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Single Locus Microsatellite Development for the Malaysian Giant Freshwater Prawns, *Macrobrachium rosenbergii*

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Keywords: Genetic diversity, *M. rosenbergii*, microsatellites

ABSTRAK

Udang genus M. rosenbergii, *Bate 1868 (Palaemonidae) adalah kumpulan pelbagai jenis krustasea decopod yang didapati di kawasan tropika, laut, estuari dan air tawar. Pada masa ini, stok liar telah digunakan untuk ternakan pengeluaran benih di kebanyakan tempat di Malaysia. Walau bagaimanapun, pengambilan stok liar secara berterusan akan mengakibatkan kepupusan dan kehilangan kepelbagaian genetik. Oleh itu adalah penting bagi seseorang mempunyai kepekaan terhadap maklumat genetik untuk tujuan pemuliharaan dan pembiakan. Kajian ini menggunakan teknik RAMS untuk menghasilkan penanda lokus mikrosatelit tunggal. Sebanyak 13 kawasan mikrosatelit telah dikesan. Hasil kajian menujukkan kejayaan menggunakan teknik ini untuk memperoleh mikrosatelit dalam* M. rosenbergii. *Kajian ini akan diteruskan untuk mencari klon yang mengandungi mikrosatelit dan mereka bentuk primer bagi setiap kawasan mikrosatelit. Jujukannya adalah sama dengan dua spesies lain iaitu ikan zebra dan* Perna viridis. *Kewujudan persamaan pada kedua-dua spesies (ikan Zebra dan* Perna viridis*) akan digunakan sebagai 'model' bagi* M. rosenbergii *untuk masa hadapan.*

ABSTRACT

Prawns of the genus Macrobrachium Bate, 1868 (Crustacea: Palaemonidae) are a highly diverse group of decopod crustaceans found in circumtropical marine-, estuarine- and freshwaters. At present, wild stocks have been used to cultivate commercial cultures in many areas in Malaysia. Such repeated harvesting from wild stocks, however, will eventually lead to extinction and loss of genetic diversity. Therefore, it is important to know the genetic make up of these wild stocks in order to manage the populations for conservation and breeding purposes. In the present study, a novel method known as Random Amplified Microsatellites (RAMS) was used to develop single locus microsatellite markers. A total of 13 microsatellite regions were detected. The findings of this study showed a successful detection of the microsatellite sequences in M. rosenbergii *using RAMS. This study will be continued to identify more clones that contain microsatellite sequences and to design the forward and reverse primers for each microsatelite region.* M. rosenbergii *sequences were homologous with Danio rerio (Zebra fish) and* Perna viridis *(green lipped mussel).*

INTRODUCTION

Macrobrachium rosenbergii, the giant freshwater prawn (Family: Palaemonoidea, subfamily: Palaemoninae) is a component of the biodiversity in Malaysia's diverse water ecosystems. It inhabits tropical and subtropical zones of the world, in lakes, rivers, swamps, irrigation ditches, canals and ponds, as well as in estuarine areas. Most species require brackish water in the initial stages of their life cycle, and a minority complete their life cycle in inland saline and freshwater lakes. Their numbers in these natural habitats has been declining due to overexploitation and habitat

alterations. At present, wild stocks have been used to cultivate commercial cultures in many areas in Malaysia. Repeated harvesting from the wild stocks will lead to extinction and eventually loss of genetic diversity. To save this important bio-resource, effective conservation and propagation-assisted rehabilitation strategies are necessary. However, this may not be feasible unless data is available for *M. rosenbergii* on stock structure and genetic variation throughout its distribution range. The identification of polymorphic markers with consistent scorable alleles is a crucial step to generate population genetics data. A study conducted by Yuzine *et al.* (1996 using isozymes as genetic markers) revealed that *M. rosenbergii* from Rompin River, Linggi River and the Hatchery Unit at Universiti Putra Malaysia have low genetic variability when compared to *P. merguiensis*. The low genetic variability showed that these populations constitute species that need to be further investigated by using DNA markers. A recent study using the mitochondrial 16S ribosomal RNA gene placed Malaysian *M. rosenbergii* into the western 'form' along with those from Thailand, Java and Vietnam. (Mather and de Bruyn 2003). This information has implications for both the aquaculture industry and the conservation of wild stocks.

Population genetic markers including allozymes, mitochondrial DNA and microsatellites can be the markers of choice for addressing issues such as genetic variability, inbreeding, parentage assignments, species and strain identification and for the construction of high resolution genetic linkage maps for aquaculture species. Thus for this study, microsatellites was the marker of choice. The great popularity of microsatellite markers is mainly due to their presence in all prokaryotic and eukaryotic genomes, and their codominant, highly polymorphic and easy to score characteristics. These characteristics make them more informative than dominant markers such as RAPD, AFLP, and others. Microsatellites were used to estimate relative levels of genetic divergence within and among populations. Microsatellites can also identify genetically distinct populations, which contribute to conservation efforts. To date, only six microsatellite loci have been developed for the eastern form of *M. rosenbergii* and they failed to show any polymorphism in the western form of *M. rosenbergii*. These six loci exhibited null alleles in the western form. (Mather, pers. comm.). Thus it will be a breakthrough in the molecular genetics of *M. rosenbergii*, if steps are taken to develop microsatellite markers by isolating microsatellite regions and developing single locus microsatellite markers for this species. At present, a well developed protocol for single locus microsatellite identification has been developed for *Perna viridis* (Teh *et al.* 2002). Single locus microsatellites are highly repeated DNA sequences that have been markers of choice in resolving genetic variability in low variability populations, genome mapping, parentage, kinship assignments and stock structures. In this study, we have attempted to develop microsatellite loci for *M. rosenbergii*.

MATERIALS AND METHODS

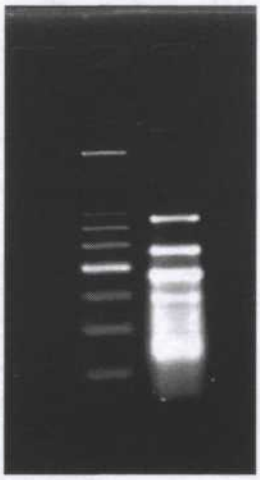
Prawns were collected from the wild stocks from Muda River (Kedah), Perak River (Perak) and Rompin River (Pahang). The samples were kept on ice for transportation and stored at -80°C for the study. Identification of the prawns collected was based on the taxonomic identification keys provided by New (2002). DNA was extracted from the muscle tissues by the protocol of Winnepenninckx *et al.* (1993). The microsatellite isolation technique followed the procedure of Teh *et al.* (2003). This involved the creation of an enriched genomic library based on the 5' anchored PCR technique. Degenerate primers were used for PCR and the DNA bands generated from each of the primers were ligated into the TOPO TA cloning vector (Invitrogen, USA). Vectors with inserts were then transformed into competent'*E. coli* cells. The transformed cells were then plated onto LB agar, and subsequently selection for white colonies were made after incubation at 37°C. Plasmid DNAs were extracted from positive clones, using the protocol of Sambrook *et al.* (1989) before

sequencing on an ABI PRISM 377 DNA sequencer.

Analysis of nucleotide sequences was carried out with BLAST-N program. This program was used to identify similarities of nucleotide sequences obtained with those of published sequences of other closely related species and to determine the significance of these sequences.

RESULTS AND DISCUSSION

A total of 16 degenerate RAMS primers were screened to check for amplification. All showed amplification. However, PCR optimization was done for some of these primers. A single degenerate primer containing $(CAG)_5$ microsatellite repeat motifs was used to detect the presence of microsatellite sequences. The primer sequence of Bp 11 (5' to 3') is $(K)_3(YH)_2Y(CAG)_5$, K= G/T, V= G/C/A, Y=T, H=A,C or T. The size of the amplified bands ranged from 200bp to 1000bp (Figure 1).



M 1

Figure 1: Banding pattern observed in one of the individuals in M. rosenbergii *using RAMS primer, Bp11. Lane M: 100 bp marker, Lane 1: Bp11 primer bands*

The whole PCR product of primer Bp-11 was cloned and almost 200 clones with an average insert size of 200 bp were isolated. A total of 12 positive clones were sequenced. Eight of the clones contained perfect CAG repeats and cryptic simple (GG)n(GGG)n repeats while the rest of the clones had no inserts (Table 1). These results suggest that the (CAG)n repeats are abundant in the genome of *M. rosenbergii.*

Nineteen cryptic simple regions were detected in the eight clones. Cryptic simple regions were found preferentially in the non-coding regions of the genomes but they are also found in the coding regions (Goldstein 1999). According to Tautz *et al.* (1986), microsatellites probably arose from cryptic simple regions. On the other hand, microsatellites may have undergone mutations to become regions of cryptic simplicity.

Random Amplified Microsatellites (RAMS) is proven to be an efficient starting point for developing single locus microsatellite markers in *M. rosenbergii.* This technique has been used to characterize species such as *Gremmeniella abietina* (Hantula *et al.* 1996); *Pinus radiata* (Fisher *et al.* 1998); *Vigna radiata* (Kumar *et al.* 2002) and blue mussel, *Mytilus edulis* (Presa *et al.* 2002). RAMS markers have provided useful information about population structures of these species. The findings of our study showed the success of using this method as a starting point for detecting microsatellite sequences in *M. rosenbergii.* The microsatellite sequences that we have obtained will be used to design primers based on the unique sequences flanking each microsatellite region. These primer pairs will then be tested for their abilities to amplify single locus microsatellite markers in *M. rosenbergii.* The development of polymorphic microsatellite loci will be useful in rebuilding wild stock populations, contributing significantly to environmental conservation and it will serve to provide valuable information for broodstock management of *M. rosenbergii.*

Blast analysis was performed to identify homology sequences between *M. rosenbergii* with its closely related species. The list of sequences between *M. rosenbergii* and other species are shown in Table 2. The analysis showed that the majority of the homologous

TABLE 1
Microsatellite loci isolated from *Macrobrachium rosenbergii* using Bp-11 RAMS clone and their GenBank accession numbers

Clone	Type of repeat units			
	Perfect	Imperfect	Compound	GenBank Accession
SUGbp11-1	$(CAG)_5$, $(TC)_4$, $(GCT)_5$, $(TAG)_3$	$(GG)_4(GGG)_2$	–	AY841999
SUGbp11-2	$(CAG)_5$	$(AG)_5$,$(GG)_5(GGG)_2$	–	AY842000
SUGbp11-3	$(CAG)_6$,$(TGC)_4$	$(GG)_5(GGG)_2(GG)_2$	–	AY842001
SUGbp11-4	$(CAG)_5$	$(CA)_2(CA)_3$	–	AY842002
SUGbp11-5	$(CAG)_5$, $(TGC)_4$	$(GG)_5(GGG)_2$	–	AY842003
SUGbp11-6	$(CAG)_7$, $(TGC)_4$	$(GG)_5$ $(GGG)_2(GG)_2$	–	AY842004
SUGbp11-7	$(CAG)_5$	$(GG)_5(GGG)_2$	–	AY842005
SUGbp11-34	$(CAG)_6$	$(GG)_5(GGG)_2$	–	AY842006

TABLE 2
Nucelotide sequence comparisons of the five clones of *M. rosenbergii* with other species

Organism		Sequences		No. of nucleotides	Homology (%)
Clone SUGbp11-1	8	cagcagcagcagcaggaagagcaggagcagtagtagtag	46		
Cepaea nemoralis microsatellite Cne1 sequence (AF139019)	343	cagcagcagcagcagcagcagcagcagtagtagtag	30	39	89
Clone SUBbp11-1	9	agcagcagcagcaggaagagcaggagcagtagtagtag	46		
Fonscochlea accepta clone Sn49 microsatellite sequence. (AY805231)	130	agcagcagcagcagcagcagcagcagtagtagtag	167	39	87
SUGbp11-2 Zebrafish DNA sequence from clone CH211-11P18 in linkage group 22,complete sequence. (AL845526)	2	tttcagcagcagcagcagaa	21	20	100
	16074	tttcagcagcagcagcagaa	160934		
SUGbp11-2 Zebrafish DNA sequence from clone DKEY-4P13 in linkage group 2, (BX255907)	4	tcagcagcagcagcagaagnagtagct	30	27	97
	183686	tcagcagcagcagcagaagcagcagct	183712		

Table 2 Cont'd

Organism		Sequences	No. of nucleotides	Homology (%)
SUGbp11-2 Danio rerio zgc:56721, mRNA (cDNA clone MGC:56721 IMAGE: 2601288), complete cds. (BC044205	2 1694	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 16752	20	100
SUGbp11-2 Danio rerio zgc:56721 (zgc:56721), mRNA. (NM_213521)	2 1694	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 167	20	100
SUGbp11-2 Argopecten irradians clone ScaE_6721 microsatellite sequence. (AY485259)	2 398	tttcagcagcagcagcagaa 21 |||||||||||||||||| tttcagcagcagcagcagaa 417	20	100
SUGbp11-2 Argopecten irradians clone ScaE_1153 microsatellite sequence. (AY496639)	2 361	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 380	20	100
SUGbp11-2 Zebrafish DNA sequence from clone BUSM1-101L20 in linkage group 2AL591481)	5 70639	cagcagcagcagcagaagnagtagct 30 |||||||||||||| || ||||||| cagcagcagcagcagcagcagtagct 70664	26	92
SUGbp11-5 Perna viridis microsatellite PV1 sequence.(AY190018)	143 24	gcctgctgctgctgctgatata 164 |||||||||||||||||||| cctgctgctgctgctgatata 3	22	100
SUGbp11-5 Zebrafish DNA sequence from clone DKEY-4P13 in linkage group 2, complete sequence.(BX255907)	8 183686	tcagcagcagcagcagaagcagtagct 34 |||||||||||||||||||| |||| tcagcagcagcagcagaagcagcagct 183712	27	96
SUGbp11-5 Zebrafish DNA sequence from clone BUSM1-101L20 in linkage group 2 (AL591481)	9 70639	cagcagcagcagcagaagcagtagct 34 ||||||||||||||| |||||||||| cagcagcagcagcagcagcagtagct 70664	26	96

Table 2 Cont'd

Organism		Sequences	No. of nucleotides	Homology (%)
SUGbp11-6 Zebrafish DNA sequence from clone CH211-245M19 in linkage group 5, complete sequence. (CR385090)	144 124120	gtttcgcctgctgctgctgctgaaataa 171 |||| |||||||||||||||||||| gtttggcctgctgctgctgctgaaataa 124147		
SUGbp11-6 Zebrafish DNA sequence from clone CH211-137C4 in linkage group 8, complete sequence. (BX248231)	151 50048	ctgctgctgctgctgaaataacca 174 |||||||||||||||| ||||| ctgctgctgctgctgaaacaacca 50071	24	96
SUGbp11-6 Zebrafish DNA sequence from clone DKEY-32D10 in linkage group 5, complete sequence.(BX469907)	149 128335	gcctgctgctgctgctgaa 167 |||||||||||||||||| gcctgctgctgctgctgaa 128353	19	100
SUGbp11-6 Perna viridis microsatellite PV3 sequence.(AY190020)	151 24	ctgctgctgctgctgaaataac 172 |||||||||||||||||||||| ctgctgctgctgctgaaataac 3	22	100
SUGbp11-6 Perna viridis microsatellite PV1 sequence. ACCESSION AY190018	149 24	gcctgctgctgctgctgaaataac 172 |||||||||||||||||| ||||| gcctgctgctgctgctgatataac 1	24	96
SUGbp11-34 Blast analysis is the same with SUGbp11-6 to the similar organism and same homologous sequences				

sequences were identified with *Danio rerio* or commonly known as zebra fish and *Perna viridis*, or green lipped muscle.

The presence of homologous sequence with zebra fish is a a very important focal point in understanding *M. rosenbergii* genome. Zebra fish is an important model system for cell fate decisions and pattern formation and for assembling molecular components of responsible pathways (Fishman 1999). By identifying more sequences from *M. rosenbergii* and with the understanding on the possible relationship that might exist between zebra fish and *M. rosenbergii*, the role of *M. rosenbergii* genes from zebra fish mutations and identification of zebra fish models for candidate genes could be established. The synthetic relationship between zebra fish and human genome is well documented by Barbazuk *et al.* (2000), and shows that similar

work can be carried out with *M. rosenbergii*. However, there is a possibility that the expected relationship might not exist between these two species as Zebra fish is a vertebrate and *M. rosenbergii* is an invertebrate. However, Ohno (1970) proposed that without duplicated genes the creation of metazoans, vertebrates and mammals from unicellular organisms would have been impossible. Ohno (1970) was not the first to suggest that genome wide redundancy could lead to new evolutionary opportunities.

Intragenome similarities through phylogeny and synteny data suggest that the common ancestor of zebra fish and puffer fish, a fish that gave rise to 22,000 species experienced a large scale gene duplication event and puffer fish has lost many duplicates that the zebra fish has retained (Taylor *et al.* 2003). Similar possible relationship could exist between Zebra fish and *M. rosenbergii* because ancestor of these two species (zebra fish and puffer fish) experienced a genome duplication event lends support to the idea that genome duplication and speciation might be casually linked (Amores *et al.* 1998). Sequence level studies on *M. rosenbergii* will help to answer possible evolutionary lineages in the development of an organism.

The homologous sequences that existed between two families, *Palaemonidae* and *Mytiloidea*, was very interesting due to the fact that similar studies on marine turtles indicated conservation of flanking sequences spanning approximately 300 million years of divergent evolution (FitzSimmons *et al.* 1995). Few primers that were obtained from Teh *et al.* (unpublished) showed positive amplification in *M. rosenbergii*. Some of the primers showed polymorphic banding patterns (Bhassu *et al.* unpublished data).

CONCLUSION

This study will be continued to test the hypothesis that microsatellite loci have great potential for broader applications such as comparative gene mapping and assessing genetic population structures within species. The use of microsatellite loci across species depends on the conservation of priming sites within flanking sequences, which enables amplification and maintenance of repeat arrays long enough to promote polymorphism (Weber 1990). In this study, the use of RAMS primer Bp11 was designed to amplify (CAG)n repeats which are trinucleotide regions that are more conserved than the dinucleotide regions because they are found in the coding region (Young *et al.* 2000) This could explain the possible existence of conservation of these sequences between species such as Zebra fish and *Perna viridis* and possibly other species.

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REFERENCES

AMORES, A., A. FORCE, Y. L YAN, L. JOLY, C. AMEMIYA, A. FRITY, R. K. HO, J. LANGELAND, V.PRINCE and Y.L. WANG. 1998. Zebrafish hox clusters and vertebrate genome evolution. *Science* **282:** 1711-1714.

BARBAZUK, W. B., I. KORF, C. KADAVI, J. HEYEN, S. TATE, E. WUN, J. BEDELL, J.D. MCPHERSON and S.L. JOHNSON. 2000. The synthetic relationship of the zebrafish and human genomes. *www.genome .org/cgi/doi/10.1101/gr.144700*

FISHER, P. J., R. C. GARDNER and T. E. RICHARDSON. 1996. Single locus microsatellite isolated using 5'- anchored PCR. *Nucleic Acids Research* **24:** 4369-4371.

FISHMAN, M. C. 1999. Zebra fish genetics. The enigma of arrival. *Proc. Natl. Acad. Sci.* **96**: 10554- 10556. USA.

FITZ SIMMON, N. N., C. MORITZ and S. S. MOORE. 1995. Conservation and dynamics of microsatellite

loci over 300 million years of marine turtle evolution. *Mol. Biol. Evol.* **12 (3)**: 432-440.

HANTULA, J., M. DUSABENYAGASANI and R. C. HAMELIN. 1996. Random amplified microsatellites (RAMS) - a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* **26:** 159-166.

GOLDSTEIN, D. B. and C. SCHLOTTERRER. 1999. *Microsatellites: Evolution and Applications.* USA: Oxford University Press.

KUMAR, S. V., S. G. TAN, S .C. QUAH and K. YUSOFF. 2002. Isolation of microsatellite markers in mungbean, *Vigna radiata. Molecular Ecology Notes* **2:** 96-98.

MATHER, P. B. and M. DE BRUYN. 2003. Genetic diversity in wild stocks of the giant freshwater prawn (*Macrobrachium rosenbergii*): Implications for aquaculture and conservation. *Aquaculture Magazine* **June**: 1-3.

NEW, M. B. 2000. History and global status of freshwater prawn farming. In *Freshwater Prawn Culture the Farming of Macrobrachium rosenbergii,* ed. M. B. New and W. C. Valenti, pp. 1-11. Oxford: Blackwell Science.

