis* sp. Hematoxylin and Eosin. Magnification: x40. Scale bar: 12 mm = 980 µm.

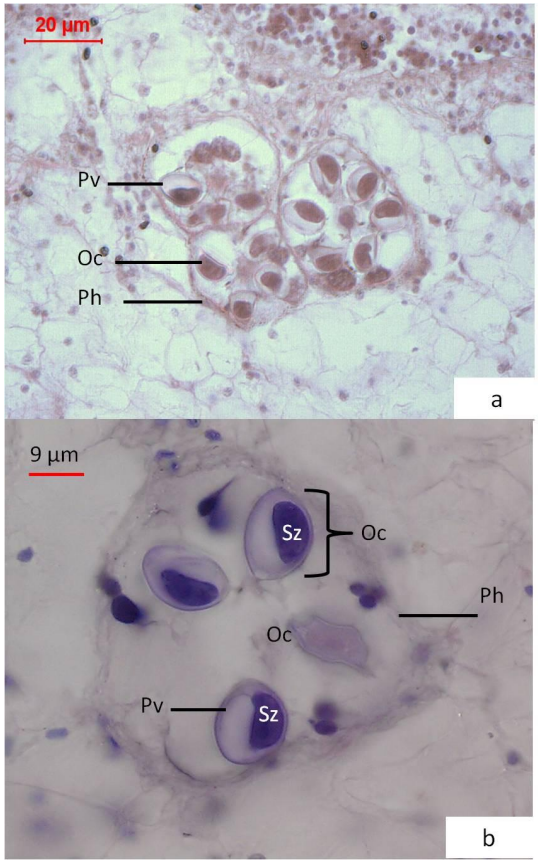


Fig.2: Histological section of connective tissue in mangrove oyster infected with *Nematopsis* sp. (a). Three phagocytes (Ph) of *C. belcheri* showing oocyts (Oc) within parasitophorous vacuoles (Pv) and (b). One phagocyte (Ph) with three oocysts (Oc), each one in a parasitophorous vacuole (Pv) with sporozoite(Sz). Hematoxylin and Eosin. Magnification: (a).x200, (b).x1000, Scale bar: (a). 3cm = 20 µm and (b). 1cm = 9 µm.

effect on *Protothaca staminea* even though cyst infestations were 70 and 100%. Uddin *et al*. (2011) showed that the blood cockles from the Straits of Malacca were infected with *Nematopsis sp*. without any sign of pathological damage. Despite wide reports of no pathogical impact of *Nematopsis sp.* infestation on bivalves, there were a few reports on the pathological significance of *Nematopsis sp.* (Azevedo & Cachola, 1992, Tuntiwaranuruk *et al*., 2004 and 2008).

In this study, pathological damage was caused by gregarine *Nematopsis* sp. Oocysts, which was comparatively minimal as compared to some irregular arrangement and disruption of gill filaments and loss of cilia in severe infection as reported by Tuntiwaranuruk *et al*. (2008). According to them, high infestation of *Nematopsis* sp. oocysts could cause a large number of phagocyte presence in the gill lumen of *Perna viridis* leading to the obstruction of water flow and indirectly reducing the filtering efficiency and food intake (Tuntiwaranuruk *et al*., 2004). The histopathology of this study was similar to previous cases on *A. granosa, P. viridis* and *A. arcuatula* and *P. undulate* reported in Thailand (Tuntiwaranuruk *et al*., 2004) and three cultivated bivalves (*A. granosa, P. viridis* and *C. iredalei)* in Malaysia .

TABLE 2
Nematopsis spp. reported from wild or cultured bivalves.

Host species	Bivalves group	Reported Nematopsis species	Location	References
Crassostrea rizophorae	*Oyster*	*Nematopsis mytella*	Brazil	Padovan *et al*., 2003
Callista chione	*Clam*	*Nematopsis* sp.	Italy	Canestri-Trotti *et al*., 2000
Anadara granosa	*Blood cockle*	*Nematopsis* sp.	Thailand	Tuntiwaranuruk *et al*., 2004
Anadara granosa	*Blood cockle*	*Nematopsis* sp.	Malaysia	Uddin *et al*., 2010
Perna viridis	Green Mussel	*Nematopsis* sp.	Thailand	Tuntiwaranuruk *et al*., 2004
Perna viridis	Green Mussel	*Nematopsis* sp.	Malaysia	Kua and Taha, 2004
Cerastoderme edule	Clam	*Nematopsis* sp.	Portugal	Azevedo and Cachola, 1992
Ruditapes decussatus	Clam	*Nematopsis* sp.	Portugal	Azevedo and Cachola, 1992
Arcuatula arcuatula	Clam	*Nematopsis* sp.	Thailand	Tuntiwaranuruk *et al*., 2004
Paphia undulate	mussel	*Nematopsis* sp.	Thailand	Tuntiwaranuruk *et al*., 2004
Crassostrea iredalei	*Tropical oyster*	*Nematopsis* sp.	Philippines	Erazo-Pagador, 2010
Crassostrea iredalei	*Tropical osyter*	*Nematopsis* sp.	Malaysia	Kua and Taha, 2004

CONCLUSION

In this study, high prevalence (99%) of mild to moderate gregarine *Nematopsis* infestation with phagocytic response was observed in the *C. belcheri* specimens imported from Thailand. This study provides important baseline information on health profile of the mangrove oyster *C. belcheri* to the research community, especially on *Nematopsis* infestation in oysters.

ACKNOWLEDGEMENTS

The authors are grateful to the Fish Quarantine Centre, Kedah, for supplying the specimens for this study.

REFERENCES

Azevedo, C., & Cachola, R. (1992). Fine structure of the apicomplexa oocyst of Nematopsis sp, of two marine bivalve mollusc. *Diseases of Aquatic Organism, 14,* 69-73.

Bower, S. M., & MacGladdery, S. E. (2001). *Synopisis of infectious Diseases and parasites of commercially exploited shellfish: Gregarine Parsitism of oysters and clam*. Retrieved from http://www-sci.pac.dfo-mpo.gc.ca/shelldis/pages/gegpoy_e.htm.

Carballal, M. J., Iglesias, D., Santamarina, J., Ferro-Soto, B., & Villalba, A. (2001). Parasites and pathologic conditions of the cockle Cerastoderma edule populations of the coast of Galicia (NW Spain). *Journal of Invertebrate Pathology, 78*, 87-97.

Department of Fisheries Malaysia (2006). *Annual Fisheries Statistics, 2006*. Department of Fisheries Malaysia (p.1-2). Retrieved from http://www.dof.gov.my/c/document_library/get_file?uuid=e7d61379-45cf-40d7-b0b1-fb1e08027518&groupId=10131

Department of Fisheries Malaysia (2007). *Annual Fisheries Statistics, 2007*. Department of Fisheries Malaysia.

Department of Fisheries Malaysia (2008). *Annual Fisheries Statistics, 2008*. Department of Fisheries Malaysia. Retrieved from http://www.dof.gov.my/html/themes/moa_dof/documents/Jadual_akuakultur_V_ii.pdf.

Department of Fisheries Malaysia (2009). *Annual Fisheries Statistics, 2009* (pp. 2-3). Department of Fisheries Malaysia. Retrieved from http://www.dof.gov.my/html/themes/moa_dof/documents/Jadual_akuakultur_V_ii.pdf

Department of Fisheries Malaysia (2010). *Annual Fisheries Statistics, 2010* (pp. 2-3). Department of Fisheries Malaysia. Retrieved from http://www.dof.gov.my/html/themes/moa_dof/documents/jadual_pendaratan_marin_%20aquaculture.pdf

Desser, S. S., & Bower, S. M. (1997). The distribution, prevalence, and morphological features of the cystic stage of an apicomplexan parasite of native littleneck clams (*Protothaca staminea*) in British Columbia. *The Journal of Parasitology*, *83*, 642-646.

Erazo-Pagador, G. (2010). A parasitological survey of slipper-cupped oyster (Crassostrea iredalei Faustino, 1932) in the Philippines. *Journal of Shellfish Research, 29*, 177-179.

FAO (1988). *Status of oyster culture in selected Asian countries. Fisheries and Aquaculture Department*. p. 97

Humsaon, G. L. (1979). *Animal tissue techniques*. San Francisco: W. H. Freeman and Company.

Imanaka, S., Itoh, N., Ogawa, K., & Wakanayashi, H. (2001). Seasonal fluctuations in the occurrence of abnormal enlargement of the ovary of pacific oyster *Crassostrea gigas* at Gokasho Bay, Mie, Japan. *Fish pathology, 36*, 83-91.

Itoh, N., Tadashi, O., Ogawa, K., & Wakanayashi, H. (2002). Identification and development of a paramyxean ovarian parasite in the pacific oyster *Crassostrea gigas. Fish pathology, 37*, 23-28.

Kua, B. C., & Taha, M.S. (2004). A Preliminary Observation of Parasitic Infestation on Blood Cockles (*Anadara granosa*) and Tropical Oyster (*Crassostrea iredalei*) in Northern Peninsular Malaysia. *Malaysian Fisheries Journal, 3,* 125-129.

Lee, J. J., Leedale, G. F., & Bradbury, P. (2000). *An illustrated guide to the protozoa* (2nd Ed.), Society of Protozoologists (pp. 190-369). USA: Allen Press.

Sindermann, C. J. (1990). Principal diseases of marine fish and shellfish (Vol. 2). *Diseases of marine shellfish* (2nd Edn). San Diego: Academic Press.

Somwang, S., Pairat, S., Janthira, K., & Toshiaki, O. (2010). Quality changes in oyster (*Crassostrea belcheri*) during frozen storage as affected by freezing and antioxidant. *Food Chemistry*, *123*, 286–290.

Taveekijakarn, P., Somsiri, T., Puttinaowarat,S., Tundavanitj, S., Chinabut, S., & Nash, G. (2008). Parasitic fauna of rock oyster (*Saccostrea forskali*) cultured in Thailand. In M. G. Bondad-Reantaso, C. V. Mohan, M. Crumlish & R. P. Subasinghe (Eds.), *Diseases in Asian aquaculture VI* (p. 335-342). Manila, Philippines: Fish Health Section. Asian Fisheries Society.

Tuntiwaranuruk, C., Chalermwat, K., Upatham, E.S., Kruatrachue, M., & Azevedo, C. (2004). Investigation of Nematopsis spp. Oocysts in 7 species of bivalves from Chonburi province, Gulf of Thailand. *Diseases of Aquatic Organisms, 58,* 47-53.

Tuntiwaranuruk, C., Chalermwat, K., Pongsakchat, V., Meepool, A., Upatham, E. S., & Kruatrachue, M. (2008). Infection of *Nematopsis* oocysts in different size classes of farmed mussel *Perna viridis* in Thailand. *Aquaculture, 281,* 12-16.

Uddin, M. Jasim., Zulfigar,Y., Munawar, K., & Shau-Hwai, A.T. (2010). Parasites of blood cockle (*Anadara granosa* Linnaeus, 1758) from the Straits of Malacca. *Journal of Shellfish Research*., *30*(3), 875-880.

Pertanika J. Trop. Agric. Sci. 36 (3): 225 - 248 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Genetic variations and population structure of the genus *Cynopterus* in Malaysia

Fong, P. H.[1]*, Yuzine, E.[1] and Abdullah, M. T.[1,2]

[1]*Molecular Ecology Laboratory, Department of Zoology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[2]*Centre For Pre-University Studies, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*

ABSTRACT

Nuclear microsatellite analysis of *Cynopterus* was aimed at characterising the microsatellite genotypes and the population structure of this genus especially in the large form of *C. brachyotis* and the small form of *C. brachyotis*. Nine pairs of existing microsatellite primers isolated from Indian *C. sphinx* were used. A total of 51 alleles and 97 genotypes were documented from four forms of *Cynopterus*. Genetic variations revealed from AMOVA analysis showed that there was low genetic variation among the four forms. The interspecies Global AMOVA comparison analysis showed that the genetic variation between the large and small forms of *C. brachyotis* was the lowest among interspecies comparisons. This resulted in low genetic structure in the UPGMA tree, and species boundary of each form was not clearly defined. This might due to the microsatellite primers that were isolated from Indian *C. sphinx* being low in sensitivity to detect variations in Malaysian cynopterans.

Keywords: Microsatellite analysis, *Cynopterus*

INTRODUCTION

Microsatellites or Simple Sequence Repeats (SSRs) are nuclear markers, as well as co-dominant Mendelian markers (DeWoody and Avise, 2000; Srikwan *et al.*, 2002; Scandura, 2004). In microsatellites, sequences are composed of repeated units of sequences; these repeats are generally two to five base pairs in length and are called di-, tri-, tetra- or pentanucleotides (Srikwan *et al.,* 2002). The dinucleotide CA repeats are most commonly found in many eukaryotes (Page & Holmes, 1998; Scandura, 2004).

ARTICLE INFO

Article history:
Received: 20 June 2011
Accepted: 13 August 2012

E-mail addresses:
fong.pooihar@gmail.com (Fong, P. H.)
eyuzine@frst.unimas.my (Yuzine, E.),
abdullahmt2@gmail.com (Abdullah, M. T.)
* Corresponding author

ISSN: 1511-3701

The mutation rate of microsatellites was estimated between 10^{-2} and 10^{-5} per gamete, per generation (Page and Holmes, 1998; Wright *et al.,* 2005), and these mutations directly influence the changes of repeat units (Degnan *et al.,* 1999). They vary greatly in copy number between individuals (Page and Holmes, 1998). This high level of diversity, together with characteristics such as neutral evolution, codominance and simple Mendelian inheritance allows microsatellites to be used to identify closely related individuals, recent migration events and sex dispersal patterns (Hedrick, 1999; Flagstad *et al.,* 2003; Hansson *et al.,* 2003).

The difference in the repeat units of nucleotides carries the information that was passed through from generations to generations from their common ancestral alleles. Recent common ancestry is shown by the similarity of the repeat number, thus the microsatellite approach can be used to examine some phylogenetic applications in mtDNA (Degnan *et al.,* 1999). The highly polymorphic nuclear loci can also be used to identify population specific polymorphisms (Bruford & Wayne, 1993).

Analysis of microsatellites can provide both paternal and maternal information (Burg *et al.,* 1999). These are important in providing more reliable genetic information such as evolutionary lineages between closely related species which cannot be answered or revealed using mtDNA based genetic markers. In one instance, Campbell *et al.* (2004) stated that without comparison of nuclear markers, they cannot rule out the possibility that *C. brachyotis* lineage may have a significantly longer and more complex evolutionary history than what was revealed from mitochondrial haplotype data.

In Malaysia, research focused on the species complex of *C. brachyotis* has been ongoing for many years. The first indication on the existence of two forms of *C. brachyotis* dates back to 1985 where Payne *et al.* (1985) noticed that there were two forms of *C. brachyotis*, namely, large-sized *C. brachyotis*, which can be found in forest edges and gardens, and small-sized *C. brachyotis*, which can be found in tall forests. Later, Francis (1990) mentioned the size difference of *C. brachyotis* captured in primary versus secondary forests. They stated that there was some morphological overlap and that the taxonomic situation of *C. brachyotis* was unresolved (Payne *et al.*, 1985; Francis, 1990).

Abdullah *et al*. (2000) and Abdullah (2003) were the first to present significant genetic data on the existence of two forms of *C. brachyotis*, with clear indication that these two forms were found in distinct habitats – *C. brachyotis* I (large-sized) in open habitat and *C. brachyotis* II (small-sized) in closed habitat – but also exist sympatrically in ecotones between forests (closed habitat) and open areas (open habitat). Abdullah (2003) also speculated the existence of hybrids in the ecotonal areas based on clustered individuals that were found in his phylogenetic analysis based on 635bp partial cytchrome *b* gene in the mtDNA. His conclusion on the existence of the two forms of *C. brachyotis* was later confirmed in similar studies by Campbell *et al.* (2004,

2006). Campbell *et al.* (2004) used 690bp partial mitochondrial cytchrome *b* gene and 576bp of partial mitochondrial control region to infer phylogenetic relationships of the cynopterans in Malaysia. Subsequently, Campbell *et al*. (2006) used 567bp of partial mitochondrial control region as mtDNA molecular marker to examine evolutionary relationships of these species. Later, these two forms of *C. brachyotis* were referred as *C.* cf. *brachyotis* "Forest" for the small size and *C.* cf. *brachyotis* "Sunda" for the large size (Campbell *et al*., 2004; 2006; Francis, 2008).

Morphological evidence was also presented by Abdullah (2003) using five external morphological characters. Furthermore, Jayaraj *et al*. (2004) collected data on 28 morphological characters and used multivariate analyses to assess the morphometrics of the *Cynopterus* complex based on Abdullah's (2003) results. Jayaraj *et al*. (2004) observed congruent results with Abdullah (2003); both studies were in-agreement with the existence of two forms of *C. brachyotis*. Jayaraj *et al.* (2004) found that the large-sized *C. brachyotis* and the small-sized *C. brachyotis* can be differentiated using forearm length as stated by Abdullah (2003), and measurements of the palatal length (Jayaraj, 2009). Later, Jayaraj *et al*. (2012) developed two predictive models with eight measurements from the skull, dental and external characters to discriminate these two forms of *C. brachyotis*. Furthermore, Abdullah and Jayaraj (2006) deduced that the type specimen was a large form of *C. brachyotis* based on limited data from the original descriptions and morphological measurements by Müller (1938).

According to Francis (2008), the small-sized *C. brachyotis* consumes mostly fruits especially figs, while the large-sized *C. brachyotis* consumes a wider range of food as compared to the small-sized *C. brachyotis*. The large-sized *C. brachyotis* consumes small fruits, bananas, nectar, pollens, and soft pulp, whereby it sucks out the juice from the pulp. In terms of habitat type, the large-sized *C. brachyotis* and small-sized *C. brachyotis* occupy different habitat types. The large-sized *C. brachyotis* is found in open habitats, such as secondary forests, agricultural lands, forest fringes and swamps, while the small-sized *C. brachyotis* is found in closed habitats, such as primary forests, old regenerated forests, and forest fringes near primary forests. However, as indicated by Abdullah (2003), these two forms of *C. brachyotis* are sympatric at forest edges where hybridisation could occur. Unfortunately, this cannot be investigated using mtDNA molecular markers as mtDNA only reflects the gene flow and dispersal pattern of the female founders in the population (García-Moreno *et al.,* 1996).

To study the species boundary and genetic variations between *C. sphinx* and *C. brachyotis* in Peninsular India, Storz (2000) developed nine pairs of microsatellite primers from *C. sphinx* (n = 413 individuals). In the preliminary study of taxonomic relationship between *C. sphinx* and *C. brachyotis*, a total of 300 cynopterans (additional 189 *C. sphinx* and 111 *C.*

brachyotis) were screened using five pairs of the microsatellite primers (CSP1, CSP2, CSP5, CSP7 and CSP9). As a result, CSP2 was found monomorphic in 20 individuals of *C. brachyotis* and the allele segregations were relatively shorter in *C. brachyotis* compared to *C. sphinx* (Storz, 2000). Besides, he also used these microsatellite primers to investigate the polygyny and social structure of *C. sphinx* using the population genetic structure (Storz, 2001; Storz *et al*., 2001a, 2001b).

This study aimed to investigate the utility and reproducibility of the existing microsatellite markers designed by Storz (2000) for population studies of *Cynopterus* in Malaysia; and subsequently to include more samples of this taxon from Malaysia especially from Sabah and Sarawak. Second, this study aimed to elucidate the possibility of hybridisation of the two forms of *C. brachyotis* occurring in the ecotone as speculated by Abdullah (2003) by using microsatellite analysis.

MATERIALS AND METHOD

Sample Identification and Collection

The samples for this study were from two sources, namely, the sample collection from the field and museum deposits in Malaysia. The distinction of the large form and small form was based on the *C. brachyotis* forearm measurements (> 60 mm = large form, < 60 mm = small form) as described in previous studies (see Abdullah, 2003; Campbell *et al.,* 2004, 2006, 2007; Jayaraj *et al.,* 2004, 2005; Francis, 2008; Jayaraj, 2009). *C. sphinx* and *C. horsfieldii* were identified based on forearm measurements and dentition as described in Payne *et al.* (1985), Lekagul and McNeely (1988), as well as Corbet and Hill (1992). Sampling locations were selected from open habitats (i.e. agricultural areas, secondary forests and swamp areas), closed habitats (i.e. primary forests and old regenerated forests) and forest edges in Peninsular Malaysia and Borneo. Museum samples were collected from the Sarawak Museum, Kinabalu Park Museum, the zoological museum of the Faculty of Resource Science and Technology (FRST) in Universiti Malaysia Sarawak (UNIMAS) (Abdullah *et al*., 2010) and the zoological museum of the Department of Wildlife and National Parks (DWNP) at Bukit Rengit, Pahang.

DNA Extraction and Microsatellite Genotyping

All instruments were sterilised by autoclave to avoid cross-contamination of DNA. DNA extractions were done using the standard cetyl trimethyl ammonium bromide (C-TAB) protocol following Grewe *et al.* (1993). Nine pairs of microsatellite primers designed by Storz (2000) were used to screen cynopterans in this study. The PCR amplifications were performed following the standard protocol as described by Sambrook *et al.* (1989). The amplifications were performed in a 25 µl of PCR mixture containing 3.5 µl of 10× PCR buffer, 1.5 µl of magnesium chloride ($MgCl_2$) (25 mM), 0.5 µl of dNTP mixture (10 mM), 1.25 µl of each primer (10 µM), 0.2 µl of

Taq polymerase and 1.0 to 2.0 µl of DNA template. Negative control (a reaction without a DNA template) was included in each PCR amplification to ensure that no DNA cross-contamination occurred during the preparation of PCR reagents.

PCR amplifications for all microsatellite primers used were done using a programmable thermal cycler (MyCycler™ by Bio-Rad) with standard-3 PCR protocol (Fong, 2011). The PCR programme was set as follows: a preliminary denaturation at 94°C for 2 minutes and 30 seconds, followed by denaturation of double stranded DNA at 94°C (30 seconds), primer annealing at optimised temperature (45 seconds), and primer extension at 72°C (50 seconds) for one complete cycle. Each cycle was repeated for 30 times and subsequently a final extension at 72°C for 2 minutes and 30 seconds was included to avoid generation of incomplete double stranded DNA.

Three percent of TBE-agarose gel was used to screen the DNA products in microsatellite analysis. All the results of the gel runs were photographed under UV illumination.

Microsatellite Data Analysis

The numbers of alleles per locus and between species were directly calculated. Overall allele frequencies for each species across loci were generated using POPGENE (version 1.31; Yeh *et al*., 1999). Probability tests for Hardy-Weinberg equilibrium (p-value < 0.05) were carried out for each locus for each species by using the algorithm of Levene (1949) in POPGENE (version 1.31; Yeh *et al*., 1999). GENEPOP (version 3.2; Raymond and Rousset, 1995) online analysis was used to test for linkage equilibrium across loci for each species.

The numbers of genotypes for each population, interspecies as well as intraspecies genotype sharing and gene diversity were generated using Arlequin (version 3.1; Excoffier *et al*., 2005). The Ewens-Watter neutrality test was performed to test the selection pressure for each locus using POPGENE (version 1.31; Yeh *et al*., 1999). The statistics in the neutrality test were calculated using 1000 simulated samples.

Global analysis of molecular variance (AMOVA) was used to perform the hierarchical analysis for overall species and inter-species level (Excoffier *et al*., 2005). Four covariance components from the total variance of the hierarchical analysis were used to compute four fixation indices generated using Arlequin (version 3.1; Excoffier *et al*., 2005). A total of 1000 permutations were used to carry out the significance tests between the covariance components and the correlated fixation indices with p-value less than 0.05.

A dendogram based on the unweighted pair group method with arithmetic mean (UPGMA) was constructed using the F_{ST} estimates between populations across the four species implemented in Molecular Evolutionary Genetics Analysis (MEGA) (version 4.0; Tamura *et al.*, 2007). The number of migrants per generation, N_m for

interspecies as well as between populations was calculated using the F_{ST} estimates as follows:

$$N_m = \frac{1 - F_{ST}}{4F_{ST}}$$

where, F_{ST} is the fixation index, and N_m is the number of migrants per generation.

RESULTS AND DISCUSSION

Allelic Diversity in Cynopterus

Overall, 173 individuals of cynopterans comprising 64 large form of *C. brachyotis*, 41 small form of *C. brachyotis*, 38 *C. horsfieldii*, and 30 *C. sphinx* were tested using nine pairs of microsatellite primers (Fig.1). Seven out of the nine microsatellite primer pairs produced distinct and reproducible genotypic patterns and showed polymorphism in all the four forms of *Cynnopterus* examined. The two remaining primer sets (CSP2 and CSP8) were excluded from the analysis as these primers did not generate any band. Similarly, the failure of both primer sets to generate bands was also observed in a similar study carried out in Peninsular Malaysia (Campbell *et al*., 2006). All nine pairs of primers utilised in the study were originally isolated from *C. sphinx* caught in India (Storz, 2000). Thus, it is safe to presume that the two primer sets failed to amplify the targeted region in the cynopterans due to inter and intraspecies variations that occurred between both sources of samples (India and Malaysia).

In reference to locus CSP4, the populations of *C. horsfieldii* and small form of *C. brachyotis* were found to be monomorphic with only two alleles produced (Table 1). In contrast, Campbell *et al.* (2006) found that CSP4 showed monomorphism in *C. brachyotis* Sunda only (referred here as large form of *C. brachyotis*) in contrast with this study where there were three alleles produced in the large form of *C. brachyotis* from Peninsular Malaysia. The allele was contributed by a sample captured from Gua Batu Puteh, Pulau Langkawi, previously not sampled by Campbell *et al.* (2006). Thus, inclusion of samples from other areas in the population as well as phylogeography study would show not only the distribution of the genotypes and gene flow of the studied species but also reveal more alleles that may not be found by restricted sampling efforts especially in bats which are highly mobile.

The allele frequencies for each locus in each species are presented in Table 1. Several private alleles were found in loci CSP3, CPS4, CSP6, CSP7 and CSP9 in each species, except the large-seized *C. brachyotis* (Table 1). This indicates that the remaining cynopterans in this study diverged from the large form of *C. brachyotis*. The founder population would have the greatest number of alleles, haplotypes, as well as genetic diversity, and shares its haplotypes with its descent groups. The descent group might hold their own unique haplotypes or private alleles as well as loss of certain haplotypes inherited from the founders due to genetic drift and other evolutionary processes during speciation (Kaestle & Smith, 2001; Merriwether *et al.*, 1996).

TABLE 1
Allele frequencies, observed heterozygosities (Obs Het), and expected heterozygosities (Exp Het) at each locus in representatives of *Cynopterus*. Bold values indicate private alleles in each species.

CSP 1

Species\Allele	200	210	220	230	240	250	Obs Het	Exp Het
Large *C. brachyotis*	0.0690	0.0345	0.5172	0.2155	0.1034	0.0603	0.3966	0.6715
Small *C. brachyotis*	0.1316	0.0789	0.2632	0.3684	0.0658	0.0921	0.7895	0.7688
C. sphinx	0.1000	0.0000	0.2333	0.2833	0.1000	0.2833	0.3000	0.7780
C. horsfieldii	0.1184	0.0000	0.2500	0.4079	0.1447	0.0789	0.3947	0.7396

CSP 3

Species\Allele	100	110	120	130	140	150	Obs Het	Exp Het
Large *C. brachyotis*	0.5678	0.0763	0.2797	0.0339	0.0424	0.0000	0.2881	0.5957
Small *C. brachyotis*	0.6148	0.1447	0.1053	0.0789	0.0526	0.0000	0.4734	0.5842
C. sphinx	0.3500	0.2333	0.3000	0.1167	0.0000	0.0000	0.2000	0.7316
C. horsfieldii	0.7895	0.0000	0.0000	0.1053	0.0000	**0.1053**	0.3158	0.3593

CSP4

Species\Allele	100	120	130	140	150	Obs Het	Exp Het
Large *C. brachyotis*	0.0169	0.0000	0.9068	0.0085	0.0678	0.1017	0.1743
Small *C. brachyotis*	0.0000	0.0000	0.9474	0.0000	0.0526	0.1053	0.1011
C. sphinx	0.0172	**0.0172**	0.2586	0.0690	0.6379	0.4138	0.5299
C. horsfieldii	0.0000	0.0000	0.9342	0.0000	0.0658	0.1316	0.1229

CSP 5

Species\Allele	130	140	150	160	170	180	190	200	Obs Het	Exp Het
Large *C. brachyotis*	0.1525	0.0085	0.4576	0.0932	0.2203	0.0169	0.0508	0.0000	0.6610	0.7132
Small *C. brachyotis*	0.1974	0.0132	0.2632	0.0395	0.3026	0.0526	0.1316	0.0000	0.7368	0.7888
C. sphinx	0.0000	0.1379	0.4483	0.1724	0.1034	0.0517	0.0345	0.0517	0.2759	0.7459
C. horsfieldii	0.0000	0.0526	0.1447	0.1974	0.2368	0.1711	0.0000	0.1974	0.7105	0.8239

CSP 6

Species\Allele	130	140	150	160	170	180	190	200	220	Obs Het	Exp Het
Large *C. brachyotis*	0.0085	0.1690	0.4237	0.0170	0.2458	0.0000	0.0508	0.1525	0.0000	0.5593	0.7297
Small *C. brachyotis*	0.0921	0.0526	0.3421	0.0921	0.1053	0.0000	0.2368	0.0789	0.0000	0.5263	0.8004
C. sphinx	0.0769	0.0000	0.6346	0.0000	0.0769	0.0577	0.0000	0.0769	**0.0769**	0.3077	0.5814
C. horsfieldii	0.0588	0.1029	0.2647	0.1029	0.3235	0.0735	0.0735	0.0000	0.0000	0.2647	0.8016

CSP 7

Species\Allele	220	230	240	250	260	270	280	290	300	310	Obs Het	Exp Het
Large *C. brachyotis*	0.0000	0.0000	0.0169	0.3220	0.1780	0.0763	0.2203	0.0424	0.1441	0.0000	0.7627	0.7641
Small *C. brachyotis*	0.0000	0.0000	0.0000	0.1447	0.2237	0.1316	0.3026	0.0132	0.1711	**0.1320**	0.7895	0.8011
C. sphinx	**0.0167**	0.0000	0.0167	0.3833	0.1667	0.0833	0.1667	0.1000	0.0667	0.0000	0.8333	0.7887
C. horsfieldii	0.0000	**0.1184**	0.0263	0.1711	0.0395	0.4079	0.2105	0.0000	0.0263	0.0000	0.4474	0.753

CSP 9

Species\Allele	280	290	300	310	320	330	350	Obs Het	Exp Het
Large *C. brachyotis*	0.2797	0.1864	0.4407	0.0254	0.0339	0.0339	0.0000	0.2203	0.6958
Small *C. brachyotis*	0.4242	0.1667	0.3182	0.0455	0.0000	0.0303	**0.0152**	0.1515	0.6984
C. sphinx	0.4821	0.0357	0.4107	0.0000	0.0714	0.0000	0.0000	0.5000	0.6032
C. horsfieldii	0.2763	0.1842	0.4211	0.0526	0.0263	0.0395	0.0000	0.2368	0.7168

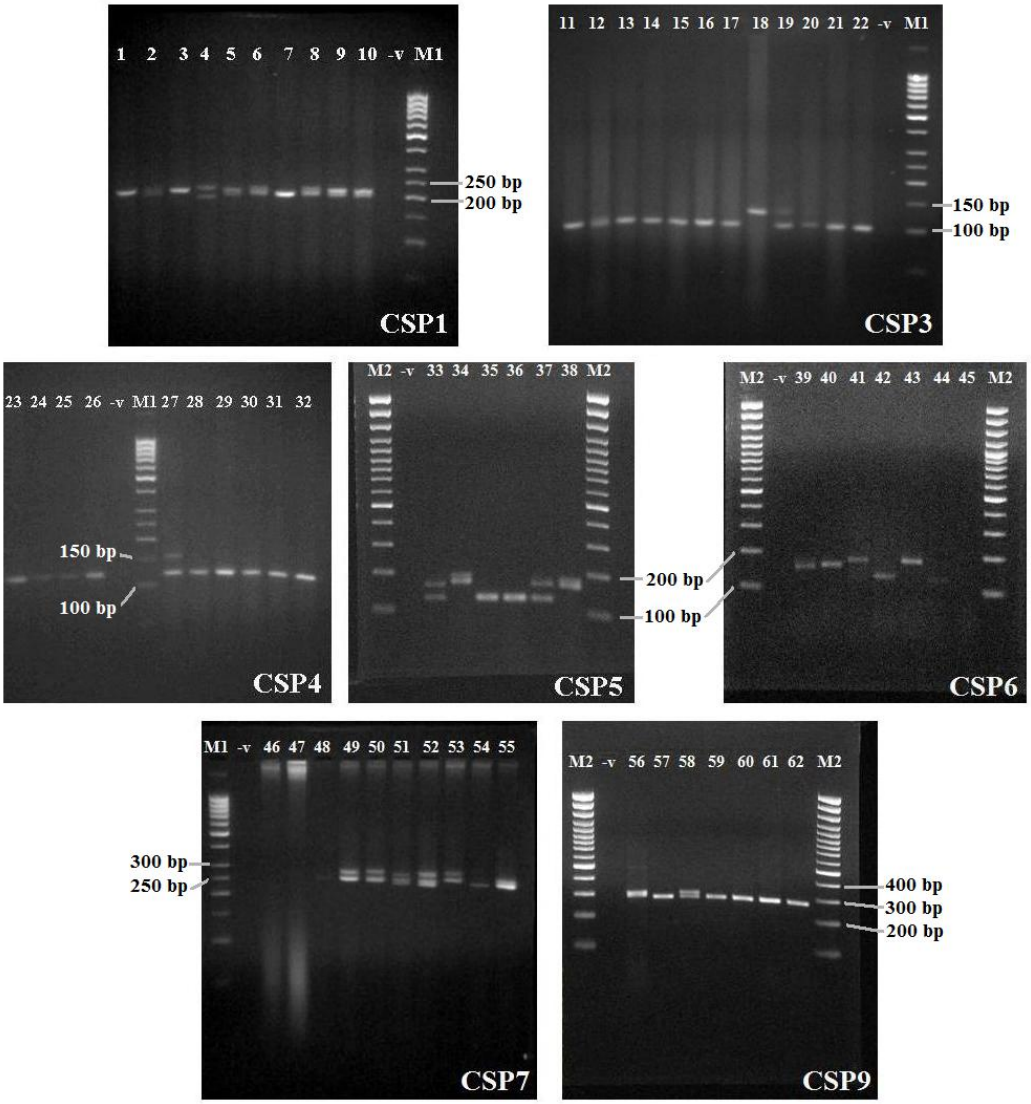


Fig.1: Representative gel pictures of each microsatellite marker used in this study. M1 indicates the GeneRuler™ 50 bp DNA Ladder, M2 is the GeneRuler™ 100 bp DNA Ladder Plus and –v is negative control. Each lane in the gels is labelled by number following the sample ID: 1 = I19; 2 = I20, 3 = I21; 4 = I22; 5 = I23; 6 = I24; 7 = I26; 8 = I29; 9 = I30; 10 = I31; 11 = H3; 12 = H4; 13 = H5; 14 = H6; 15 = H7; 16 = H8; 17 = H9; 18 = H10; 19 = H11; 20 = H12; 21 = H13; 22 = H14; 23 = K31; 24 = K32; 25 = K40; 26 = K92; 27 = I19; 28 = I20; 29 = I21; 30 = I22; 31 = I23; 32 = I24; 33 = PB026; 34 = PB012; 35 = B1; 36 = B2; 37 = B12; 38 = 01274; 39 = I20; 40 = I21; 41 = I23; 42 = I26; 43 = I30; 44 = H2; 45 = H7; 46 = FK1; 47 = FK2; 48 = FK3; 49 = FK4; 50 = FK5; 51 = FK6; 52 = FK7; 53 = FK8; 54 = FK9; 55 = FK10; 56 = S131; 57 = S184; 58 = S192; 59 = S185; 60 = S220; 61 = S221; 62 = S222.

There were 51 alleles documented in this study, where the highest number of alleles was found in the large form of *C. brachyotis* (42 alleles), while the lowest number of alleles was found in *C. horsfieldii* (36 alleles). The average observed heterozygosity ranged from 0.3574 in *C. horsfieldii* up to 0.5103 in the small form of *C. brachyotis* populations (Table 2). All loci in this study deviated from Hardy-Weinberg equilibrium across the four *Cynopterus* species (data not shown). No linkage disequilibrium was detected in all loci across all four cynopterans.

Deviation from Hardy-Weinberg expectations was detected across species over seven screened loci. However, not all genotype frequencies over seven loci deviated from Hardy-Weinberg expectation when observed within the populations of all species in this study. In this study, the populations with small sample size (less than or equal to seven individuals) were prone to follow Hardy-Weinberg equilibrium. Nevertheless, larger sample size for each region is still required to show the actual situation in the test of Hardy-Weinberg equilibrium. Non-deviation from Hardy-Weinberg equilibrium does not mean there were no evolutionary forces acting on these populations (Halliburton, 2004). Hardy-Weinberg principle is a null hypothesis to describe an ideal population; however, in reality, there are many evolutionary processes or factors that may influence genotype frequencies and cause deficiency and excess of heterozygosity in a population of a particular species (Snustad & Simmons, 2010; Halliburton, 2004).

A total of 97 unique genotypes were found across the four forms of *Cynopterus* in 15 regions (28 sites) of this study (Table 3). There were three common genotypes across the four forms. Among the four forms of cynopterans, the large and small forms of *C. brachyotis* shared the most genotypes compared to others. Besides, null alleles were found in all cynopteran bats (Table 3).

Population Structure

High genotype numbers were detected in the populations of large and small forms of *C. brachyotis* from southern Sarawak. *C. sphinx* from Perak had higher number of genotypes (44 genotypes) compared to the population from Perlis (38 genotypes), whereas in *C. horsfieldii*, the population from southern Peninsular Malaysia had

TABLE 2
Genetic variability of cynopterans across seven microsatellite loci and average observed (Obs) and expected (Exp) Hardy-Weinberg heterozygosities (Het)

Species	Total visible alleles	Average allelic genotypes	Average Obs Het	Average Exp Het
Large *C. brachyotis*	42	118	0.4271	0.6206
Small *C. brachyotis*	40	75	0.5103	0.6490
C. sphinx	39	58	0.4044	0.6798
C. horsfieldii	36	75	0.3574	0.6167

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus*

Genotype	KLCb (6)	NSLCb (20)	SSLCb (21)	NPMLCb (10)	SPMLCb (7)	NPMSCb (5)	SSSCb (23)	NSSCb (10)	SPMSCb (3)	NSCh (2)	SSCh (13)	NPMCh (7)	SPMCh (16)	PLCs (16)	PKCs (14)
100/100	6	8	8	5	3	2	8	4	3	2	5	7	10	4	5
110/100			4	1			3	2							
110/110		2			2	1	2	1						2	4
120/100			3	1	1		2	3							
110/120														1	
120/120		6	6		1		1							6	2
120/150														1	
130/100							4				4			2	2
130/110															1
130/120		1		1										1	
130/130	7	18	21		4	7	24	10	3	2	15	6	14	1	5
130/160			1	10			1								
130/170							1								
140/100		2		2		1	2								
140/120		1					1								
140/130								1							
140/140		1	1				1				1	1	2	1	3
150/100											2		6		
150/130	4	3	3	1	3	2	5	3			1	1	3	3	5
150/140			1	1		1								1	1
150/150	1	11	7	4	4	1	5	4	4	2	4	1	4	24	13
160/140							1							1	
160/150		2	6				2	1							1
160/160	1	2		2			1				1	1	4	1	2
170/130	1						1								
170/140										1			2		1
170/150	1	9	9	6	5		6	2			1	2			2
170/160						1	1						4		
160/160		1	5	1	2		2				3	1	4	1	2
180/160		3						2			3		1	3	1
180/160				1								2	1		2
180/170				1			3						3		
180/180								1				2	1		
190/130		1													
190/150			1			2	1	1							
190/160			1	1			1								
190/170			6		2	2	5	3	2	1	2		2		

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus* (cont.)

190/180							1									
190/190							5							1		
200/150	2	4	1	2	1		2							1	1	
200/160		1									3					
200/170		1									1	3	2			
200/180		1									1		1			
200/200	1	7			1		1	4			6			2	1	
210/210		1	1						1							
220/150															1	
220/170															1	
220/200			2	1			3	1							1	
220/220	6	5	11				3				1		4	2	4	
230/200						1	1	1					1			
230/210						2			2							
230/220		2	1		1		4	4			1		6		1	
230/230		6	2	1		1	2			1	7		1	5	2	
250/230							1									
260/230													1			
270/230													1			
240/200		1			1		1							2		
240/220				4	3		1					2				
240/230							2	1		1		1	3			
240/240					1							1	1		2	
250/200							1							1		
250/220			2	1			1	1						1	1	
250/230			2	1		1	2								2	
250/240				1												
250/250	2	2	1	2	1		3		1		2	3	3	6	4	
260/220														1		
260/250				1	1	1										
260/260		2	1	1	1				2					1		
270/230											1		2			
270/240											1		1		1	
270/250		1						2					2	2	1	
270/260							1									
270/270				1				2			5	4	2			
280/230											3		1			
280/240			2													
280/260	2	7	6		2		1	1		1				8	1	
280/260		4	3			2	4	3		1		1			1	
280/270							2						1			
280/280	6		3	6			9	6			2	3	9	6	2	

TABLE 3
Genotype distribution of nuclear microsatellite in 15 populations of four forms of *Cynopterus* (cont.)

290/250														1	2
290/260		2	2											1	2
290/270			1				1								
290/290		6	8	1			3	3		2	2	1	1		
300/250				3											
300/260	1		1	1			1							1	2
300/270	1	2	1	1	1	1	1	2							1
300/280			4	2			8				1			4	4
300/290														1	1
300/300		12	4		6	4	6	1			4	5	5	3	3
310/300											1				
310/260						1	3								
310/290			2								2				
320/300		1		1	2								2	1	2
330/300		1	3				1				1		1		1
330/310											1				
350/330							1								
Null alelle				1		1	1		3		3	1		7	1

the highest number of genotypes among all the other populations of *C. horsfiledii* in this study (Table 4). Average gene diversity over the loci of the large form of *C. brachyotis* showed that the population in southern Peninsular Malaysia had the highest diversity (0.6421), while the lowest diversity was recorded in the population from Kalimantan. The highest average gene diversity among seven loci was found in southern Sarawak populations of the small form of *C. brachyotis*. The average gene diversity over the loci in the population from Perak was higher than the population from Perlis in *C. sphinx*. The populations of *C. horsfieldii* from southern Sarawak had the highest average gene diversity in the overall loci among the remaining three populations.

The Ewens-Watterson neutrality test indicated that loci CSP1 and CSP5 were under selection pressure as the observed F-values were lower than 95% (L95) confidence limits of expected F-values in all seven microsatellite loci of cynopterans in this study. However, these two loci were included in further analysis as the observed F-values were within the interval of standard errors (Table 5).

The interspecies Global AMOVA comparison analysis showed that there was low genetic variation (0.53%) in the populations between the large and small forms of *C. brachyotis*. This was followed by genetic variation for the populations between the small form of *C. brachyotis* versus *C. horsfieldii* (3.12%) and the populations between the large form of *C. brachyotis* versus *C. horsfieldii* (3.59%). Although there were significant differences between the small form of *C. brachyotis*

TABLE 4
Number of sample size, genotypes, gene diversity, average gene diversity over loci (with standard deviation in brackets) for each region across four species

Species	Region	Sample size	Number of genotype	Gene diversity	Average gene diversity over loci
Large form *C. brachyotis*	northern Sarawak	20	37	0.9977 (0.0094)	0.5307 (0.3068)
	southern Sarawak	21	39	0.9872 (0.0114)	0.5817 (0.3360)
	northern Peninsular Malaysia	10	34	0.9895 (0.0193)	0.5667 (0.3380)
	southern Peninsular Malaysia	7	22	1.0000 (0.0270)	0.6421 (0.3775)
	Kalimantan	6	15	0.9848 (0.0403)	0.2966 (0.2006)
Small form *C. brachyotis*	northern Sarawak	10	28	1.0000 (0.0270)	0.5981 (0.3548)
	southern Sarawak	23	58	0.9990 (0.0048)	0.6707 (0.3713)
	northern Peninsular Malaysia	5	20	1.0000 (0.0625)	0.5306 (0.3410)
	southern Peninsular Malaysia	3	9	1.0000 (0.0625)	0.4167 (0.2854)
C. sphinx	Perlis	16	38	0.9960 (0.0090)	0.6820 (0.4133)
	Perak	14	44	1.0000 (0.0095)	0.7297 (0.4137)
C. horsfieldii	northern Sarawak	2	10	1.0000 (0.1768)	0.3095 (0.2557)
	southern Sarawak	13	33	0.9938 (0.0126)	0.5882 (0.3445)
	northern Peninsular Malaysia	7	21	1.0000 (0.0270)	0.4524 (0.2867)
	southern Peninsular Malaysia	16	37	1.0000 (0.0078)	0.6005 (0.3406)

TABLE 5
The overall Ewens-Watterson test for neutrality. The k-values were the number of visible alleles in each locus.

Locus	k	Min F	Max F	Mean*	L95*	U95*	Obs. F	SE*
CSP1	6	0.1667	0.9700	0.4920	0.2519	0.8885	0.2480**	0.0284
CSP3	6	0.1667	0.9702	0.4964	0.2519	0.8723	0.3988	0.0298
CSP4	5	0.2000	0.9759	0.5517	0.2844	0.9402	0.6802	0.0327
CSP5	8	0.1250	0.9582	0.4063	0.2029	0.7510	0.2007**	0.0213
CSP6	9	0.1111	0.9503	0.3683	0.1841	0.7068	0.2323	0.0185
CSP7	10	0.1000	0.9469	0.3392	0.1743	0.6702	0.1839	0.0169
CSP9	7	0.1429	0.9627	0.4498	0.2330	0.8558	0.3099	0.0279

* These statistics were calculated using 1000 simulated samples;
**Deviation from neutrality.

versus *C. horsfieldii* and the large form of *C. brachyotis* versus *C. horsfieldii,* variations between the populations were less than 5%. This may indicate that the populations' split was significant but the variations were not meaningful. This might be due to the small sample size, resulting in insufficient alleles and genotypes generated to show meaningful variations between them.

Both forms of *C. brachyotis* showed that they were more distantly related to *C. sphinx* as compared to *C. horsfieldii*. The percentage of variation showed that the populations of *C. sphinx* and *C. horsfieldii* had the highest genetic variation among the four species (Table 6).

A UPGMA tree was constructed based on the F_{ST} estimates values between 15 populations across four forms of cynopterans as listed in Table 7 (Fig.2). Among the 15 populations, *C. brachyotis* from Kalimantan was distantly related to other populations. Clustering of populations of the large and small forms of *C. brachyotis* did not conform to expected regions and forms (large versus small form), instead they were clustered into two clades (Clade A and Clade C). Precisely, Clade A consisted of the large form of *C. brachyotis* from northern Peninsular Malaysia, the small form of *C. brachyotis* from northern Sarawak, the small form of *C. brachyotis* from southern

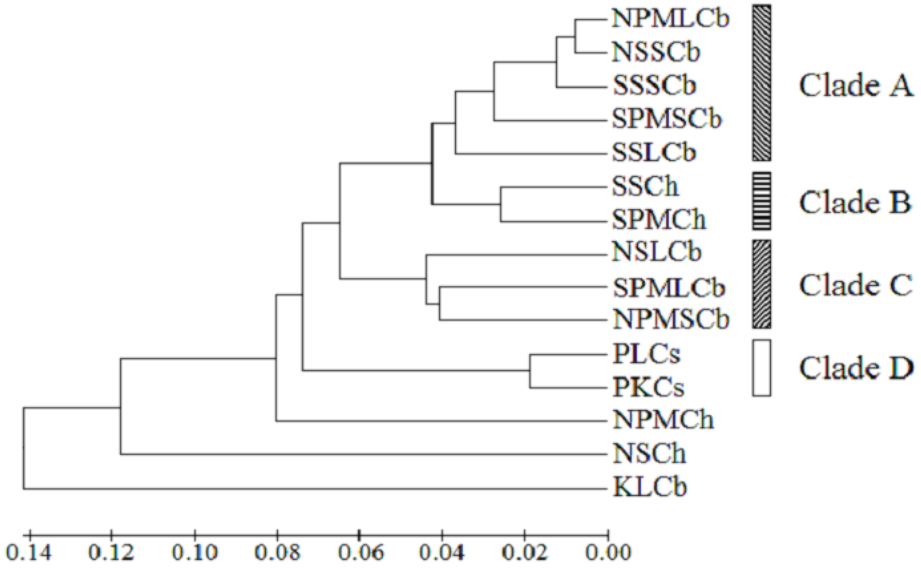


Fig.2: Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree based on interbreed genetic distance, F_{st} estimates. Branches labelled by region of each species and populations; NPMLCb = northern Peninsular Malaysia large form *C. brachyotis*, NSSCb = northern Sarawak small form *C. brachyotis*, SSSCb = southern Sarawak small form *C. brachyotis*, SPMSCb = southern Peninsular Malaysia small form *C. brachyotis*, SSLCb = southern Sarawak large form *C. brachyotis*, SSCh = southern Sarawak *C. horsfieldii*, SPMCh = southern Peninsular Malaysia *C. horsfieldii*, NSLCb = northern Sarawak large form *C. brachyotis*, SPMLCb = southern Peninsular Malaysia large form *C. brachyotis*, NPMSCb = north Peninsular Malaysia small form *C. brachyotis*, PLCs = Perlis *C. sphinx*, PKCs = Perak *C. sphinx*, NPMCh = north Peninsular Malaysia *C. horsfieldii*, NSCh = northern Sarawak *C. horsfieldii*, KLCb = Kalimantan *C. brachyotis*.