OHNO, S. 1970. *Evolution by Gene Duplication.* New York: Springer-Verlag.

PRESA, P., M. PEREZ and A. P. DIAZ. 2002. Polymorphic microsatellite markers for the blue mussels (*Mytilus* spp.) *Conservation Genetics* **3**: 441-443.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS. 1989. *Molecular Cloning: A Laboratory Mannual.* 2 edn. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor.

TAUTZ, D., M. TRICK and G. A. DOVER. 1986. Cryptic simplicity in DNA is a major source of genetic variation. *Nature* **322:** 652-656.

TAYLOR, J. S., I. BRAASCH, T. FRICKEY, A. MEYER and Y. Y. DE PEER. 2003. Genome duplication, a trait shared by 22,000 species of ray-finned *fish.www.genome.org/cgi/doi/10.1101/gr.640303.*

TEH, C. H., B. H. CHUA, S. Y. YONG, S. G. TAN and K. M. YUSOFF. 2003. Development of single locus microsatellite markers for the Green-Lipped Mussel, *Perna viridis* using a Random Amplified Microsatellites (RAMS) based technique. In *Aquatic Resource and Environmental Studies of the Straits of* Malacca*: Managing the Straits through Science and Technology,* ed. J.S. Bujang, A. Arshaad, M. H. Zakaria and Kawamura, pp. 351-356.

WEBER, J. L. 1990. Informativeness of human 9dC-dA)n –(dG-dT)n polymorphisms. *Genomics* **7**: 524-530.

WINNEPENNINCKX, B., T. BACKELJAU and R. D. WACHTER. 1993. Extraction of high molecular weight DNA from mollusks. *Trends in Genetics* **9:** 407.

YUZINE, E., I. PATIMAH, S. G. TAN and P. JOTHY. 1996. Genetic polymorphism of local population of udang galah (*Macrobrachium rosenbergii de Man*) and Udang Putih (*Penaeus merguiensis de Man*). In *Second National Congress on Genetics,* ed. M. Osman, M. M. Clyde and Z. Zamrod, pp. 451-457.

YOUNG, E.T., J. S. SLOAN and K. VAN RIPER. 2000. Trinucleotide repeats are clustered in regulatory genes in *Saccharomyces cerevisiae. Genetics* **154:** 1053-1068.

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Bacteria from an Oil Palm Agricultural System and Their Interactions with *Ganoderma boninense* and *Trichoderma harzianum*

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Keywords: ***Ganoderma boninense*, *Trichoderma harzianum*, biological control, bacteria, oil palm**

ABSTRAK

Kulat Trichoderma harzianum *(pencilan FA 1132) telah mempamerkan potensi sebagai agen kawalan biologi terhadap patogen kelapa sawit,* Ganoderma boninense. *Kulat ini kini sedang dihasilkan pada skala besar sebagai kompos-Trichoderma untuk ujian di lapangan. Kajian ini telah memencilkan bakteria daripada kompos tersebut dan juga daripada rizosfera kelapa sawit. Kesemua bakteria pencilan disaring untuk tindak balas antagonis terhadap* G. boninense, *selepas itu stren-stren terpilih akan diuji untuk keserasian dengan FA 1132. Empat daripada enam spesies bakteria yang dipencilkan telah menunjukkan aktiviti fungistatik terhadap* G. boninense. Corynebacterium urealyticum *memberi peratus perencatan radius pertumbuhan (PIRG) paling tinggi pada 86.6%, tetapi stren ini menunjukkan ketidakserasian dengan FA1132. Kecuali* C. urealyticum, *kesemua pencilan bakteria menunjukkan keserasian dengan FA 1132. Kajian ini merumuskan* Chromobacterium violaceum *dan* Burkholderia cepacia *masing-masing mungkin mempunyai keupayaan untuk bertindak terus sebagai inokulan tersendiri untuk merencatkan pertumbuhan* G. boninese *di samping juga mempunyai potensi digunakan sebagai ko-inokulan di dalam konsortium bersama* T. harzianum *FA 1132, untuk peningkatan keberkesanan di dalam sesuatu formulasi agen kawalan biologi.*

ABSTRACT

The fungus Trichoderma harzianum *(isolate FA 1132) has shown potential as a biological control agent of the oil palm pathogen,* Ganoderma boninense. *It is currently being produced on a large scale as Trichoderma-infused compost for field trials. This study isolated bacteria from such a compost as well as from oil palm rhizospheres. They were screened for antagonistic reactions towards* G. boninense *after which the selected strains were tested for compatibility with FA 1132. Four out of six species of bacteria showed fungistatic activity towards* G. boninense. *Corynebacterium urealyticum gave the highest percentage inhibition of radial growth (PIRG) at 86.6%, but was incompatible with FA 1132. Except for* C. urealyticum, *all the other isolates showed compatiblity with FA 1132. This study deduced that* Chromobacterium violaceum *and* Burkholderia cepacia *may have the capacity to act as individual inoculants to inhibit the growth of* G. boninense, *as well as have the potential to be used as co-inoculants in consortium with* T. harzianum *FA 1132, for enhanced performance in a biological control formulation.*

INTRODUCTION

Ganoderma boninense (Pat.) is a fungal pathogen which causes basal stem rot (BSR) of oil palms. Chemical treatments carried out on a trial basis on infected palms showed somewhat limited success (MPOB 2003) and the disease still persists in regions where the palms are grown. Ilias and Abdullah (1998) conducted an *in vitro* screening of soilborne fungi and found one strain of *T. harzianum* and *T. virens*, that showed promise as biocontrol agents of *G. boninense.* Repeated greenhouse trials using *T. harzianum* (isolate FA 1132) as a potential biocontrol agent of BSR confirmed the efficacy of this strain (Ilias 2000; Abdullah *et al.* 2003; Nagappan 2005; Sundram 2005).

Strains of bacteria also do behave as biocontrol agents. Among others, *Bacillus*

subtilis have been investigated for the biocontrol of Cercospora leaf spot in sugar beet (Collins and Jacobsen 2003); against the pathogenic *Fusarium oxysporum* (Knox *et al.* 2000) and against crown rot of tomato in the field (Nemec *et al.* 1996). Although some bacteria are used individually as biocontrol agents, many others are used as synergistic co-inoculants in a biocontrol system (Bennet *et al.* 2003; De Jensen *et al.* 2002; Knox *et al.* 2000). Many bacteria-based biocontrol products are already commercialized; among them is Deny® which has *Burkholderia cepacia* in its basic formula and is used against the fungal pathogens *Fusarium*, *Pythium* and *Rhizoctonia*. *Burkholderia cepacia* is again found in Leone®, which is used against *Botrytis* and *Phytophthora* diseases of potatoes as well as *Septoria* on wheat (Driesche and Bellows 1996; Fravel *et al.* 1998; McPartland *et al.* 2000; Khetan 2001). Products that contain *Trichoderma* as well have been formulated for commercial purposes. An example is Trichodex™, which contains *T. harzianum* as the active ingredient, is used against post harvest rot of apple. Another product, Binab-T™, which is a combination of *T. harzianum* with *T. polysporum*, is used in the control of wood rots (Samuels 1996).

To date and to our best knowledge, no studies have yet been conducted where a bacterial strain is used against *G. boninense*; it is thus one aspect worth exploring. Another unexplored avenue is the use of a compatible bacterial strain as a co-inoculant to a known fungal biocontrol agent for enhanced synergistic performance.

Nagappan (2005) reported that *T. harzianum* (isolate FA 1132) performed best when applied individually to artificially-infected oil palm seedlings. However, when the isolate was applied as 1:1 combination with *T. longibrachiatum*, or 1:1 combination with *T. virens*, or 1:1:1 combination of all 3 species, the control performance declined significantly. Sundram (2005) also reported that strain FA 1132 exhibited excellent efficacy when used individually in greenhouse trials, but it performed poorly when used as a 1:1 combination with another strain of *T. harzianum* (FA 1166). Thus the possibility of using bacterial strains compatible with FA 1132 instead of in combination with members of the genus *Trichoderma*, is one avenue pursued in this investigation.

The first objective of this study was to isolate and identify resident bacteria from two sources, namely soils from several oil palm rhizospheres and samples from a *Trichoderma*–infused compost. The latter is the end-product of a pilot scale trial in the production of a *Trichoderma*-based biocontrol product, for application onto mature palms infected with *Ganoderma* (Abdullah *et al.* 2005). The second objective was to screen and select for bacterial isolates that showed antagonistic properties towards *G. boninense*. The third was to screen the selected antagonistic bacteria for compatibility with *T. harzianum*, strain FA 1132. It is expected that from this study, a bacterial species that simultaneously showed an acceptable degree of antagonistic intimidation towards *G. boninense* and good compatibility with *T. harzianum* will be found. A combination of these two characteristics in a bacterial strain may give added value to its possible use as a co-inoculant in consortium with FA1132 in the end-product, for use against *G. boninense*.

MATERIALS AND METHODS

Sampling Site

Bacteria were isolated from two sources, namely from oil palm rhizospheres and from 25 kg packed bags of *Trichoderma*-infused compost meant for biocontrol trials. Both sources were from Sedenak, Johor. A custom–made soil auger was used to collect the soil samples, taken at 15 cm depth and from four cardinal points per palm, for eight randomly-selected palms. About 200 g of each composite sample per source was obtained and put into labeled containers and brought back to the laboratory for processing.

Isolation of Bacteria

Composite samples for each source were prepared by pooling and mixing for homogenous distribution, after which a stock solution of 10:100 (w:v) sample to water was made. The solutions were maintained as shake cultures at 100 rpm, under ambient laboratory conditions of temperature (28° ± 2°C) and light (12 hours light, 12 hours darkness). An aliquot of 1ml solution was subjected to a ten-fold serial dilution, from which 0.5ml at dilution 10^{-8} was dispensed onto a Nutrient Agar (NA) culture plate. An L-shaped glass rod was used to spread the cultures evenly and the plates were then incubated for 48 hours under ambient conditions. The morphologies of each colony-forming unit (CFU) that emerged were recorded to aid species characterisation. The bacteria from each colony type was then streaked onto fresh NA and incubated for 48 hours to obtain pure single colonies.

Biolog® *Identification of Bacteria*

Single colonies obtained from the isolation process were subjected to a series of tests before placing on the Biolog® plate reader for species identification. Bacterial isolates were picked up with a sterile loop and stirred into 1 ml of KOH solution. Samples that agglutinated to the loop indicate Gram Negative (GN) bacteria, while those that did not, were Gram Positive (GP). The GN samples were next subjected to an oxidative test wherein a loopful of bacteria was mixed into a solution of oxidative agent and observed for colour change. The formation of a dark blue color was recorded as GN–NENT (Gram Negative–non enteric).

A bacterial suspension was then made, from which a loopful was mixed into an inoculant solution and then put onto a turbidimeter. The Biolog® turbidimeter percentage of transmission was pre-set to accommodate the GN or GP status. The cultures were next inoculated into GN or GP microplates (based on the status determined from previous tests) at 145 µl per well of the 96 well micro plates. These were then covered and incubated at 28° to 30°C for 24 hours. Finally, the microplates were placed into the automated plate reader installed with the Biolog® identification software, which identifies bacteria up to species level based on percentage similarity. Details of the protocol and procedures for bacterial identification were in accordance with that outlined in the Biolog® identification system.

Antagonistic Studies Against Ganoderma boninense *(FA 5011)*

Strain FA 5011 was previously isolated from an infected oil palm in Banting, Selangor, which was the infecting agent used in the nursery trials of Abdullah *et al.* (2003), Nagappan (2005) and Sundram (2005). Two single bacterial streaks were made on Potato Dextrose Agar (PDA) on the upper and lower part of the plate, after which a 6-mm mycelial plug of FA 5011 cut from a freshly-growing colony and was placed centrally in between the streaks. The cultures were incubated under ambient laboratory conditions for 8 days. The linear growth of the fungus was observed and compared to its growth on the unchallenged control plate. Bacterial colonies which exhibited antagonistic properties towards *G. boninense* were selected for subsequent quantitative assessment by dual culture bioassays.

For the bioassay, a single bacterial streak was made at 5 cm away on one side from the edge of the petri plate, after which one 5 mm-diameter culture plug of *G. boninense* (isolate FA 5011) was centrally placed on the plate. Growth measurements were made of the linear mycelial growth away from the bacteria (R1) and that facing the bacteria (R2). Readings were recorded at 8, 16, 24 and 32 days after incubation (i.e. until static fungal growth was observed).

The percentage inhibition of radial growth (PIRG) of *G. boninense* by the bacteria was determined based on the formula by Skidmore and Dickinson (1976), given as:

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

where R1 = radius of the colony growing away from the bacteria.
R2 = radius of the colony growing towards the bacteria.

At 32 days, a 6 mm-diameter plug of the challenged *G. boninense* on the side facing the bacteria was cut and replated onto a fresh PDA plate. A resultant fungal regrowth would indicate a fungistatic property while a non-growth would indicate a fungicidal property exerted by the bacteria.

Compatibility Tests with T. harzianum *(FA 1132)*

Strain FA 1132 was originally isolated from soils of an oil palm plantation in Gemencheh, Negeri Sembilan. It proved to be a good biocontrol agent based on previous nursery trials and is the fungus being produced on a large scale for field applications (Abdullah *et al.* 2005).

Bacterial isolates that inhibited the mycelial growth of *G. boninense* were selected and subjected to a dual culture bioassay against *T. harzianum*. A single linear streak of each of the selected bacterial strain was made on PDA and a culture disc of *T. harzianum* (FA 1132) was plated centrally on the agar. The cultures were incubated for seven days after which radial extensions were measured and the PIRG calculated. At the same time, the challenged bacterial colony was picked up with a sterile loop, streaked onto NA and observed for subsequent bacterial growth. A normal regrowth would indicate compatibility whereas an abnormal or a non-growth would indicate lysis of the bacterial colony by the *Trichoderma* isolate.

RESULTS

Bacterial Identification

Four species of bacteria were isolated from the oil palm rhizospheres. They were identified as *Corynebacterium urealyticum* with a population density of 1.27 x 10^9 cfu/ml, *Pseudomonas spinosa* (8.7 x 10^8 cfu/ml), *Chromobacterium violaceum* (5.6 x 10^8 cfu/ml) and *Burkholderia cepacia* (1.07 x 10^8 cfu/ml). With the excpection of *C. urealyticum*, all species were Gram Negative (GN).

Three bacterial species were obtained from the *Trichoderma*-infused compost. These were *Corynebacterium urealyticum* with a population density of 5.8 x 10^8 cfu/ml, *Corynebacterium nitrilophilus* (2.1 x 10^8 cfu/ml) and *Actinomyces radingae* (3.2 x 10^8 cfu/ml). All the three bacterial species above were Gram Positive (GP).

Antagonistic Reactions towards G. boninense *(FA 5011)*

Three out of six bacterial isolates were antagonistic towards FA 5011. They were *Corynebacterium urealyticum, Chromobacterium violaceum* and *B. cepacia*. The non-antagonistic bacteria were *Corynebacterium nitrophilus* and *A. radingae* from the *Trichoderma*-infused compost and *P. spinosa* from the oil palm rhizospheres. Dual culture bioassays showed that except for the *P. spinosa* control isolate, the three other species inhibited the growth of FA 5011 by more than 56% on the eighth day (Table 1).

The mycelial growth of FA 5011 on the control plate reached its maximum at eight days after incubation and started to show declined growth thereafter. For the others, the PIRG of FA 5011 peaked and levelled off at day 24 with no further increases by day 32 (*Fig. 1*). The highest and most significant PIRG at day 24 was shown by *Corynebacterium urealyticum* at 88.20%, followed by *Chromobacterium violaceum* (83.45%) and *B. cepacia* (76.92%), each being significantly different from each other. When the challenged *G. boninense* cultures were replated on PDA, two out of the three cultures grew back at a rate similar to the control plate. The exception was *Corynebacterium urealyticum*, which exhibited retarded growth on PDA. Thus, all the bacterial antagonists were deduced to be fungistatic towards *G. boninense*, with *C. urealyticum* showing very strong fungistasis, possibly fungicidal.

TABLE 1
Percentage inhibition of radial growth (PIRG) of *G. boninense* by selected bacterial isolates at 8, 16, 24 and 32 days of bioassay

Bacterial species / Days of Incubation	8	16	24	32
1. *Corynebacterium urealyticum*	68.42^{aC}	87.50^{aB}	88.20^{aA}	88.20^{aA}
2. *Choromobacterium violaceum*	63.33^{bC}	82.50^{bB}	83.45^{bA}	83.45^{bA}
3. *Burkholderia cepacia*	56.52^{cC}	71.79^{cB}	76.92^{cA}	76.92^{cA}
4. *Pseudomonas spinosa*	7.50^{dA}	0.00^{dB}	0.00^{dB}	0.00^{dB}

Mean separation was done using Tukey HSD. Different letters in the lowercase denote a significant difference ($P \leq 0.05$) between isolates while different letters in the uppercase denote a significant difference ($P \leq 0.05$) between days of incubation.

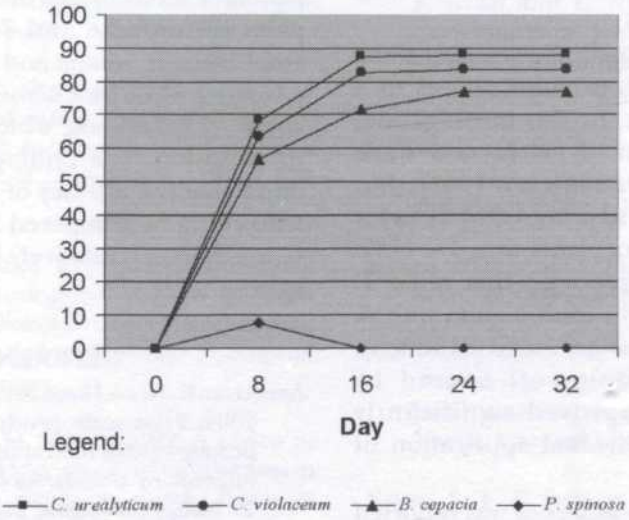

Fig. 1: Percentage inhibition of radial growth (PIRG) of G. boninense *by four bacterial species*

Compatibility with FA 1132

When tested against *T. harzianum*, only *Corynebacterium urealyticum* showed incompatibility towards FA1132. When all of the *Trichoderma*-challenged bacteria were re-isolated and recultured on NA, each of the cultures grew back normally, at a rate similar to the controls.

Of the bacteria isolated from the *Trichoderma*-compost, only *Corynebacterium urealyticum* showed a strongly antagonistic reaction towards FA 5011. *A. radingae* and *Corynebacterium nitrilophilus* both showed good compatibility with FA 1132; however they did not inhibit FA 5011. Thus, these two isolates, together with *Corynebacterium urealyticum* were not good candidates for the selection of potential *Trichoderma* co-inoculants.

DISCUSSION AND CONCLUSION

Bacteria are a good source of microbes for use as biocontrol agents of plant diseases and insect pests. This study successfully isolated and identified a total of seven strains belonging to six genera of bacteria, from two sources closely associated with the oil palm environment. *Pseudomonas spinosa, Actinomyces radingae* and *Corynebacterium nitrilophilus* showed no

antagonistic reaction towards *G. boninense*. The antagonistic isolates were *Corynebacterium urealyticum*, *Chromobacterium violaceum* and *Burkholderia cepacia*; with all three species showing significant differences between each other and a highly significant difference from the control, *P. spinosa*. The PIRG of these three strains increased with time and started to level off at day 24 onwards. However, their antagonistic interactions were fungistatic although *C. urealyticum* showed a reaction that was close to fungicidal. Nevertheless, all the interactions could not match the fungicidal property recorded for *T. harzanium* (strain FA 1132) against *G. boninense* by Abdullah *et al.* (2003) and Ilias (2000).

Besides its individual application, some bacteria are effective as co-inoculants in a microbial consortium. In this investigation, only *B. cepacia* and *C. violaceum* were compatible with *T. harzianum* (FA 1132), thus presenting themselves as prime candidates for use as potential co-inoculants with FA 1132. Roberts *et al.* (2004) reported that when *T. virens* was formulated in combination with *B. cepacia* and *B. ambifaria* as a seed treatment, the control of damping-off caused by *Rhizoctonia solani* improved significantly compared to the individual application of these microbes.