TABLE 6
Interspecies Global AMOVA based on seven loci

Source of variation	Sum of squares	Variance components	Variation (%)	Fixation Indices	p-value
Large form *C. brachyotis* vs Small form *C. brachyotis*					
Among species	6.470	0.01019	0.52967	F_{CT}: 0.00530	0.13978
Within species	29.418	0.11185	5.81455	F_{SC}: 0.05846	0.00000*
Within populations	183.572	0.28847	14.996	F_{IS}: 0.16012	0.00000*
Within individuals	146.50	1.51316	78.659	F_{IT}: 0.21340	0.00000*
Total	365.960	1.92367			
Large form *C. brachyotis* vs *C. sphinx*					
Among species	24.307	0.19059	7.54064	F_{CT}: 0.07541	0.01271*
Within species	41.252	0.23548	9.31699	F_{SC}: 0.10077	0.00000*
Within populations	221.492	0.64708	25.60197	F_{IS}: 0.30793	0.00000*
Within individuals	127.500	1.45432	57.54040	F_{IT}: 0.42460	0.00000*
Total	414.487	2.52748			
Large form *C. brachyotis* vs *C. horsfieldii*					
Among species	16.321	0.08369	3.59461	F_{CT}: 0.03595	0.01173*
Within species	51.839	0.23742	10.19767	F_{SC}: 0.10578	0.00000*
Within populations	228.011	0.61860	26.56971	F_{IS}: 0.30821	0.00000*
Within individuals	133.500	1.38849	59.63801	F_{IT}: 0.40362	0.00000*
Total	429.670	2.32820			
Small form *C. brachyotis* vs *C. sphinx*					
Among species	22.042	0.27156	11.79640	F_{CT}: 0.11796	0.00196*
Within species	14.026	0.05786	2.51354	F_{SC}: 0.02850	0.00098*
Within populations	149.713	0.48287	20.97591	F_{IS}: 0.24479	0.00000*
Within individuals	100.000	1.48973	64.71415	F_{IT}: 0.35286	0.00000*
Total	285.781	2.30202			
Small form *C. brachyotis* vs *C. horsfieldii*					
Among species	9.180	0.06119	3.11801	F_{CT}: 0.03118	0.00587*
Within species	21.492	0.08641	4.40305	F_{SC}: 0.04545	0.00000*
Within populations	146.624	0.37623	19.17190	F_{IS}: 0.20731	0.00000*
Within individuals	108.500	1.43860	73.30704	F_{IT}: 0.26693	0.00000*
Total	285.796	1.96243			
C. sphinx vs *C. horsfieldii*					
Among species	30.262	0.37074	13.81301	F_{CT}: 0.13813	0.00098*
Within species	21.582	0.11204	4.17452	F_{SC}: 0.04844	0.00000*
Within populations	184.744	0.87952	32.77125	F_{IS}: 0.39959	0.00000*
Within individuals	88.000	1.32162	49.24122	F_{IT}: 0.50759	0.00000*
Total	324.588	2.68396			

*Significantly different at 95% confident interval.

TABLE 7
F_{ST} estimates (below diagonal) as a measure of genetic distance between four species of cynopterans and number of migrants per generation, N_m (above diagonal and bolded)

Pop	NSLCb	SSLCb	NPMLCb	SPMLCb	KLCb	NSSCb	SSSCb	NPMSCb	SPMSCb	PLCs	PKCs	NSCh	SSCh	NPMCh	SPMCh
NSLCb		**2.3447**	**1.3519**	**2.5899**	**0.5558**	**1.2048**	**2.5625**	**2.6382**	**1.6374**	**1.0504**	**1.5866**	**0.8031**	**1.4674**	**1.1879**	**1.3110**
SSLCb	0.0964		**2.6853**	**1.6086**	**1.1984**	**3.0543**	**8.6027**	**1.4156**	**2.1319**	**0.9549**	**1.7772**	**1.7629**	**1.8419**	**1.8737**	**2.0211**
NPMLCb	0.1561	0.0852		**1.7073**	**2.1962**	**15.6029**	**7.9360**	**1.2709**	**6.5881**	**1.2714**	**2.9692**	**0.8913**	**2.0131**	**2.4167**	**3.8618**
SPMLCb	0.0880	0.1345	0.1277		**0.4624**	**1.0784**	**2.5919**	**2.8243**	**2.0834**	**1.6422**	**5.0321**	**0.6447**	**1.3105**	**1.4209**	**1.4526**
KLCb	0.3103	0.1726	0.1022	0.3510		**1.9386**	**1.2819**	**0.3347**	**0.4472**	**0.4881**	**0.8228**	**0.2295**	**0.5793**	**0.6154**	**0.9874**
NSSCb	0.1719	0.0757	0.0158	0.1882	0.1142		**12.5247**	**1.2504**	**2.2267**	**1.1847**	**1.9286**	**0.9989**	**2.1114**	**1.7837**	**2.7570**
SSSCb	0.0889	0.0282	0.0305	0.0880	0.1632	0.0196		**3.5645**	**8.8608**	**1.4183**	**2.5315**	**1.6754**	**3.9503**	**4.1283**	**3.9467**
NPMSCb	0.0866	0.1501	0.1644	0.0813	0.4276	0.1666	0.0655		**3.5151**	**0.8052**	**1.8537**	**0.5977**	**2.0736**	**2.0344**	**1.6790**
SPMSCb	0.1325	0.1050	0.0366	0.1071	0.3586	0.1009	0.0274	0.0664		**0.9383**	**2.2368**	**0.5549**	**2.3089**	**1.6664**	**1.7666**
PLCs	0.1923	0.2075	0.1643	0.1321	0.3387	0.1743	0.1499	0.2369	0.2104		**6.3831**	**0.5319**	**0.8179**	**0.7962**	**0.8105**
PKCs	0.1361	0.1233	0.0777	0.0473	0.2330	0.1148	0.0899	0.1188	0.1005	0.0377		**0.7418**	**1.6146**	**1.6594**	**1.9708**
NSCh	0.2374	0.1242	0.2191	0.2794	0.5214	0.2002	0.1298	0.2949	0.3106	0.3197	0.2521		**1.2726**	**0.8837**	**0.7253**
SSCh	0.1456	0.1195	0.1105	0.1602	0.3015	0.1059	0.0595	0.1076	0.0977	0.2341	0.1341	0.1642		**3.4055**	**3.2445**
NPMCh	0.2107	0.1684	0.1431	0.1659	0.3754	0.1809	0.0935	0.1349	0.2069	0.2814	0.1645	0.3240	0.1228		**5.9643**
SPMCh	0.1497	0.0922	0.0496	0.1444	0.1839	0.0690	0.0470	0.1206	0.1003	0.2197	0.1044	0.2149	0.0517	0.0626	

NSLCb = northern Sarawak large form *C. brachyotis*, SSLCb = southern Sarawak large form *C. brachyotis*, NPMLCb = northern Peninsular Malaysia large form *C. brachyotis*, SPMLCb = southern Peninsular Malaysia large form *C. brachyotis*,KLCb = Kalimantan *C. brachyotis*, NSSCb = northern Sarawak small form *C. brachyotis*, SSSCb = southern Sarawak small form *C. brachyotis*, NPMSCb = north Peninsular Malaysia small form *C. brachyotis*, SPMSCb = southern Peninsular Malaysia small form *C. brachyotis*, PLCs = Perlis *C. sphinx*, PKCs = Perak *C. sphinx*, NSCh = northern Sarawak *C. horsfieldii*, SSCh = southern Sarawak *C. horsfieldii*, NPMCh = north Peninsular Malaysia *C. horsfieldii*, SPMCh = southern Peninsular Malaysia *C. horsfieldii*.

Sarawak, the small form of *C. brachyotis* from southern Peninsular Malaysia, and the large form of *C. brachyotis* from southern Sarawak. Meanwhile, Clade C comprised the large form of *C. brachyotis* populations from northern Sarawak, the large form of *C. brachyotis* from southern Peninsular Malaysia, and the small form of *C. brachyotis* from northern Peninsular Malaysia. *C. horsfieldii* from southern Peninsular Malaysia and *C. horsfieldii* from southern Sarawak were clustered in Clade B, whereas both populations of *C. sphinx* were clustered together in Clade D.

Based on the UPGMA tree constructed using the seven pairs of microsatellite primers, all cynopterans within this genus were very closely related to each other. There was a lack of distinct clusters for each form, dissimilar with what was revealed in mtDNA data. Besides, greater number of migrants per generation (N_m) between the large form and small form of *C. brachyotis* was observed compared with other forms of cynopterans (Table 7). Thus, the populations of both forms of *C. brachyotis* were mixed up, while *C. sphinx* and *C. horsfieldii* were grouped within their own phylogroups in the tree. This basically shed some light on the paternal inheritance in the genus of *Cynopterus*.

The low genetic structure in the bi-parental phylogenetic tree in the genus *Cynopterus* might be caused by low genetic diversity and limited Y chromosome inheritance in the male germ line. Several studies have shown that there is low genetic diversity of Y chromosome in most mammalians including humans (Hellborg & Ellegren, 2004; Handley *et al*., 2006; Goetting-Minesky & Makova, 2006). The low genetic variations in the Y chromosome are caused by the non-recombinant nature in the Y chromosome, where only the common genealogy is shared in the male germ line (Charlesworth & Charlesworth, 2000; Hellborg & Ellegren, 2004; Handley *et al*., 2006). In addition, selections and mutations, especially favourable mutations, may contribute to the reduction of the diversity of Y chromosome where these mechanisms could reduce the effective population size of the Y chromosome in a random mating population (Boissinot & Boursot, 1997; Charlesworth & Charlesworth, 2000; Hellborg & Ellegren, 2004).

Besides, low structuring in the microsatellite data might be caused by low sensitivity of microsatellite primers used in this study to examine Malaysian cynopterans. The existing primers used in this study were designed by Storz (2000) using *C. sphinx* from India, and currently, there are no existing microsatellite primers for Malaysian cynopterans. Different evolutionary forces may have been acting on Indian *C. sphinx* as compared to Malaysian *C. sphinx*. The study done by Campbell *et al*. (2006) on Malaysia *Cynopterus* spp. showed a similar result, where no distinctive genetic structure was generated using the same microsatellite loci used in this study. Several factors such as geographical barriers, the climate and forest ecology between India and Malaysia might be the possible explanation to the different genetic

profiles in the cynopterans. In Chen *et al*. (2010), little genetic variation of *C. sphinx* was found using eight out nice microsatellite loci by Storz (2000) although there was greater genetic mtDNA differentiation between the *C. sphinx* samples from India and those from China and Vietnam. In addition, the sample size used in this study for each form of cynopterans was unequal and small, thus the generated allelic profiles might result in the loss of other alleles as well as private alleles. Large and equal population sizes have great influence in determining the number of private alleles and distinct alleles in a population, which in turn can reveal genetic diversity and relationships (Szpiech *et al*., 2008).

CONCLUSION

The objective of this study was to investigate the utility of the existing microsatellite markers designed by Storz (2000) for the population study of *Cynopterus* in Malaysia. We found that seven out of the nine existing microsatellite markers isolated from *C. sphinx* can be utilised on cynopterans in Malaysian and Kalimantan samples. These seven primer pairs produced mixed results of distinctiveness, polymorphism and monomorphism as opposed to the two remaining primers that did not produce any band.

Subsequently, the utility of these microsatellite markers on *Cynopterus* was tested using the samples from Malaysia, especially from Sabah and Sarawak. The results showed that there were a total of 51 alleles and 97 genotypes generated from four forms, large and small forms of *C. brachyotis*, *C. sphinx* and *C. horsfieldii*. Although these primers were able to produce microsatellite bands, the data generated were not enough to clearly differentiate both forms of *C. brachyotis*, and thus, these primers cannot be used to clearly define the species boundary of these two forms of *C. brachyotis* as suggested by previous authors.

Second, on elucidating the possibility of hybridisation of both forms of *C. brachyotis* occurring in the ecotone as the contact zone that was speculated by Abdullah (2003), intermixing between the two forms of *C. brachyotis* could not be observed as most of the microsatellite alleles were shared amongst the species. Besides, genetic variations between the species were also low without any significant genetic difference. Thus, no distinctive genetic structure was exhibited between the four species using microsatellite analysis and without clear indication of hybridisation happening between the small and large forms of *C. brachyotis*.

Based on the results of previous and the current studies, the existing microsatellite primers isolated from *C. sphinx* by Storz (2000) could not clearly define the species boundary of both forms of *C. brachyotis*. Most of the alleles generated in this study were common among all cynopterans leading to no genetic structuring using the bi-parental genetic data. Thus, it is recommended that new microsatellite primers should be designed using local cynopterans especially the large form of *C. brachyotis*, which is the founder and

its cryptic species in this region. More polymorphisms between the two forms of *C. brachyotis* as well as *C. sphinx* and *C. horsfieldii* may be detected and can be used to further elucidate the genotype and genetic structure from bi-parental inheritance.

ACKNOWLEDGEMENTS

The main research funding for this study came from the Ministry of Science and Technology, the Environment (MoSTI) Zamalah Pascasiswazah to FPH, and IRPA grant 09-02-09-1022 EA001 led by Professor Mohd. Tajuddin Abdullah, with co-researchers Dr. Yuzine Esa and Dr. Awg. Ahmad Sallehin Awg. Husaini. We would like to express our gratitude to the zoological museum of the Faculty of Resource Science and Technology, as well as the zoological museum of the Department of Wildlife and National Parks (DWNP) for allowing us to examine the specimens and tissue samples; Sarawak Forestry Department and Sarawak Forestry Corporation for the data collection and research permits; the Molecular Ecology Laboratory (MEL) staff; and the undergraduate students of the Animal Resource Science Programme for collecting the bat specimens used in this study. Special thanks also go to Dr. Jessica Worthington Wilmer for her precious comments and suggestions in this study. We would also like to express our gratitude to the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, for the various administrative and logistic supports.

REFERENCES

Abdullah, M. T. (2003). *Biogeography and variation of* Cynopterus brachyotis *in Southeast Asia*. (PhD thesis dissertation). The University of Queensland, St. Lucia, Australia.

Abdullah, M. T., & Jayaraj, V. K. (2006). Preliminary investigation on the relationship of the nominate *C. brachyotis* with the small-sized and large-sized *C. brachyotis* using clustering analysis. *Sarawak Museum Journal, 62*(83), 223-236.

Abdullah, M.T., Moritz, C., Grigg, G. C., & Hall, L. S. (2000). Evidence of cryptic species within *Cynopterus brachyotis* by using mtDNA sequence. In Yaacob, Z., Moo-Tan, S. and Yorath, S. (Eds.). *Proceedings of the International Conference on* In-Situ *and* Ex-Situ *Biodiversity Conservation in the New Millenium*. Yayasan Sabah, Kota Kinabalu.

Abdullah, M. T., Wong, S. F., & Ketol, B. (2010). *Catalogue of Mammals of UNIMAS Zoological Museum*. Universiti Malaysia Sarawak, Kota Samarahan.

Boissinot, S., & Boursot, P. (1997). Discordant phylogeographic patterns between the Y chromosome and mitochondrial DNA in the house mouse: Selection on the Y chromosome? *Genetics*, *146*, 1019-1034.

Bruford, M. W., & Wayne, R. K. (1993). Microsatellites and their application to population genetic studies. *Current Biology*, *3*, 939-943.

Bruford, M. W., & Wayne, R. K. (1993). Microsatellites and their application to population genetic studies. *Current Biology*, *3,* 939-943.

Burg, T. M., Trites, A. W., & Smith, M. J. (1999). Mitochondrial and microsatellite DNA analyses of harbour seal population structure in the northeast Pacific Ocean. *Canadian Journal of Zoology*, *77*, 930-943.

Campbell, P., Schneider, C. J., Adnan, A. M., Zubaid, A., & Kunz, T. H. (2004). Phylogeny and phylogeography of Old World fruit bats in the *Cynopterus brachyotis* complex. *Molecular Phylogenetics and Evolution*, *33*, 764-781.

Campbell, P., Schneider, C. J., Adnan, A. M., Zubaid, A., & Kunz, T. H. (2006). Comparative population structure of *Cynopterus* fruit bats in Peninsular Malaysia and southern Thailand. *Molecular Ecology*, *15*, 29-47.

Campbell, P., Schneider, C. J., Zubaid, A., Adnan, A. M., & Kunz, T. H. (2007). Morphological and ecological correlates of coexistence in Malaysian fruit bats (Chiroptera: Pteropodidae). *Journal of Mammalogy*, *88*(1), 105-118.

Charlesworth, B., & Charlesworth, D. (2000). The degeneration of Y chromosomes. *Philosophical Transactions B*, *355*, 1563-1572.

Chen J., Rossiter S. J., Flanders J. R., Sun Y., Hua P., Miller-Butterworth, C., Liu, X. Rajan, K. E., & Zhang, S. (2010). Contrasting genetic structure in two co-distributed species of Old World fruit bat. *PLoSONE 5*(11), e13903. doi:10.1371/journal.pone.0013903.

Corbet, G. B., & Hill, J. E. (1992). *The Mammals of the Indomalayan Region: A Systematic Review*. Oxford: Oxford University Press.

Degnan, S. M., Owens, I. P. F., Clegg, S. M., Moritz, C. C., & Kikkawa, J. (1999). MtDNA, microsatellites and coalescence: Tracing the colonisation of Silvereyes through the southwest Pacific. In N. J. Adams, & R. H. Slotow (Eds.), *Proceeding of 22 International Ornithology Congress, Durban: 1881-1898.* Johannesburg: BirdLife South Africa.

DeWoody, J. A., & Avise J. C. (2000). Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, *56*, 461-473.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, *1*, 47-50.

Flagstad, Ø., Walker, C. W., & Vilá, C. (2003). Two centuries of the Scandinavian wolf population: Patterns of genetic variability and migration during an era of dramatic decline. *Molecular Ecology*, *12*, 869-880.

Fong, P. H. (2011). *Genetic variation and population structure of the genus* Cynopterus *in Malaysia.* (MSc thesis dissertation). Universiti Malaysia Sarawak, Kota Samarahan.

Francis, C. M. (1990). Trophic structure of bat communities in the understorey of lowland dipterocarp rain forest in Malaysia. *Journal of Tropical Ecology*, *6*(4), 421-431.

Francis, C. M. (2008). *A Field Guide to the Mammals of South-East Asia*. New Holland Publishers (UK) Ltd., London.

García-Moreno, J., Matocq, M. D., Roy, M. S., Geffen, E., & Wayne, R. K. (1996). Relationships and genetic purity of the endangered Mexican wolf based on analysis of microsatellite loci. *Conservation Biology*, *10*, 376-389.

Goetting-Minesky, M. P., & Makova, K. D. (2006). Mammalian male mutation bias: Impacts of generation time and regional variation in substitution rates. *Journal of Molecular Evolution*, *63*, 537-544.

Grewe, P. M., Krueger, C. C., Aquadro, C. F., Bermingham, E., Kincaid, H. L., & May, B. (1993). Mitochondrial variation among Lake Trout (*Salvenilus namaycush*) strains stocked into Lake Ontorio. *Canadian Journal of Fisheries and Aquatic Science*, *50*, 2397-2403.

Halliburton, R. (2004). *Introduction to Population Genetics*. New Jersey: Pearson Education Inc.

Handley, L. J. L., Berset-Brändli, L., & Perrin, N. (2006). Disentangling reasons for low Y chromosome variation in the Greater White-toothed Shrew (*Crocidura russula*). *Genetics*, *173*, 935-942.

Hansson, B., Bensch, S., & Hasselquist, D. (2003). A new approach to study dispersal: Immigration of novel alleles reveals female biased dispersal in Great Reed Warblers. *Molecular Ecology*, *12*, 631-637.

Hedrick, P. W. (1999). Highly variable loci and their interpretation in evolution and conservation. *Evolution*, *53*, 313-318.

Hellborg, L., & Ellegren, H. (2004). Low levels of nucleotide diversity in mammalian Y chromosomes. *Molecular Biology and Evolution*, *21*(1), 158-163.

Jayaraj, V. K. (2009). *The phylogenetic relationships of Megachiroptera in Malaysia inferred from morphological and DNA analyses*. (MSc thesis dissertation). Universiti Malaysia Sarawak, Kota Samarahan.

Jayaraj, V. K., Laman, C. J., & Abdullah, M. T. (2004). Morphological variation in the genus *Cynopterus* of Peninsular Malaysia and Borneo. In Ismail A. I. M., Koh H. L., & Hasan Y. A. (Eds). *Proceedings of the Regional Conference on Ecological and Enviromental Modelling (ECOMOD 2004)*. , pp. 69-81.

Jayaraj V. K., Laman C. J., & Abdullah M. T. (2005). Application of multivariate techniques in determining morphological variation in the genus *Cynopterus* of Peninsular Malaysia and Borneo. In Tuen, A. A., & Das, I. (Eds.). *International Conference on Biogeography and Biodiversity: Wallace in Sarawak – 150 years later*. , pp. 226.

Jayaraj, V. K., Laman, C. J., & Abdullah, M. T. (2012). A predictive model to differentiate *C. brachyotis* from *C.* cf. *brachyotis* Forest (Chiroptera: Pteropodidae) from Malaysia using Multivariate Analysis. *Zoological Studies*, *51*(2), 259-271.

Kaestle, F. A., & Smith, D. G. (2001). Ancient mitochondrial DNA evidence for prehistoric population movement: the numic expansion. *American Journal of Physical Anthropology*, *115*, 1-12.

Lekagul, B., & McNeely, J. A. (1988). *Mammals of Thailand.* Sahankarnbhat, Bangkok.

Levene, H. (1949). On a matching problem in genetics. *Annals of Mathematical Statistics*, *20*, 91-94.

Merriwether, D. A., Hall, W. W., Vahlne, A., & Ferrell, R. E. (1996). MtDNA variation indicates Mongolia may have been the source for the founding population for the New World. *American Journal of Human Genetics*, *59*, 204-212.

Müller, S. (1838). Over eenige nieuwe zoogdieren van Borneo [On some new mammals from Borneo]. *Tijdschrift voor Natuurlijke Geschiedenis en Physiologie, 5*, 134-150.

Page, R. D. M., & Holmes, E. C. (1998). *Molecular Evolution: A Phylogenetic Approach*. Oxford: Blackwell Science Ltd.

Payne, J., Francis, C. M., & Phillipps, K. (1985). *A Field Guide to the Mammals of Borneo*. The Sabah Society and World Wildlife Fund Malaysia, Kota Kinabalu.

Raymond, N., & Rousset, F. (1995). GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, *86*, 248-249.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (2nd edn.). New York: Cold Spring Harbor Laboratory Press.

Scandura, M. (2004). *The use of microsatellites in the study of social structure in large mammals: Italian wolf and fallow deer as case studies*. (PhD thesis dissertation). University of Bielefeld, Bielefeld.

Snustad, D. P., & Simmons, M. J. (2010). *Principle of Genetics* (5th Edn.). New York: John Wiley and Sons Inc.

Srikwan, S., Hufford, K., Eggert, L., & Woodruff, D. S. (2002). Variable microsatellite markers for genotyping tree shrews, *Tupaia*, and their potential use in genetic studies of fragmented populations. *Science Asia*, *28*, 93-97.

Storz, J. F. (2000). Variation at tri- and tetranucleotide repeat microsatellite loci in the fruit bat genus *Cynopterus* (Chiroptera: Pteropodidae). *Molecular Ecology*, *9*, 1198-2200.

Storz, J. F., Bhat, H. R., & Kunz, T. H. (2001a). Genetic consequences of polygyny and social structure in an Indian fruit bat, *Cynopterus sphinx*. I. Inbreeding, outbreeding, and population subdivision. *Evolution*, *55*(6), 1215-1223.

Storz, J. F., Bhat, H. R., & Kunz, T. H. (2001b). Genetic consequences of polygyny and social structure in an indian fruit bat, *Cynopterus sphinx*. II. Variance in male mating success and effective population size. *Evolution*, *55*(6), 1224-1232.

Szpiech, Z. A., Jakobsson, M., & Rosenberg, N.A. (2008). ADZE: A rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics*, *24*(21), 2498-2504.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596-1599.

Wright, T. F., Rodriguez, A. M., & Fleischer, R. C. (2005). Vocal dialects, sex-biased dispersal, and microsatellite population structure in the Parrot, *Amazona auropalliata. Molecular Ecology*, *14*, 1197-1205.

Yeh, F. C., Yang, R., & Boyle, T. (1999). *A Microsoft Window Based Freeware for Population Genetic Analysis*. Version 1.31. University of Alberta, Canada.