Chromobacterium violaceum was reported to have shown good properties as a biocontrol agent of the soilborne *Fusarium solani* against damping off of aubergine by Park *et al.* (1995). However, this violet-pigmented bacteria was also recorded as a human pathogen (Betts *et al.* 2004; Chattopadhyay *et al.* 2002). *C. violaceum* was reported by Duran and Menck (2002) as an opportunistic pathogen but of a type which can cause extreme virulence to humans and animals when infected. Further studies will need to be conducted to determine its biosafety as a biological control agent. Similarly, *B. cepacia* has also been recorded as a human pathogen but studies by Richardson *et al.* (2002) confirmed that the *B. cepacia* used for the control of plant pathogens was of a different strain to that which caused cystis fibrosis in humans. In another study, Alias and Tan (2005) reported that *B. cepacia* has the ability to biodegrade polyhydroxyalkanoate (PHA).

Bacteria is also known to exert its influence in the rhizosphere competency of other biocontrol agents. Kredics *et al.* (2003) reported that strains of soil bacteria can actually suppress the activity of *Trichoderma* biofungicides in agricultural soils. Thus the success of *Trichoderma* biocontrol agents applied to soil is highly dependent on the resident soil bacteria for rhizosphere competency.

In conclusion, this study was able to isolate a total of seven strains of bacteria from an oil palm rhizosphere and *Trichoderma*-compost combined. *B. cepacia* and *C. violaceum* have a potential as co-inoculants to the biocontrol agent *T. harzanium*, which warrants further investigation. The findings of this study also imply that the efficacy of *T. harzanium* in the field could be hampered if the population of *C. urealyticum* is relatively high in the oil palm rhizospheres.

REFERENCES

ABDULLAH, F., NOOR HAIDA SEBRAN and STEVEN CHONG. 2004. Pilot scale production of *Trichoderma* propagules for field application onto oil palms infected by *Ganoderma boninense*. In Abstracts, *2nd Biology Colloquium. Biology: Unity and Diversity 2004*, organized by the Department of Biology, Universiti Putra Malaysia.

ABDULLAH, F., M. Z. N. A. IZZATI, G. N. M. ILIAS, M. NELSON and U.K. YUSUF. 2003. Disease assessment and efficacy of *Trichoderma* as a biological control agent of basal stem rot of oil palm. *Science Putra Research Bulletin* **11(2):** 31–33.

ALIAS, Z. and I. K. P. TAN. 2005. Isolation of palm oil-utilising, polyhydroxyalkanoate (PHA)-producing bacteria by an enrichment technique. *Bioresource Technology* **96(1):** 1229-1234.

BENNET, A. J., C. LEIFERT and J. M. WHIPPS. 2003. Survival of the biocontrol agents *Coniothyrium minitans* and *Bacillus subtilis* MBI600 introduced into pasteurized, sterilized and

non-sterile soils. *Soil Biology and Biochemistry* **31(3):** 422-427.

BETTS, H. J., R. R. CHAUDRI and M. J. PALLEN. 2004. An analysis of type-III secretion gene clusters in *Chromobacterium violaceum*. *Trends in Microbiology* **12(11):** 476-482.

CHATTOPADHYAY, A., V. KUMAR, N. BHAT and P. L. N. G. RAO. 2002. A rare but frequently fatal disease in *Chromobacterium violaceum* infection. *Journal of Pediatric Surgery* **37(1):** 108-110.

COLLINS, D. P. and B. J. JACOBSEN. 2003. Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet Cercospora leaf spot. *Biological Control* **26(2):** 153-161.

DE JENSEN, C. V., J. A. PERCICH and P. H. GRAHAM. 2002. Integrated management strategies of bean root rot with *Bacillus subtilis* and *Rhizobium* in Minnesota. *Field Crops Research* **74(2):** 107-115.

DRIESCHE, V. R. G. and T. S. J. BELLOWS. 1996. Methods for biological control of plant pathogens. In *Biological Control*. Washington: Chapman & Hall. International Thompson Publishing Company.

DURAN, N. and C. F. M. MENCK. 2002. A review of pharmacological and industrial perspectives in *Chromobacterium violaceum*. *Critical Review in Microbiology* **27(3):** 201-222.

FARIDAH ABDULLAH, NOR HAIDA SEBRAN and MOHD. HEFNI RUSLI. 2005. Production and field application of *Trichoderma harzianum* on oil palms. In *Proceedings of the 27th Symposium of the Malaysian Society for Microbiology: Innovation through Microbes*, pp. 77 – 79. Organised by the Malaysian Society for Microbiology.

FRAVEL, D. R., W. J. JR. CONNICK and J. A. LEWIS. 1998. Formulation of microorganisms. In *Formulation of Microbial Biopesticides*, ed. H.D. Burges, pp. 198-202. Dordrecht: Kluwer Academic Publishers.

ILIAS, G. N. M. 2000. *Trichoderma (Pers. Ex Fr.)* and its efficacy as a biological control agent of basal stem rot of oil palm *(E. guineensis Jacq.)*. Ph.D. Thesis, Universiti Putra Malaysia.

ILIAS, G. N. M. and F. ABDULLAH. 1998. Diversity of *Trichoderma* from its natural habitat and its *in vitro* studies against *G. boninense*. Abstracts of *International Science Conference (ISC 98)*, 7-9 May 1999.

KHETAN, S. K. 2001. *Biofungicides*. New York: Marcel Dekker Inc.

KNOX, O. G. G., K. KILHAM and C. LEIFERT. 2000. Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. *Journal of Bioscience and Bioengineering* **91(2):** 173-177.

KREDICS, L., Z. ANTAL, L. MANCZINGER, A. SZEKERES, F. KEREI and E. NAGY. 2003. Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. *Food Technology and Biotechnology* **41(1):** 37-42.

MCPARTLAND, J. M., R. C. CLARKE and D. P. WATSON. 2000. *Hemp Diseases and Pests: Management and Biological Control*. UK: CABI Publishing.

MPOB. 2003. *Annual Research Report 2002*. Bangi, Selangor, Malaysia: Malaysian Palm Oil Board.

NAGAPPAN, J. 2005. The efficacy of three species of *Trichoderma* as a biological control agent of basal stem rot on oil palm seedlings. MSc. Thesis, Universiti Putra Malaysia.

NEMEC, S., L. E. DATNOFF and J. STRANDBERG. 1996. Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. *Crop Protection* **15(8):** 735-742.

PARK, S. K., YEON and K. K. CHUNG. 1995. Antagonistic effect of chitinolytic bacteria on soil borne plant pathogens. *Korean Journal of Plant Pathology* **11(1):** 47-52.

RICHARDSON, J., J. ELPHINSTONE, D. STEAD and R. COUTTS. 2002. Differentiation of *Burkholderia cepacia* isolates by fatty acids profiling and PCR for biocontrol of *Ralstonia solanacearum*. In *Proceedings of the 7th International Congress of Plant Pathology*, Scotland.

ROBERTS, D. P., S. M. LORHKE, S. L. F. MEYER, J. S. BUYER, J. H. BOWERS, C. J. BAKER, J. T. DE SOUZA, J. A. LEWIS and S. CHUNG. 2004. Biocontrol agents applied individually and in

combination for suppression of soilborne diseases of cucumber. *Crop Protection* **24(2):** 141-155.

SAMUELS, G. J. 1996. Centenary review. *Trichoderma*: A review of biology and systematic of the genus. *Mycological Research* **100**: 923-935.

SKIDMORE, A. M. and C. H. DICKINSON. 1976. Colony interaction and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Transactions of The British Mycological Society* **66(1):** 57-64.

SUNDRAM, S. 2005. Performance of *Trichoderma harzianum Rifai* as biological control agent of basal stem rot of oil palm *(Elaeis guineensis Jacq.)* caused by *Ganoderma boninense* on oil palm seedlings. MSc. Thesis, Universiti Putra Malaysia.

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Population Dynamics and Exploitation Level of Green-Lipped Mussel (*Perna viridis*) Using FiSAT from the Offshore Island of the Cox's Bazar Coast of Bangladesh

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ABSTRAK

Perancangan dan pengurusan sumber moluska memerlukan pengetahuan pelbagai parameter populasi dan tahap eksploitasi (E) populasi tersebut dalam lokasi khusus yang tertentu. Aggaran parameter populasi seperti kepanjangan asimptot (L∞), pekali pertumbuhan (K), kematian, tahap eksploitasi (E) dan corak rekruitmen kupang Perna viridis *di luar pesisir pantai Pulau Cox's Bazar Bangladesh telah dikaji dengan menggunakan frekuensi kepanjangan berdasarkan analisis perisian FiSAT. Kepanjangan asimptot (L∞) adalah 19.43 cm dan pekali pertumbuhan (K) dianggarkan 0.56 setahun. Jumlah kematian (Z) adalah 1.44 setahun untuk* P. viridis. *Kematian semula jadi (M) dan kematian penangkapan (F) masing-masing adalah 1.38 setahun dan 0.06 setahun. Tahap eksploitasi (E)* P. viridis *adalah 0.04 manakala nilai had eksploitasi maksimum yang dibenarkan (E_{max}) adalah 0.68. Tahap eksploitasi (<0.50) menunjukkan bahawa stok* P. viridis *mungkin kurang dieksploitasi di luar pesisir pantai Pulau Cox's Bazar Bangladesh.*

ABSTRACT

*Planning and management of molluscan resources require knowledge of various population parameters and exploitation level (E) of the population in a particular location. Estimation of population parameters like asymptotic length (L∞), growth co-efficient (K), mortalities, exploitation level (E) and recruitment pattern of green-lipped mussel (*Perna viridis*) in the offshore Island of Cox's Bazar coast of Bangladesh were studied by using the length frequency based analysis of FiSAT software. Asymptotic length (L∞) was 19.43 cm and growth co-efficient (K) was estimated at 0.56/yr. Total mortality (Z) was 1.44/yr. Natural mortality (M) and fishing mortality (F) were 1.38/yr and 0.06/yr, respectively. Exploitation level (E) of* P. viridis *was 0.04 while the maximum allowable limit of exploitation value(E_{max}) was 0.68. The exploitation level (< 0.50) indicates that the stock of* P. viridis *might underexploited in the offshore Island of the Cox's Bazar coast of Bangladesh.*

INTRODUCTION

The majority of fishermen in coastal communities of Bangladesh are poor and do not own or have access to any land. Many of them are engaged in the fishing industry as temporary labour. Women and children particularly are involved in the collection of shrimp post larvae (PL) by push net in the shallow coast. Their livelihood options are limited and unstable and some are currently being threatened with the recommended bans on PL collection and shallow water fishing using bag nets. Alternative income generating activities are also limited. Recent surveys, reviews and workshops (TAANGO 2002; Luu 2002; Frankenberger 2002) have identified a number of potential alternative livelihood options that could have a considerable and

positive impact to the poor as well as enhancing foreign earnings. One of these options is the development of a molluscan fishery, in particular the bivalves. The coastal water of Bangladesh is one of the most productive zones in the world and rich in fish and shell-fishes including molluscs (Ahmed *et al.* 1978). Along the coastal area, varieties of marine habitats such as sandy, muddy and rocky grounds, mangrove areas and coral reefs are inhabited by the bivalves, and thus are potentially viable for the development of shellfish fishery. Commans (1940) was the first to report the presence of some species of molluscs in the St. Martin's Island from the Bay of Bengal. Ali (1975) later reported that several taxa including 33 species exist within the St. Martin's Island. Ali and Aziz (1976) later described 33 species under three different taxa from the same Island. Ahmed (1990) subsequently identified 301 molluscs species belonging to 151 genera, 79 families, 16 orders and 4 classes from the Cox's Bazar, Moheskhali Channel, Teknaf, St. Martin's Island, Sundarban reserve forest and deep water of the Bay. This survey was conducted mainly to identify the marine molluscs available in the Bay of Bengal. But there is no report regarding the status of exploitation levels despite a market demand from foreign countries such as Japan, Thailand and China.

For planning and management of mollusc resources, knowledge of various population parameters and exploitation level (E) of their population is required. There are many tools for assessing exploitation level and status of stock. FAO-ICLARM Stock Assessment Tools (FiSAT) is one which has been most frequently used for estimating population parameters of fin-fish and shell-fish (Amin *et al.* 2001, 2002; Angell 1986; Cha *et al.* 2002; Mancera and Mendo 1996; Tuaycharoen *et al.* 1988; Vakily 1992) because it needs only length-frequency data. The advantage of this technique is that within a year it is possible to assess of any fish stock if you have sufficient length-frequency data.

The commercially important mollusc species green-lipped muscle (*P. viridis*), is available in Cox's Bazar coast. Tribal communities are currently exploiting them for local consumption. The marine mussels are popular food items in many other countries around the globe. There has been no published report on population dynamics and status of exploitation of *P. viridis* in Bangladesh prior to this study. Hence, the estimation of population parameters and exploitation level of this species of bivalves from the coast of Bangladesh is very important.

The objective of this study was to estimate the population parameters and exploitation level of *P. viridis* to assess the stock position of the species from the offshore Island (Moheskhali) of Cox's Bazar coast of Bangladesh using FiSAT.

MATERIALS AND METHODS

The study was conducted in Moheskhali Channel (N21^0 28$^/$ and N21^0 46$^/$, E91^0 57$^/$ and E92^0 03$^/$) of south-eastern coast of Bangladesh (*Fig. 1*). Random sampling was done monthly between June 2003 and May 2004. Specimens of *P. viridis* were attached with the stone on inter-tidal zone of Moheskhali Channel and Chaufaldandi coastal area of Cox's Bazar, Bangladesh. An iron rod was utilized during sampling for separating the specimens from the stone. Samples were preserved with 10% formalin at field level immediately after collection. In the laboratory, total shell length was measured with the help of a meter scale to the nearest millimeter and total weight was taken by an electronic balance of 0.001 g accuracy. A total of 981 specimens were collected. The data from two stations were then pooled month-wise and subsequently grouped into length classes by 1 cm interval. Then the data were analyzed using FiSAT software as explained in detail by Gayanilo Jr. *et al.* (1996).

Asymptotic length (L_∞) and growth co-efficient (K) of the von Bertalanffy growth equation were estimated by means of ELEFAN-1 (Pauly and David 1981; Saeger and Gayanilo 1986). The estimates of L_∞ and K were used to estimate the growth performance index (φ') (Munro and Pauly 1983; Pauly and Munro 1984) of *P. viridis* using the equation:

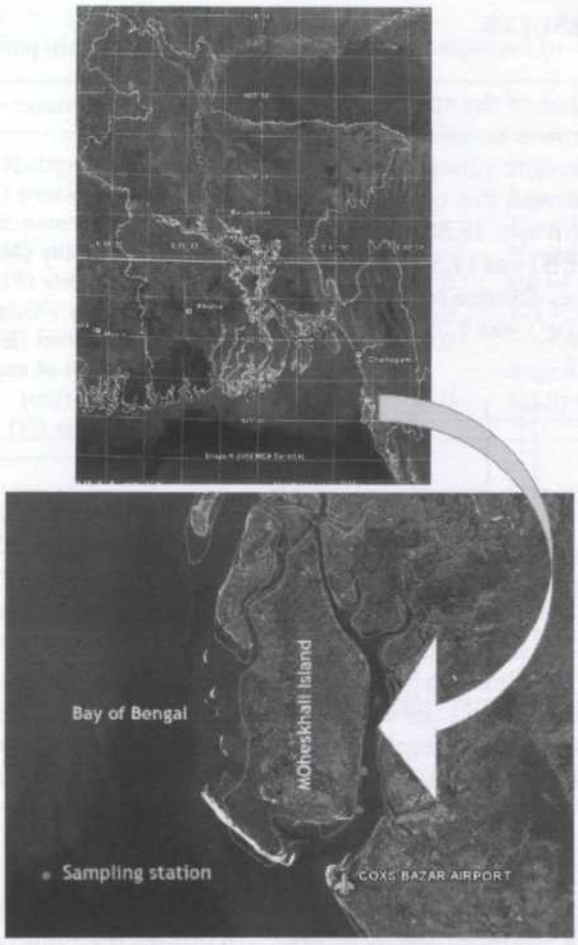

Fig. 1: Sampling stations of the study area

$$\varphi' = 2 \log_{10} L\infty + \log_{10} K$$

The total mortality (Z) was estimated by length converted catch curve method (Pauly 1984, 1990). Natural mortality rate (M) was estimated using the empirical relationship of Pauly (1980):

$$\text{Log}_{10}M = -0.0066 - 0.279\text{Log}_{10}L_{\infty} + 0.6543.\ \text{Log}_{10}K + 0.4634\ \text{Log}_{10}T$$

where T is the mean annual habitat temperature 0c of the water in which the stocks live. Once Z and M were obtained, then fishing mortality (F) was estimated using the relationship:

$$F = Z\text{-}M$$

where Z is the total mortality and F fishing mortality. The exploitation level (E) was obtained by the relationship of Gulland (1971):

$$E = F/Z = F/\ (F+M)$$

The recruitment pattern of the stock was determined by backward projection on the length axis of the available length frequency data set as described in FiSAT. This routine reconstructs the recruitment pulse from a time series of length-frequency data to determine the number of pulses per year and the relative strength of each pulse. Input parameters were L_{∞}, K and t_0 (t_0=0).

RESULTS

Growth Parameters

Asymptotic length (L_∞) of the von Berttalenfy was 19.43 cm and growth co-efficient (K) was 0.56/yr for *P. viridis*. The observed extreme length was 18.50 cm and the computer predicted extreme length was 19.38 cm (*Fig. 2*). The confidence interval was 17.35 to 21.42 cm (95% probability of occurrence and the growth performance index (φ') was 2.32.

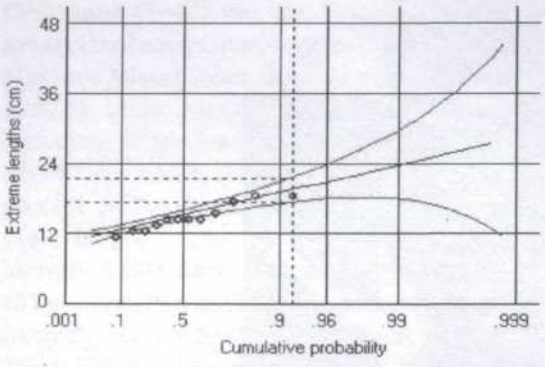

Fig. 2: Maximum size estimation of Perna viridis

Mortalities

Length converted catch curve analysis produced total mortality estimates of Z = 1.44/yr for *P. viridis* (*Fig. 3*). Natural mortality (M) was 1.38/yr and fishing mortality (F) was 0.06/yr for the species (Table 1).

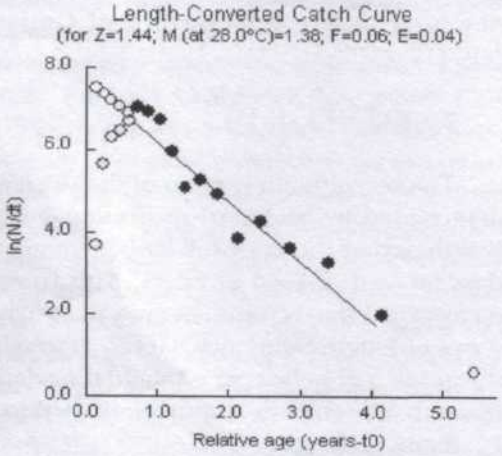

Fig. 3: Length converted catch curve of Perna viridis

TABLE 1
Population parameters of *Perna viridis*

Population parameters	*Perna viridis*
Asymptotic length (L_∞) in cm	19.43
Growth co-efficient (K)/yr	0.56
Growth performance index (φ')	2.32
Natural mortality (M)/yr	1.38
Fishing mortality (F)/yr	0.06
Total mortality (Z)/yr	1.44
Exploitation level (E)	0.04
Allowable limit of exploitation (E_{max})	0.68
Length range (cm)	1.50-18.50
Sample number (N)	981

Exploitation Level (E)

Exploitation level (E) of *P. viridis* was 0.04 and the maximum allowable limit of exploitation (E_{max}) value was 0.68 (Table 1). Results in Table 1 show that exploitation level (E) of *P. viridis* alone in comparison to its E_{max} was lower than 94%. It clearly shows that there was a definite case of under exploitation of the total stock of the species.

Recruitment Pattern

The recruitment pattern of *P. viridis* was continuous but there were two major peaks in a year; the highest peak observed in March-May and second peak occurred in August-October (*Fig. 4*).

Fig. 4: Recruitment pattern of Perna viridis

TABLE 2
Population parameters of the *P. viridis* and other bivalves as reported in other countries

Location	Species	L_∞ (cm)	K/yr	φ'	T (°c)	Source
Bangladesh	*P. viridis*	19.43	0.56	2.32	28	Present study
Hong Kong	*P. viridis*	10.19	0.30	-	-	Lee (1985)
Thailand	*P. viridis*	11.20	1.00	-	-	Tuaycharoen *et. al.*, (1988)
India	*P. viridis*	18.46	0.25	-	-	Narasimham (1981)
USA	*C. virginica*	12.58	0.50	3.90	11.0	Vakily (1992)
India	*C. madrasensis*	11.90	0.77	4.04	28.0	Vakily (1992)
Colombia	*C. rhizophorae*	14.90	0.90	4.30	30.0	Mancera and Mendo (1996)
Venezuela	*C. rhizophorae*	7.60	3.96	4.34	-	Angell (1986)
Korea	*C. gigas*	10.37	2.35	4.40	16.0	Vakily (1992)

DISCUSSION

The estimated asymptotic length (L_∞) was 19.43 cm and growth co-efficient (K) 0.56/yr for *P. viridis* in this study. The comparison with growth parameters obtained in other studies show differences for *P. viridis* from different areas of the world (Table 2). The highest value of (L_∞) (19.43 cm) was obtained from this study in Bangladesh coast waters and the lowest value (10.19 cm) was from Hong Kong (Lee 1985). The highest K value (1.0/yr) was obtained in Thailand (Tuaycharoen *et. al.* 1988) and the lowest value (0.25/yr) was reported from India (Narasimham 1981). It was observed that the L_∞ of *P. viridis* from Bangladesh coastal waters is higher than other countries (Table 2) but K is more or less very close to *P. viridis* of India and Hong Kong waters. Higher L_α indicates plenty of oyster resources in the Moheskhali Island of Bangladesh.

Higher natural mortality (1.38/yr) observed as opposed to fishing mortality (0.06/yr) of *P. viridis* observed in this study indicates an imbalance in the stock. The yield is optimized when fishing mortality (F) = natural mortality (M) (Gulland 1971).

The lower value of E indicates an 'under fishing' status during the study period. Theoretically when E= 0.50, then the stock of any aquatic species is at the optimum level. According to Gulland (1971), the yield is optimized when F = M; therefore, when E is more than 0.5, the stock is over fished. Sparre and Venema (1992) advocated the use of Beverton and Holt's E_{max} (E_{MSY}) to decide the state of under or over exploitation and suggest management measures, if necessary, because the hypothetical ideal E value of 0.5 is only possible if natural and fishing mortality is equal, and this is unsual for any exploited fish population. From this study, it could be concluded that the stock of *P. viridis* is virgin in the study area.

The recruitment pattern suggests that annual recruitment consist of two seasonal pulses (*Fig. 4*), i.e. two cohorts are produced per year; the highest peak occurs in March-May followed by a second peak occuring in August-October. However, studies on larval abundance and spat collections in the St. Martin coast of Bangladesh (Hossain *et al.* 2004) showed that green mussel larvae settle through the year but the highest peak was found in October and the second highest in March. The recruitment peaks detected in this study should correspond to the first and second larval settlement.

CONCLUSION

Higher natural mortality (1.38/yr) was observed compared to fishing mortality (0.06/yr) and the stock of *P. viridis* is underexploited in the study area. It could be concluded that the stock of green-lipped mussel has a great potential in the Cox's Bazar coast of Bangladesh. More exploitation is possible and

it could be an option for the livelihood of poor coastal communities of Bangladesh.

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REFERENCES

AHMED, A. T. A. 1990. Identity and abundance of molluscan fauna of the Bay of Bengal. pp. 1-85. Bangladesh Agricultural Research Council, Dhaka.

AHMED, A.T.A, R. ISLAM and M. SANAULLAH. 1978. A preliminary report on the molluscan fauna of the Bay of Bengal with notes on their ecology. *Journal of Asiatic Society of Bangladesh* (Sc.) **3(2):** 59-82.

ALI, S. 1975. Notes on a collection of shells from St. Martin's Island. *Bangladesh Journal of Zoology* **3(2):** 153-154.

ALI, S. and K.M.S. AZIZ. 1976. A systematic account of molluscan fauna of the St. Martin's Island. *Bangladesh Journal of Zoology* **4(2):** 23-33.

AMIN, S. M. NURUL, A. K. YOUSUF HAROON and M. ALAM. 2001. A study on the population dynamics of *Labeo rohita* (Ham.) in the Sylhet basin, Bangladesh. *Indian Journal of Fisheries* **48(3):** 291-296.

AMIN, S. M. N., M. A. RAHMAN, G. C. HALDAR, M. A. MAIZE and D. MILTON. 2002. Population dynamics and stock assessment of Hilsa shad, *Tenualosa ilisha* in Bangladesh. *Asian Fisheries Science* **15:** 123-128.

ANGELL, C.L. 1986. The biology and culture of tropical oysters. *ICLARM Studies Reviews* **12**: 37.

CHA, K. H., O. C. OH, S. Y. HONG and K. Y. PARK. 2002. Reproduction and population dynamics of *Penaeus chinensis* (Decapoda: Penaeidae) on the western coast of Korea, Yellow Sea. *Fisheries Research* **56:** 25-36.

COMMANS, H. E. 1940. The marine molluscs of St. Martin. Lesser Articles, collected by H. B. Krebss. Stud. Faura. *Aure Cao.* **16 (1):** 38-87.

FRAKENBERGER, T. R. 2002. A livelihood analysis of shrimp fry collections in Bangladesh: future prospects in relation to a wild fry collection ban. 56pp.

GAYANILO, JR. F. C., P. SORIANO and D. PAULY. 1996. The FAO-ICLARM stock assessment tools (FiSAT) users guide. FAO Computerised Information Series (Fisheries), No. 8, 266p. Rome, FAO.

GULLAND, J. A. 1971. Estimation of mortality rates. Annex to artic fisheries working group report. ICES C. M. Doc. 3 (mimeo).

HOSSAIN, M. S., M. SHAMSUDDOHA and M. AKHTERUZZAMAN. 2004. Mollusc aquaculture for sustainable coastal livelihood. Support for University Fisheries Education and Research (SUFER) project, DFID, University Grant Commission, Dhaka, Bangladesh. Final report. 94p.

LEE, S. Y.1985. The population dynamics of the green mussel, *Perna viridis* (L) in Victoria Harbour, Hong Kong-Dominance in a polluted environment. *Asian Marine Biology* **2:** 107-118.

LUU, L. T. 2002. First mission report of empowerment of coastal fishing communities for livelihood security project. 104p.

MANCERA E., J. MENDO. 1996. Population dynamics of the oyster *Crassostrea rhizophorae* from the Cienaga Grande de Santa Marta, Colombia. *Fisheries Research* **26:** 139-148.

MUNRO, J. L. and D. PAULY. 1983. A simple method for comparing the growth of fishes and invertebrates. *ICLARM Fishbyte* **1(1):** 5-6.

NARASIMHAM, K. A. 1981. Dimentional relationships and growth of green mussel *Perna viridis* in Kakinada Bay. *Indian Journal of Fisheries* **28 (1&2):** 240-248.

TANGO INTERNATIONAL, INC. 2002. A livelihood assessment of poor communities in south-east Bangladesh. 97pp.

TUAYCHAROEN, S, J. M. VAKILY, A. SAELOW and E.W. MCCOY. 1988. Growth and maturation of the green mussel (*Perna viridis*) in Thailand. In *Bivalve Mollusc Culture Research in Thailand*, ed. E.W. McCoy and T. Chongpeepien, pp. 88-101.

PAULY, D. 1980. On the interrelationships between natural mortality, growth parameters and mean environmental temperature in 175 fish stocks. *J. Cons. CIEM* **39(3):** 175-192.

PAULY, D. 1984. Length-converted catch curves: a powerful tool for fisheries research in the tropics (Part II). *ICLARM Fishbyte* **2(1):** 17-19.

PAULY, D. 1990. Length-converted catch curves and the seasonal growth of fishes. *ICLARM Fishbyte* **8(3):** 33-38.

PAULY, D. and J. L. MUNRO.1984. Once more on the comparison of growth in fish and invertebrate. *Fishbyte* **2:** 21.

PAULY, D. and N. DAVID. 1981. ELEFAN-I BASIC program for the objective extraction of growth parameters from length frequency data. *Meeresforsch.* **28(4):** 205-211.

SAEGER, J. and F. J. GAYANILO. 1986. A revised and graphics oriented version of ELEFAN-I and II basic programs for use on HP/86/87 microcomputers. No. 8. Tech. Rep. Dept. Marine Fish.

SPARRE, P. and S.C. VENEMA. 1992. Introduction to tropical fish stock assessment. Part 1-Manual. FAO Fisheries Technical Paper 306/1, 376pp.

VAKILY, J. M. 1992. Determination and comparison of bivalve growth, with emphasis on Thailand and other tropical areas. ICLARM Technical Report 36, 125 pp.

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Determination of Flavonoid Components from *Morinda citrifolia* (Mengkudu) and Their Antioxidant Activities

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Keywords: ***Morinda citrifolia*, flavanoid**

ABSTRAK

Lima komponen flavonoid dapat diekstrak dan dikenal pasti daripada daun Morinda citrifolia*: quercetin 3,7-O-dimethyl ether, quercetin 3-O-methyl ether, kaempferol 3,4'-O-dimethyl ether, kaempferol 5,7-O-diarabinoside dan apigenin. Pengasingan komponen flavonoid dijalankan menggunakan teknik kertas kromatografi dengan empat sistem pelarut; BAW (n-butanol:asid asetik:air; 4:1:5), 15% asid asetik (15% HoAc), BEW (n-butanol:etanol:air; 4:1:2.2) dan air. Flavonoid dikenal pasti melalui warna di bawah cahaya ultra violet (UV) dengan atau tanpa wap NH_3, nilai R_f, hidrolisis asid kepada flavonoid aglikon dan gula, perbandingan ko-kromatografi dengan penanda piawai, analisis spektral dengan menggunakan beberapa bahan reagen. Kesemua komponen flavonoid pertama kali dilaporkan dalam genus Morinda. Aktiviti antioksidan bagi komponen flavonoid ditentukan dengan menggunakan kaedah pelunturan β-karotena. Butylated hydroxyl toloul (BHT) dan quercetin digunakan sebagai bahan penanda piawai positif. Kesemua komponen flavonoid menunjukkan penurunan aktiviti antioksidan sepanjang tempoh uji kaji. Pada minit yang ke 40, quercetin 3-O-methyl ether menunjukkan aktiviti antioksidan yang tertinggi (77.67%); diikuti oleh quercetin 3,7-O-dimethyl ether (77.00%), kaempferol 3,4'-O-dimethyl ether (66.47%), apigenin (58.10%) dan kaempferol 5,7-O-diarabinoside (56.14%). Ujian statistik ANOVA satu hala dari perisian SPSS versi 11.0 menunjukkan kesemua aktiviti antioksidan komponen flavonoid adalah berbeza secara signifikan daripada aktiviti antioksidan BHT dan quercetin ($p < 0.05$).*

ABSTRACT

Five flavonoid components were isolated and identified from Morinda citrifolia's *leaves; quercetin 3,7-O-dimethyl ether, quercetin 3-O-methyl ether, kaempferol 3,4'-O-dimethyl ether, kaempferol 5,7-O-diarabinoside and apigenin. The isolation of flavonoid components was carried using paper chromatography methods with three different system solutions namely, BAW (n-butanol:acetic acid:water; 4:1:5), 15% acetic acid (15% HoAc), BEW (n-butanol:ethanol:water; 4:1:2.2) in addition to pure water. Flavonoid was identified through their colors under ultra violet (UV) with/without NH_3, R_f values, acid hydrolysis to aglycone and sugar, co-chromatographic comparison with standard markers and spectral analysis by using a series of shift reagents. All of these flavonoid components were firstly reported in the genus Morinda. The antioxidant activities of flavonoid components were evaluated by using the β-carotene bleaching method. Butylated hydroxyl toloul (BHT) and quercetin were used as positive markers. All isolated flavonoid components showed declining antioxidant activities through out the experiment. At the 40th minute, quercetin 3-O-methyl ether showed the highest antioxidant activities (77.67%); followed by quercetin 3,7-O-dimethyl ether (77.00%), kaempferol 3,4'-O-dimethyl ether (66.47%), apigenin (58.10%) and kaempferol 5,7-O-diarabinoside (56.14%). Statistical analysis was done using ANOVA with SPSS version 11.0 software. The results showed that all antioxidant activities belonging to the flavonoid components were significantly different from BHT and quercetins' antioxidant activites ($p < 0.05$).*

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INTRODUCTION

Flavonoid is a phenolic compound and was discovered as early as the 1930s. At that time, it was known as vitamin P (Nijveldt *et al.* 2001) because of its nutritional properties. Flavonoid is abundant in plants and can be found in all plant parts; bark, leaves, fruit and stem because flavonoid is a secondary metabolite in plants. There were five major groups of flavonoid; flavone, flavanone, isoflavone, flavonol, and anthocyanin (Wang and Halliwel 2001). Based on *in vitro* experiments, flavonoid was found to have anti-inflammatory, anti-allergy, antiviral and anti-carcinogenic properties (Middleton 1998). From a biological activity perspective, flavonoids have the ability to scavenge oxygen-derived free radicals and can act as antioxidant. According to Nijveldt *et al.* (2001), flavones and catechins are the most powerful antioxidant against reactive oxygen species (ROS).

An antioxidant is a substance that can prevent the process of lipid peroxidation; a process whereby free radicals (FR) can damage membrane cells. The damaged cells can lead to many degenerative diseases (Mohd Zin *et al.* 2002). In plants, antioxidants are produced/developed to defend their structures against ROS attack during photosynthesis. By taking antioxidant supplements such as vitamins or foods high in antioxidants such as vegetables and green leaves can help counter detrimental effects of free radicals (Faridah *et al.* 2003).

Morinda citrifolia or Mengkudu is commonly used as a Malay traditional remedy and its young shoots are eaten as *ulam* or raw. *Morinda citrifolia* has been used a lot especially by post-natal women for healing menstrual problems and curing diabetes (Zainatul Suhaida 2003). The plant belongs to the *Rubiaceae* family and originated from tropical Asia especially the coast of South East Asia (Dharma 1987). Apart from its utilization in traditional medicine, the pulp is also used as hair shampoo (Jaganath *et al.* 2000). Many scientific and modern research studies have been conducted to investigate the active compounds and special abilities of this plant. From previous research that has been conducted, few pure active compounds have been revealed in the leaf of *Morinda citrifolia* like iridoids (Sang *et al.* 2003) and anthraquinone (Thomson 1987) while the biological activities that has been discovered from *Morinda citrifolia* are antioxidative (Mohd Zin *et al.* 2002), and anti-inflammatory (Li *et al.* 2003) activities.

MATERIALS AND METHODS

Plant Sources

The leaves of *Morinda citrifolia* were collected at Universiti Putra Malaysia's campus and dried in an oven at 50°C for four–five days.

Extraction of Flavonoid

Air-dried leaves of *Morinda citrifolia* were extracted using three solvents; 80% methanol, petroleum ether (60-80°C boiling point) and chloroform. 300-600 g dried leaves were pulverized and extracted using the solvents. After standing overnight for methanol and three-four days for petroleum ether (60-80°C boiling point) and chloroform, the extracts were concentrated using a rotary evaporator.

Isolation of Flavonoid

The concentrated extracts were separated by chromatography on 3 mm paper (46 x 57 cm) in BAW (4:1:5, top layer). Bands that appeared dark under UV light, changed to yellow-green or unaffected by fuming with NH_3 vapour were eluted. The concentrated eluates were separated on 3 mm paper in 15% HOAc and the same processes were repeated with BEW (*n*-butanol: ethanol:water; 4:1:2.2) and water. Flavonoid components were identified through their colors under ultra violet (UV) with/without ammonia vapor, comparison R_f values with standard markers, acid hydrolysis and spectral data (Markham 1982). The isolated compounds were also identified based on the reported compounds in the previous studies (Harborne 1967).

Quercetin 3,7 O-dimethyl ether

The $^{a}_{max}$ values for this component are 349, 300 (shoulder) and 265; + NaOAc 393, 305 (sh) 269; +NaOH 408, 325 (sh), 273; + Al_2Cl_3 348, 300 (sh), 397 (iv), 273 nm. The absence of a NaOAc shift indicates that the 7-position is blocked. The component gave a dark colour under UV light but changed to yellow-green when fumed with ammonia vapor (NH_3). On partial acid hydrolysis, it produced quercetin, quercetin 3-*O*-methyl ether and quercetin 7-*O*-methyl ether. Quercetin showed yellow to yellow reaction in UV + NH_3 and showed identical spectral and R_f properties to the standard marker. Quercetin 3-*O*-methyl ether showed dark to yellow color reaction in UV + NH_3. The location of the methyl group at position 3 followed from the UV spectral analysis: $^{a}_{max}$ in MeOH at 359, 266, 256 (sh), in MeOH + NaOH 385, 359 (sh), 270. Quercetin 7-*O*-methyl ether was identified because of the R_f value, colour reaction under UV + NH_3 and Co-chromatogram comparision standard marker.

Quercetin 3-O-methyl Ether

Quercetin 3-O-methyl ether was dark when viewed under UV light on paper and yellow with NH_3. The $^{a}_{max}$ values for this component are 359, 299 (sh), 255, 268 (sh). The sodium hydroxide UV spectral data established the presence of a hydroxyl group on C_3 was changed (320 nm as band III). On acid hydrolysis no sugar was detected and tentatively this component was identified as Quercetin 3-*O*-methyl ether.

Kaempferol 3,4'-O-dimethyl Ether

The color of the component was dark under UV light and unchanged after fuming. Spectral analysis; in MeOH 350, 295sh; 266, in MeOH + NaOH 406, 325 (iii), 274 nm (decompose), indicated that there was free C_3 and C_4. After hydrolysis with acid, three derivatives were isolated: kaempferol that showed unchanged yellow under UV/UV+NH_3, (366, 317sh; 266 nm), kaempferol 3-*O*-methyl ether with dark to yellow reaction under UV/UV+NH_3 and kaempferol 4'-*O*-methyl ether also known as kaempferide,'which showed unchanged dark reaction under UV/UV+NH_3 as well as the decompose reaction after NaOH was added to neutralise the sample for spectral data indicates free C_4. Identification *kaempferol*, *kaempferol 3-O-methyl ether* and *kaempferide* was confirmed from their spectral data, R_f values, color reaction under UV/UV+NH_3, Co-chromatography comparison with standard marker and bibliography data by Harborne (1967). This component was identified as *kaempferol 3,4'-O-dimethyl ether* because the sugar test produced negative results.

Apigenin

This component was isolated from petroleum ether (60-80°C boiling point) extract and identified using standard procedures. It produced a dark color under UV and changed to yellow after fuming with NH_3 vapor.

Kaempferol 5,7-O-diarabinoside

Kaempferol 5,7-*O*-diarabinoside was isolated from chloroform and its color under UV light was dark and turned yellow after fuming with NH_3. Its neutral spectral in MeOH was 360, 283sh; 268, 257sh. No shift between neutral spectral and spectral after addition of NaOAc reagent on band II (268 nm) indicates that the hydroxyl group at C_7 was not free. The hydroxyl group at C_5 was not free because there was no shift between the neutral spectral and the spectral after addition of Al_2Cl_3 reagent on band I (361 nm). After hydrolisis with acid, three derivatives were isolated. The first derivative was identified as kaempferol because the yellow color under UV did not change after NH_3 fuming. Its R_f and spectral data were identical to the purchased kaempferol. The second derivative was recognized as kaempferol 7-*O*-arabinose based on the spectral characteristics, R_f value, and color reaction under UV/UV+NH_3. The sugar test detected arabinose and tentatively this component is known as kaempferol 5,7-*O*-diarabinoside.

Antioxidant Assay (β-carotene Bleaching Method)

Fresh leaves were used to determine the antioxidant activities. The leaves were crushed by using mortar and extracted using 80% methanol for 2 hours by stirring with a magnetic stirrer. The antioxidant activity of the crude extract and pure flavonoid compounds were evaluated by the β-carotene bleaching method (Veliglou 1998) with some modification to suit the equipment in the laboratory. A solution of β-carotene was prepared by dissolving 1 mg of powder β-carotene in 5 ml of chloroform. 60 µl of 95% linoleic acid, between 20 and 600 µl of the sample or control (80% methanol) or standard marker were pipetted into a 250 ml round bottom flask and covered with aluminium foil. Three ml of β-carotene prepared previously was added into the same flask. After chloroform was removed by vacuum using a rotary evaporator, 150 ml of distilled water was added to the flask and shaked vigorously for 60 seconds. Aliquots (5 ml) of this emulsion were transferred into test tubes to make triplicates. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The tubes were placed at 50°C in dri-block. Absorbance measurements were recorded every 20 minutes for 2 hours. Butylated hydroxyl toloul (BHT) and quercetin were used as positive standard markers for comparative purposes. The antioxidant activities of the methanolic extract and flavonoid compounds were determined by using the following formula. The antioxidant activity was calculated in terms of percent inhibition relative to the control after 40 minutes incubation.