Pertanika J. Trop. Agric. Sci. 36 (3): 249 - 260 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Vitrification of Dikaryotic Mycelial Cells from *Lignosus rhinocerus*

Lai Wei Hong[1]*, Ninie Noor Diana Enche Baharuddin[2], Shu San Loo[1], Azura Amid[2], Fauzi Daud[3], Abas Mazni Othman[4] and Norihan Mohd Saleh[1]

[1]Agro-Biotechnology Institute Malaysia (ABI), Ministry of Science, Technology and Innovation, c/o Malaysian Agricultural Research and Development Institute, 43400 Serdang, Selangor, Malaysia
[2]Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia
[3]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[4]Strategic Livestock Research Centre, Malaysian Agricultural Research and Development Institute, 43400 Serdang, Selangor, Malaysia

ABSTRACT

In Malaysia, *Lignosus rhinocerus* is one of the few important traditional medicinal mushrooms being used by indigenous communities to treat diseases. Currently, this rare mushroom can be found in the deep forest in Peninsular Malaysia, but its number is insufficient to meet the increasing local demand. Therefore, a vitrification technique previously used in the cryopreservation of actinomycetes was adapted in this study to preserve and maintain the commercially potential *L. rhinocerus* strain in a viable state. In this study, combinations of different sucrose concentrations and exposure time were experimented without serial washing phase after thawing. In addition, electron microscopy and comet assay were applied to study the cryoinjury and genotoxity of vitrified mycelial cells. Mycelial cells incubated for 10 minutes in 1.6 M sucrose of Plant Vitrification Solution 2 (PVS2) yielded largest radial mycelial growth with 100% survival rate. Scanning electron microscopy results indicated the swelling of mycelial cells due to osmotic shock which occurred from thawing procedure, while transmission electron microscopy findings revealed fusion of two nucleus membranes of dikaryotic mycelium. Comet assay suggested insignificant differences ($p > 0.05$) of comet formation between the normal and vitrified mycelial cells, suggesting cryoprotectants used in vitrification will

ARTICLE INFO

Article history:
Received: 1 November 2011
Accepted: 4 October 2012

E-mail addresses:
weihong@mosti.gov.my (Lai Wei Hong)
niniediana@yahoo.co.uk (Ninie Noor Diana Enche Baharuddin),
loss@mosti.gov.my (Shu San Loo),
azura@yahoo.co.uk (Azura Amid),
fauzid@ukm.my (Fauzi Daud),
abasmazni@mosti.gov.my (Abas Mazni Othman),
(Norihan Mohd Saleh)
* Corresponding author

ISSN: 1511-3701

not cause genotoxity to mycelial cells of *L. rhinocerus*. In conclusion, the current vitrification technique is suitable to cryopreserve the dikaryotic mycelial cells of *L. rhinocerus* with 100% regeneration and without trace of genotoxicity.

Keywords: Lignosus rhinocerus, vitrification, electron microscopy, comet assay

INTRODUCTION

Various organisms in nature are found to be able to survive at low temperature (Cannon & Block, 1988; Storey *et al*., 1988). Those that have adapted to the subzero temperatures are generally classified as either freeze tolerant or freeze avoidant organisms (Cannon & Block, 1988). Freeze avoidant organisms are able to prevent their body fluid from freezing together whilst freeze tolerant counterparts can survive body fluid freezing (Duman, 2001). The cold tolerance strategy raise the interest among cryobiologists to understand these mechanisms which are important for the cryopreservation of mammalian tissues and organs, environment security, as well as the sustainable use of biological resources.

Maintenance of active cultures is expensive, time consuming and prone to contamination for many types of biological samples. Thus, the most practicable way is to cryopreserve the cells. Cryopreservation has long been developed since 50 years ago from the studies of cell freezing to the application in living plant cells. Sakai (1960) first initiated the plant cryopreservation by studying the response of dormant plant buds to liquid nitrogen, and subsequently, freezing of callus tissues in liquid nitrogen was done by Quatrano (1968). This was followed by the first storage of differentiated plant tissues, such as the shoot tips of strawberry (Kartha *et al.,* 1980) and potato (Schafer-Menuhr, 1996). Vitrification (Langis *et al.,* 1990; Sakai *et al*., 1991) was developed and sharply increased the number of species cryopreserved over the years (Sakai *et al.,* 1990, 1991; Sakai & Engelmann, 2007).

Vitrification happens when a highly concentrated cryoprotective solution supercools to very low temperatures (at or below glass transition temperature, T_g) and eventually molecular motion ceases and a glassy solid is formed without undergoing crystallization (Fahy *et al.,* 1984). As a glass, it is extremely viscous, thus at this stage, all chemical reactions that require molecular diffusion will stop and lead to metabolic inactivity, and stability can be sustained over prolonged time (Burke, 1986). Vitrification is generally applied for cryopreservation of cultured plant materials (Sakai *et al.,* 1990, 1991; Niino *et al.,* 1990). Normally, serial dilution is not often practised in vitrification of plant materials such as shoot tips during rewarming procedure, but it still gives promising growth and survival rate (Martin & González-Benito, 2005; Panis *et al.,* 2005; Kami *et al.,* 2009). Therefore, Engelmann's (2009) vitrification protocol for shoot tips in combination with vitrification protocol for Actinomycetes by Stalper and Tan (1996) were adapted to cryopreserve the dikaryotic mycelial cells of Tiger's Milk Mushroom (*Lignosus rhinocerus*).

Lignosus is a macrofungal genus that belongs to Polyporaceae family in Basidiomycota phylum. *Lignosus* comprises 5 species, namely, *L. dimiticus, L. ekombitii, L. goetzii, L. rhinocerus,* and *L. sacer* (Douanla-Meli & Langer, 2003; Núñez & Ryvarden, 2001; Ryvarden & Johansen, 1980), until just recently, a new species called *L. hainanensis* was discovered in the tropical forest of Hainan Province, southern China (Cui *et al.*, 2010). In Malaysia, *L. rhinocerus* is singled out as one of the most popular medicinal mushrooms sought by indigenous communities upon request by herbalists (Lee *et al.*, 2009). The local community has been using *L. rhinocerus* for medicinal purposes since 1930 as described by a local in Pahang, Tuan Haji Mat Yusop (Corner, 1989). *L. rhinocerus* is believed to have therapeutic properties and is used as treatment for asthma, breast cancer, cough, fever, food poisoning and wound healing, as well as a tonic to maintain health (Chang & Lee, 2001).

However, *L. rhinocerus* is rarely found in nature and the amount of wild mushrooms is not sufficient for commercial exploitation and research purposes. Thus, vitrification of mycelial cells for this mushroom was studied. In addition, electron microscopy observation and comet assay were employed to study the effect of vitrification to the ultra-structure and genetic materials of mycelial cells.

MATERIALS AND METHODS

Mycelial Growth

The fruit bodies and tubers of *L. rhinocerus* were collected at the state of Pahang, Malaysia, in June 2009. Pure cultures of *L. rhinocerus* were obtained from the sterilized surface of small pieces of pileus, stipe, and tuber, which were then innoculated to Potato Dextrose Agar (PDA) supplemented with streptomycin (200 µg/L) and incubated in the dark condition for 15 days at 25°C to be used as inoculums. The radial mycelial growth was observed and measured after 10 days. All experiments in this study were conducted in triplicates.

Vitrification

Vitrification experiment was designed using Design-Expert version 6 (DX6) with conduction of two parameters (sucrose concentration and exposure time) and one response (mycelia diameter) as shown in Table 1. Vitrification procedure was applied to a modified procedure of Tan and Stalper (1996). The mycelia from the solid media were placed into 2 mL cryovials followed with loading solution (2 M glycerol and 0.4 M sucrose) for 20-30 min at 25°C. Two millilitres of Plant Vitrification Solution 2 (PVS2) solution containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and different concentrations (0.4, 1.0, and 1.6 M) of sucrose at pH 5.8 was added and gently mixed vigorously for 5 min after removing the loading solution. Replacement of another 2 mL fresh PVS2 was held at

25°C for different lengths of time without shaking followed by reducing the PVS2 until 0.5 mL in the cryovials, which was subsequently plunged directly into the liquid nitrogen (LN, -196°C) for at least 1 h. Cryovials were rapidly transferred to sterile distilled water in a water bath (37°C – 40°C) for 1.5 min rewarming with vigorous shaking. Immediately, the PVS2 solution was drained from the cryovials and replaced with a 2-mL basal culture medium (Potato Dextrose Broth) supplemented with 1.2 M sucrose which was then left for 20 min. The basal medium was used for 10 min washing at room temperature prior to the growth performance analysis by measuring the mycelia diameter.

TABLE 1
Vitrification experiment design using Design-Expert version 6 (DX6)

Design	Sucrose concentration (M)	Exposure time (min)
1	0.40	10
2	1.00	10
3	1.60	10
4	0.40	20
5	1.00	20
6	1.60	20
7	0.40	30
8	1.00	30
9	1.60	30
10	1.00	20
11	1.00	20
12	1.00	20
13	1.00	20

Electron Microscopy of Vitrified Mycelia

Revived mycelial cells with optimal radial growth were submitted to electron microscopy study. Starting with primary fixation, 1 mm^3 and 1 cm^3 slides of tissues were stored in separate vials for transmission electron microscopy (LEO 912AB-TEM) and scanning electron microscopy (JEOL JSM-6400-SEM), and then they were fixed in fixative (4% glutaraldehyde) for 2 days at 4°C. The slides were washed 3 times with 0.1 M sodium cacodylate buffer at 30 min. Post-fixation was done in 1% osmium tetraoxide for 2 hr at 4°C, and the same washing step was repeated once. Dehydration was performed by a series of acetone with 35%, 50%, 75%, and 95% for 30 min each followed by three (3) changes of 100% at 1 hr.

In the SEM preparation, the specimens were allowed for critical point drying (CPD) by transferring them into a specimen basket, and then they were put into a critical dryer for about half (½) day. Before coating with gold coating in a sputter coater for SEM viewing, the specimens were stuck onto a stub using a double-sided tape or colloidal silver. For TEM viewing, a mixture of acetone and resin was used to infiltrate the specimens before placing them into beam capsules which were then filled up with resin. Polymerization was allowed in an oven set at 60°C for 1-2 days before going through the thick and ultra-thick sectioning. The section was stained with uranyl acetate for 10 min.

Comet Assay of Vitrified Mycelia

In addition to electron microscopy study, the revived mycelial cells with optimal radial growth were also submitted to comet assay. Approximately 0.03 g of untreated and cryopreserved mycelia from the agar medium was added into 1 mL of Y1 buffer (1 M sorbitol, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7.4 and 0.1% beta-mercaptoethanol) before the addition of 0.25 mg/mL enzyme chitinase (Torres-Bauza & Riggsby, 1980) and incubated at 37°C with mild shaking for 2 hr.

Comet assay was performed with minor modification from Singh *et al.* (1988). A base layer of 1.0% agarose on a microscope slide was added with 50 µL of the cell agarose mixture (50 µL of 0.7% agarose mixed with 10 µL of spheroplasts in Y1 buffer) and covered with a cover slip. The slides were then allowed to dry on a flat surface on ice for 2 min before the addition of the last layer of 0.7% agarose. Again, the agarose was allowed to solidify on ice for 2 min. The uncovered slides were then immersed in a freshly made lysing solution composed of a 100-mL stock solution (1.25 M NaCl, 50 mM EDTA, 100 mM Tris base, adjusted to pH 10 and topped up with 0.1% sodium dodecyl sulphate). Subsequently, 1.5 mg/mL proteinase K was added and incubated at 37°C overnight. After lysis, the slides were transferred to the electrophoresis unit, filled up with a fresh electrophoresis solution (300 mM NaOH, 1 mM EDTA, 0.2% DMSO and 0.1% hydroxyquinoline) and left for 20 min. A current of 12 V (0.4 V/cm, 250 mA) was applied for 20 min before the slides were immerged in a neutralization buffer (50% ethanol, 20 mM Tris-HCl, pH 7.4) for 30 min. Followed by another 30 min in a new buffer, they were then oven dried at 50°C. Lastly, the slides were stained with the SYBR-Green dye and evaluated at 20x magnification using a ZeissAxiovert 200M fluorescence microscope.

DNA damage on the untreated and cryopreserved mycelial cells was evaluated using the Tritek Comet Score™ (Ver. 1.5) software. During evaluation, the percentage of DNA in the tail of each cell was recorded. The comets were categorized into four classes (0, 1, 2, and 3), namely; (0) tail size with no tail, (1) short tail length smaller than the diameter of the head (nucleus), (2) tail length between 1 and 2 times the head diameter, and (3) long tail more than twice the diameter of the head. The comets with no head and very wide tail observed were excluded from the analysis which represented dead cells (Hartmann & Speit, 1997).

RESULTS AND DISCUSSIONS

Radial Growth of Vitrified Mycelial Cells

The current results suggest that the mycelial cells exposed to PVS2 at 1.6 M sucrose and 10 min yielded the largest radial mycelial growth diameter, while those treated with 0.4 M sucrose at 30 min yielded the smallest radial mycelial growth diameter (Table 2). PVS2 plays an important role in obtaining a sufficient level of dehydration, which allows growth after cryopreservation (Yamada *et*

al., 1991) and protects the mycelia from damaging effects due to freezing. Higher sucrose concentration is ideal because the hydroxyl group present in sucrose will interact with phospholipid membrane by hydrogen bonding and membrane stabilization imparting during dehydration (Crowe & Crowe, 1986). In addition, shorter incubation period is ideal because PVS2 is potentially harmful due to phytotoxicity or osmotic stress damages (Rajnesh *et al.*, 2008). This current study indicates that higher sucrose concentration in combination with shorter incubation period is ideal for the revival of vitrified mycelia cells of *L. rhinocerus*.

Electron Microscopy Observation of Vitrified Mycelia from L. rhinocerus

Cryopreserved mycelial cells can be regenerated with no significant growth difference compared to normal mycelial cells. However, it is unclear on the effects of vitrification without serial dilution washing to the ultra-structure of cryopreserved mycelial. Hence, SEM and TEM experiments were conducted to study the ultra-structure of cryopreserved mycelial cells with the optimal regrowth rate (design 3, treatment of PVS2 containing 1.6 M sucrose at 10 min).

The SEM images indicated that the ultra-structures of cryopreserved hyphaes were bigger compared to normal hyphaes (Fig.1A and Fig.1B). This phenomenon could be due to osmotic shock and ice-crystal formation during freezing (Karlsson & Toner, 1996; Farrant, 1980). Throughout cooling process, ice formation will lead to the occurrence of osmotic imbalance or shock in which water migrates across cell membrane and cell wall (Ferrant, 1980). In addition, lipid is potentially deleted from

TABLE 2
Survival rates of cryopreserved mycelial cells based on mycelia diameter in triplicate measurement

Design	Sucrose concentration (M)	Exposure time (min)	Mycelium diameter		Mycelia regrowth on plate (%)	Survival rate (% ± S.E)
			Actual	Predicted		
1	0.40	10	2.0	2.005172	67	67 ± 0.004
2	1.00	10	2.7	2.656322	100	100 ± 0.031
3	1.60	10	2.9	2.938506	100	100 ± 0.192
4	0.40	20	1.6	1.622989	100	100 ± 0.163
5	1.00	20	2.4	2.324138	67	67 ± 0.054
6	1.60	20	2.7	2.656322	67	67 ± 0.031
7	0.40	30	1.4	1.371839	67	67 ± 0.020
8	1.00	30	2.1	2.122989	100	100 ± 0.515
9	1.60	30	2.5	2.505172	100	100 ± 0.004
10	1.00	20	2.3	2.324138	67	67 ± 0.017
11	1.00	20	2.4	2.324138	67	67 ± 0.054
12	1.00	20	2.3	2.324138	67	67 ± 0.017
13	1.00	20	2.2	2.324138	67	67 ± 0.088

Mean±SD (n= 3)

membrane during osmotic dehydration, which reduces the ability for the cell to return its isotonic volume during rehydration (Steponkus *et al.,* 1983).

Complimentary to SEM, TEM was conducted to study the cell wall, membrane plasma, nucleus and cytoplasmic content of the cryopreserved mycelial cells. The TEM observation of both normal and cryopreserved mycelial cells (Fig2A and Fig.2B) suggested that the cell wall of vitrified mycelium was ruptured. The current findings could be due to the formation of cell wall made from chitin which possesses priority in terms of strength and rigidity and can withstand mechanical stress. This

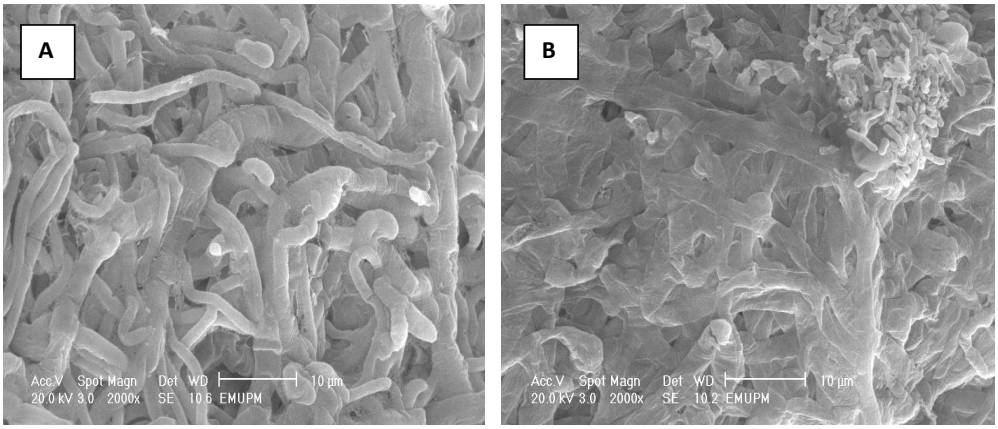


Fig.1: Ultrastructure of mycelial cells: (A) normal mycelial cells (control); (B) cryopreserved mycelial cells with enlarged and swollen hyphae due to cryoinjury. Magnification: 2000x (A, B).

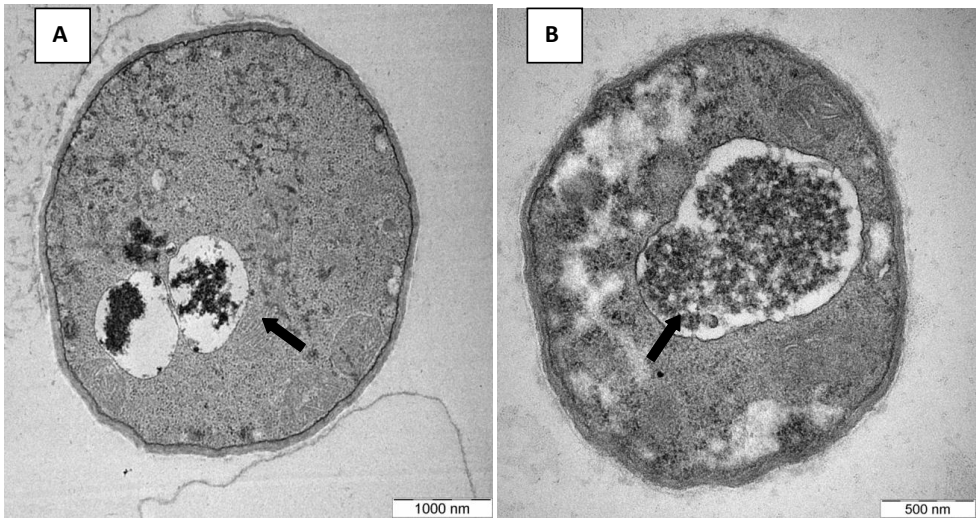


Fig.2: Cross section of mycelium: (A) normal mycelium (control); arrow shows two nucleus (n + n); (B) cryopreserved mycelium with ruptured cell wall and arrow shows fusion of two nucleus membranes. Scale bars = 1000 nm (A), 500 nm (B).

could be due to branched polysaccharide linked by α-1,4 and β-1,4 glycosidic bond in chitin that is composed of amino sugar N-acetyl-D glucosamine [GlcNAc] (Ibrahim *et al.,* 2000), which is not in cellulose that can strengthen the bond and cell wall structure. Thus, the cell wall is strong enough to withstand the osmotic pressure from the difference in solute concentration between the cell interior and distilled water (Howland, 2000), which in turn makes the cell wall remain intact.

In addition, Fig.2B illustrates the fusion of nuclear membrane from two nucleuses, and the occurrence of this phenomenon was unclear. The white region probably represented non-dehydrated zone where water still remained in the cell after vitrification. Farrant (1980) suggested that if too much water remains inside the cell, damage due to ice crystal formation and recrystallization during warming might occur.

Comet Assay of Vitrified Mycelia from L. rhinocerus

Table 3 summarizes the percentage of comet tails of the untreated and cyropreserved *L. rhinocerus* mycelial cells with 10.506 ± 0.898 and 10.836 ± 0.807, respectively. Statistical analysis demonstrated insignificant differences ($p > 0.05$) of comet formation between the untreated and cryopreserved *L. rhinocerus* mycelial cells, indicating that the DNA damage inflicted by cryopreservation to the mycelial cells was minimal.

Vitrification is an alternative cryopreservation method for those organisms that cannot stand at slow cooling as studying species. The cooling rate is important for a period of time before cooling to liquid nitrogen temperatures. This process enhances the dehydration of the cytoplasm prior to freezing. The noxious effects of toxic vitrification solution are minimized by performing the exposure towards PVS2 as brief as possible (Tan & Stalper, 1996). The choice of a cryoprotective agent is dependent upon the type of cell to be preserved. Cryoprotectants work as an antifreeze to prevent formation of ice crystals and aid in the process of vitrification. As in PVS2 solution, a mixture of cryoprotectants (glycerol, ethylene glycol, and DMSO) was used. When used together, cryroprotection was enhanced as much as double than when used alone, possibly because of a decrease in toxic effect (Ulrich *et al*., 1979). They work by diffusing into cells and replacing much of the cells' water. This indicates that PVS2 solution is an applicable cryoprotectant for vitrification of *L. rhinocerus*.

TABLE 3
The percentage of DNA in comet tail of the untreated (control) and cryopreserved *L. rhinocerus* mycelial cells

Samples	Percentage of DNA in tail (%) Mean ± SD
Untreated (control)	10.506 ± 0.898
Cryopreserved	10.836 ± 0.807

Mean±SD (n= 3).
*$p > 0.05$; t test.

CONCLUSION

The current study elucidates the efficiency of vitrification technique and subsequent impact towards dikaryotic mycelial cells of *L. rhinocerus*. Although serial dilution during the washing stage was not applied in the thawing phase, all vitrified samples were successfully regenerated. The current findings suggest that vitrification method remains an easy and inexpensive method for long-term preservation of mycelial cells from Basidioamycetes or more specifically Polyporaceae.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Technology, and Innovation of Malaysia (ABI R&B Initiative Fund: MOSTI/08-05-ABI-PB030). We greatly appreciate the assistance from the Department of Orang Asli Affairs, and most of all, the local indigenous communities that have been very generous in sharing their knowledge and providing us with the wild Tiger's milk mushroom specimens.

REFERENCES

Burke, M. J. (1986). The glassy state and survival of anhydrous biological systems. In A.C. Leopold (Ed.), *Membrane, Metabolism and Dry Organisms, Cornell University Press* (pp. 358-364). Ithaca, NewYork.

Cannon, R. J. C., & Block, W. (1988). Cold tolerance of microarthropods. *Biological Reviews, 63,* 23–77.

Chang, Y. S., & Lee, S. S. (2001). *Utilisation of wild mushrooms by the Temuans in Selangor, Malaysia.* Poster presented at CFFPR 2001, 100 Year Celebration of Forestry Research, 1-3 October 2001, Nikko Hotel, Kuala Lumpur.

Corner, E. J. H. (1989). Ad Polyporaceae IV. *Nova Hedwigia Beihefte*, *97,* 147.

Cui, B. K., Wang, Z., & Dai, Y. C. (2010). *Albatrelluspiceiphilus* sp. nov.on the basis of morphological and molecular characters. *Fungal Diversity, 28,* 41–48.

Crowe, J. H., & Crowe, L. M. (1986). Stabilisation of membranes in anhy-drobiotic organisms. In C. Leopold (Ed.), *Membranes, metabolism and dry organisms* (pp. 188-209). Ithaca: Comstock Publishing Associates.

Douanla-Meli, C., & Langer, E. (2003). A new species of *Lignosus* (Polyporaceae) from Cameroon. *Mycotaxon, 86*, 389–394.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual Review of Physiology*, *63,* 327–357.

Engelmann, F. (2009). Cryopreservation for Long-term Conservation of Agrobiodiversity: *Progress and Prospects.* France: IRD.

Fahy, G. M., MacFarlane, D. R., Angell, C. A., & Meryman, H. T. (1984). Vitrification as an approach to cryopreservation. *Cryobiology, 21,* 407-426.

Farrant, J. (1980). General observations on cell preservation. In M. J. Ashwood-Smith & J. Farrant (Eds.), *Low Temperature Preservation in Medicine and Biology* (pp. 1 – 18). Kent, England: Pitman Medical Limited.

Hartmann, A., & Speit, G. (1997). The contribution of cytotoxicity to effects seen in the alkaline comet assay. *Toxicology Letters, 90,* 183-188.

Howland, J. L. (2000). *The Surprising Archaea: Discovering Another Domain of Life*. Oxford: Oxford University Press. pp. 69–71.

Ibrahim, G. H., Smartt, C. T., Kiley, L. M., & Christensen, B. M. (2000). Cloning and

characterization of a chitin synthase cDNA from the mosquito *Aedesaegypti*. *Insect Biochemistry and Molecular Biology, 30,* 1213-1222.

Kami, D., Shi, L., Sato, T., Suzuki, T., & Oosawa, K. (2009). Cryopreservation of shoot apices of hawthorn in vitro cultures originating from East Asia. *Scientia Horticulturae, 120,* 84–88.