Antioxidant activity (%) formula:

1. $$dr = \frac{(\ln a/b)}{t}$$

ln = natural log
dr = degradation rate
a = absorbance value at 0 minute
b = absorbance value at observation time (0, 20, 40, 60, 80, 100, 120 minute)
t = observation time (0, 20, 40, 60, 80, 100, 120 minute)

2. Antioxidant activity (%)

$$= \frac{\text{dr control} - \text{dr sample}}{\text{dr control}} \times 100$$

The principle of β-CB determines the oxidative degradation of β-carotene in an emulsion containing linoleic acid. To measure these products, spectrophotometry has been used (Cruz *et al.* 1999). β-carotene in the model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules loose their double bonds, the system loses its characteristic orange colour, which can be monitored using spectrophotometry. The presence of a flavonoid as an antioxidant can hinder the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system.

RESULT AND DISCUSSION

Flavonoid Identification

In this study, five flavonoid components were isolated and identified from the leaves of *Morinda citrifolia* viz; quercetin 3,7-*O*-dimethyl ether, quercetin 3-*O*-methyl ether, kaempferol 3,4'-*O*-dimethyl ether, kaempferol 5,7-*O*-diarabinoside and apigenin. Quercetin 3,7-*O*-dimethyl ether, quercetin 3-*O*-methyl ether and kaempferol 3,4'-*O*-dimethyl ether were isolated from methanolic extract. Kaempferol 5,7-*O*-diarabinose and apigenin were isolated from chloroform and petroleum ether extracts respectively. The color reactions under UV/

TABLE 1
R_f value, color and spectral data of flavonoid component from leaves of *Morinda citrifolia*

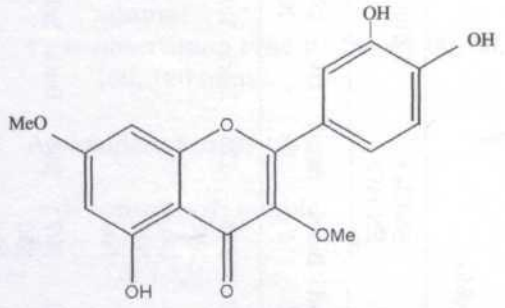
	R_fs value				Spectrum in 80% MeOH		+NaOAc		+NaOAc +H_3BO_3		+NaOH		+$AlCl_2$		+$AlCl_2$ + 2N HCl		Color	
	BAW	BEW	15% HOAc	H_2O	Band I	Band II	Band I	Band II	Band I	Band II	Band I	Band II	Band I	Band II	Band I	Band II	UV	UV + NH_3
Qu 3,7-*O*-diMethyl ether	47	64	69	54	349 297 (sh)	265	393 305 (sh)	269	354 299 (sh)	265	408 325 (sh)	273	348 300 (sh) 397 (iv)	273	343 300 (sh) 396 (iv)	273	Dark	Yellow
Qu 3-*O*-Methyl ether	58	49	54	33	359 299 (sh)	255 268 (sh)	379 319 (sh)	268 389	376	260	406 320 (sh)	274	401 341 (sh) 299 (sh)	271	397 350 (sh) 299 (sh)	269	Dark	Yellow
Km 3,4'-*O*-dimethyl ether	67	51	60	39	350 295 (sh)	266	386 308 (sh)	273	352 297 (sh)	266	406 325 (sh) 386 (iv)	274	348 300 (sh) 395 (iv)	272	346 300 (sh)	273	Dark	Yellow
Km 5,7-*O*-diarabinoside	75	83	57	46	342 328 (sh) 297 (sh)	265	341 303 (sh)	269	346 296 (sh)	266	383 321 (sh)	272	340 381 (sh) 303 (sh)	273	322.6	269 242 (sh)	Dark	Dark
Apigenin	75	67	65	22	347 282 (sh)	266	381 304 (sh) 360 (sh)	272	357 299 (sh)	268	399 328 (sh)	275	340 381 (sh) 303 (sh)	273	383 300 (sh)	274	Dark	Yellow

UV+ NH_3 vapor, R_f values and spectral properties of the identified flavonoid components are shown in Table 1.

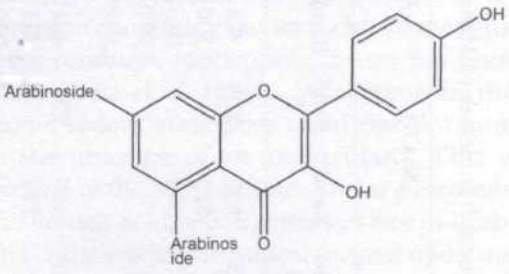
Flavonoids that have been revealed in *Rubiaceae* family in previous study were rutin and kaempferol 3-*O*-rutinoside from the leaves of *Randia formosa* (Sahpaz *et al.* 2000). Cimanga *et al.* (1995), identified quercetin, quercetin 7,4'-*O*-dimethyl ether, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, quercetin 3-*O*-rhamnoside, kaempferol 3-*O*-rhamnoside, quercetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside and chrysoeriol neohesperidoside from the leaves of *Morinda* morindoides. From a similar study, Siti Juhaida (2000) found quercetin 3-*O*-rhamnoside, kaempferol 3-*O*-rutinoside, myricetin 3-*O*-galactoside from the leaves of *Morinda citrifolia*. The difference between the results of previous studies and this study is the flavonoid's rate of syhthesis and acumulation. There were many factors that contribute to both processes such as light, pH and soil's salinity (Onyilagha *et al.* 2003). The condition of the plants played a major role too, as plants infected with any kind of disease will tend to synthesize flavonoids in higher concentration as a mechanism of protection. (Manthey *et al.* 2000). According to Manach *et al.* (1996) the concentration of flavonoids varies for different plant organs.

Quercetin 3,7-*O*-dimethyl ether has been found before in the leaves and trichome of *Nicotiana attenuata* (wild tabacco) (Roda *et al.* 2003). Quercetin 3-*O*-methyl ether has been found in the fruits of *Rhamnus disperma* (Marzouk *et al.* 1999), stem and fruit of *Optunia ficus-indica* var. saboten (Go *et al.* 2003). To the best fo our knowledge, all the five flavonoid components isolated from the leaves *of M. citrifolia* in this study are pioneer findings for the genus *Morinda*.

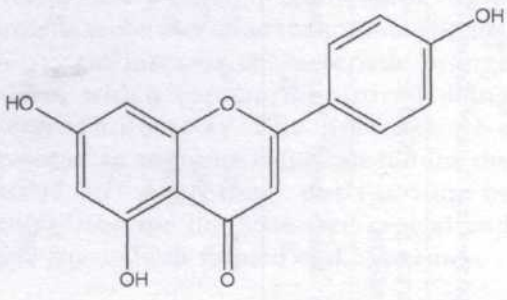
Structure of Isolated Flavonoid from Morinda citrifolia



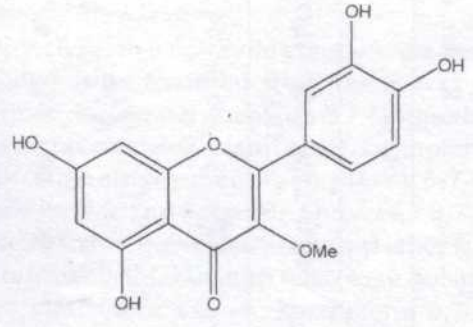
Qu 3,7-*O*-dimethyl ether



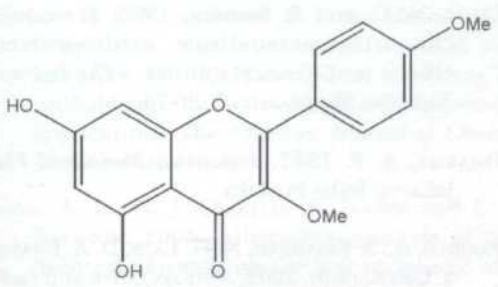
Km 5,7-*O*-diarabinoside



Apigenin



Qu 3-*O*-methyl ether



Km 3,4'-*O*-dimethyl ether

emulsion containing linoleic acid and flavonoid methylated antioxidant properties with time. The correlations indicated decreasing antioxidant activity during the reaction for all flavonoid components isolated from *Morinda citrifolia* leaves (*Fig. 1*). For the purpose of comparison, the 40th minute was selected and all antioxidant activities of the samples and standards for that time were compared (*Fig. 2*). Unfortunately, antioxidant

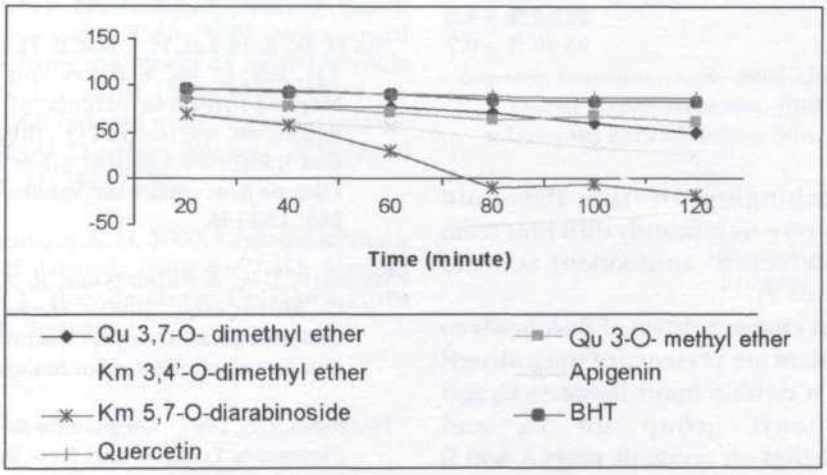


Fig. 1: Flavonoid components' antioxidant activities during 120 minutes

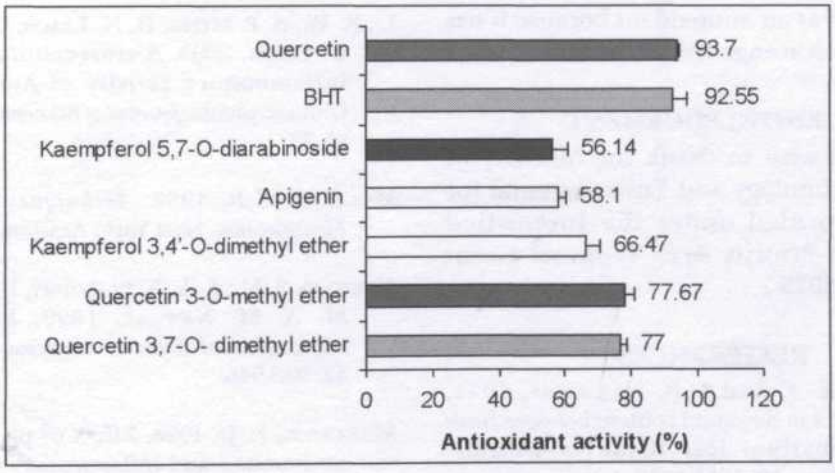


Fig. 2: Comparison of flavonoid antioxidant activities at 40 minutes

Antioxidant Activities

Based on the antioxidant activities formula, there were inverse correlations between the oxidative degradation of β-carotene in an

properties of all the samples were lower than the standards; quercetin (93.7%) and BHT (92.55%). Based on the statistical test comparing all the mean values, all antioxidant

TABLE 2
Differences of antioxidant properties values between samples and standards

Samples	Antioxidant properties values (%)
Quercetin 3,7-*O*-dimethyl ether	77.00 % ± 1.4[a]
Quercetin 3-*O*-methyl ether	77.67 % ± 3.4[a]
Kaempferol 3,4'-*O*-dimethyl ether	66.47 % ± 4.2[a]
Kaempferol 5,7-*O*-diarabinoside	58.10 % ± 2.5[a]
Apigenin	56.14 % ± 4.6[a]
BHT	92.55 % ± 4.0
Quercetin	93.70 % ± 0.7

a: $p<0.05$

activities belonging to the flavonoid components were significantly different from BHT and quercetins' antioxidant activites ($p<0.05$) (Table 2).

The main characteristics of flavonoids to be an antioxidant are presence of the hydroxyl group at C_3, a double bond between C_2 and C_3, a carbonyl group on C_4 and polyhydroxylation on aromatic rings A and B (Cook and Samman 1996). A study by Go *et al.* (2003) showed that quercetin 3-methyl ether can act as an antioxidant because it has the ability to scavenge free radicals.

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REFFERENCES

BATE-SMITH, E. C and J. B. HARBORNE. 1971. Differences in flavonoid content between fresh and herbarium leaf tissue in *Dillenia*. *Phytochemistry* **10**: 1055-1058.

CIMANGA, K., T. DE BRUYNE, A. LASURE, Q. LI, L. PIETERS, M. CLAEYS, D.V. BERGHE, K. KAMBU, L. TONA and A. VLIETINCK. 1995. Flavonoid *O*-glycosides from the leaves of *Morinda morindoides*. *Phytochemistry* **38**: 1301-1303.

COOK, N. C. and S. SAMMAN. 1996. Flavonoid – chemistry, metabolisme, cardioprotective effects and dietary sources. *The Journal of Nutrition Biochemistry* **7**: 66-76.

DHARMA, A. P. 1987. *Indonesia Medicinal Plant*. Jakarta: Balai Pustaka.

FARIDAH, A., S. KHOZIRAH, N. H. LAJIS, D. A. ISRAF and Y. UMI KALSOM. 2003. Antioxidative and radical scavenging properties of the constituents isolated from *Cosmos caudatus* Kunth. *Natural Product Sciences* **9:** 245-248

GO. H. D., K. H. LEE, H. J. KIM, E. H. LEE, Y. S. SONG, Y.H. LEE, C. JIN, Y. S. LEE and J. CHO. 2003. Neuroprotective effects of antioxidant flavonoids, quercetin, (+) - dihydroquercetin and quercetin-3-methyl ather isolated from *Optunia ficus - indica* var. *saboten*. *Brain Research* **965**: 130-136.

JAGANATH, I. B., S. MOHD NASIR, R. A. RAHMAN and C. MUTHUVELU. 2000. *Herba Berpotensi di Malaysia*. Kuala Lumpur: Institut Penyelidikan dan Kemajuan Pertanian Malaysia.

HARBORNE, J. B. 1967. *Comparative Biochemistry of the Flavonoids*. London and New York: Academic Press.

LI, R. W., S. P. MYERS, D. N. LEACH, G. D. LIN and G. LEACH. 2003. A cross-cultural study: anti-inflammatory activity of Australian and Chinese plants. *Journal of Ethnopharmacology* **85**: 25-32.

MARKHAM, K.R. 1982. *Techniques of Flavonoid Identification*. New York: Academic Press.

MARZOUK, S. M., S. A. A. EL-TOUMY, I. MERFORT and M. A. M. NAWWAR. 1999. Polyphenolic metabolites of *Rhamnus disperma*. *Phytochemistry* **52**: 943-946.

MIDDLETON, E. Jr. 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Adv. Exp. Med. Biol.* **439:** 175–182.

MOHD ZIN, Z., A. ABDUL-HAMID and A. OSMAN. 2001. Antioxidative activity of extracts from Mengkudu (*Morinda citrifolia* L.) root, fruit and leaf. *Food Chemistry* **78:** 227-231.

NIJVELDT R. J., E. VAN NOOD, D. F. VAN HOORN, P. G. BOELENS, K. VAN NORREN and P. A. M VAN LEEUWEN. 2001. Flavonoids: a review of probable mechanisms of action and potential applications *The American Journal of Clinical Nutrition* **74:** 418-425.

RODA, A. L., N. J. OLDHAM, A. SVATOS and I. T. BALDWIN. 2003. Allometric analysis of the induced flavonols on the leaf surface of wild tabacco (*Nicotiana attenuata*). *Phytochemistry* **62**: 527-536.

SANG, S., G. LIU, K. HE, N. ZHU, Z. DONG, Q. ZHENG, R. T. ROSEN and C. T. HO. 2003. New unusual iridoids from the leaves of noni (*Morinda citrifolia* L.) show inhibitory effect on ultraviolet B-induced transcriptional activator protein-1 (AP-1) activity. *Bioorganic & Medicinal Chemistry* **11**: 2499-2502.

SITI NOR JUHAIDA, S. A. H. 2000. Ekstraksi sebatian flavonoid daripada daun dan buah *Morinda citrifolia* L. Bac. Sc. Tesis, Universiti Putra Malaysia, Selangor.

THOMSON, R. H. 1987. *Naturally Occurring Quinones III. Recent Advances*. London: Chapman & Hall.

VELIGLOU, Y. S., G. MAZZA., L. GAO and B. D. OOMAH. 1998. Antioxidant activity and total phenolics in selected fruits, vegetable and grain products. *Journal Agricultural Food Chemistry* **46**: 4113-4117.

WANG, H. and K. HELLIWELL. 2001. Determination of flavonols in green and black tea leaves and green tea infusions by high-performance liquid chromatography. *Food Research International* **34:** 223-227.

ZAINATUL SHUHAIDA, A. R. 2003. *Herba dan Ubatan Tradisi Zaman-berzaman*. **Jilid 1:** 64-65. Kuala Lumpur: Zebra Editions Sdn. Bhd.

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Morphometric Analysis of Malaysian Oxudercine Goby, *Boleophthalmus boddarti* (Pallas, 1770)

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Keywords: Morphometric, *Boleophthalmus boddarti*, Principal Component Analysis (PCA), gobies, mudskipper

ABSTRAK

Ikan gobi Oxudercine dikenali sebagai ikan belacak atau tembakul. Ikan eurihalin ini merupakan antara ikan belacak di Malaysia yang boleh bernafas di udara. Boleophthalmus boddarti merupakan satu daripada ikan belacak yang biasa terdapat di kawasan pamah pasang surut, paya bakau, kuala sungai dan pesisir pantai. Data morfometrik biasa, morfometrik truss dan meristik yang diperolehi daripada 85 sampel lima populasi B. boddarti (Pulau Pinang, Kuala Selangor, Banting, Port Dickson dan Melaka) dianalisis dengan menggunakan kaedah statistik ANOVA sehala dan Analisis Komponen Prinsipal (PCA). Lima belas ciri morfometrik, 28 ciri morfometrik truss dan 9 ciri meristik dianalisis untuk menentukan darjah keserupaan antara kelima-lima populasi ini. Semua ciri morfometrik mempunyai perbezaan bererti ($P<0.05$) bagi dalam dan antara populasi. Berdasarkan analisis komponen prinsipal ke atas ciri morfometrik biasa, populasi B. boddarti dikelompokkan kepada 3 kumpulan, di mana populasi Banting dan Melaka berada dalam satu kumpulan, Pulau Pinang dan Kuala Selangor dalam kumpulan lain, manakala populasi Port Dickson dalam satu kumpulan lain yang berbeza dengan kumpulan lain. Berdasarkan kepada ciri morfometrik truss, tiga kumpulan juga dihasilkan tetapi kelompoknya adalah berbeza di mana populasi Kuala Selangor dan Banting dalam satu kumpulan, Port Dickson dengan Melaka dalam satu kumpulan lain, manakala Pulau Pinang pula jauh berbeza dengan kumpulan-kumpulan lain. Keputusan kajian ini menunjukkan bentuk badan ikan belacak di kawasan utara sangat berbeza dengan yang terdapat di kawasan tengah dan selatan Semenanjung Malaysia. Berdasarkan kepada ciri mersitik, semua populasi belacak adalah memusat dan tiada kelompok yang boleh dikenal pasti. Hubungan panjang-berat bagi B. boddarti dinyatakan sebagai: $\log W = \log 0.754 + 1.029 \log TL$.

ABSTRACT

The Oxudercine gobies or mudskippers are locally known as "belacak" or "tembakul". These euryhaline fish are amongst the air breathing gobies found in Malaysia. Boleophthalmus boddarti is one of the common mudskippers inhabiting tidal flats, mangrove swamps, estuarines and coastal areas. Conventional and truss morphometrics as well as meristic data from 85 samples in five populations (Pulau Pinang, Kuala Selangor, Banting, Port Dickson and Melaka) of B. boddarti were analyzed using one-way ANOVA and Principal Component Analysis (PCA). Fifteen morphometric, 28 truss morphometric and 9 meristic data were analyzed to examine the degree of similarity among the five populations. All morphometric characters within and between the populations were significantly different ($P<0.05$). Using the conventional morphometric data analysed by PCA, the populations of B. boddarti are clustered into 3 groups, where Banting and Melaka populations form the first group, Pulau Pinang and Kuala Selangor populations the second group and Port Dickson population the third group. In contrast, 3 groups were also clustered based on the truss morphometric data but the grouping was different. The three groups from the truss morphometric data consist of Kuala Selangor and Banting populations in the first group, Melaka and Port Dickson populations the second group and the Pulau Pinang population the third group which is distantly separated from the other two groups. The results indicate that the shape of mudskippers

in the northern part is distinct from the middle and southern parts of Peninsular Malaysia. Based on meristic data, all populations were centric and no grouping was identified. The length-weight relationship for B. boddarti in this study was described as log W = log 0.754 + 1.029 log TL.

INTRODUCTION

Oxudercine gobies are most notable among the amphibious air breathing gobies, consisting of at least 40 species (Murdy 1989). All oxudercine are at least occasional burrow dwellers and several genera are known as mudskippers or locally named as "Belacak" or "Tembakul". *Boleophthalmus boddarti* (Pallas, 1770) is one of the mudskippers that spend much of its time out of water. It is also known as Boddart's goggle-eye goby or blue spotted mudskipper. This species is widely distributed in the Indo-West Pacific, from India to New Guinea and north to China. *B. boddarti* is characterised by an elongated and laterally compressed body covered with cycloid scales. The head is slightly flat, warty skin and entirely covered with scales. The eyes are very close, movable to dorsal profile of head, and having well developed lower eyelids. The snout is blunt with slightly oblique mouth. There are two dorsal fins, fused pelvic fins, scaled base muscular pectoral fins, and an asymmetrical caudal fin where the upper half is slightly longer than the lower half. *B. boddarti* is marked by lighter colour of the first dorsal fin, the dark edge of the pectoral fins, large blue spots on the head and a number of darker dorso-ventrally stripes along the body.

A large number of *B. boddarti* inhabit brackish water of estuaries, mangrove swamps and intertidal mudflats in Malaysia. During low tides, *B. boddarti* are often aggregated at the water edge. During high tide, they hide themselves in the submerged burrows to avoid being attacked by predatory fish that forage for food on the mudflats (Takita *et al.* 1999). The submerged burrows made by this species have directly or indirectly increased the productivity of mangrove swamp and mudflat areas.

Morphological characters are commonly used in fisheries biology to measure discreteness and relationships among various taxa and have long been used to delineate stocks of fish. Morphometric characters are continuous characters describing the body shape, which have provided evidence for stock discreteness as documented by Corti *et al.* (1988) and Murta (2000). Meristic characters are the number of discrete serially countable structures, and often being used for species identification because they are considerably less affected by environmental changes. Morphometric and meristic analyses can thus be a first step in investigating the stock structure of species with a large sized population.

However, there is a major limitation in using morphological characters at the intra-specific level, in which phenotypic variation is not directly controlled by genetic factors but rather subject to environmental changes (Ihssen *et al.* 1981). However, both environmental and genetic components are now believed to contribute to phenotypic variation in organisms. To improve the use of morphometric analysis, truss morphometry has been developed especially for stock differention (Corti *et al.* 1988; Roby *et al.* 1991). Truss morphometry has proven to be more powerful in describing morphological variation between closely related fish taxa (e.g. stock) than conventional morphometry (Strauss and Bookstein 1982; Rohlf 1990). The size of a fish population is best estimated by length-weight relationship, which can also be used in determining allometric growth in a fish species. Thus, this study was carried out to examine morphological variation and length-weight relationships of *B. boddarti* in Peninsular Malaysia.

MATERIALS AND METHODS

A total of 85 samples of *B. boddarti* were collected from five different locations, namely Pulau Pinang, Kuala Selangor, Banting, Port Dickson and Melaka. Samples were collected

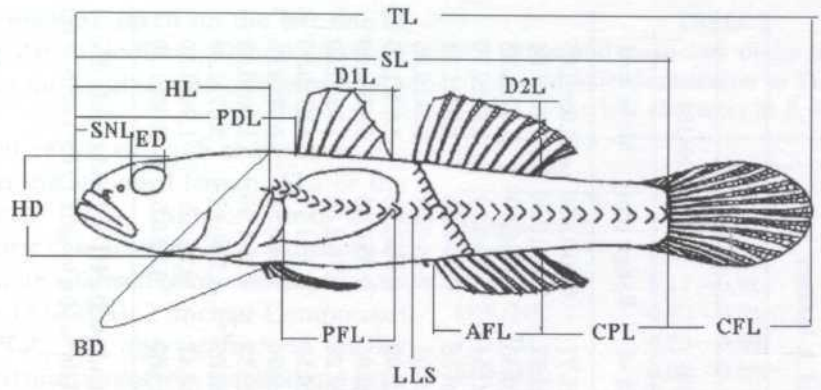

Fig. 1: Morphometric characters used for Boleophthalmus boddarti

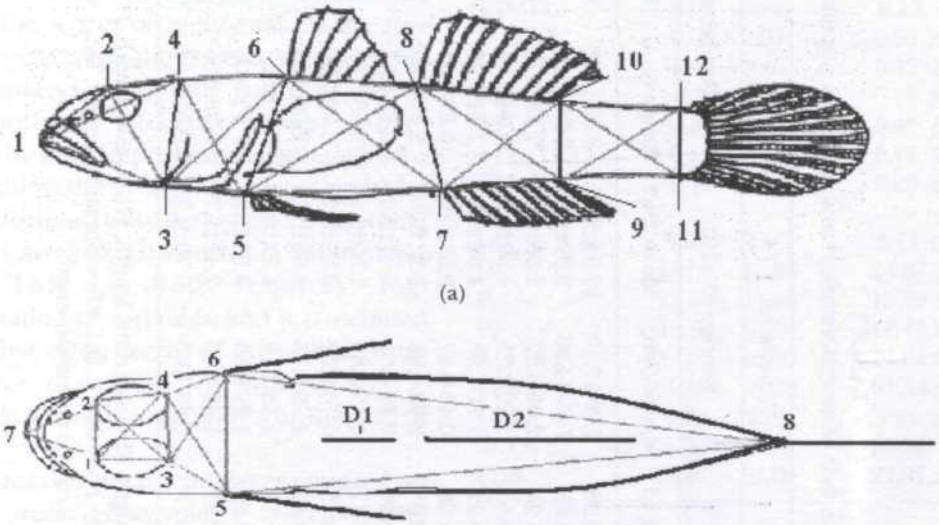

Fig. 2: The body landmarks used for the truss morphometric characters in B. boddarti*: (a) body section (b) head section*

by hook and line, casting net and traps as well as by hand and scope net. The measurements taken for mophometric (to the nearest 0.01 cm), and meristic studies follow the methods of Murdy (1989). Sexes were pooled for all analyses since the sexual dimorphism was unknown in this species.

Fifteen selected conventional morphometric characters were measured using vernier calipers for each sample (*Fig. 1*). The following morphometric characters were measured: total length (TL), standard length (SL), head length (HL), head width (HW), head depth (HD), snout length (SNL), predorsal length (PDL), eye diameter (ED), body depth (BD), first dorsal fin length (D_1L), second dorsal fin length (D_2L), pectoral fin length (PFL), anal fin length (AFL), caudal fin length (CFL), and caudal peduncle length (CPL) (*Fig. 1*). To reduce the allometric effects and make the results more comparable, each measurement was expressed as a ratio to the standard length or head length.

For truss morphometric data, 12 landmarks were chosen based on the methods described by Strauss and Bookstein (1982). All

TABLE 1
Range and mean ± standard deviation (sd) of morphometric characters in five populations of *B. boddarti*

MC	Population									
	P. Pinang (N=16)		K. Selangor (N=15)		Banting (N=20)		N. Sembilan (N=18)		Melaka (N=16)	
	Range (cm)	Mean ± sd	Range (cm)	Mean ± sd	Range (cm)	Mean ± sd	Range (cm)	Mean ± sd	Range (cm)	Mean ± sd
TL	8.80-12.90	10.53±1.07	7.71-11.50	9.61±0.96	9.00-17.10	12.04±2.47	8.50-14.50	10.75±1.77	9.00-18.50	13.05±2.85
SL	7.05-11.30	8.44±1.08	6.14-9.50	7.71±0.85	7.55-14.90	9.99±2.14	7.05-12.50	9.11±1.57	7.15-16.00	10.79±2.84
HL	1.78- 2.50	2.16±0.18	1.67-2.66	2.11±0.21	2.02-3.57	2.58±0.43	1.89-3.76	2.44±0.55	2.00-6.50	3.07±1.23
HW	1.08-1.75	1.32±0.16	0.99-1.68	1.19±0.51	1.20-2.25	1.59±0.70	1.04-2.66	1.52±0.51	1.20-3.10	1.93±0.70
HD	1.00-1.55	1.25±0.70	0.90-1.45	1.12±0.47	1.12-1.98	1.62±0.27	1.11-2.90	1.35±0.18	0.94-2.23	1.68±1.51
SNL	0.30-0.53	0.42±0.07	0.27-0.59	0.47±0.08	0.43-0.67	0.52±0.09	0.27-0.61	0.43±0.01	0.43-0.59	0.51±0.05
PDL	1.80-4.30	2.48±0.07	2.12-2.96	2.50±0.03	2.02-4.90	3.32±0.08	2.00-4.80	3.89±0.09	0.60-6.60	3.83±1.75
ED	0.27-0.49	0.37±0.06	0.32-0.47	0.39±0.04	0.33-0.61	0.47±0.09	0.32-0.57	0.39±0.06	0.35-0.63	0.44±0.09
BD	0.84-1.63	1.30±0.21	0.84-1.63	1.20±0.17	1.15-2.68	1.60±0.40	0.85-1.86	1.26±0.28	0.76-2.86	1.75±0.60
D_1L	0.61-1.47	1.09±0.21	0.58-1.02	0.99±0.13	0.76-2.02	0.89±0.29	0.73-1.96	1.11±0.34	0.75-1.74	1.21±0.29
D_2L	2.26-4.21	3.42±1.38	2.75-3.80	3.25±0.42	2.99-5.43	4.08±0.25	2.98-6.00	3.73±0.96	1.11-6.50	4.07±0.96
PFL	1.40-1.98	1.59±0.19	1.45-1.89	1.67±0.14	1.42-2.10	1.72±0.23	0.70-2.17	1.33±0.37	1.30-1.95	1.56±0.22
AFL	2.60-4.22	3.42±0.37	2.43-3.90	3.08±0.39	1.92-5.66	3.03±1.20	1.92-5.06	3.10±0.09	1.78-6.90	3.87±1.39
CFL	0.39-2.30	1.35±0.55	1.38-2.24	1.80±0.21	1.33-6.50	2.41±1.79	0.38-2.05	1.31±0.47	0.38-6.5	1.98±1.50
CPL	0.50-2.20	0.90±0.65	0.41-1.02	0.66±0.16	0.48-1.06	0.79±0.02	0.55-2.50	1.64±0.65	0.82-6.5	2.80±1.39

Abbreviations: MC = Morphometric character, TL = total length, SL = standard length, HL = head length, HW = head width, HD = head depth, SNL = snout length, PDL = predorsal length, ED = eye diameter, BD = body depth, D_1L = first dorsal fin length, D_2L = second dorsal fin length, PFL = pectoral fin length, AFL = anal fin length, CFL = caudal fin length, CPL = caudal peduncle length.

measurements were taken on the left side of the fish (*Fig. 2a*). In addition, 8 landmarks were also taken in the head section as shown in *Fig. 2b.*

Data on ratios of each morphometric character to the standard length (SL) or the head length (HL), meristic and truss morphometric characters in all populations of *B. boddarti* were analysed using one-way analysis of variance (ANOVA). Principal Component Analysis (PCA) was also performed on the conventional morphometric, meristic and truss morphometric data. The mean values (centroids) and 95% asymptotic confidence limits of the scores of individual on the first two principal components were computed for each sample to classify the fish into one of several mutually exclusive groups and to establish the most important characteristics for distinguishing the groups. The truss variables (log-transformed) were corrected for size using Burnaby's method (Darroch and Mosimann 1985). This method requires log-transformation of variables, and it is assumed that the first eigen vector of the within-group covariance matrix of log-morphometric variables is a multivariate index of the size of fish

Nine meristic characters were counted on fresh and preserved samples. These characters include the numbers of first dorsal fin rays (D_1), second dorsal fin rays (D_2), pectoral fin rays (P_1), pelvic fin rays (P_2), anal fin rays (A), lateral line scales (LLS), upper lateral line scales (ULLS), lower lateral line scales (LLLS), and predorsal scales (PDS).

Linear regression analysis was also performed to describe length-weight relationship of *B. boddarti* in Malaysia using logarithmic transformation. The relationship is expressed as: $\log W = \log a + b \log L$, where W is the weight (g), L is the total length (cm), a is the intercept of the regression curve and b is the regression coefficient (slope). The statistical significance of the regression was assessed using analysis of variance (ANOVA).

TABLE 2
Range and mean ± sd of the ratios of each morphometric character to TL or HL and meristic character in *B. boddarti*

Characters	Range	Mean ± sd
Morphometric		
SL/TL	0.79 - 0.90	0.83 ±0.026
HL/TL	0.17 - 0.42	0.22 ±0.033
HW/HL	0.49 - 0.85	0.62 ±0.067
HD/HL	0.23 - 0.89	0.58 ±0.099
SNL/HL	0.09 - 0.28	0.20 ±0.040
ED/HL	0.08 - 0.26	0.17 ±0 .037
PDL/TL	0.07 - 0.29	0.12 ±0.038
BD/TL	0.05 - 0.16	0.13 ±0.017
D_1L/TL	0.08 - 0.09	0.09 ±0.023
D_2L/TL	0.40 - 0.46	0.42 ±0.014
PFL/TL	0.10 - 0.22	0.16 ±0.024
AFL/TL	0.38 - 0.47	0.42 ±0.024
CFL/TL	0.11 - 0.21	0.17 ±0.025
CPL/TL	0.12 - 0.24	0.20 ±0.020
Meristic		
D_1	5.00 – 7.00	5.11 ±0.420
D_2	23.00 – 27.00	24.62 ±0.816
P_1	15.00 – 20.00	18.39 ±0.914
P_2	15.00 – 19.00	18.42 ±0.971
A	24.00 – 26.00	24.61 ±0.709
LLS	59.00 – 78.00	67.54 ±4.314
ULLS	6.00 – 10.00	7.66 ±0.919
BLLS	8.00 – 13.00	10.54–± 0.814
PDS	25.00 – 35.00	27.91 ±2.831

Abbreviations: TL = total length, SL = standard length, HL = head length, HW = head width, HD = head depth, SNL = snout length, PDL = predorsal length, ED = eye diameter, BD = body depth, D_1L = first dorsal fin length, D_2L = second dorsal fin length, PFL = pectoral fin length, AFL = anal fin length, CFL = caudal fin length, CPL = caudal peduncle length, D_1 = first dorsal fin ray, D_2 = second dorsal fin ray, P_1 = pectoral fin ray, P_2 = pelvic fin ray, A = anal fin ray, LLS = lateral line scale, ULLS = upper lateral line scale, BLLS = below lateral line scale, PDS = predorsal scale.

RESULTS

Morphometric

The range and mean ± standard deviation values of morphometric characters for *B. boddarti* are presented in Table 1. The total

TABLE 3
Summary of one -way ANOVA for each ratio of morphometric data to the TL in *B. boddarti*

Variable	Between populations		Within population	
	F value	P	F value	P
SL/TL	7.101	.000**	2.476	.067
HL/TL	1.845	.128 ns	.237	.870
HW/HL	2.934	.026*	1.747	.164
HD/HL	3.115	.020*	4.148	.009*
SNL/HL	3.032	.022*	1.943	.129
ED/HL	2.091	.090 ns	1.464	.231
PDL/TL	.658	.623 ns	.827	.429
BD/TL	2.024	.099 ns	2.624	.056
D_1/TL	3.548	.010*	3.237	.026*
D_2/TL	2.067	.093 ns	2.447	.068
PFL/TL	4.611	.002**	3.567	.018*
AFL/TL	3.715	.008*	3.688	.015*
CFL /TL	5.800	.000**	2.486	.067
CPL/TL	1.061	.381ns	.851	.470

ns= not significant (P>0.05); * significant at p < 0.05; ** highly significant at p < 0 .005

length of the 85 samples of *B. boddarti* ranged from 7.71 to 18.5 cm with a mean of 11.268 ± 4.214 cm and the standard length ranged from 6.14 to 16.0 cm with a mean of 9.327 cm. The weight ranged from 41 to 82 g, with a mean of 54.28 ± 9.461 g. The biggest individual was found in the Melaka population whilst the smallest was from Kuala Selangor population. The ratios of each morphometric character to TL or HL for *B. boddarti* are shown in Table 2. The standard length (SL) is about 80-90% of the total length (TL). The body depth (BD) was about 5-16% of the TL.

The ANOVA showed that the ratios of standard length (SL), pectoral fin length (PFL) and caudal length (CFL) to TL showed highly significant differences (P<0.005) among the populations. The ratios of head width (HW), head depth (HD), snout length (SNL) to the head length (HL), and the ratios of first dorsal fin length (D_1L) and anal fin length (AFL) to TL were also significantly different (P<0.005) among the populations.

Head length (HL), predorsal length (PDL), body depth (BD), second dorsal fin length (D_2L) and caudal fin length (CFL) to TL, and the ratio of eye diameter (ED) to HL were not significantly different among the populations of *B. boddarti.*

The values of the first four principal components performed on the 15 raw morphometric data and weight of *B. boddarti* are presented in Table 4. The positive and negative values indicate shape variation. The negative value was not considered a good discriminant as shown by predorsal length (-0.177) in the first component. The total correct classification rate was 80.43% which is considered a good discrimination. The component loadings (Table 4) were also not very high for most of the variables accounted for by the first principal component, which described 54.43% of the cumulative variance within the samples.

Based on Principal Component Analysis (PCA) on morphometric data, the populations of *B. boddarti* are clustered into 3 groups, wherein Banting and Melaka populations are clustered in one group, Pulau Pinang and Kuala Selangor populations in another group, and both groups are well separated from the Port Dickson population (*Fig. 3*).

TABLE 4
Values of the first four components obtained through a PCA performed on raw morphometric data of *B. boddarti*

Morphometric character (cm)	Component			
	1	2	3	4
Weight (g)	.835	.199	-.066	.299
TL	.957	-.055	.039	.046
SL	.959	-.149	.004	.007
HL	.713	-.107	.563	.059
HW	.877	-.213	.202	-.196
HD	.907	-.030	-.115	-.204
SNL	.550	.364	-.060	.594
PDL	-.177	.388	-.533	.002
ED	.514	.489	.382	-.140
BD	.907	-.027	-.188	.062
D_1L	.539	-.277	.331	-.487
D_2L	.778	-.001	-.464	-.165
PFL	.483	.628	.155	-.063
AFL	.918	-.108	-.199	.072
CFL	.498	.512	-.079	-.211
CPL	.564	-.526	.460	.423
Eigen value	8.710	1.658	1.048	1.453
Variance explained (%)	54.435	10.365	9.081	6.549
Cumulative variance (%)	54.435	64.800	73.881	80.430

Truss Morphometry

Of 28 truss morphometric characters, only 5 did not differ significantly ($P<0.05$) among the five populations of *B. boddarti* as shown in Table 5. Highly significant differences ($P<0.005$) of truss morphometric characters among the populations were mostly found in the head region.

Three components were extracted from the 28 truss morphometric data (Table 6). The first component accounted for 68.6% of the total variance. The component loadings were also higher (81.6%) than those of morphometric and meristic characters. Based on these data, populations of *B. boddarti* were also clustered into 3 groups although the grouping is slightly different from that of the conventional morphometric data. In this grouping, Kuala Selangor and Banting populations are in the first group, while Port Dickson and Melaka populations in the second group, and the Pulau Pinang population by itself is distantly isolated from the other two groups (*Fig. 4*).

Meristic

These five populations were significantly different ($P<0.05$) in their meristic characters except for the second dorsal fin ray count (D_2) (Table 7).