Karlsson, J. O. M., & M. Toner. (1996). Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials, 17*, 243–256.

Kartha, K. K., Leung, N. L., & Pahl, K. (1980). Cryopreservation of strawberry meristems and mass propagation of plantlets. *Journal of the American Society for Horticultural Science, 105,* 481-484.

Langis, R., Schnabel-Preikstas, B. J., Earle, E. D., & Steponkus, P. L. (1990). Cryopreservation of carnation shoot tips by vitrification. *Cryobiology*, *27*, 657-658.

Lee, S. S., Chang, Y. S., & Noraswati, M. N. R. (2009). Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia.Forest Research Institute Malaysia. *Forest Ecology and Management*, *257*, 2062–2065.

Martin, C., & González-Benito, M. E. (2005). Survival and genetic stability of *Dendranthemagrandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydration. *Cryobiology*, *51,* 281–289.

Niino, T., Sakai, A., & Yakuwa, H. (1990). Cryopreservation of *in vitro* grown shoot tips of apple and pear shoots. *Plant Cells Tissues Organs Culture*, *28,* 261–266.

Núñez, M., & Ryvarden, L. (2001). East Asian polypores 2.Polyporaceae s. lato. *Synopis Fungorum, 14,* 170–522.

Panis, B., Piette, B., & Swennen, R. (2005). Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. *Plant Science, 168,* 45–55.

Quatrano, R. S. (1968). Freeze preservation of cultured flax cells utilisingdimethylsulfoxide. *Plant Physiology, 43*, 2057-2061.

Rajnesh, S., Bart, P., Mary, T., & Anand, T. (2008). Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (*Colocasiaesculenta* var. *esculenta*) accessions. *Plant Cell, Tissue and Organ Culture, 92*(1)*,* 107-111.

Ryvarden, L., & Johansen, I. (1980). A preliminary polypore flora of East Africa. *Fungi flora.* Oslo. 1-636

Sakai, A., & Engelmann, F. (2007). Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *CryoLetters, 28,* 151-172.

Sakai, A., Kobayashi, S., & Oiyama, I. (1991). Survival by vitrification of nucellar cells of navel orange *Citrus-sinensis*-var-*brasiliensis* Tanaka cooled to minus 196˚C. *Journal of Plant Physiology, 137,* 465-470.

Sakai, A., Kobayashi, S., & Oiyama, I. (1990). Cryopreservation of nucellar cells of navel orange (*Citrus sinensis*Osb. Var. *brasiliensis*Tanaka) by vitrification. *Plant Cell Report, 9,* 30-33.

Sakai, A. (1960). Survival of the twig of woody plants. *Nature*, *185,* 393-394.

Sakai, A., Matsumoto, T., Hirai, D., & Niino, T. (2000). Newly developed encapsulation-dehydration protocol for plant cryopreservation. *Cryo Letters*, *21,* 53-62.

Schafer-Mehuhr, A. (1996). Refinement of cryopreservation techniques for potato. *Final report for the period September 1991-1993 August 1996.International Plant Genetic Resources Institute.* Rome.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175,* 184-191.

Storey, K. B., Storey, J. M., Brooks, S. P. J., Churchill, T. A., & Brooks, R. J. (1988). Turtles survive freezing during winter hibernation. *Proceedings of National Academy of Sciences, USA, 85,* 8350–8354.

Steponkus, P. L., Dowgert, M. F., & Gordon-Kamm, W. J. (1983). Destabilization of the plasma membrane of isolated plant protoplast during freezing–thaw cycle: the influence of cold acclimation. *Cryobiology, 20,* 448–465.

Tan, C. S., & Stalpers, J. A. (1996). Vitrification of fungi. In A. Cimerman and N. Gunde-Cimerman (Eds.), *Biodiversity, International Biodiversity Seminar ECCO XIV meeting* (pp. 189–193). ECCO, Ljubljana, Slovenia.

Torres-Bauza, L. J., & Riggsby, W. S. (1980). Protoplasts from Yeast and Mycelial Forms of Candida albicans. *Journal of General Microbiology, 119,* 341-349.

Ulrich, J. M., Finkle, B. J., Moore, P. H., Ginoza, H. (1979). Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of callus cultures of a tropical plant. *Cryobiology*, *16*(6), 550–556.

Yamada, H., Sun, X. B., Matsumoto, T., Ra, K. S., Hirano, M., & Kiyohara, H. (1991). Purification of anti-ulcer polysaccharides from the roots of *Bupleurum falcatum. Planta Medica, 57(6),* 555-9.

Pertanika J. Trop. Agric. Sci. 36 (3): 261 - 274 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Feed Restriction and Realimentation on Performance and Carcass Characteristics of Growing Rabbits in a Humid Environment

O. A. Adeyemi*, O. O. Ajeboriogbon and S. A. Aderinoye

Department of Animal Production and Health, College of Animal Science and Livestock Production, Federal University of Agriculture, P.M.B 2240 Abeokuta, Ogun State, Nigeria

ABSTRACT

The effects of feed restriction for three weeks followed by a four-week realimentation on performance and carcass characteristics were investigated in growing rabbits in a derived savannah vegetation zone of South West Nigeria. Thirty (30) growing rabbits of mixed breeds and sexes with an average weight range of 750 – 800g were randomly distributed into five restriction regimes which served as the study treatments. The rabbits were divided into five groups of six rabbits each. Each treatment was replicated three times with two rabbits per replicate. The experiment was carried out using the completely randomized design (CRD). The rabbits in Treatment 1 (T_1) (control) were fed *ad libitum*, whereas those in Treatment 2 (T_2) and Treatment 3 (T_3) were restricted to 80% and 60% of *ad libitum* respectively throughout the 7-week study period. Meanwhile, the rabbits in Treatment 4 (T_4) were restricted to 80% of *ad libitum* for three weeks and later fed *ad libitum* for the remaining three weeks, whereas those in Treatment 5 (T_5) were restricted to 60% of *ad libitum* for three weeks and later fed *ad libitum* for the remaining four weeks. The trial lasted for 7 weeks after which the rabbits were starved overnight and slaughtered. Weight gain over the entire period, average daily gain and average daily feed intake over the 49 days of experimental period were significantly ($P<0.05$) influenced by the feeding regime employed. The least average daily weight gain over the entire period of the experiment was observed on the rabbits subjected to 60% of *ad libitum* feeding throughout the experimental duration. The rabbits in Treatment 4 (which were restricted to 80% of *ad libitum* feeding for 3 weeks followed by 4 weeks of *ad libitum* feeding showed a consistent similarity in performance with the rabbits on *ad libitum* feeding (Control). Feed restriction regimes significantly influenced ($P<0.05$) dressed weight and dressing out

ARTICLE INFO

Article history:
Received: 20 February 2012
Accepted: 10 September 2012

E-mail address:
olajideadeyemi@gmail.com (O. A. Adeyemi)
* Corresponding author

ISSN: 1511-3701

percentage (DOP); however, there were no effects (P>0.05) on the forelimb, loin, hind limb and thoracic cage weights of the rabbit carcasses. In terms of overall performance, the rabbits in Treatment 4, however, required less feed to gain a unit weight as compared to the rabbits in other treatments. Based on the findings of this study, it was concluded that feed restriction did not significantly influence carcass yield and relative organs investigated. It is recommended that growing rabbits can be subjected to a three-week feed restriction of not more than 20% provided at least four weeks of *ad libitum* feeding is allowed for compensatory growth.

Keywords: Feed restriction, realimentation, rabbits, performance, and carcass characteristics

INTRODUCTION

Interest in rabbit production has been on the increase in recent years. Rabbit occupies a unique niche in that it is a mini livestock that is easy to manage, highly prolific and has a short generation interval. The cost of feeding rabbits is however very high, a condition that also prevails for other Nigerian livestock species (Adeyemi *et al*., 2008). Currently, there has been an increased interest in studying feed restriction in rabbits as a means of reducing the cost of production. Growing rabbits usually have unlimited access to the feed and eat *ad libitum.* In a restricted feeding system, either the access of the animals to the feed is limited, or a fixed amount of feed is given. There are two methods for restricting feed intake, namely, qualitative and quantitative. In a qualitative feed restriction, the total amount of feed distributed to each animal is the same, but the feed composition can be changed, such as by increasing the fibre level and reducing the digestible energy content (Feugier, 2002). Quantitative feed restriction can be achieved by limiting the time for access to the feeder or by reducing the quantity of feed distributed (Feugier, 2002; Szendrö *et al*., 2000).

Restricted rabbits are reported to have improved feed efficiency (Maertens and Peeters, 1988; Perrier and Ouhayoun, 1996; Tůmová *et al*., 2002, 2003). Improved digestibility of nutrients at restricted feeding period was found in rabbits by Ledin (1984a, b). Feed restriction also reduces carcass fat deposition. Limiting feed intake depresses growth during the period of restriction, but reduced growth can be later compensated by realimentation. This phenomenon of accelerated growth following a period of feed restriction is termed "compensatory growth" (Tumova *et al*., 2002).

Most of the restriction studies carried out in rabbits were conducted in temperate regions of the world, thus there is a need to investigate the situation in a tropical climate. The present study was carried out to investigate the effect of quantitative restriction with or without *ad libitum* feeding after a restriction period on the growth and carcass indices under a tropical environment.

MATERIALS AND METHODS

Experimental Site

The experiment was carried out at the rabbitry unit of the Teaching and Research Farm, Directorate (TREFAD), Federal University of Agriculture, Abeokuta, Nigeria. The location lies within the rainforest vegetation zone of South West Nigeria with a mean annual rainfall of 1100 mm, a temperature of 34.7^{0}C, and a relative humidity of 82%. It is located in the region 70 m above sea level, with latitude 7^{0}5' to 7^{0}8'N and longitude 3^{0}11.2'E (Federal University of Agriculture, Abeokuta, Meteorological Station).

Rabbit Management and Housing

Thirty (30) growing rabbits of mixed breeds (Chinchilla x Dutch x California White) and sexes (20 males and 10 females) with an average weight of 750-800 g were selected for this experiment from a larger pool of 80 rabbits in the weight classification of 700 to 900 g in such a manner to minimize variations in initial weights between replicate pens. The rabbits were housed two per cells, in which there were three groups made up of two groups of two males and one group of two females per treatment in single tier hutches that had been washed and properly disinfected. The hutches raised 90 cm above the floor were housed in an open sided house that allowed for flow through ventilation. The hutches made of wood and wire mesh were divided into pens with a dimension of 120 x 50 x 45 cm each. Two flat bottom 20 cm wide earthen pots with inner lips to prevent wastage were placed in each pen, one serving as a feeder while the other one as a drinker. The rabbits were treated for endo and ectoparasites using Ivomec® at 1ml/50 kg live weight.

Experimental Design

The experiment was carried out as a completely randomized design with five treatments. Each treatment group was replicated three times with two rabbits housed in the same cell serving as a replicate. The treatments were as follows:

T1: *ad libitum* feeding

T2: 80% of *ad libitum* feeding for the entire feeding trial

T3: 60% of *ad libitum* feeding for the entire feeding trial

T4: 80% of *ad libitum* (0-3 weeks) and *ad libitum* for the remaining four (3-7) weeks

T5: 60% of *ad libitum* (0-3 weeks) and *ad libitum* for the remaining four (3-7) weeks

Ad libitum feed intake of rabbits of the same weight class had been previously determined in a preliminary study. Briefly, 20 rabbits were allowed to acclimate for 3 days on *ad libitum* feeding and allowed free access to water at all times. After the acclimation period, the rabbits were offered feed *ad libitum* for 21 days during which *ad libitum* feed intake for each week was established. It is this established weekly *ad libitum* feed intake that was utilized in the present study. The composition of the diet is shown in Table 1. The ingredient

composition (%) of the diet was on *as-fed* basis. The diet was based on feed composition used for growing rabbits on the Federal University of Agriculture Abeokuta, Teaching and Research Farm, which was developed in line with the recommendations of Hall (2010) and Merck (2011). The major ingredients (i.e. maize, groundnut cake and soy bean meal) were milled through a screen mesh size of 3.5 mm in a hammer mill. Other ingredients were already in milled forms at the point of purchase. The various ingredients were individually weighed out in their milled form into a rotary feed mixer and mixed to get the experimental diet. The experimental feed was mixed in the research feed mill. The feed was fed in mash form.

DATA COLLECTION AND ANALYSIS

Feed Intake

The feed intake per replicate cage was determined by collecting the left-over feed from the feeders each morning at 08:00 hrs before feeding. The daily collection of the left-over feed from each replicate was stored in marked nylon bags and kept in airtight plastic containers. These were bulked together at the end of each week, weighed and subtracted from the addition of daily feed supplied to get the feed intake per replicate cage. The value obtained was divided by the number of rabbits in the pen (2) to get the feed consumption per rabbit per week. The daily intake was derived by dividing the weekly intake by 7. Feed and water were supplied on a daily basis after removing the left-over feed each morning.

$$\textit{Feed intake (g)} = \frac{\textit{Feed supplied - Feed left over}}{\textit{No of rabbit in the replicate}}$$

Body Weight and Weight Gain

The rabbits in each replicate cage were weighed together at the beginning of the trial and weekly thereafter. The gain for each week was obtained by the value difference. The value obtained was divided by the number of rabbits in each replicate to get the weight gain per rabbit per week. From this, the weight gain per day was calculated.

TABLE 1
Composition of experimental diet (% as-fed)

Ingredient	**(%)**
Maize	47.50
Groundnut cake	10.00
Soybean meal	8.00
Wheat offal	31.00
Bone meal	3.00
Salt	0.25
Vitamin/mineral premix*	0.25
Total	**100.00**
Determined Analysis (% DM)	
Dry matter	89.45
Protein	18.74
Ether extract	4.58
Crude Fibre	15.68
NDF	34.29
ADF	20.54
ADL	3.31
Ash	4.25
Metabolisable Energy^{+} (KJ /Kg)	10.93

*contains *Vit. A 4000000IU; Vit. D. 800000IU; Vit. E 40000mg; Vit. K_3 800mg; Vit. B_1 1000mg; Vit. B_2 6000mg; Vit.B_6 5000mg; Vit.B_{12} 25mg; Niacin 6000mg; Pantothenic acid 20000mg; Folic acid 200mg; Biotin 8mg; Manganese 300000mg; Iron 80000mg; Zinc 20000mg; Cobalt 80mg; Iodine 400mg; Selenium 40mg; Choline 800000mg*
$^{+}$=Calculated

$$\text{Average weight gain (g/rabbit)} = \frac{\text{Final weight - Initial weight}}{\text{No of rabbit in the replicate}}$$

$$\text{Average daily weight gain}\left(\frac{g}{\text{rabbit}}/\text{day}\right) = \frac{\text{Final weight} - \text{initial weight}}{\text{No of rabbit in the replicate x No of days on trial}}$$

Feed Conversion Ratio (FCR) was determined as follows:

$$\text{Feed conversion ratio} = \frac{\text{Average Feed Intake}(g/\text{rabbit})}{\text{Average Daily Weight Gain}(g/\text{rabbit})}$$

Carcass Characteristics

Since there were two rabbits per replicate cage, one rabbit was taken from each replicate. This constituted 50% of the replicate and was thus used. The three selected rabbits were individually weighed and slaughtered after a 24-hour fast. The slaughtered animals were dressed by flaying, eviscerating and splitting according to Blasco and Ouhayoun (1993), as well as Blasco *et al*. (1993). The cut parts, namely, head, fore limb, thoracic cage, loin and hind limb, were dissected according to Blasco and Ouhayoun (1993) as described below:

The head was separated from the body by cutting it though the section between occiput and atlas vertebra.

The fore limb was separated by cutting it through section between the 7th and 8th thoracic vertebra following the prolongation of the rib when cutting the thoracic wall.

The thoracic cage was taken as a section between the last thoracic and the first lumber vertebra following the prolongation of the 12th rib when cutting the thoracic wall.

The loin section was between the 6th and 7th lumber vertebra cutting the abdominal wall transversely to the vertebral column.

The hind legs was separated by cutting it through the os coxae and posterior part of m. iliopsoas, m. psoas major and m. iliacus.

The paws were removed at the carpal and tarsal joints. The parts were weighed and recorded.

The dressed weight and dressing percentage were calculated as follows:

Dressed weight = Live Weight - Offal weight

where,

Offal weight = Gastro intetinal weight (GIT) + Internal Organs weight

Dressing out Percentage (DOP) % = (Dressed weight/Live weight) x 100

The weight of the internal organs, such as spleen, liver, kidney, lungs, heart and gastro intestinal tract, were also taken. The gastro intestinal tract was taken as the digestive tube from the point of decapitating the head to the anus comprising oesophagus, stomach, small intestine and the large intestine.

Cost Analysis

The prevailing market prices of the feed ingredients at the time of the experiment were used to estimate the unit cost of the experimental diet. Feed cost per kilogramme and cost per kilogramme of weight gain were calculated. The percentage feed cost saving of rabbits on restriction compared to the *ad libitum* fed rabbits was also determined (at the time of the experiment, one hundred and fifty five Naira (^), Nigeria National Currency was equivalent to One United States Dollar (^ 155.00 = US$ 1.00).

Data Analysis

The experimental diet samples were subjected to proximate analysis according to the methods of AOAC (1995). The detergent components were determined by the procedure developed by Goering and Van Soest (1970). Metabolisable energy (ME) value of the test diet was calculated by the method of Wardeh (1981). All data collected were subjected to statistical analysis appropriate for a completely randomized design layout using Minitab Analytical Computer Package (Minitab Inc., 1999). Significant differences between the treatment means were determined using the Duncan's Multiple Range Test (Steel & Torrie, 1990).

RESULTS AND DISCUSSION

The growth performance of rabbits subjected to varying dietary feeding regimes is presented in Table 2.

Final live weight, weight gain over the entire period, average daily gain and average daily feed intake over the 49-day experimental period were significantly ($P<0.05$) influenced by the feeding regimes employed. The least average daily weight gain over the entire period of the experiment was observed on the rabbits subjected to 60% *ad libitum* feeding throughout the experimental duration.

The average daily weight gain of these rabbits which was on the most severe restriction was 25.72% less than the average weight gain on the control feed regimen. The rabbits in Treatment 4 (80% *ad libitum* feeding for 3 weeks followed by 4 weeks of *ad libitum* feeding) showed a consistent similarity in performance with the rabbits on *ad libitum* feeding (Control). In terms of overall performance, the rabbits in Treatment 4, however, required less feed to gain a unit weight compared to the rabbits in the other treatments.

The rabbits on the Control treatment required significantly ($P<0.05$) more quantity of feed to gain a unit weight, an indication that it is less efficient. The nature of average weight gain over the period of feed regimentation is presented in Fig.1. As expected, from Week 1 through Week 3 (end of the restriction period), the rabbits restricted to 80 and 60% of the *ad libitum* intake presented a lower live weight than those fed *ad libitum*.

The rabbits previously restricted but later reverted to *ad libitum* feeding showed a steep rise in average daily weight gain from Week 4 onwards. The average daily gains during restriction were 11.32 and 9.72 g/day for the rabbits fed 80 and 60% of the *ad*

TABLE 2
Effect of feed restriction and realimentation on rabbit performance

	Treatments				
	T1 *Ad libitum* Feeding for 7 weeks	**T2** 80% *Ad libitum* Feeding for 7 weeks	**T3** 60% *Ad libitum* Feeding for 7 weeks	**T4** 80% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding	**T5** 60% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding
Av. Initial Weight (g)	800.10± 2.55	783.05± 6.80	766.00± 6..80	800.00± 6.50	783.10± 4.67
Av. Final Weight (g)	1545.92^{a}± 28.87	1385.00^{ab}± 57.74	1253.76^{b}± 43.34	1531.20^{b}± 16.67	1312.26^{ab}± 26.17
Weight Gain (g)	745.92^{a}± 18.02	602.00^{b}± 13.33	487.67^{c}± 13.64	731.20^{a} ± 10.02	609.28^{b}± 11.35
Av. DailyWeight Gain (g/day)	15.18^{a}± 0.57	12.29^{b}± 0.88	9.95^{c}± 1.02	14.92^{a}± 0.44	12.43^{b}± 0.91
Av. Daily Feed Intake (g/day)	92.67^{a}±4.46	61.95^{c}±16.55	52.83^{c}±8.19	73.71^{ab}±2.84	67.06^{b}±7.79
Feed: Gain	6.09^{b}± 0.57	5.04^{a}± 0.88	5.31^{ab}± 1.09	4.94^{a}± 0.21	5.39^{ab}± 0.90
*Cost/ Kg feed (^)	65.25	65.25	65.25	65.25	65.25
Cost/ Kg Weight Gain (^)	397.37^{c}± 10.57	328.86^{a}± 12.05	346.48^{b}± 9.07	322.34^{a}± 8.50	351.70^{b}± 8.78
% cost / kg gain compared to ad libitum	100.00	82.78	87.19	81.12	88.51

$^{a, b, c}$ Means within the same row with differing superscripts are significantly different (P<0.05)

(^) = Naira, Nigeria National Currency ^ 155.00 = US$ 1.00

libitum intake; however, in the last 4 weeks when *ad libitum* feeding was restored, average daily weight gains of 17.62 and 14.47% were obtained, respectively. These results were in agreement with the report by Perrier (1998), where a reduced level of intake (50% and 70% of the *ad libitum* intake) provoked a reduction of live weight (-32.9 and –20.5%, respectively) at the end of the restriction period (35-56 days).

Foubert *et al*. (2008) reported that a restricted feeding (70% of the *ad libitum* level) resulted in a lower live weight (-8.8%) at the end of restriction period (32-53 days). Tumova *et al*. (2002) also reported that during the restriction period, weight gain in the restricted rabbits was about 60-70% lower than in the *ad libitum* fed rabbits. The current results also confirm the results of Boisot *et al.* (2003), who observed that feed restriction (60% and 80% of the *ad libitum* level) elicited a lower live weight (respectively, -17.5 and -7%,) at the end of the restriction period (34-54 days).

The average daily feed intake for the entire period was significantly influenced by feeding strategy. The rabbits on continuous *ad libitum* feeding consumed significantly more feed than those in other feeding regimes. In addition, the rabbits on previously restricted but later *ad libitum* fed consumed significantly more feed than their continuously restricted counterparts. The feed intake pattern during the experiment is shown in Fig.2. The trend showed that the feed intake was consistently lower for

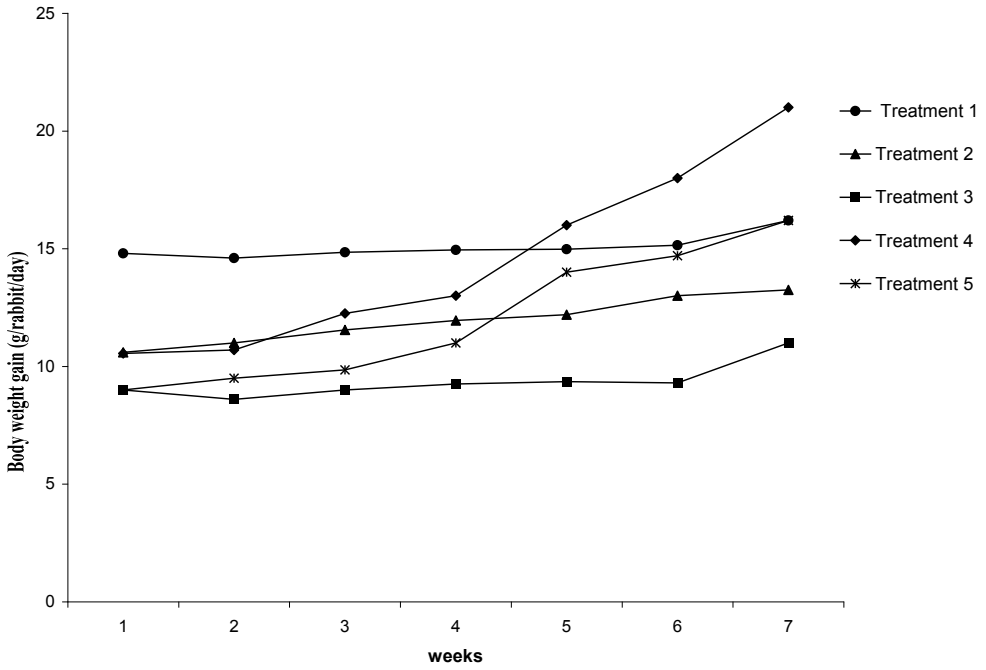


Fig.1: Weight change trend of rabbits subjected to feed restriction

the restricted-realimented rabbits compared with the continuously *ad libitum* fed rabbits. This observation was at variance with the report of Ledin (1984), that the restricted-fed rabbits during realimentation showed a tendency to consume more feed per day than those that were fed continuously *ad libitum*. On the contrary, the finding was in agreement with Boisot *et al.* (2003), who observed that the restricted rabbits (80% of *ad libitum* intake) had even lower daily feed intake (-18.4%, P<0.001) than the *ad libitum* ones during realimentation.