Three principal components were extracted from 7 meristic data. The component loadings were not very high for most variables in the first component (26.9%) of the total variance within the samples (Table 8). As expected there was an overlap among Pulau Pinang, Kuala Selangor and Banting populations as shown in *Fig. 5*.

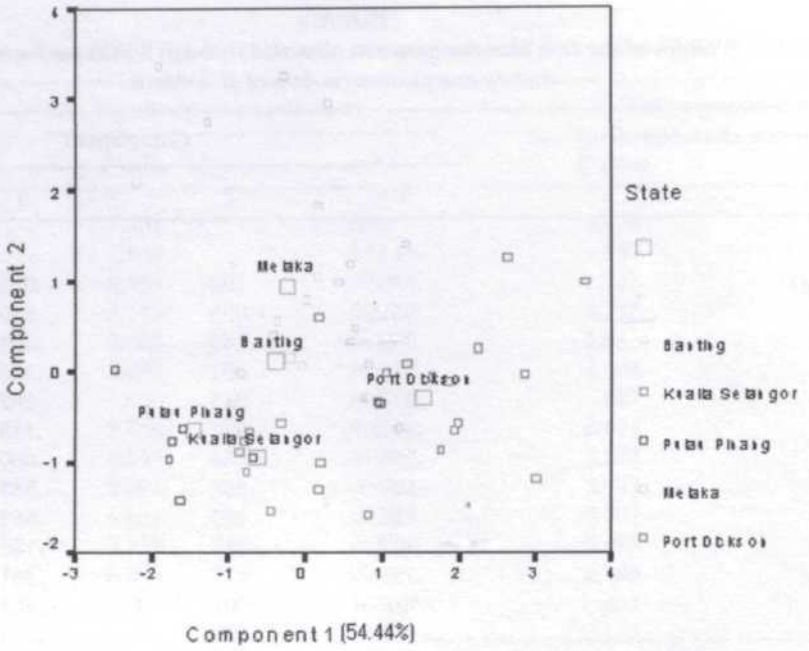

Fig. 3: Plots of the coordinates of individuals of B. boddarti *according to the first two discriminant functions, obtained from morphometric data*

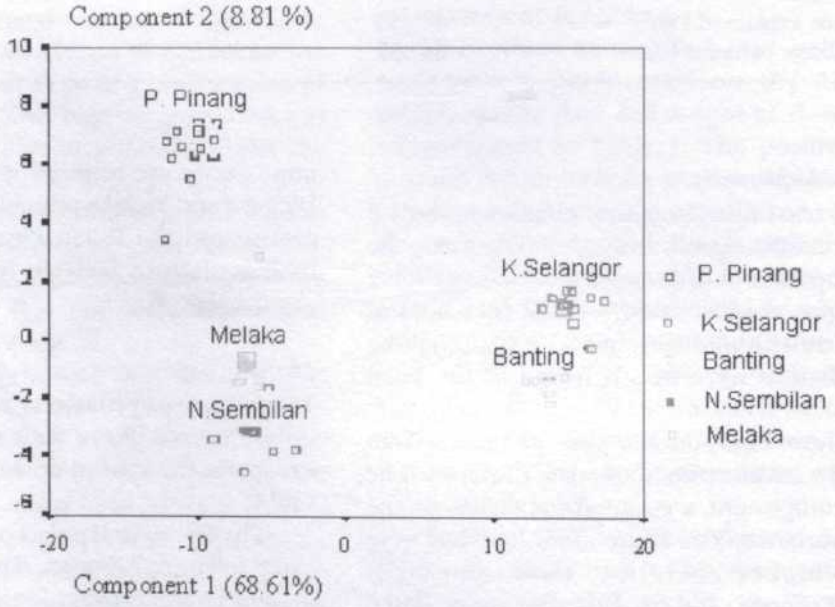

Fig. 4: Plots of the coordinates of individuals of B. boddarti *according to the first two discriminant functions, obtained from truss morphometric data*

TABLE 5
Summary of One -Way ANOVA for each truss character in *B. boddarti* populations. The variables are referred to *Fig. 2a*

Variable	Between populations		Within population	
	F value	P	F value	P
B1 to 2	5.823	.001**	21.008	.000**
B2 to 4	10.646	.000**	15.956	.000**
B2 to 6	78.984	.000**	30.682	.000**
B1 to 4	7.532	.000**	19.287	.000**
B1 to 6	4.871	.002**	10.949	.002**
B6 to 5	6.045	.000**	14.745	.000**
B4 to 5	2.178	.084 ns	.029	.866
B6 to 8	5.165	.001**	9.009	.003**
B8 to 7	2.048	.100 ns	2.551	.116
B7 to 5	3.093	.023*	4.659	.035*
B6 to 7	4.882	.002*	12.095	.001**
B5 to 8	6.957	.000**	19.923	.000**
B8 to 10	1.128	.351ns	.816	.370
B10 to 9	4.289	.004**	10.890	.002**
B9 to 7	2.803	.034*	6.747	.012*
B8 to 9	2.898	.030*	6.482	.014*
B7 to 10	3.455	.014*	8.776	.001**
H7 to 2	7.808	.000**	13.165	.001**
H7 to 1	5.409	.001**	8.883	.004**
H1 to 2	6.512	.000**	22.437	.000**
H2 to 4	2.979	.027*	7.652	.008*
H4 to 3	4.582	.027*	9.278	.004**
H3 to 1	2.913	.029*	7.675	.008*
H2 to 3	1.650	.031*	3.909	.053
H1 to 4	1.386	.251ns	4.613	.036*
H4 to 6	1.692	.165ns	2.154	.104
H6 to 8	3.210	.019*	8.051	.006*
H5 to 8	4.540	.003**	11.369	.001**

ns = not significant (P>0.05); * significant at $p < 0.05$; ** highly significant at $p < 0$ .005

TABLE 6
Values of the first three components obtained through a PCA performed on raw 26 truss morphometric data of *B. boddarti*

Truss character	Component		
	1	2	3
B1 to 2	865	-.161	.110
B2 to 4	.901	-.223	.054
B2 to 6	.962	-.126	-.077
B1TO4	.956	-.171	.023
B1TO6	.822	.433	-.065
B6TO5	.836	-.121	-.034
B1TO5	.460	-.072	.761
B4TO5	.903	.165	-.181
B6TO8	.804	.049	.181
B8TO7	.968	-.074	.013
B7TO5	.932	-.200	.069
B6TO7	.869	.306	-.255
B5TO8	.936	.052	-.166
B8TO10	.953	.062	-.097
B10TO9	.903	-.154	-.118
B9TO7	.678	.004	-.005
B8TO9	.827	-.271	.016
B7TO10	.863	-.267	-.134
H7TO2	.667	-.272	-.040
H7TO1	.753	.512	-.067
H1TO2	.727	.440	.341
H2TO4	.621	.680	-.024
H3TO1	.387	.602	.289
H2TO3	.930	-.042	-.131
H4TO6	.962	-.106	-.036
H6TO5	.688	-.507	.221
Eigen value	17.839	2.299	1.085
Variance explained (%)	68.610	8.841	4.173
Cumulative variance (%)	68.610	77.45	81.624

TABLE 7
Summary of One-Way ANOVA for each meristic character in *B. boddarti*

Variable	Between population		Within population	
	F value	P	F value	P
D_1	3.390	.013*	4.391	0.007*
D_2	1.655	169 ns	2.193	0.095ns
P_1	4.490	.037*	4.490	0.037*
P_2	4.304	.003**	4.192	0.008*
A	7.482	.000**	9.975	0.000**
LLS	6.084	.000**	8.045	0.000**
ULLS	2.834	.030*	3.503	0.019*
BLLS	3.082	.021*	3.352	0.023*
PDS	6.876	.000**	8.880	0.003**

ns = not significant (P>0.05); *significant at p < 0.05; ** highly significant at p < 0 .005

TABLE 8
Values of the first three components obtained through a PCA performed on 7 raw meristic data of *B. boddarti*

Meristic character	Component		
	1	2	3
D_1	.720	- .513	.202
D_2	.548	.480	.533
A	.447	.480	- .448
P_1	- .262	.077	.696
P_2	.665	- .624	.115
LLS	- .125	.210	.639
PDS	.583	.605	- .088
Eigen value	1.885	1.528	1.439
Variance explained (%)	26.925	21.835	20.560
Cumulative variance (%)	26.925	48.760	69.320

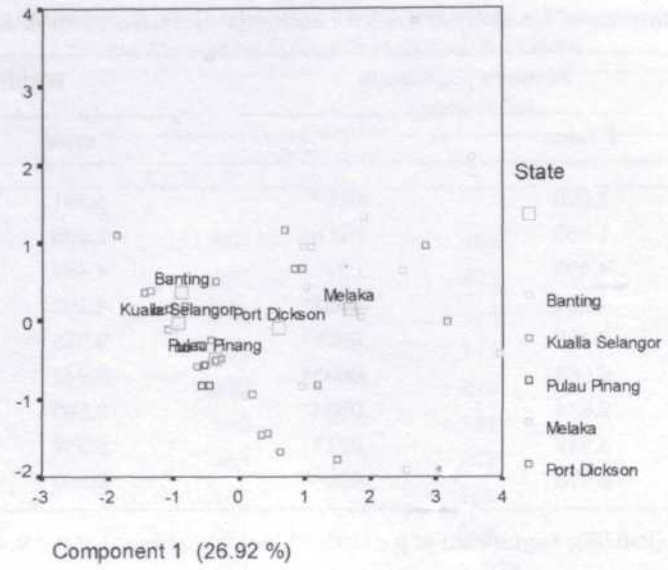

Fig. 5: Plots of the coordinates of individuals of B. boddarti *according to the first two discriminant functions, obtained from meristic data*

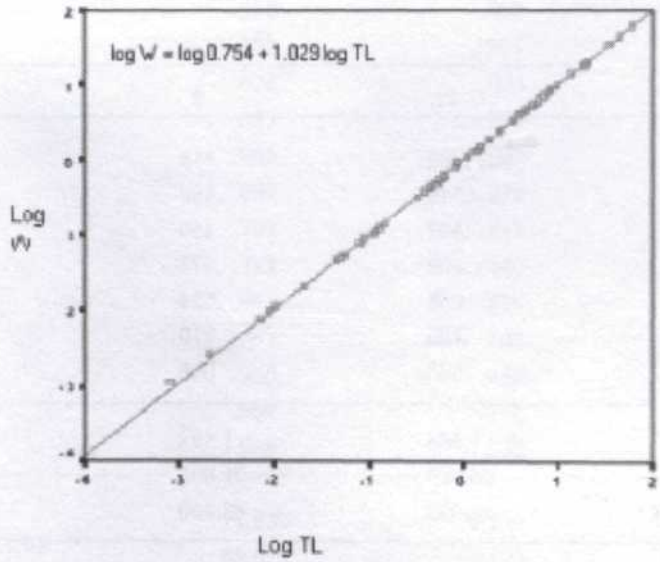

Fig. 6: Length-weight relationship for B. boddarti *populations in Peninsular Malaysia (R^2 = 0.937; n = 85)*

Length-weight Relationship

The length-weight relationship for *B. boddarti* in this study is described as: log W = log 0.754 + 1.029 log TL and the regression curve is presented in *Fig. 6*. The linear regression between total length and weight for *B. boddarti* was highly significant (P<0.005) with a R^2 value of 0.937.

DISCUSSION

Some morphometric and meristic characters of *B. boddarti* have been recorded by Murdy (1989) based on samples from India, Indonesia, Thailand and Malaysia. In this study, more comprehensive conventional morphometric, truss morphometric and meristic data were collected and analysed to determine the most appropriate method to be used for handy fish stock identification and assessment.

The size of *B. boddarti* varies from one population to another. Of the five populations, the Melaka population had the biggest sized individuals ranging from 9.0 to 18.5 cm with a mean of 13.05 cm in TL, whilst the population of Kuala Selangor comprised the smallest sized individuals, ranging from 7.7 to 11.5 cm with a mean of 9.61 cm in total length. Various factors may be responsible for the differences such as food availability, environmental conditions, stage of maturity and seasonal changes, such as dry and rainy seasons.

The results of ANOVA and PCA are complementary to each other as shown by the results of the predorsal length of *B. boddarti*. The ANOVA showed that the PDL/TL was not significantly different ($P>0.05$) in the populations, and similarly for PCA, the predorsal length value was negative, suggesting that this character was not a good criterion to discriminate populations of *B. boddarti*.

The first component coefficient of morphometric data had positive and negative values, indicating shape variation. Based on PCA, the populations of *B. boddarti* are clustered into 3 groups (*Fig. 3*). Although the populations of *B. boddarti* were also clustered into 3 groups based on truss morphometric data, the grouping was different from that for the conventional morphometric data. The grouping based on truss morphometric data was more reasonable and meaningful as the geographically closer populations were grouped together. The results support the hypothesis that the shape of *B. boddarti* in the northern part is distinct from the middle and the southern parts of Peninsular Malaysia. These differences could be based on physical characteristics of each habitat, such as water temperature and currents (McElroy and Douglas 1995).

The first component of meristic characters only consists of 26.5% of the cumulative variance which is not good enough for stock identification (Doherty and McCarthy 2004). All populations were centric and no grouping was identified. Thus the populations of *B. boddarti* could not be differentiated using meristic characters.

In the length-weight relationship study, the estimated value of b was less than 3 ($b = 1.029$), indicating that allometric negative growth occurred in *B. boddarti*. In general, the exponent b from length-weight regression equation in fish and other invertebrates often lies between 2.5 and 3.5, and usually close to 3 for symmetrical or isometric growth (Gonzales *et al.* 2000; Atar and Secer 2003). In contrast, the length-weight relationship recorded for *B. boddarti* is $\log W = \log 0.0156 + 3.0 \log SL$ (*http://www.fishbase.org*).

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REFERENCES

ATAR, H. H. and S. SECER. 2003. Width/length-weight relationships of the blue crab (*Callinectes sapidus* Rathbun 1896) population living in Beymelek lagoon lake. *Turk. J. Vet. Anim. Sci.* **27**: 443-447.

CORTI, M., R. S. THORPE. L. SOLA. V. SBORDONI and S. CATAUDELLA. 1988. Multivariate morphometric in aquaculture: A case study of six stocks of the common carp (*Cyprinus carpio*) from Italy. *Can. J. Fish. Aquat. Sci.* **45**: 1548-1554.

DARROCH, J. N. and J. E. MOSIMANN. 1985. Canonical and principal components of shape. *Biometrika* **72**: 241-252.

DOHERTY, D. and T. K. MCCARTHY. 2004. Morphometric and meristic characteristics analysis of two western Irish population of Arctic Char, *Sivelinus alpinus*. In *Royal Irish Academy Biology and Environment*, **1**: 75-85. Royal Irish Academy.

GONZALES, B. J., H. P. PALLA and H. MISHINA. 2000. Length-weight relationship of five serranids from Palawan Island, Philippines. *Naga, The ICLARM Quarterly* **23(3)**: 26-28.

IHSSEN, P. E., H. E. BOOKE, J. M. MCGLADE, N. R. PAYNE and F. M. UTTER. 1981. Stock identification. *Can. J. Fish. Aquat. Sci.* **38**: 1838–1855.

JOHNSON, R. A. and D. W. WICHERN. 1998. *Applied Multivariate Statistics*. 4th ed. New Jersey: Prentice Hall.

MCELROY, D. M. and M. C. DOUGLAS. 1995. Patterns of morphological variation among endangered populations of *Gila robusta* and *Gila cypha* (Teleostei: *Cyprinidae*) in the upper Colorado River basin. *Copeia* **3**: 636–649.

MURDY, E. O. 1989. A taxonomic revision and cladistic analysis of the Oxudercine gobies (Gobiidae: Oxudercinae). *Res. Australian Mus.Suppl.* **11**: 1-93.

MURTA, A. G. 2000. Morphological variation of horse mackerel (*Trachurus trachurus*) in the Iberian and North African Atlantic: Implications for stock identification. *ICES Journal of Marine Science* **57**: 1240–1248.

ROBY, D., J. D. LAMBERT and J. M. SEVIGNY. 1991. Morphometric and electrophoretic approaches to discrimination of capelin (*Mallotus villosus*) populations in the estuary and Gulf of St. Lawrence. *Can. J. Fish. Aquat. Sci.* **48**: 2040-2050.

ROHLF, F. J. 1990. Morphometrics. *Ann. Rev. Ecol. Syst.* **21**: 299–316.

SILVA, A. 2003. Morphometric variation among sardine (*Sardina pilchardus*) populations from the northeastern Atlantic and the western Mediterranean *ICES Journal of Marine Science* **60(6)**: 1352-1360.

STRAUSS, R. E. and C. E. BOND. 1990. Taxonomic methods: Morphology. In *Methods for Fish Biology*, ed. C. B. Schreck and P. B. Moyle, p. 109-140. Maryland: American Fisheries Society.

STRAUSS, R. E. and F. L. BOOKSTEIN. 1982. The truss: Body form reconstruction in morphometrics. *Syst. Zool.* **31**: 113–135.

TAKITA, T., AGUSNIMAR and A. B. ALI. 1999. Distribution and habitat requirement of Oxudercine gobies(Gobiidae: Oxudercinae) along the Strait of Malacca.–*Ichtyol. Res.* **46 (2)**: 131-138.

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Orchids of Perlis: New Records and Distribution

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ABSTRAK

Satu kajian diversiti orkid secara intensif telah dijalankan di Perlis terutamanya di dalam kawasan Taman Negeri Perlis dari tahun 2003 hingga 2004. Melalui banyak kerja lapangan yang dilaksanakan, sebanyak 1783 sampel orkid telah berjaya dikumpulkan dari 12 bukit (11 bukit batu kapur dan satu gunung separa batu kapur dan granit). Sampel ini telah dikenal pasti kepada 119 spesies dalam 50 genera yang diwakili oleh 4 subfamili. Daripada jumlah ini, 62 spesies dalam 20 genera adalah rekod baru untuk Perlis and 9 spesies dan satu genus Panisea merupakan rekod baru untuk Malaysia. Diversiti orkid di Perlis menunjukkan pertalian yang rapat dengan flora orkid di Thailand, iaitu Kawasan Flora Indo-Malaya atau Kawasan Flora Thai-Myanmar berbanding dengan kawasan Malaya. Pertalian rapat ini disumbangkan oleh keadaan iklim (monsun utara yang kering), kedudukan geografi (bersempadan dengan Semenanjung Thailand) dan jenis habitat iaitu batu kapur yang sememangnya terkenal dengan kadar keendemikan spesies yang tinggi. Sebanyak 90% daripada rekod baru ini dijumpai di kawasan berdekatan sempadan Thailand dan juga di Gunung Perlis yang merupakan puncak paling tinggi di Perlis (733m).

ABSTRACT

An intensive study on orchid diversity was conducted in Perlis especially within the Perlis State Park during the period 2003 – 2004. During the numerous field trips and studies, a total of 1,783 orchid specimens where collected from the 12 hills (11 limestone hills and one partly granite stone mountain). These samples were identified and differentiated into 119 taxa in 50 genera which are represented by 4 subfamilies. Ninety were identified to species level and the remaining 29 were only identified to genus level as the specimens were incomplete, because of lack of flowers. From these numbers, 62 species in 20 genera are new records for Perlis and 9 species and one genus, Panisea, are new records for Malaysia. The diversity of orchids in Perlis is characteristically closely related to Thailand's orchid flora, which is Indo-Malayan Floristic Region or Thailand – Burmese Floristic Region as compared to the other parts of Malaya which is Malayan Floristic Region. This can be to the climatic conditions (northern dry – monsoon), geographical location (bordering Peninsular Thailand) and the limestone habitat which is known to habour a high rate of species endemism. As much as 90% of these new records of orchids were collected from near the Malaysian-Thailand border and from Gunung Perlis, the highest peak in Perlis (733m).

INTRODUCTION

Perlis is the smallest and farthest north state in Peninsular Malaysia, bordered by Thailand in the north and Kedah in the south. Perlis covers an area of 80,302 ha with about 12,048 ha of total forested land scattered into seven permanent forest reserves. Four large forest reserves, namely, Wang Mu, Bukit Bintang, Mata Ayer, and Kurong Batang Forest Reserves are located on the Nakawan Range, which is part of the Setul Formation that lies between the Perlis – Thailand border. It extends to the eastern part of Langkawi Islands and extends to the north into Thailand where it is known as Tung Song Formation. Nakawan Range is the oldest, and the longest continuous limestone Range in Peninsular Malaysia, aged from Ordovician to Devonian (450 – 350

millions years ago). Perlis has unique flora and known to have many species unique only to limestone and endemic to the northern part of Peninsular Malaysia and Perlis.