Feed conversion was significantly affected by the feeding regimens over the entire feeding period. The rabbits on continuous *ad libitum* feeding required more feed to attain a unit live weight gain compared to the rabbits on continuous feed restriction and those on restriction-realimented intake. The pattern of feed conversion measured as feed:gain is shown in Fig.3. In the restriction period (Week 1 - Week 3), the rabbits in Treatment 2 and Treatment 4 (80% *ad libitum*) had similar feed:gain. The same situation also occurred between the rabbits in Treatment 3 and Treatment 5 (60% *ad libitum*). While the feed:gain values for the rabbits on 80% *ad libitum* were higher than the values for the rabbits on *ad libitum* feeding, the values for the two groups on 60% *ad libitum* were lower than the values for the *ad libitum* fed rabbits in Week 1 and Week 2. However, from Week 3 up to the end of the feeding strategy, the rabbits on *ad libitum*

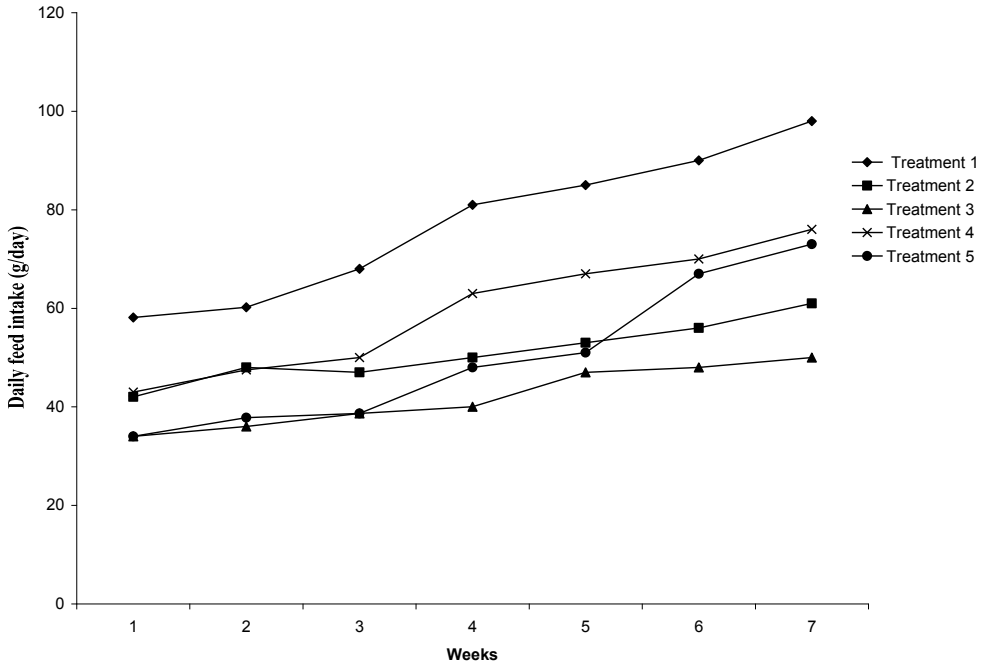


Fig.2: Feed intake trend of rabbits on different feeding strategies (g/rabbit/day)

feeding appeared to be less efficient as they required more feed to gain a unit weight. Similarly, improvements were noted in the feed conversion in the weight of the rabbits in Treatment 4 and Treatment 5 (80 and 60% *ad libitum* feeding during restriction followed by *ad libitum* feeding in the 4-week realimentation period). It was thus clear that during the realimentation (4-7 weeks), there was a favourable effect of feed restriction on feed conversion. This finding was in consonance with the previous reports of Boisot *et al.* (2003) and Foubert *et al*. (2008), in which significant favourable effects of feed restriction level were observed on feed conversion in the total fattening period.

The results were, however, at variance with that of Ledin (1984), who reported that in the entire growth period, the feed conversion was similar for the restricted-realimented and continuously *ad libitum* animals because the differences in the feed conversion efficiency in both periods (restriction and realimentation) tended to cancel each other. Meanwhile, Perrier (1998) found significant differences, but just for animals having a level of 50% intake of *ad libitum.*

The effect of treatments on economy of production measured as cost per kilogram weight gain indicated that restricting the feed by 20 and 40% without realimentation (T2 and T3) resulted in a reduction of 17.24 and 12.81% respectively as compared with the *ad libitum* fed control group. For the rabbits subjected to realimentation after the restriction period of 3 weeks, the savings in

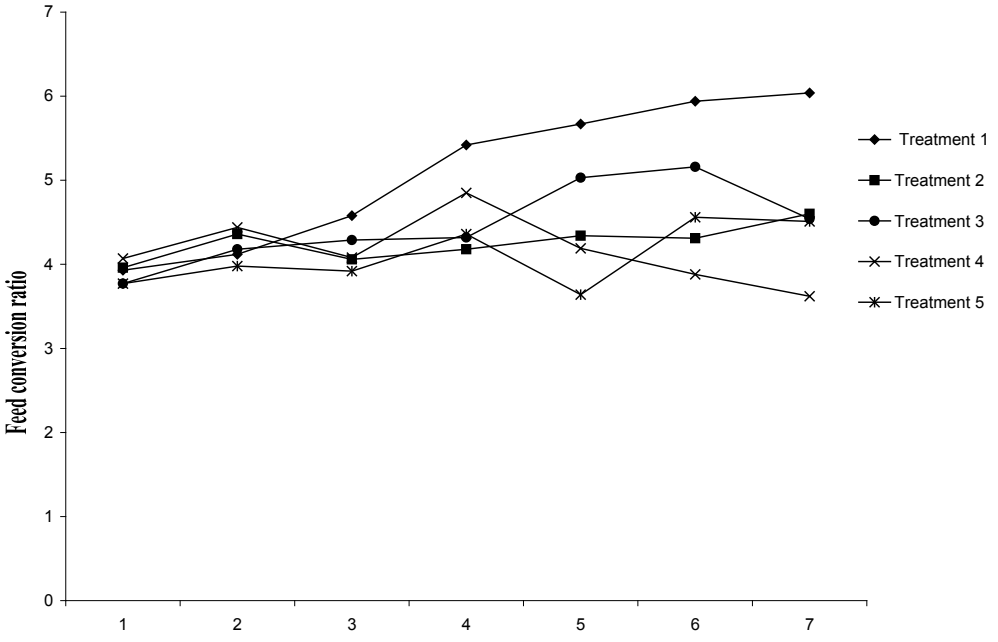


Fig.3: Feed conversion trend of rabbits on different feeding strategies (g feed: g weight)

feed cost per kilogramme weight gain were ^75.03 and ^ 45.67 representing 18.89 and 11.50% savings for T4 and T5 respectively compared with the control treatment (T1). It was observed that in terms of economic efficiency, the rabbits fed at 80% *ad libitum* feed intake (T4) were better than those on other treatments and the control. The superiority of the rabbits in T4 compared to the rabbits in the control (T1) arose from the fact that, although weight gain was similar between the two treatments, the amounts of feed required to attain the weight were however dissimilar.

The effects of treatments (feeding regimes) on carcass and retail cuts of the rabbits are presented in Table 3. Feed restriction regimes significantly influenced ($P<0.05$) dressed weight and dressing out percentage (DOP); however, there was no effect ($P>0.05$) on the fore limb, loin, hind limb and thoracic cage weights of the rabbit carcasses expressed as percentages of live weight.

The rabbits subjected to 80% *ad libitum* feeding in the first 3 weeks of feeding followed by *ad libitum* feeding in the last 4 weeks (T_4) had the highest dressed weight and dressing out percentage. The dressed weight and dressing out percentage obtained from the rabbits on *ad libitum* feeding throughout the feeding trial and those rabbits on 60% *ad libitum* feeding in the first 3 weeks of feeding followed by *ad libitum* feeding in the last 4 weeks were not different from each other. Similar results of no significant effect on dressed weight and dressing out percentages were obtained for the rabbits subjected to 80 and 60% *ad libitum* feeding throughout the 7-week duration. However, the values recorded for these two treatments were significantly lower than the values obtained from the other treatments. Perrier (1998) found lower dressing out percentage in cases of stricter restriction, which is similar to the observation in this study. The result of DOP was however in contrast with the results of Ferreira and Carregal (1996), Tůmova *et al.* (2003, 2006) and Boisot *et al*. (2004), who reported that restriction did not affect dressing percentage.

Other carcass traits were not affected by the feeding programmes, a finding that was in agreement with the report of Gidenne *et al*. (2009). Some earlier studies have shown little effect of feed restriction on relative organ weights, carcass portions, and meat quality (Matics *et al*., 2008; Tůmova *et al*. (2007).

The effects of feeding regimen on the organ weights of the rabbits are shown in Table 4. Feed restriction had no significant ($P>0.05$) influence on relative organ weights across the treatments. The present result was in consonance with the findings of Tumova *et al.* (2004), that there were no significant differences in the relative organ weights among the rabbits on *ad libitum* feeding and those on feed restriction.

CONCLUSION AND RECOMMENDATIONS

Restricting the amount of feed could be a suitable method for reducing feed intake and improving feed conversion. Compensatory

TABLE 3
Carcass and retail cuts weights of rabbit on different feed restriction regimes

Carcass characteristics	**T1** *Ad libitum* Feeding for 7 weeks	**T2** 80% *Ad libitum* Feeding for 7 weeks	**T3** 60% *Ad libitum* Feeding for 7 weeks	**T4** 80% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding	**T5** 60% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding
Liveweight (g)	$1440.90^{a} \pm 23.00$	$1369.10^{ab} \pm 17.12$	$1231.10^{b} \pm 18.28$	$1480.53^{a} \pm 23.15$	$1380.00^{ab} \pm 21.38$
Dressed weight (g)	$871.31^{b} \pm 16.62$	$764.32^{bc} \pm 14.14$	$681.11^{c} \pm 12.28$	$951.54^{a} \pm 11.37$	$850.49^{b} \pm 14.31$
Dressing out percentage (%)	$60.47^{b} \pm 4.33$	$55.83^{c} \pm 5.15$	$55.33^{c} \pm 7.41$	$64.27^{a} \pm 4.48$	$61.63^{b} \pm 5.72$
Head (% LW)	7.83 ± 0.12	8.03 ± 0.08	8.10 ± 0.10	8.23 ± 0.10	7.83 ± 0.12
Forelimb (% LW)	8.39 ± 0.11	7.81 ± 0.13	9.05 ± 0.13	8.37 ± 0.19	7.11 ± 0.14
Loin (% LW)	10.10 ± 0.10	10.87 ± 0.11	10.50 ± 0.11	12.20 ± 0.11	10.23 ± 0.12
Hind limb (% LW)	19.37 ± 2.33	18.57 ± 1.49	18.43 ± 3.20	20.23 ± 2.78	19.00 ± 2.40
Thoracic (% LW)	10.30 ± 0.30	10.23 ± 0.22	9.70 ± 0.35	11.83 ± 0.24	10.65 ± 0.20

[a, b, c] Means within the same row with differing superscripts are significantly different ($P<0.05$)

TABLE 4
Relative organ weight of rabbit on varying durations of feed restriction (% Liveweight)

Organs	**T1** *Ad libitum* Feeding for 7 weeks	**T2** 80% *Ad libitum* Feeding for 7 weeks	**T3** 60% *Ad libitum* Feeding for 7 weeks	**T4** 80% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding	**T5** 60% *Ad libitum* feeding for 3 weeks followed by 4 weeks *ad libitum* feeding
Liver (% LW)	2.54 ± 0.35	3.29 ± 0.28	2.10 ± 0.29	3.00 ± 0.31	3.09 ± 0.26
Kidney (% LW)	0.68 ± 0.06	0.63 ± 0.08	0.53 ± 0.03	0.50 ± 0.05	0.55 ± 0.06
GIT* (% LW)	20.57 ± 0.27	20.23 ± 0.32	19.50 ± 0.26	21.17 ± 0.22	20.90 ± 0.24
Spleen (% LW)	0.14 ± 0.03	0.17 ± 0.01	0.50 ± 0.35	0.14 ± 0.03	0.15 ± 0.02
Heart (% LW)	0.43 ± 0.02	0.41 ± 0.01	0.40 ± 0.02	0.42 ± 0.01	0.41 ± 0.02

- Gastro intestinal tract

growth was observed in the rabbits subjected to restriction but later *ad libitum* fed. Body weight compensation was almost complete in the rabbits of T4, which was similar to the weight obtained on the control treatment (*ad libitum* feeding). Concerning the carcass traits, the observed decrease in dressing out percentage with increasing stiffness of restriction is an indication of the fact that feed restriction may have its own disadvantages except when realimentation is allowed for adequate period.

From the findings of this study, it is recommended that growing rabbits can be subjected to a three-week feed restriction of not more than 20% provided at least four weeks of *ad libitum* feeding is allowed for compensatory growth.

REFERENCES

A. O. A. C. (1995). *Official Methods of Analysis*. Association of Official Analytical Chemists (16th Edn.). Washington D.C.

Adeyemi, O. A., Sobayo, R. A., Aluko, F. A., & Oke, D. B. (2008). Utilisation of rumen filtrate fermented corn-cobs by weaner rabbits. *Nig. J. Anim. Prod*., *35*(1), 69-75.

Blasco, A.,& Ouhayoun, J. (1993). Harmonization of criteria and terminology in rabbit meat research. Revised proposal. *World Rabbit Sci*., *4*(2), 93-99.

Blasco A., Ouhayoun, J., & Masoero, G. (1993). Harmonization of criteria and terminology in rabbit meat research. *World Rabbit Sci., 1,* 3-10.

Boisot, P., Licois, D., & Gidenne, T. (2003). *Feed restriction reduces the sanitary impact of an experimental reproduction of Epizootic Rabbit Enteropathy Syndrom (ERE), in the growing rabbit*. 10ème Journées Recherche. Cunicoles Française. p. 267-270.

Boisot P., Duperray, J., Dugenetais, X., & Guyonvarch, A. (2004). *Interest of hydric restriction times of 2 and 3 hours per day to induce feed restriction in growing rabbits*. In Proc. 8th World Rabbit Congress, September7–10, Puebla, Mexico. 759–764.

Ferreira R. G., & Carregal, R. D. (1996). *A note on carcass characteristics of rabbits fed on a restricted system*. In Proc. 6th World Rabbit Congress, July 9–12, Toulouse, France. 163–165.

Feugier, A. (2002). *Incidence d'un rationnement quantitatif sur la croissance, la digestion et l'état sanitaire du lapin*. Ed. ISARA. 1- 41.

Foubert, C., Boisot, P., Duperray, J., & Guyonvarch, A. (2007). *Interêt d'un accès limité à la mangeoire de 6h, 8h et 10 h pqr jour pour engendrer un rationnement alimentaire chez le lapin en engraissement*. 12èmes Journées de la recherche Cunicole. 123-126.

Gidenne, T., Combes, S., Feugier, A., Jehl, A., Arveux, P., Boisot, P., Briens, C., Corrent, E., Fortune, H., Montessuy, S., & Verdelhan, S. (2009). Feed restriction strategy in the growing rabbit. 2. Impact on digestive health, growth and carcass characteristics. *Animal*, *3*(4), 509–515.

Goering, H. K., & van Soest, P. J. (1970). Forage analysis *(Apparatus, Reagents, Proceedings and some applications)*. Agric. Handbook No.379 ARS. USDA, Washington D. C.

Halls, A. E. (2010). Nutritional Requirements of Rabbits. Retrieved from http://www.nutrecocanada.com/docs/shur-gain---specialty/nutritional-requirements-of-rabbits

Ledin, I. (1984a). Effect of restricted feeding and realimentation on growth, carcass composition and organ growth during the first seven days of realimentation in rabbit. *Acta Agric. Scand. 34*, 54-66.

Ledin, I. (1984b). A note on the effect of different feeding levels on the rate of digesta passage in rabbits. *Acta Agric. Scand*., *34*, 67-70.

Maertens, L., & Peeters, J. E. (1988). Effect of a feed restriction after weaning on fattening performances and caecal traits of early weaned rabbits. 6. Arbeitstagung über Peltztiere, Kaninchen und Heimtier production und Krankenheit. *Celle 2-4 Juni*, 158-169.

Matics, Z. S., Dalle Zotte, A., Radnai, I., Kovács, M., Metzger, Z. S., & Szendrı, Z. S. (2008). *Effect of restricted feeding after weaning on the productive and carcass traits of growing rabbits.* In the Proceeding of 9th World Rabbit Congress – June 10-13, 2008 – Verona – Italy pp 741-745

Merck Manual (2011). *Nutritional Requirements of Rabbits*. Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc. Whitehouse Station, New Jersey, USA. Retrieved from http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/171315.htm

Minitab Inc. (1999). *Minitab Data Analysis System*. Release 7.1 Standard Version Serial. Minitab Inc.

Perrier, G. (1998). Influence de deux niveaux et de deux durées de restriction alimentaire sur l'efficacité productive du lapin et les caractéristiques bouchères de la carcasse. 7ème *Journées. Recherche. Cunicole Française,* 179-182.

Perrier, G., & Ouyayoun, J. (1996). Growth and carcass traits of the rabbit: a comparative study of three modes of feed rationing during fattening. *6th World Rabbit Congress, 3*, 225-231.

Steel, R. G. D., & Torrie, J. H. (1980). *Principles and Procedures of Statistics* (2nd Edn.). USA: McGraw Hill Book Co., Inc.

Szendrõ, Z. S., Mihálovics, G. Y., Milistis, G., Biró-Németh, E., & Radnai, I. (2000). Effect of reduction of feeding time on the performances and carcass quality of growing rabbits. 7th *World Rabbit Congress, 6*.

Tumova, E., Sk Ivan, M., Sk Ivanová, V., & Kacerovská, V. (2002). Effect of early feed

restriction on growth in broiler chickens, turkeys and rabbits. *Czech Journal of Animal Science, 10*, 418-428.

Tůmová, E., Skřivanová, V., & Skřivan, M. (2003). Effect of restricted feeding time and quantitative restriction in growing rabbits. *Arch. Geflűgelkde*, *67*, 182 -190.

Tůmova E., Zita, L., & Štolc, L. (2006). Carcass quality in restricted and *ad libitum* fed rabbits. *Czech J. Anim. Sci., 51*(5)*, 214–219.*

Tumová, E, Zita, L., Skrivanová, V., Fucíková, A., Skrivan, M., and Burešová, M. (2007). Digestibility of nutrients, organ development and blood picture in restricted and ad libitum fed broiler rabbits. *Arch.Geflügelk., 71*(1), S. 6–12.

Wardeh, M. F. (1981). *Methods for estimating energy and protein utilization of feed*. PhD Dissertation. Utah State University. Logan. US.

Pertanika J. Trop. Agric. Sci. 36 (3): 275 - 284 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Management of Root Knot Nematodes (*Meloidogyne incognita*) on Cowpea with Plant Extracts

Chimbekujwo, I. B.[1]*, Bukar, A. M.[2] and Channya, F. K.[3]

[1]*Department of Plant Sciences, Modibbo Adama University of Technology, P.M.B. 2076, Yola, Adamawa State, Nigeria*
[2]*Department of Biological Sciences, Bama College of Education, Borno State, Nigeria*
[3]*Department of Plant Sciences, Modibbo Adama University of Technology, Yola, Adamawa State, Nigeria*

ABSTRACT

Screen house experiment was conducted to test the efficacies of leaf powder of neem (*Azadirachta indica*), kassod tree (*Cassia siamea*), eucalyptus (*Eucalyptus gigantea*) and locust bean tree (*Parkia biglobosa*) in the management of *Meloidogyne incognita* on cowpea yield. In this study, 50g of each of the leaf powder was separately mixed with 4 kg of soil in a 25-cm diameter plastic pot. Non-amended pots served as the control. Cowpea seeds were sown in each pot and each seedling was inoculated with about 3000 freshly hatched juveniles of *Meloidogyne incognita* two weeks after sowing. The experiment was laid out in a completely randomised design with five replications. The obtained data were analysed using ANOVA and means, while the significance values were separated using the Duncan multiple test. The result of the study showed that all the treatments significantly ($p<0.001$) reduced root galling and nematode population, as well as improved plant growth and yield of cowpeas. Although all treatments were effective in reducing root galls and nematode population, application of *Azadirachta indica* leaf powder gave the highest reduction in root galls (0.293) and nematode population (24), followed by *Cassia siamea*, *Eucalyptus gigantea* and *Parkia biglobosa*. The findings recorded root galls and nematode population of 28.25 and 37.34, 29 and 48, and 30 and 53.34 respectively as compared to the control treatment, which recorded root galls of 107.75 and nematode population of 189.34.

ARTICLE INFO

Article history:
Received: 18 May 2011
Accepted: 2 November 2011

E-mail addresses:
chimbe2007@yahoo.com (Chimbekujwo, I. B.)
bukarmodu@ymail.comr (Bukar, A. M.),
farbinahchannya60@yahoo.com (Channya, F. K.)
* Corresponding author

Keywords: Azadirachta indica, cowpea, *Cassia siamea*, *Eucalyptus gigantea*, *Parkia biglobosa* and *Meloidogyne incognita*

ISSN: 1511-3701

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp) is a dicotyledonous plant belonging to the *Fabaceae* family. It is the most important grain legume in West Africa, which provides a source of profitable revenue between 23 and 29% of the selling price (Langyintuo *et al.,* 2000). It is also valued as the cheapest dietary and high quality vegetable protein of about 25-43% (Nielson *et al.,* 1997), accounting for up to 80% of total protein intake in Nigeria (Rachie & Rawal, 1975). *Meloidogyne* spp is a major problem of cowpeas in most crop growing regions of the world (Caveness, 1992). Losses on cowpeas are attributable to *Meloidogyne* species in some West African countries, which have been put between 10% and 89% including total crop losses in some cases (Adesiyan *et al*., 1990). Iheukwumere *et al*. (1995) recognized *Meloidogyne* spp as one of the plant-parasitic nematodes of economic importance in legume production in Nigeria. The root knot nematode is estimated to cause losses ranging from 10% to 69% in Nigeria (Olowe, 2009). The symptoms of nematode infection include formation of root galls which results in growth reduction, nutrient and water uptake reduction, increased wilting, mineral deficiency, as well as weak and poor yielding plants. The use of synthetic nematicides is considered the most effective practical means of combating the menace of plant-parasitic nematodes in cowpeas (Adesiyan, 1992). However, chemical control of root knot nematodes leads to environmental hazards because of the high toxicity and persistence in the soil (Anastasiadis *et al*., 2008). As an alternative, organic soil amendment has been found to be cheaper, less harmful to man and effective in the management of plant-parasitic nematodes (Olabiyi *et al.,* 2007). In view of this, the present investigation was undertaken to assess the nematicidal activity of four plant species, namely; *Azadirachta indica, Cassia siamea, Eucalyptus gigantean* and *Parkia biglobosa*, in the management of *Meloidogyne* spp in cowpeas.

MATERIALS AND METHODS

Soil Collection and Sterilization

Top soil of 0–30 cm depth was used for the experiment in this study. The soil was collected from the Biological Garden of Modibbo Adama University of Technology, Yola, in Adamawa state, Nigeria, located at latitude 9□ 14′N and longitude 12□ 27′E. The soil, composed of 55.6% sand, 19.4% silt, 25% clay, pH 6.8 and 0.98% organic matter, was steam-sterilized by heating with lighted firewood in a large aluminium pot to a temperature of 100^{o}C and maintained for one hour. The soil was then allowed to cool and later stored in jute sacs to rest for six weeks to regain its stability.

Collection and Preparation of Leaves

Mature leaves of *Azadirachta indica, Cassia siamea, Eucalyptus gigantea, Parkia biglobosa* and *Calotropis procera* were used for the experiment. The leaves were separately collected from different plants at different locations within the premises of

Modibbo Adama University of Technology, Yola, and spread on polythene sheets in an open protected area for one week to dry. The dried leaves were ground separately to fine particles using a mortar and stored in a sealed container for use.

Amendment Applications Rate

The ground leaves were separately mixed with 4 kg of steam-sterilized soil at the rate of 50 g. The mixtures were transferred into 25-cm diameter perforated plastic pots.

Sowing of Seeds

Seeds of cowpeas, cv. "Kanannado", were obtained from Monday market in Maiduguri, Borno, Nigeria. The seeds were sown into plastic pots filled with the mixture of steam-sterilized soil and ground leaves. Meanwhile, the control pots contained only steam-sterilized soil. Three seeds were sown per pot at a depth of 2 cm, but the seedlings were thinned to one per pot six days after emergence to ensure uniform plant vigour. The pots were watered regularly once a day, and the potted soil around the base of the plants was loosened from time to time with a hand fork to avoid compacting without disturbing the plant roots.

Collection of Root Knot Nematode Samples

The samples of root knot nematodes (*Meloidogyne* spp.) were collected from tomato plants under irrigation in various farms with the permission of the farmers at Lake Alau in Borno. The roots of the diseased plants showing characteristic symptoms of 15 root knot nematodes were carefully uplifted with a trowel up to 15–20 cm depth from the rhizosphere of the diseased plants together with approximately 1 kg of soil . The samples were then placed in polythene bags and brought to the laboratory for analysis.

Identification of Root Knot Nematode Species

The root knot nematode species was identified on the basis of perineal pattern characteristics of mature female as described by Eisenback *et al*. (1981). A pure culture of root knot nematode, *Meloidogyne incognita*, was raised from a single egg mass obtained from a root knot nematode-infected tomato plant. The single egg mass was propagated on the tomato plant by inoculating the tomato seedlings grown in steam-sterilized soil. Further sub-cultures were made from the initial culture to increase the nematode population .

Preparation of Inoculum

Second-stage juvenile nematodes were used as the inoculum. Eggs of *Meloidogyne incognita* were collected from a pure culture and maintained on tomato roots using sodium hypochlorite technique (Hussey & Barker, 1973). The eggs were placed in the tap water in a Petri dish and incubated for 24 hours at room temperature for hatching. After hatching, the second-stage juveniles were collected and larval suspension was prepared in tap water.

Inoculation Procedure

The cowpea plants were inoculated two weeks after planted in the pots. The population of about 3000 juvenile nematodes per plant was used. Four holes about 2 cm deep and 1 cm wide each were made in the soil around each seedling to expose the roots. The second-stage juvenile nematode suspension was applied into each hole with a syringe and the holes were filled with moist soil. Each treatment was replicated five times and the pots were laid out in a completely randomized experimental design in the screen house. The experiment was terminated sixty days after sowing.

Data Collection

Collection of data was carried out during harvest, whereby the shoot height was ascertained using a measuring tape, the numbers of seeds per plant were counted, and the fresh weight of shoots and grain yield per plant were determined using an electronic balance. Furthermore, the populations of nematodes in the soil and roots, as well as the number of galls and gall index were also identified.

Estimation of Nematode Population in Soil

The population of nematodes in the soil was determined using modified Baermann's funnel extraction technique (Barker, 1985).

Assessment of Galling Index

The roots were rated for the amount of galls using a rating scheme described by Ogbuji (1981), as follows:

0 = 0 gall (no infection)

1 = 1 – 3 galls (rare infection)

2 = 4 – 10 galls (light infection)

3 = 11 - 30 galls (moderate infection)

4 = 31 - 100 galls (severe infection)

5 = > 100 galls (very severe infection)

Estimation of Nematode Population in Roots

The population of nematodes in the roots was determined using the maceration method followed by Baermann's funnel technique (Southey, 1970). The reproduction rate of nematodes was calculated by using the formula, R= Pf/Pi, where Pf represents the final nematode population and Pi represents the initial nematode population (Oostenbrink, 1966).

Measurement Plant Height

The plant height was measured from the base of the plant to the tip of the top leaf on the main shoot. Measurements were taken from three plants in each treatment and the average height was calculated and expressed in cm.

Measurement of Shoot Weight

The weight of three randomly selected fresh shoots was measured on electronic balance and the average weight was taken as the shoot weight per plant.

Estimation of Number of Seeds per Plant

The total number of seeds produced in the three plant samples were counted, and the

average number was taken as the number of seeds per plant.

Estimation of Cowpea Grain Yield

After threshing, the grain produce obtained from each plant was cleaned and weighed in grams on electronic balance, and the average weight for each treatment was taken as the grain yield per plant.

RESULTS AND DISCUSSION

The results showed that all the treatments significantly (P<0.001) suppressed the development of *Meloidogyne incognita* population in the soil as compared to the control. The highest population of 733.34 was recorded in the control treatment, while the population dropped to as low as 213.34 in the soil treated with *Azadirachta indica* leaf powder, representing 70.91% of reduction over the control. This was followed by the leaf powder of *Cassia siamea* (306.67) with 58.18% reduction, *Eucalyptus gigantean* (320.00) with 56.36% reduction, and *Parkia biglobosa* (440.00) with 40.00% reduction as compared to the control (Fig.1). The highest population of *M. incognita* in the roots (189.34) was recorded in the control plants, while the lowest population (24.00) representing 87.32% reduction over the control was recorded in the plants grown in the soil amended with the leaf powder of *Azadirachta indca*. Likewise, this was followed by *Cassia siamea* (37.34) with 80.28% reduction, *Eucalyptus gigantea* (48.00) with 74.65% reduction, and *Parkia biglobosa* (53.34) with 71.83% reduction as compared to the control treatment (Fig. 2).

The reproductive capacity of the nematodes was also significantly (P<0.001) affected by the treatments imposed on the plants (Fig.3). The highest rate of reproduction of *Meloidogyne incognita* (1.041) was observed in the control plants, which was significantly different from other treatments. The highest reduction in the reproduction rate (0.293) representing 71.85% reduction as compared to the control was recorded in plants administered with the leaf powder of *Azadirachtaindica*. This was followed by *Cassia siamea* (0.422) with 59.46% reduction, *Eucalyptus gigantea* (0.443) with 57.47 % reduction, and *Parkia biglobosa* (0.605) with 41.88% reduction as compared to the control treatment. The number of root galls incited by *Meloidogyne incognita* on the roots of cowpeas peaked at 107.75 in the control (Fig.4), but ranged from as low as 19.50 in the *Azadirachtaindica* leaf powder treated plants to 30.00 in the *Parkia biglobosa* leaf powder treated plants. The plants treated with *Azadirachta indica* leaf powder had the highest reduction efficacy (81.9%), followed by *Cassia siamea* leaf powder (73.78%), *Eucalyptusgigantea* leaf powder (73.09%) and *Parkia biglobosa* leaf powder (72.16%). Fresh shoot weight, shoot height and number of leaves per plant were significantly (P<0.001) higher in the pots treated with *Azadirachta indica, Parkiabiglobosa, Eucalyptus gigantea* and *Cassia siamea* than in the control treatment. The lowest shoot weight (10.18g) was recorded in the control plants, whereas the highest fresh shoot weight (46.12g) representing 353.05% increase over the

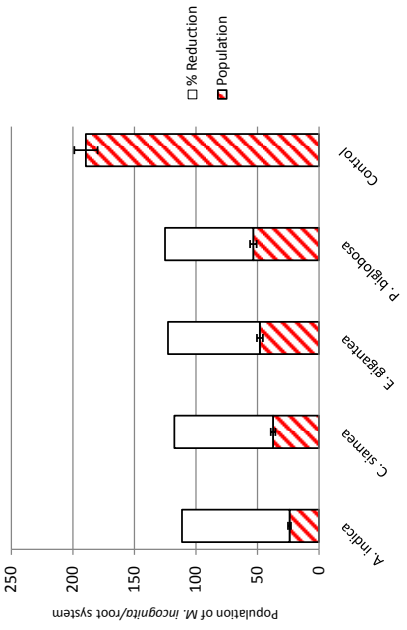


Fig.1: The effects of organic soil amendments on the population of *M. incognita* in the rhizosphere of cowpea

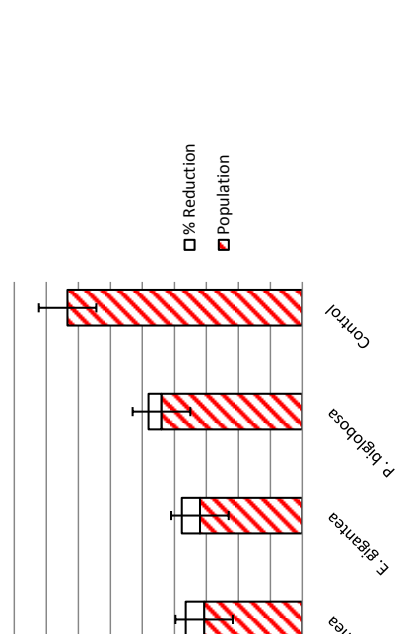


Fig.2: The effects of organic soil amendments on the population of *M. incognita* in the root system of cowpea

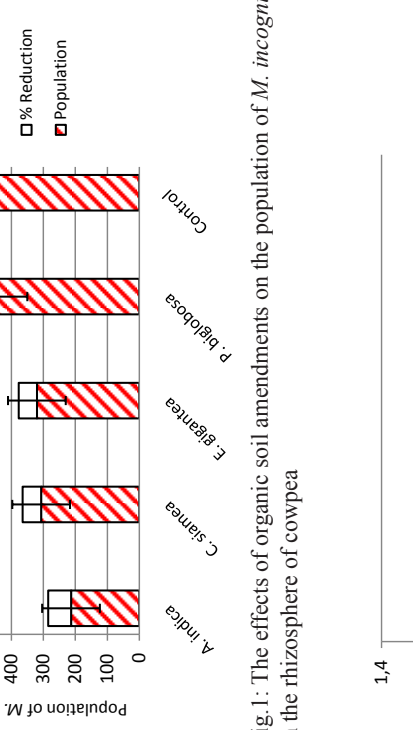


Fig.3: The effects of organic soil amendments on *M. incognita* reproduction factor in the root system of cowpea

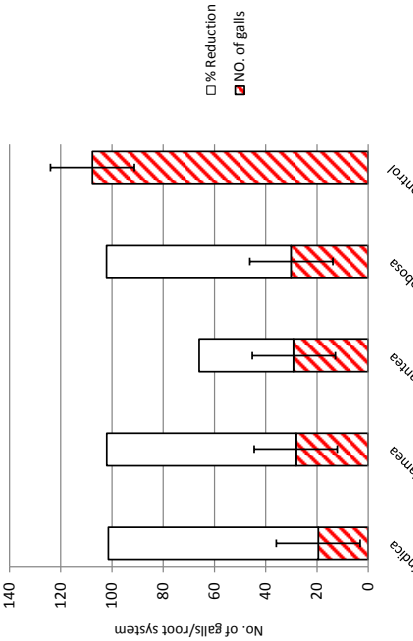


Fig.4: The effects of organic soil amendments on gall formation in *M. incognita*-infected root system of cowpea

control was recorded in the plants treated with the leaf powder of *Azadirachta indica*.

Meanwhile, the plants treated with the leaf powder of *Cassia siamea, Eucalyptus gigantea* and *Parkia biglobosa* recorded 208.35% (31.39g), 87.33% (19.07g), and 157.17% (26.18g) of shoot weight increase respectively over the control treatment (Table 1). The lowest shoot height (10.50cm) was noted in the control plants, while the maximum shoot height (49.87cm) representing 374.95% increase over the control was recorded in the plants treated with the leaf powder of *Azadirachta indica*. This was followed by *Cassia siamea* (45.34cm) with 331.81% increase, *Eucalyptus gigantea* (42.20cm) with 301.9% increase and *Parkia biglobosa* (36.00cm) with 242.86% increase over the control treatment (Table 1). The number of seeds per plant and grain yield per plant were significantly ($P<0.001$) higher in the plants treated with organic materials than in the control plants. The highest number of seed per plant (49.25) was obtained from the plants treated with the leaf powder of *Azadirachta indica*. This was followed by *Cassia siamea* (44.50), *Eucalyptus gigantea* (36.50) and *Parkia biglobosa* (28.75). The lowest number of seeds per plant (10.00) was recorded in the control plants (Table 1). The lowest grain yield (2.26g) was recorded in the control plants, while the highest grain yield per plant (10.60g) representing 369.03% increase as compared to the control was recorded in the plants treated with the leaf powder of *Azadirachta indica*. The plants treated with the leaf powder of *Cassia siamea* recorded 9.58g representing 323.89% increase, while the plants treated with the leaf powder of *Eucalyptus gigantea* and *Parkia biglobosa* recorded 225.22% (7.35g) and 192.48% (6.61g) yield increase, respectively (see Table 1).

Means in the same column followed by the same letter do not differ statistically between themselves at 5% probability level, as indicated by Duncan multiple range tests.

Figures in parentheses indicate percentage increase as compared to the control treatment.

The results of this study showed that amending the soil with the leaf powder of *A. indica, P. biglobosa, E. gigantea* and *C. siamea* suppressed the population of *M. incognita* both in the soil and roots of cowpea plants with a concomitant increase in growth and yield of cowpeas. These results were in agreement with the previous findings of Ahmad *et al.* (2007) and Adegbite

TABLE 1
Effect of organic soil amendments on the growth and yield of *Meloidogyne incognita*-infected cowpea plant.

Treatments	Fresh Shoot wt.(g)	Shoot Height (cm)	No. of seeds/ plant	Grain yield/ plant (g)
A. indica	46.12^{b} (353.05)	49.87^{de} (374.95)	49.25^{cd} (392.5)	10.60^{cd} (369.03)
C. siamea	31.39^{fg} (208.35)	45.34^{fg} (331.81)	44.50^{cd} (345.0)	9.58^{ef} (323.89)
E .gigantea	19.07^{jl} (87.33)	42.20^{gh} (301.9)	36.50^{ef} (265.0)	7.35^{gh} (225.22)
P. biglobos	26.18 (157.17)	36.00 (242.86)	28.75 (187.5)	6.61 (192.48)
Control	10.18^{m}	10.50^{m}	10.00^{i}	2.26^{j}

(2011a), who reported that application of botanicals as soil amendment causes significant reduction in root knot nematode infestation which consequently leads to the increase in growth of different plants. The nematicidal potential of *Azadirachta indica* found in this study was supported by Yasmin *et al*.'s (2003) findings, which reported that fresh extracts of seeds, leaves and barks of neem inhibited the hatching of *Meloidogyne incognita*. Boiled water extract of fresh neem leaves was reported to be toxic to eggs and juveniles *of M. incognita* (Claudius *et al.,* 2010). The neem constituents, namely, nimbin, salanin, thionemone, azadirachtin and various flavonoids, have been reported to have a nematicidal action (Akhtar and Malik, 2000).

The results of the present study also showed that the incorporation of *Eucalyptus gigantea* leaf powder significantly suppressed the development of *Meloidogyne incognita* population both in the soil and root systems of cowpea, reduced root gall formation, and improved the growth of cowpea plant. Similar findings were also reported by Shahnazdawar *et al.* (2007) that aqueous and ethanol extracts of different parts of *Eucalyptus gigantean*, namely, leaf, stem, bark and fruit showed a nematicidal effect against *Meloidogyne javanica* on mung bean and chick pea plants when used as soil amendment.

The findings of this study also showed that amending the soil with *Cassia siamea* leaf powder significantly reduced the development of *Meloidogyne incognita* population in the soil and root of cowpea, reduced root knot disease and improved growth of cowpea plant. This result was in agreement with the findings of Bello *et al.* (2006), who reported that water extract of seed, leaf and bark of *Cassia siamea* significantly inhibited larval hatch of *Meloidogyne incognita*.

The results of this study also showed that amending the soil with *P. biglobosa* leaf powder significantly reduced nematode population, as well as improved plant growth and grain yield. This result was in agreement with the findings of Fatoki and Oyedunmade (1996), who reported the effective control of *Meloidogyne incognita* with chopped leaves of *Parkia biglobosa*. Olabiyi *et al.* (2007) also reported that the leaf extract of *Parkia biglobosa* effectively inhibited the hatching of *M. incognita* eggs.

CONCLUSION

In conclusion, the findings of this study showed that the leaf powder of *Azadirachta indica, Parkia biglobosa, Eucalyptus gigantea* and *Cassia siamea* has strong nematicidal properties. Their addition to the soil controls the population build up of *Meloidogyne incognita*, which results in better growth of cowpeas. This finding is very important from the point of view of controlling the root knot nematodes affecting cowpeas since the use of synthetic nematicides by subsistence farmers is plagued with several limitations, such as prohibitive cost, lack of technical expertise in their applications, and the environmental pollution they likely cause.

REFERENCES

Adegbite, A. A. (2011a**)**. Assessment of Yield Loss of Cowpea (*Vigna unguiculata* L.) due to Root Knot Nematode, *Meloidogyne incognita* under Field Conditions. *American Journal ofExperimental Agriculture, 1*(3), 79-85.

Adesiyan, S. O., Caveness, F. E., Adeniji, M. O., & Fawole, B. (1990). *Nematode Pests of tropical crops*. Heinemann Educational Books (Nigeria) Limited.

Adesiyan, S. O. (1992). *Chemical Control of nematode pests of some economic crops*. In proceedings of the first regional symposium of the Biology and control of nematode pests of food crops in Africa University of Ibadan, Nigeria.

Ahmad, F., Rather, M. A., & Siddiqui, M.A. (2007). Impact of organic soil amendments and nematicides on *Meloidogyne javanica* infecting tomato. *Indian Journal of Nematology, 37*, 55-57.

Akhtar, M., & Malik, A. (2000). Role of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. *Biores. Technol.*, *74*, 35-47.

Anastasiadis, I. A., Giannakou, I. O., Prophetou-Athanasiadou, D. A., & Gowen, D. A. (2008). The combined effect of the application of a biocontrol agent *Paecilomyces lilacinus*, with various practices for the control of root-knot nematodes. *Crop Protection, 27*, 352-361.

Barker, K. R. (1985). Nematode extraction and bioassays. In K. R. Barker, C. C., Carter & J. N. Sasser (Eds.)**,** *An Advance Treatise on Meloidogyne* (Vol. 2) (pp. 19-35). Methodology. North Carolina State University Graphics.

Bello, L. Y., Chindo, P. S., Marley, M. D., & Alegbejo, M. D. (2006). Effect of some plant extracts on egg hatching of *Meloidogyne incognita. Archives of Phytopathology and Plant Protection, 39*, 253-257.

Caveness, F. E. **(**1992). Nematological Researchat IITA. A summary of investigationconducted between 1969 and 1988. In J. Lowe (Ed). *Plant health management Researchmonograph* p. 17-19.

Claudius-Cole, A. O., Aminu, A. E., & Fawole, B. (2010). Evaluation of plant extracts in the management of rootknotnematode Meloidogyne incognita on cowpea [Vigna unguiculata (L) Walp] *Mycopath, 8*(2), 53-60.

Eisenback, J. D., Hirschmann, H., Sasser, J. N., & Trianttaphyllou, A.C. (1981).*A Guide to the Four Most Common Species of Root Knot Nematodes* (*Meloidogyne* species) with Pictorial Key. International Meloidogyne Project. Department of Plant Pathology, North Carolina University, Raleigh, N.C.

Fatoki, O. K., & Oyedunmade, E. E. A. (1996). Controlling effect of some plant leaves on the root knot nematode, *Meloidogyne incognita* attacking tomato. *Nigerian Journal of Plant Protection, 16*, 59 – 65.

Hussey, R. S., & Barker, K. R. (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp including a new technique. *Plant Disease Reporter, 57*, 1025 – 1028.

Iheukwumere, C. C., Atiri, G. I., Fawole, B., & Dashiell, K. E. (1995). Evaluation of some commonly grown soybean cultivars for resistance to root-knot nematodes and soybean mosaic virus in Nigeria. *Fitopatologia Brasileria, 20*, 190-193.

Langyintuo, A. S., Lowenberg-De Boer, J., Faye, M., Lambert, D., Ibro, G., Moussa, B., Kergna, A., Kushwaha, S., Musa, S., & Ntoukam, G. (2000). *Cowpea Supply and Demand in West and Central Africa*. In the Proceedings of the Third world Cowpea Conference. International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. September 4-7, 2000. 2000: 1-35.

Nielson, S. S., Ohler, T. A., & Mitchell, C. A. (1997). Cowpea leaves for human consumption. In B. B. Singh, D. R. Mohan Raj, K. E. Dashiell, & L. E. N. Jackaj (Eds), *Advances in Cowpea Research*. Co-publication IITA and JIRCAS, IITA, Nigeria.

Ogbuji, R. O. (1981). Infectivity of three *Meloidogyne* sp. on soyabean in Nigeria. Der*tropenlandwrit, 88*, 149-152.

Olabiyi, T. I., W. B. Akanbi, & Adepoju, I. O. (2007). Control of certain nematode pest with different organic manure on cowpea. *American-Eurasian Journal of Agricultural and Environmental Sciences, 2*(5), 523- 527.

Olowe, T. (2009). Cowpea Germplasm Resistant to*Meloidogyne arenaria* Race 1, *Meloidogyne incognita* Race 4 and *Meloidogyne javanica. European Journal of Scientific Research, 28*(3), 338-350.

Oostenbrink, M. (1966). Major characteristics of the relation between nematodes and plants. *Meded. Landbouwhogesch. Wageningen, 66*, 4 – 46.

Rachie, K. O., & Rawal, K. A. (1975). Integrated approaches to improving cowpea, *Vigna unguiculata* (L) Walp. *Technical Bulletin* 1975, p. 5.

Shahnazdawar, S., Younus, M., & Javidzaki, M. (2007). Use of *Eucalyptus* spp.in the control of *Meloidogyne javanica* root knot nematode. *Pakistan Journal of Botany*, *39*(6), 2209-2214.

Southey, J. F. (1970). *Laboratory methods for work with plant and soil nematodes*. Ministry of Agriculture, Fisheries and Food. *Technical Bulletin* 2. Her Majesty's Stationary Office.

Yasmin, L. Rashid, M. H. Nazim Uddin, M. Hossain, M. S. Hossain, M. E., & Ahmed M. U. (2003). Use of Neem Extract in Controlling Root-knot Nematode (*Meloidogyne javanica*) of Sweet-gourd. *Plant Pathology Journal, 2, 161-168.*

Pertanika J. Trop. Agric. Sci. 36 (3): 285 - 288 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Case Study

Electroejaculation in a Perineal Urethrostomy Goat

Sringam, S.[1]*, Suwanathada, P.[1], Sangkaew A.[1], Thuangsanthia, A.[2] and Leingchareon, N.[2]

[1]Department of Veterinary Surgery and Theriogenology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen -40002, Thailand.
[2]Embryo Transfer Technology Research Center, Department of Livestock Development, Pakchong, Nakhonratchasima -30130, Thailand

ABSTRACT

This report describes semen collection by electroejaculation (EE) method from an urethrostomy goat. The buck used in this study underwent the urethrostomy four months before being incapacitated. The semen was collected by EE two times at 30 min apart. Generally, all the semen characteristics were very poor. The semen volume, concentration, motility and viability of the first ejaculate were higher than those of the second ejaculation. EE was useful for semen collection from a genetically superior goat, incapacitated due to urethrostomy.

Keywords: Electroejaculation, urethrostomy, goat

INTRODUCTION

Semen collection is an essential step for utilizing the artificial insemination technology in farm animals. In collecting the semen from the males, there are several methods used for various situations, such as artificial vagina (AV), electroejaculation (EE) and epididymal spermatozoa recovery (from the dead animals) (Memon *et al*., 1986; Ritar *et al*., 1992; Datta *et al*., 2009). Basically, the use of AV is considered as the best method for semen collection in domestic animals. However, the EE method has been employed successfully both in domestic and wildlife animals when

ARTICLE INFO

Article history:
Received: 14 June 2012
Accepted: 28 August 2012

E-mail addresses:
sarsri@kku.ac.th (Sringam, S.)
ponsuw@kku.ac.th (Suwanathada, P.),
adisak@kku.ac.th (Sangkaew A.),
biotech4@dld.go.th (Thuangsanthia, A.),
biotech4@dld.go.th (Leingchareon, N.)
* Corresponding author

ISSN: 1511-3701

the AV method is inapplicable. The EE method can be used for various purposes, such as in animals having problems with mounting due to musculoskeletal diseases or injury, animals outside their breeding season, or paralyzed animals due to spinal cord injury. This study reports a buck that was incapacitated and unable to serve the does after recovering from its perineal urethrostomy, despite having reasonably good libido. Therefore, the EE method was applied to collect the semen from this valuable buck.

CASE DESCRIPTION

Case History

A 30-month-old male Kalahali Red goat weighing 65 kg belonging to the Embryo Transfer Technology Research Centre, Department of Livestock Development, Nakhon Ratchasima, Thailand, was used in this study. It was fed with a commercial diet (16% protein, CP, Thailand) at approximately 600g/day and free access to dry Pangola grass, water and vitamin/mineral block. The goat semen was collected by using AV once a week to produce frozen semen and distributed to the farmers for 6 months. The post-thaw motility of frozen semen was at least 40%. During the last 4 months, the animal had developed a severe difficulty in urinating. After a complete physical examination, X-ray and ultrasonography, perineal urethrostomy without penile transection was performed at the Veterinary Teaching Hospital, the Faculty of Veterinary Medicine, Khon Kaen University, Thailand. A new urethral opening was reconstructed at the perineal area of the animal.

Electroejaculation Procedure

After surgery, the buck urinated via the new urethral opening and had a good health. Although the buck regained its libido completely, semen collection with AV was not successful. Semen collection was conducted in February 2012, 4 months after the surgery. The buck was subjected to electroejaculation procedure as previously described by Sundararaman *et al*. (2007). Briefly, the buck was restrained in a lateral recumbency position, faeces were removed and the urethral opening was cleaned. A lubricated rectal probe (2.5 cm in diameter with three longitudinal electrodes) was inserted approximately 10 cm into the rectum with the electrodes facing down and then connected to an electro-ejaculator. By using the manual control knob of the instrument, the stimulation power was increased from 0 to 20 mA and held for 2 to 3 sec and brought to 0. This procedure was repeatedly performed after a short resting period, with increasing stimulation power by 20 mA in every attempt until ejaculation occurred. This buck was able to ejaculate when using the stimulation power at 40 mA. Semen was collected into a 15 ml sterile tube, which was immediately evaluated for appearance and volume. Equal volume of egg yolk tris (3.025 g tris (hydroxymethyl)-aminomethane, 1.7 g sodium citrate monohydrate, 1.25 g glucose, 100 mg benzylpenicillin, 100

mg dihydrostreptomycin sulphate, freshly collected egg yolk 20% (v/v) and distilled water into 100 ml) was added to the ejaculate and kept in a Styrofoam box to protect it from light and maintained at an ambient temperature of between 30-32ºC. Then, the semen was adjusted into the final concentration of 500 X 10^6 cells/ml. The second ejaculation was later collected at approximately 30 min after the first ejaculation by using the same procedure. The semen samples were slowly cooled down to 5ºC at the rate of -0.5ºC/min using drop wise of small pieces of ice.

Semen Evaluation

The samples were sent to the laboratory within 3½ hrs. The motility of the diluted semen was evaluated using a light microscope (Howard, 1993). The total sperm per ejaculate was determined by multiplying the sperm concentration/ml with the undiluted ejaculate volume. Sperm viability was assessed using eosin-nigrosin staining (World Health Organization, 2010) and 200 spermatozoa per sample were examined. Sperm morphology was assessed at 1000x using a phase contrast microscope (Olympus, Tokyo, Japan) and 200 spermatozoa were evaluated per sample.

RESULTS AND DISCUSSION

The semen volumes of the first and second ejaculation were within the normal range for a goat (Oyeyemi *et al*., 2001; Carter *et al*., 1990). However, the concentration, total sperm and sperm motility were generally poor. Nonetheless, the semen characteristics of the first ejaculation were found to resemble those from a paralysed goat (Sundararaman *et al*., 2007). On the contrary, those from the second ejaculation were much lower. The low quality of semen might have been caused by the pathological change of urinary tract or less sperm production due to the prolong period of sexual inactivity. Pellati *et al.* (2008) suggested that spermatozoa can be affected by urogenital infections at different levels from the development to maturation and transport. The study of Levita *et al.* (2005) showed a significant decrease in the percentage of human sperm motility and normal morphology on days 11–14 of sexual abstinence. Typically, the semen collected by the EE method contains a greater amount of seminal plasma than the semen collected by the AV method. From the study of Memon *et al.* (1986), the characteristics of goat semen collected using AV were greater in concentration and mass motility than EE. Furthermore, the data of Ritar *et al*. (1992) showed that the frequent ejaculation within days in breeding season had an effect on the number of spermatozoa. Urethral epithelial cells, red blood cells and polymorphonuclear cells were also detected in the semen, indicating that a certain level of inflammation had remained in the urinary tract. However, EE did help to harvest a few more samples with a moderate quality. Unfortunately, the buck in this study gradually suffered from an unknown illness and died 1 month after semen collection. As a result, the semen could not be repeatedly collected to provide us with the complete data.

TABLE 1
Semen characteristics of the urethrostomy goat on electroejaculation

Parameters	1st ejaculate	2nd ejaculate
Volume (ml)	0.9	0.65
Concentration (x10^6 spermatozoa/ml)	1,450	660
Total sperms (spermatozoa x10^6 cells)	1,305	429
Motility (%)	20	5
Viability (%)	43	38
Morphology (%)		
Normal	54	62
Abnormal head	39	28
Abnormal midpiece	0	2
Abnormal tail	7	8

CONCLUSION

EE offers an alternative approach to collect semen from an incapacitated urethrostomy goat.

REFERENCES

Carter, P. D., Hamilton, P. A., & Duffy, J. H. (1990). Electroejaculation in goats. *Australian Veterinary Journal, 67*(3)*,* 91-93.

Datta, U., Chandra-Sekar, M., Hembram, M. L., & Dasgupta, R. (2009). Development of a new method to preserve caprine cauda epididymal spermatozoa in-situ at -10 degrees C with electrolyte free medium. *Journal of Assisted Reproduction and Genetics, 26*(8)*,* 467-473.

Howard, J. G. (1993). Semen collection and analysis in carnivores. In M. E., Fowler (Ed), *Zoo and Wildlife Medicine* (3rd edn.) (p. 390-398). Philadelphia, USA: WB Saunders.

Levitas, E., Lunenfeld, E., Weiss, N., Friger, M., Har-Vardi, I., Koifman, A., & Potashnik, G. (2005). Relationship between the duration of sexual abstinence and semen quality: analysis of 9489 semen samples. *Fertil Steril, 83,* 1680–1686.

Memon, M. A., Bretzlaff, K. N., & Ott, R. S. (1986). Comparison of semen collection techniques in goats. *Theriogenology, 26,* 823-827.

Oyeyemi, M. A., Akusu, M. O., & Ola-Davies, O. E. (2001). Effect of successive ejaculation on the spermiogram of West African dwarf goats. *Israel Veterinary Medicine Association Journal, 56,* 1-5.

Pellati, D., Mylonakis, I., Bertoloni, G., Fiore, C., Andrisani, A., Ambrosini, G., & Armanini, D. (2008). Genital tract infections and infertility. *European Journal of Obstetrics & Gynecology and Reproductive Biology, 140,* 3–11.

Ritar, A. J., Mendoza, G., Salamon, S., & White, I. G. (1992). Frequent semen collection and sperm reserves of the male Angora goat (*Capra hircus*). *Journal of Reproduction and Fertility, 95*, 97-102.

Sundararaman, M. N., Kalatharan, J., & Edwin, M. J. (2007). Attempts to achieve semen collections from incapacitated Boer bucks by electro-ejaculation. *Asian Journal of Animal Veterinary Advances, 2*(4)*,* 244-246.

World Health Organization (2010). *WHO laboratory manual for the examination of human semen* (5th Ed). Geneva, Switzerland: WHO Press. 286 p.

REFEREES FOR THE PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

VOL. 36(3) AUG. 2013

The Editorial Board of the Journal of Tropical Agricultural Science wishes to thank the following for acting as referees for manuscripts published in this issue of JTAS.

Anuchai Pinyopummin
(Kasetsart University, Thailand)

Baharuddin Salleh
(USM, Malaysia)

Dahlan Ismail
(UPM, Malaysia)

Faizah Shaharom
(UMT, Malaysia)

Hadi Hajarian
(Razi University, Iran)

Jugah Kadir
(UPM, Malaysia)

Mohd Zamri Saad
(UPM, Malaysia)

Mojtaba Dashtizad
(National Institute of Genetic Engineering and Biotechnology, Iran)

Najiah Musa@Zakaria
(UMT, Malaysia)

Odunsi Adeyinka Adeniyi
(Ladoke Akintola University of Technology, Nigeria)

Rajinder Singh
(Malaysia Palm Oil Board, Malaysia)

Shaik Mohd Amin
(UPM, Malaysia)

Sundararaman M. N.
(Madras Veterinary College, India)

Tan Soon Guan
(UPM, Malaysia)

Tan Yee How
(UPM, Malaysia)

Yong Hoi Sen
(UM, Malaysia)

Zary Shariman Yahaya
(USM, Malaysia)

UPM- Universiti Putra Malaysia
UM- Universiti Malaya
USM- Universiti Sains Malaysia
UMT- Universiti Malaysia Terengganu

Special Acknowledgement

The **JTAS Editorial Board** gratefully *acknowledges* the assistance of **Doreen Dillah**, who served as the English language editor for this issue.

While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Chief Executive Editor, *Pertanika* Journals at nayan@upm.my.

Any inclusion or exclusion of name(s) on this page does not commit the *Pertanika* Editorial Office, nor the UPM Press or the University to provide any liability for whatsoever reason.

Pertanika

Our goal is to bring high quality research to the widest possible audience

Journal of Tropical Agricultural Science

INSTRUCTIONS TO AUTHORS

(Manuscript Preparation & Submission Guidelines)

Revised: February 2013

We aim for excellence, sustained by a responsible and professional approach to journal publishing.
We value and support our authors in the research community.

Please read the guidelines and follow these instructions carefully; doing so will ensure that the publication of your manuscript is as rapid and efficient as possible. The Editorial Board reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

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* began publication in 1978 as a Journal of Tropical Agricultural Science and became a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other *Pertanika* series include Journal of Science and Technology (JST) and Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published four times a year, i.e. in **February**, **May**, **August** and **November**.

Goal of *Pertanika*

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Indexing of *Pertanika*

Pertanika is now over 33 years old; this accumulated knowledge has resulted in Pertanika JTAS being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge (BIOSIS & CAB Abstracts), EBSCO, DOAJ, Google Scholar, CABI, AGRICOLA, ISC and MyAIS.

Future vision

We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: *agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance,* and *veterinary medicine*.

Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Guidelines for Authors

Publication policies

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in Proceedings. Please refer to Pertanika's **Code of Ethics** for full details.

Editorial process

Authors are notified on receipt of a manuscript and upon the editorial decision regarding publication.

Manuscript review: Manuscripts deemed suitable for publication are sent to the Editorial Board members and/or other reviewers. We encourage authors to suggest the names of possible reviewers. Notification of the editorial decision is usually provided within to eight to ten weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

Author approval: Authors are responsible for all statements in articles, including changes made by editors. The liaison author must be available for consultation with an editor of *The Journal* to answer questions during the editorial process and to approve the edited copy. Authors receive edited typescript (not galley proofs) for final approval. Changes **cannot** be made to the copy after the edited version has been approved.

Manuscript preparation

Pertanika accepts submission of mainly four types of manuscripts. Each manuscript is classified as **regular** or **original** articles, **short communications**, **reviews**, and proposals for **special issues**. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Acceptable English usage and syntax are expected. Do not use slang, jargon, or obscure abbreviations or phrasing. Metric measurement is preferred; equivalent English measurement may be included in parentheses. Always provide the complete form of an acronym/abbreviation the first time it is presented in the text. Contributors are strongly recommended to have the manuscript checked by a colleague with ample experience in writing English manuscripts or an English language editor.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

The instructions for authors must be followed. Manuscripts not adhering to the instructions will be returned for revision without review. Authors should prepare manuscripts according to the guidelines of *Pertanika*.

1. Regular article

Definition: Full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Should not exceed 5000 words or 8-10 printed pages (excluding the abstract, references, tables and/or figures). One printed page is roughly equivalent to 3 type-written pages.

2. Short communications

Definition: Significant new information to readers of the Journal in a short but complete form. It is suitable for the publication of technical advance, bioinformatics or insightful findings of plant and animal development and function.

Size: Should not exceed 2000 words or 4 printed pages, is intended for rapid publication. They are not intended for publishing preliminary results or to be a reduced version of Regular Papers or Rapid Papers.

3. Review article

Definition: Critical evaluation of materials about current research that had already been published by organizing, integrating, and evaluating previously published materials. Re-analyses as meta-analysis and systemic reviews are encouraged. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field.

Size: Should not exceed 4000 words or 7-8 printed pages.

4. Special issues

Definition: Usually papers from research presented at a conference, seminar, congress or a symposium.

Size: Should not exceed 5000 words or 8-10 printed pages.

5. Others

Definition: Brief reports, case studies, comments, Letters to the Editor, and replies on previously published articles may be considered.

Size: Should not exceed 2000 words or up to 4 printed pages.

With few exceptions, original manuscripts should not exceed the recommended length of 6 printed pages (about 18 typed pages, double-spaced and in 12-point font, tables and figures included). Printing is expensive, and, for the Journal, postage doubles when an issue exceeds 80 pages. You can understand then that there is little room for flexibility.

Long articles reduce the Journal's possibility to accept other high-quality contributions because of its 80-page restriction. We would like to publish as many good studies as possible, not only a few lengthy ones. (And, who reads overly long articles anyway?) Therefore, in our competition, short and concise manuscripts have a definite advantage.

Format

The paper should be formatted in one column format with at least 4cm margins and 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font. Be especially careful when you are inserting special characters, as those inserted in different fonts may be replaced by different characters when converted to PDF files. It is well known that 'μ' will be replaced by other characters when fonts such as 'Symbol' or 'Mincho' are used.

A maximum of eight keywords should be indicated below the abstract to describe the contents of the manuscript. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should preferably be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout.

Every page of the manuscript, including the title page, references, tables, etc. should be numbered. However, no reference should be made to page numbers in the text; if necessary, one may refer to sections. Underline words that should be in italics, and do not underline any other words.

We recommend that authors prepare the text as a **Microsoft Word** file.

1. Manuscripts in general should be organised in the following order:

    - **Page 1: Running title.** (Not to exceed 60 characters, counting letters and spaces). This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript.

      In addition, the **Subject areas m**ost relevant to the study must be indicated on this page. Select the appropriate subject areas from the Scope of the Journals provided in the Manuscript Submission Guide

    - A **list** of n**umber of black and white / colour figures and tables** should also be indicated on this page. Figures submitted in color will be printed in colour. See "*5. Figures & Photographs"* for details.

    - **Page 2: Author(s) and Corresponding author information**. This page should contain the **full title** of your paper with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, fax number and e-mail address) for editorial correspondence. The names of the authors **must** be abbreviated following the international naming convention. e.g. Salleh, A.B., Tan, S.G., or Sapuan, S.M.

      Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

      George Swan[1] and Nayan Kanwal[2]
      [1]Department of Biology, Faculty of Science, Duke University, Durham, North Carolina, USA.
      [2]Office of the Deputy Vice Chancellor (R&I), Universiti Putra Malaysia, Serdang, Malaysia.

    - **Page 3**: This page should **repeat** the **full title** of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication). **Keywords** must also be provided on this page (Not more than eight keywords in alphabetical order).

    - **Page 4 and subsequent pages**: This page should begin with the **Introduction** of your article and the rest of your paper should follow from page 5 onwards.

    Abbreviations. Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

    Footnotes. Current addresses of authors if different from heading.

2. **Text.** Regular Papers should be prepared with the headings **Introduction**, **Materials and Methods**, **Results and Discussion, Conclusions** in this order. Short Communications should be prepared according to "*8. Short Communications."* below.

3. **Tables.** All tables should be prepared in a form consistent with recent issues of *Pertanika* and should be numbered consecutively with Arabic numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a separate page. (Note that when a manuscript is accepted for publication, tables must be submitted as data - .doc, .rtf, Excel or PowerPoint file- because tables submitted as image data cannot be edited for publication.)

4. **Equations and Formulae.** These must be set up clearly and should be typed triple spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

5. **Figures & Photographs.** Submit an original figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a separate sheet and numbered consecutively with Arabic numerals. Appropriate sized numbers, letters and symbols should be used, no smaller than 2 mm in size after reduction to single column width (85 mm), 1.5-column width (120 mm) or full 2-column width (175 mm). Failure to comply with these specifications will require new figures and delay in publication. For electronic figures, create your figures using applications that are capable of preparing **high resolution** TIFF files acceptable for publication. In general, we require **300 dpi or higher resolution for coloured and half-tone artwork** and **1200 dpi or higher for line drawings** to be submitted in separate electronic files.

   For review, you may attach low-resolution figures, which are still clear enough for reviewing, to keep the file of the manuscript under 5 MB. Illustrations may be produced at no extra cost in colour at the discretion of the Publisher; the author could be charged Malaysian Ringgit 50 for each colour page.

6. **References.** Literature citations in the text should be made by name(s) of author(s) and year. For references with more than two authors, the name of the first author followed by *'et al*.' should be used.

   Swan and Kanwal (2007) reported that …
   The results have been interpreted (Kanwal *et al.,* 2009).

   - References should be listed in alphabetical order, by the authors' last names. For the same author, or for the same set of authors, references should be arranged chronologically. If there is more than one publication in the same year for the same author(s), the letters 'a', 'b', etc., should be added to the year.

   - When the authors are more than 11, list 5 authors and then *et al*.

   - Do not use indentations in typing References. Use one line of space to separate each reference. The name of the journal should be written in full. For example:

     - Jalaludin, S. (1997a). Metabolizable energy of some local feeding stuff. *Tumbuh, 1,* 21-24.
     - Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Malayan Agriculturist, 11,* 29-31.
     - Tan, S. G., Omar, M. Y., Mahani, K. W., Rahani, M., & Selvaraj, O. S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, *32,* 415 - 422.

   - In case of citing an author(s) who has published more than one paper in the same year, the papers should be distinguished by addition of a small letter as shown above, e.g. Jalaludin (1997a); Jalaludin (1997b).

   - Unpublished data and personal communications should not be cited as literature citations, but given in the text in parentheses. 'In press' articles that have been accepted for publication may be cited in References. Include in the citation the journal in which the 'in press' article will appear and the publication date, if a date is available.

7. **Examples of other reference citations**:

   Monographs: Turner, H. N., & Yong, S. S. Y. (2006). *Quantitative Genetics in Sheep Breeding*. Ithaca: Cornell University Press.

   Chapter in Book: Kanwal, N .D. S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Ed.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

8. ***Proceedings***: Kanwal, N. D. S. (2001). Assessing the visual impact of degraded land management with landscape design software. In Kanwal, N. D. S., & Lecoustre, P. (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

9. **Short Communications** should include **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Headings should only be inserted for Materials and Methods. The abstract should be up to 100 words, as stated above. Short Communications must be 5 printed pages or less, including all references, figures and tables. References should be less than 30. A 5 page paper is usually approximately 3000 words plus four figures or tables (if each figure or table is less than 1/4 page).

   *Authors should state the total number of words (including the Abstract) in the cover letter. Manuscripts that do not fulfill these criteria will be rejected as Short Communications without review.

STYLE OF THE MANUSCRIPT

Manuscripts should follow the style of the latest version of the Publication Manual of the American Psychological Association (APA). The journal uses American or British spelling and authors may follow the latest edition of the Oxford Advanced Learner's Dictionary for British spellings.

SUBMISSION OF MANUSCRIPTS

All articles should be submitted electronically using the ScholarOne web-based system. ScholarOne, a Thomson Reuters product provides comprehensive workflow management systems for scholarly journals. For more information, go to our web page and click "**Online Submission**".

Alternatively, you may submit the electronic files (cover letter, manuscript, and the **Manuscript Submission Kit** comprising *Declaration* and *Referral* forms) via email directly to the Chief Executive Editor. If the files are too large to email, mail a CD containing the files. The **Manuscript Submission Guide** and **Submission Kit** are available from the *Pertanika*'s home page at http://www.pertanika.upm.edu.my/home.php or from the Executive Editor's office upon request.

All articles submitted to the journal **must comply** with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication.

Please do **not** submit manuscripts to the editor-in-chief or to any other office directly. All manuscripts must be **submitted through the chief executive editor's office** to be properly acknowledged and rapidly processed at the address below:

Dr. Nayan KANWAL
Chief Executive Editor
Pertanika Journals, UPM Press
Office of the Deputy Vice Chancellor (R&I)
IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

E-mail: nayan@upm.my; journal.officer@gmail.com
tel: + 603-8947 1622.
or visit our website at http://www.pertanika.upm.edu.my/home.php for further information.

Authors should retain copies of submitted manuscripts and correspondence, as materials cannot be returned. Authors are required to inform the Chief Executive Editor of any change of address which occurs whilst their papers are in the process of publication.

Cover letter

All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is original and the content has not been published or submitted for publication elsewhere. This must be stated in the cover letter.

The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

The cover letter of the paper should contain (i) the title; (ii) the full names of the authors; (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed on a cover sheet.

Peer review

Pertanika follows a **double-blind peer-review** process. Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts. Authors are encouraged to indicate in the **Referral form** using the **Manuscript Submission Kit** the names of three potential reviewers, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Manuscripts should be written so that they are intelligible to the professional reader who is not a specialist in the particular field. They should be written in a clear, concise, direct style. Where contributions are judged as acceptable for publication on the basis of content, the Editor reserves the right to modify the typescripts to eliminate ambiguity and repetition and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

The Journal's review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The executive editor asks them to complete the review in three weeks and encloses two forms: (a) referral form B and (b) reviewer's comment form along with reviewer's guidelines. Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.

3. The executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Editorial Board, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.

5. The executive editor sends the revised paper out for review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, the paper is sent to that Press and the article should appear in print in approximately three months. The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, only essential changes are accepted. Finally, the article appears in the pages of the Journal and is posted on-line.

English language editing

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Thus all authors are required to get their manuscripts edited by **professional English language editors**. Author(s) **must provide a certificate** confirming that their manuscripts have been adequately edited. A proof from a recognised editing service should be submitted together with the cover letter at the time of submitting a manuscript to Pertanika**. All costs will be borne by the author(s)**.

This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Author material archive policy

Authors who require the return of any submitted material that is rejected for publication in the journal should indicate on the cover letter. If no indication is given, that author's material should be returned, the Editorial Office will dispose of all hardcopy and electronic material.

Copyright

Authors publishing the Journal will be asked to sign a declaration form. In signing the form, it is assumed that authors have obtained permission to use any copyrighted or previously published material. All authors must read and agree to the conditions outlined in the form, and must sign the form or agree that the corresponding author can sign on their behalf. Articles cannot be published until a signed form has been received.

Lag time

A decision on acceptance or rejection of a manuscript is reached in 3 to 4 months (average 14 weeks). The elapsed time from submission to publication for the articles averages 5-6 months.

Hardcopies of the Journals and off prints

Under the Journal's open access initiative, authors can choose to download free material (via PDF link) from any of the journal issues from Pertanika's website. Under "Browse Journals" you will see a link entitled "Current Issues" or "Archives". Here you will get access to all back-issues from 1978 onwards.

The **corresponding author** for all articles will receive one complimentary hardcopy of the journal in which his/her articles is published. In addition, 20 off prints of the full text of their article will also be provided. Additional copies of the journals may be purchased by writing to the executive editor.

BACKGROUND

Pertanika began publication in 1978 as the Journal of Tropical Agricultural Science (JTAS).

In 1992, a decision was made to streamline *Pertanika* into **3 journals**. i.e.,

1. Journal of Tropical Agricultural Science (JTAS)
2. Journal of Science and Technology (JST)
3. Journal of Social Sciences and Humanities (JSSH)

BENEFITS TO AUTHORS

PROFILE: *Pertanika* publishes original academic articles rapidly. It is fully committed to the Open Access Initiative and provides free access to all articles as soon as they are published.

QUALITY: Articles submitted to *Pertanika* undergo rigid originality checks. Our double-blind peer review procedures are fair and open.

AUTHOR SERVICES: We ensure that your work reaches the widest possible audience in print and online rapidly. Submissions are through **ScholarOne** system by Thomson Reuters.

SUBMISSION GUIDELINES

The Journal accepts articles as **regular**, **short communication** or **review papers**.

The article should include the following:

- An abstract of not more than 300 words;
- Up to 8 related keywords;
- Name(s), Institutional affiliation(s) and email(s) of each author.
- The maximum length of your article must not exceed:
  - approximately 6000 words or 27 pages, including abstract, diagrams tables and references for full research papers,
  - 2000 words for short communication papers, or
  - 4000 words for review papers
- References should be listed in APA style.

SUBMISSION DEADLINE

You may submit your articles at any time of the year. The journal is now accepting papers for its 2013-14 issues.

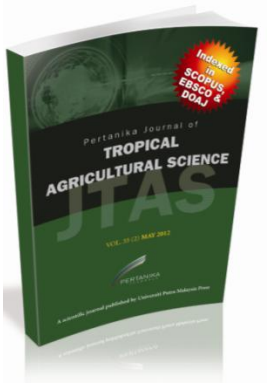
CONTACT US

For guidance on the submission process, or for any questions regarding submissions, you may contact the **Chief Executive Editor** at: nayan@upm.my

Call for Papers 2013-14

now accepting submissions…

About the Journal



- An international multidisciplinary peer-reviewed leading Malaysian journal.
- Publishes articles in **English** quarterly. i.e., *February, May, August* and *November*.
- The elapsed time from submission to publication for the articles averages 5 to 6 months. A decision on acceptance of a manuscript is reached in 3 to 4 months (average 14 weeks).
- Indexed in **SCOPUS** (Elsevier), **Thomson** (BIOSIS), **EBSCO**, **DOAJ**, **Agricola**, **CABI**, **Google Scholar**, **MyAIS** & **ISC**.

Scope of Journal

- Pertanika JTAS aims to provide a forum for high-quality research related to **tropical agricultural research** dealing with issues of worldwide relevance.
- Refer to our website for detailed scope areas. http://www.pertanika.upm.edu.my/scope.php

Format for Paper Submission

- Articles should include the following:
  - problem formulation
  - conceptual framework
  - methodology/ approach
  - research design (if applicable)
  - statistical analysis (if applicable)
  - main findings
  - overall contribution
  - conclusions & suggestion for further research
  - acknowledgements (if applicable)

Rapid research publication…

Pertanika is the resource to support you in strengthening your research.

View current issue

View journal archives

Submit your manuscript to
http://mc.manuscriptcentral.com/upm-jtas

PERTANIKA JOURNALS

Journal's profile: http://www.pertanika.upm.edu.my/

Pertanika Journal of Tropical Agricultural Science
Vol. 36 (3) Aug 2013

Contents

Pertanika Editorial Office
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.pertanika.upm.edu.my/
E-mail: executive_editor.pertanika@upm.my
Tel: +603 8947 1622 / 1619

PENERBIT UPM
UNIVERSITI PUTRA MALAYSIA
PRESS

http://penerbit.upm.edu.my
E-mail : penerbit@putra.upm.edu.my
Tel : +603 8946 8855 / 8854
Fax : +603 8941 6172

ISSN 1511-3701
08 >
9 771511 370234