These massive limestone hills contain a variety of vegetations that support a great diversity of limestone flora. However, botanical records for Perlis are poor compared to the limestone areas of other Malaysian states, as most floral studies of flora were focused on Langkawi Island (Kiew *et al.* 1993).

The history of botanical collection in Perlis started in 1896 by Ridley, who collected plants from Bukit Lagi and Bukit Chuping. This was followed by Henderson (between 1923 and 1937) who explored Bukit Chuping, Bukit Lagi, Wang Tangga and Tebing Tinggi. A local collector, Kiah (1938) did some collecting from Wang Tangga. In 1965, Burtt and Woods collected plants from Bukit Bintang. These collections are summarized in Chin (1977, 1979, 1983a, b) who also recorded 80 species from Perlis (Kiew *et al.* 1993). Several collections were done between 1990s to present; in 1992 by the World Wild Fund (WWF) Malaysia team headed by Sharma who collected from Bukit Rongkit and Yong, Rahimatshah and Tan collected from Sg. Anak Chelong and Wang Kelian (Sharma 1992); Kiew *et al.* (1993) who recorded 215 species of plants in 164 genera and 65 families (recorded 22 species of orchids). During a scientific expedition conducted in 1999 covering the Wang Kelian area, Faridah Hanum *et al.* recorded 164 species in 129 genera and 65 families of non herbaceous flowering plants; Rusea *et al.* has recorded 45 species from 21 families of herbaceous plants including 6 species of orchids (Latiff *et al.* 2001). In the year 2000, another scientific expedition was conducted covering the Wang Mu Forest Reserve, which recorded 145 species from 116 genera and 54 families (with only one orchid species recorded) (Latiff *et al.* 2002). Shakirah (2003) recorded 41 species of orchids on a single limestone hill, Bukit Pelarit.

This study is focused on limestone hills grouped as Setul Limestone Formation, the oldest limestone formation in Peninsular Malaysia (Ordovician to early Devonian) except for Bukit Chabang and Bukit Mata Air which belong to Chuping limestone formation (early Permian to late Triassic). Most of the limestone hills are located in the Perlis State Park (Latiff *et al.* 2002) (*Fig. 1*).

SITE DESCRIPTION AND METHODS

Perlis

Perlis is situated at latitude 6° 15' N and longitude 100° 6' to 100° 23'E. Perlis shares borders with Thailand in the north and the state of Kedah in the south. The climate in Perlis is warm and dry from January to April with temperatures ranging from 21° to 3°C and an average annual rainfall ranging between 2000 mm to 2500 mm. The wet season is between September and December. The rainfall peak is in October and between April – May (Rahimatsah–*et al.* 2001).

Most of the forest in Perlis is the semi-deciduous type influenced by the northern and dry monsoon element from Thai-Burmese that differentiate the flora in Perlis from the in other parts of Peninsular Malaysia (Mathew *et al.* 1993). Ridley (1911) mentioned that the difference between the flora, especially the limestone flora, at lower Thailand (including Perlis which was part of Thailand at that time) and the other states of Peninsula Malaysia south of Alor Setar may be due to the distinct dry season (December to February) in the extreme north of Peninsular Malaysia (Chin 1977).

Perlis has about 10,631 ha of forested area scattered in 7 forest reserves and a proposed forest reserve. The Perlis State Park with an area greater than 5,000 ha was established in 1997 and comprises Mata Ayer, Wang Mu and Wang Tangga Forest Reserves. The state park was established to conserve and protect the limestone biodiversity. The limestone hills in Perlis contain a high number of endemic and rare plants including the Orchidaceae (Latiff 2002; Wong 2002).

Plate 1. New orchid species records for Perlis* and Malaysia**. A. *Thelasis pymae**, B. *Dendrobium kentrophyllum**, C. *Dienia ophrydis**, D. *Acampe rigida**, E. *Eria ochracea***, F. *Oberonia langbianensis***, G. *Cymbidium ensiformis** and H. *Thrixspermum pensile***

Methods

Four botanical surveys or field studies were carried out on limestone hills in Perlis between 2003 and 2004. Orchids were collected from eleven limestone hills and a limestone/granite hill, in Gunung Perlis. Specimens were collected along the trails. Each specimen was assigned a collection number under the author series (Wendy Yong 1-478). However, if there were too few surviving plants of a species in the collection site, the species was not collected but all its characteristics were recorded in the field notebook and noted as seen on site and photographed as evidence. Notes on morphology and habitat for each specimen collected or observed *in situ* were documented. Photographs were taken whenever possible. All specimens collected were processed according to standard herbarium specimen preparation techniques outlined in Forman (1989). Specimens were identified using characters described and identification keys in Br.hl (1926), Holttum (1957), Seidenfaden and Smitinand (1959 – 1961), Seidenfaden (1968, 1973, 1975, 1976, 1977, 1978a, 1978b, 1979, 1980), Banerji (1978), Teo (1985), Seidenfaden and Wood (1992), Vermeulen (1991), Wood (1997, 2001) and Comber (1990, 2001). All herbarium specimens collected during this study were deposited at the Herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia.

RESULTS AND DISCUSSION

A total of 119 species of orchids belonging to 50 genera were identified from 12 hills surveyed during this study (Table 1), of which 44 species (37%) are new records for Perlis (Plate 1) and 11 species (9.2%) are new records for Malaysia (Plate 2), an addition to the existing checklist (Tables 2 and 3). Therefore, the total orchid species in Perlis (excluding the 11 new recorded species for Malaysia) represented 12.3% of 878 species of the total orchids species recorded from Peninsular Malaysia (Schuiteman 1999). This result clearly shows the great diversity of orchids for the smallest state, Perlis, which covers an area of 81, 063 hectares only.

The genus *Dendrobium* is the largest with about 16 species which stands 13.4% of the orchids in Perlis followed by *Flickingeria* with 11 species (9.2%), then *Eria* with 9 species (7.6%) and *Bulbophyllum* with 8 species (6.7%) (Table 4). A noteworthy discovery during this study is that 6 species (54.5%) out of 11 new recorded species for Peninsular Malaysia were actually found on a single hill, Bukit Rongkit, where it was also discovered that an abundance of *Flickingeria* spp. grows on the exposed limestone rocks (some grows on tree trunks) along the way to the hill top. The *Flickingeria* spp. is well adapted to the extreme hot and dry conditions which enable them to survive well in the extreme climate.

However the distribution and the diversity of orchids in Perlis are more related to the Indo-Malayan orchids compared to the Malayan orchids. Most of the orchid species found are restricted to the northern part of Peninsular Malaysia (Langkawi Island, Kedah, Kelantan and Perlis) and Peninsular Thailand, which do not occur elsewhere outside this range such as *Habenaria carnea*, *Paphiopedilum niveum* and *Eria ornata*.

TABLE 1
Orchids distribution in Perlis

Species	Bukit Rongkit	Bukit Merah	Bukit Bintang	Bukit Genting Hantu	Bukit Wang Mu	Bukit Teluk Tapu	Bukit Ayer	Bukit Gua Ikan	Bukit Chabang	Bukit Wang Tangga	Bukit Wang Pisang	Gunung Perlis
Acampe rigida		✓										
Aerides odorata	✓	✓			✓							
Agrostophyllum sp.												
Apotasia nuda												✓
Ascocentrum miniatum	✓	✓				✓		✓				
Ascocentrum sp.											✓	
Biermannia ciliata											✓	
Bulbophyllum dentiferum					✓							
Bulbopyhllum microglossum						✓						
Bulbophyllum mutabile												✓
Bulbophyllum purpurascens	✓	✓						✓		✓		
Bulbophyllum taeniophyllum												✓
Bulbophyllum sp. 1	✓											
Bulbophyllum sp. 2	✓		✓									
Bulbophyllum sp. 3												✓
Calanthe sp.	✓											
Ceratostylis radiata												✓
Ceratostylis subulata												✓
*Chamaeanthus brachystachys***											✓	
Cleisostoma discolor						✓						
Cleisostoma williamsonii			✓									
Coelogyne trinervis	✓										✓	
Coelogyne sp. 1		✓										
Coelogyne sp. 2												✓
Coelogyne sp. 3								✓				
Cymbidium aloifolium						✓		✓	✓			
Cymbidium ensifolium ssp. *haematodes*	✓			✓		✓						
Cymbidium lancifolium												✓
Cymbidium sp. 1	✓			✓								
Cymbidium sp. 2	✓											
Dendrobium acerosum	✓											

TABLE 1 (Continued)

Species	Bukit Rongkit	Bukit Merah	Bukit Bintang	Bukit Genting Hantu	Bukit Wang Mu	Bukit Teluk Tapu	Bukit Ayer	Bukit Gua Ikan	Bukit Chabang	Bukit Wang Tangga	Bukit Wang Pisang	Gunung Perlis
Dendrobium aloifolium		✓										
Dendrobium anosmum					✓							
Dendrobium concinnum		✓										
Dendrobium crumenatum	✓											
Dendrobium hughii												✓
Dendrobium indivisum					✓							
Dendrobium indivisum var pallidum	✓	✓										
Dendrobium kentrophyllum												✓
Dendrobium leonis	✓	✓								✓		
Dendrobium linguella		✓								✓	✓	
Dendrobium salaccense		✓		✓							✓	
Dendrobium secundum	✓	✓									✓	
Dendrobium setifolium												✓
Dendrobium trinervium				✓	✓	✓						
Dendrobium truncatum											✓	
Dienia ophrydis		✓									✓	
Eria sp.												✓
Eria floribunda												✓
Eria javanica		✓	✓	✓							✓	
Eria mucronata				✓							✓	
Eria nutans Lindl.												✓
Eria ochracea **											✓	
Eria ornata	✓	✓		✓								
Eria tenuiflora												✓
Eria sp.												✓
Eulophia andamanensis			✓	✓								✓
Flickingeria angustifolia												✓
Flickingeria bancana												✓
Flickingeria convexa												✓
Flickingeria fimbriata	✓											
Flickingeria pallens	✓											
Flickingeria xantholeuca				✓								
Flickingeria sp. 1	✓											
Flickingeria sp. 2				✓								

TABLE 1 (Continued)

Species	Bukit Rongkit	Bukit Merah	Bukit Bintang	Bukit Genting Hantu	Bukit Wang Mu	Bukit Teluk Tapu	Bukit Ayer	Bukit Gua Ikan	Bukit Chabang	Bukit Wang Tangga	Bukit Wang Pisang	Gunung Perlis
Flickingeria sp. 3		✓										
Flickingeria sp. 4			✓									
Flickingeria sp. 5												
Gastrochilus hainanensis **		✓										
Gastrodia javanica												✓
Grosourdya incurvicalar											✓	
Grosourdya muscosa												✓
Habenaria carnea	✓	✓	✓								✓	
Habenaria reflexa				✓							✓	
Kingidium deliocosum	✓	✓	✓	✓				✓			✓	
Liparis aurita **											✓	
Liparis caespitosa												✓
Liparis viridiflora		✓										
Luisia sp.	✓											
Macodes petola												✓
Malaxis calophylla											✓	
Malaxis prasina											✓	
Malaxis sp.1											✓	
Malaxis sp.2											✓	
Malaxis sp.3												✓
Nephelaphyllum pulchrum												✓
Nervilia plicata				✓							✓	
Nervilia punctata											✓	
Oberonia ensiformis **		✓										
Oberonia langbianensis **	✓											
Oberonia sp.		✓										
*Panisea uniflora***											✓	
Paphiopedilum niveum	✓	✓	✓									
Pennilabium struthio	✓	✓				✓					✓	✓
Pholidota imbricata												✓
Pholidota sp.	✓											
Podochilus lucescens	✓	✓	✓	✓	✓						✓	

TABLE 1 (Continued)

Species	Bukit Rongkit	Bukit Merah	Bukit Bintang	Bukit Genting Hantu	Bukit Wang Mu	Bukit Teluk Tapu	Bukit Ayer	Bukit Gua Ikan	Bukit Chabang	Bukit Wang Tangga	Bukit Wang Pisang	Gunung Perlis
Polystachya concreta	✓											
Pomatocalpa andamanica	✓	✓			✓	✓					✓	
Pomatocalpa spicata					✓		✓				✓	
Porpax sp.											✓	
Renanthera sp.												✓
Renantherella histrionica		✓										
Spathoglottis plicat a					✓							
Stresosandra javanica												✓
Taeniophyllum sp.												✓
Tainia speciosa												✓
Thelasis pygmae		✓	✓									
*Thelasis rhomboglossa***	✓											
Thelasis sp.											✓	
Trichoglottis bipunctata				✓	✓			✓				
Trichoglottis cirrhifera	✓	✓			✓	✓		✓				
Trichotosia gracilis												✓
*Thrixspermum pensile***												✓
Tropidia cucurligoides	✓			✓								
Tropidia sp.		✓										
Tuberolabium odoratissium **	✓											

TABLE 2
New records for Perlis

No.	*Species*	Notes
1.	*Acampe rigida*	Previously only known from Langkawi Island and Penang.
2.	*Biermannia ciliata*	Previously found at Sungai Siput, Perak, Sungai Sat in Pahang and Kemaman.
3.	*Bulbopyhllum microglossum*	Previously found in Gunung Tahan and Cameron Highlands.
4.	*Bulbophyllum mutabile*	Previously only collected at Bukit Fraser, Ulu Kali (Pahang) and Bukit Larut, Perak.
5.	*Bulbophyllum purpurascens*	Previously not recorded from Perlis.
6.	*Bulbophyllum taeniophyllum*	Previously found on limestone in Kelantan
7.	*Ceratostylis radiata*	Previously only known from Langkawi Island
8.	*Coelogyne trinervis*	Previously found at Pahang, Kelantan, Pinang and Langkawi Island.
9.	*Cymbidium aloifolium*	Previously only recorded from Langkawi Island.
10.	*Cymbidium ensifolium ssp. haematodes*	Previously only recorded from Langkawi Island.
11.	*Dendrobium hughii*	Previously found in Gunung Raya in Langkawi Island, Gunung Jerai, Kedah, Bukit Fraser, Ulu Kali, Gunung Tahan and Gunung Benom.
12.	*Dendrobium kentrophyllum*	Previously found on Taiping Hills, Cameron Highlands, Bukit Fraser and Gunung Ulu Kali.
13.	*Dendrobium setifolium*	Previously only found in Pahang.
14.	*Dendrobium truncatum*	Previously found in Kedah, Perak, Selangor, Pahang and Pulau Tioman.
15.	*Dienia ophrydis*	Previously found in Gunung Raya, Langkawi Island and Negeri Sembilan.
16.	*Eria floribunda*	-
17.	*Eria javanica*	Previously found in Pahang and Terrengganu.
18.	*Eria mucronata*	Previously found in Gua Musang, Kelantan and Bukit Takun, Selangor.
19.	*Eria nutans*	-
20.	*Eria tenuiflora*	-
21.	*Flickingeria angustifolia*	
22.	*Flickingeria bancana*	
23.	*Flickingeria convexa*	Previously found at Pontian, Johor and Gunung Ulu Kali, Selangor.
24.	*Flickingeria xantholeuca*	Previously found in Langkawi Island, Perak, Pahang, Johor.
25.	*Gastrodia javanica*	-
26.	*Grosourdya incurvicalar*	Previously found in Tembeling.
27.	*Grosourdya muscosa*	Previously found in Pahang.
28.	*Habenaria reflexa*	Previously found in forest on limestone in Pahang and Perak.
29.	*Kingidium deliocosum*	-
30.	*Liparis cespitosa*	-
31.	*Liparis viridiflora*	Previously found in Perak, Penang and Pahang.
32.	*Macodes petola*	Found from north to Penang.
33.	*Malaxis calophylla*	found on Bukit Bendera, Penang, on limestone at Baling, Kedah, at Gua Ledang, Johor and at Gua Musang, Kelantan.
34.	*Nephelaphyllum pulchrum*	-
35.	*Nervilia punctata*	Previously found in Langkawi Island, Perak and Penang.
36.	*Pholidota imbricata*	Common on limestone.
37.	*Pomatocalpa andamanica*	Previously recorded from Batu Caves, Selangor.
38.	*Pomatocalpa spicata*	Previously found in Perak, Pahang and Negeri Sembilan.
39.	*Spathoglottis plicata*	-
40.	*Stresosandra javanica*	Previously found in the north part and Penang.
41.	*Tainia speciosa*	Previously recorded from Genting Highlands and Fraser's Hill.
42.	*Thelasis pygmae*	-
43.	*Trichotosia gracilis*	-
44.	*Tropidia cucurligoides*	-

TABLE 3
New records for Malaysia

No.	Species	Notes
1.	*Chamaeanthus brachystachys*	Previously recorded in Java and south Thailand.
2.	*Eria ochracea*	Previously recorded as an endemic species to Thailand.
3.	*Gastrochilus hainanensis*	Previously recorded from Hainan and Thailand.
4.	*Liparis aurita*	Previously recorded from Thailand and Timor.
5.	*Oberonia ensiformis*	Previously recorded from Thailand, Northwest Himalaya, Deccan, Myanmar, China and Indochina.
6.	*Oberonia langbianensis*	Previously recorded from Thailand and Vietnam
7.	*Panisea uniflora*	Previously recorded from Yunnan, Bhutan, Cambodia, India, Laos, Myanmar, Nepal, Thailand and Vietnam.
8.	*Pholidota recurva*	Previously recorded from Sikkim, Nepa, Burma (Tenasserim), Thailand and Vietnam
9.	*Thelasis rhomboglossa*	Previously recorded as an endemic species to Sumatera.
10.	*Thrixspermum pensile*	Previously recorded from Java and Thailand.
11.	*Tuberolabium odoratissium*	Previously recorded from Java and Sumatera.

TABLE 4
Summary of orchids found in Perlis

Genera	No. of species
Acampe	1
Aerides	1
Agrostophyllum	1
Apotasia	1
Ascocentrum	2
Biermannia	1
Bulbophyllum	8
Calanthe	1
Ceratostylis	2
Chamaeanthus	1
Cleisostoma	2
Coelogyne	4
Cymbidium	5
Dendrobium	16
Dienia	1
Eria	9
Eulophia	1
Flickingeria	11
Gastrochilus	1
Gastrodia	1
Grosourdya	2
Habenaria	2
Kingidium	1
Liparis	3
Luisia	1

Table 4 (Continued)

Genera	No. of species
Macodes	1
Malaxis	5
Nephelaphyllum	1
Oberonia	3
Panisea	1
Paphiopedilum	1
Pennilabium	1
Pholidota	3
Podochilus	1
Polystachya	1
Pomatocalpa	2
Porpax	1
Renathera	2
Renantherella	1
Spathoglottis	1
Stresosandra	1
Taeniophyllum	1
Tainia	1
Thelasis	3
Trichoglottis	2
Trichotosia	1
Thrixspermum	1
Tropidia	2
Tuberolabium	1
Total 50 genera	Total 119 species

CONCLUSION

Perlis shows a great diversity of orchids including 119 species collected during this study (from year 2003 – 2004). A total of 44 new species were recorded for Perlis and 11 are new records for Malaysia (Plate 1). These results simultaneously increase the number of orchid species found in Peninsular Malaysia from 878 species (Schuiteman 1999) to 889 species. Nine species from the 11 new records for Malaysia are also recorded in Thailand. This shows a strong connection between the Perlis flora and the Thailand flora especially the orchidaceae family.

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REFERENCES

BANERJI, M. L. 1978. *Orchids of Nepal*. New Delhi: International Bioscience Publisher.

CHIN, S. C. 1977. The limestone flora of Malaya 1. *Gard. Bull. Sing.* **30:** 165 — 219.

CHIN, S. C. 1979. The limestone flora of Malaya 2.— *Gard. Bull. Sing.* **32:** 64 — 203.

CHIN, S. C. 1983a. The limestone flora of Malaya 1. *Gard. Bull. Sing.* **35:** 137 — 190.

CHIN, S. C. 1983b. The limestone flora of Malaya 1. *Gard. Bull. Sing.* **36:** 31 — 91.

COMBER, J.B. 1990. *Orchids of Java*. Royal Botanic Garden, Kew: Bentham-Moxon Trust.

COMBER, J.B. 2001. *Orchids of Sumatera*. Malaysia: Natural History Publications (Borneo).

FARIDAH-HANUM I., MAT-SALLEH K., RAHIM OTHMAN A., RIZAL KHALIT A., SHAMSUL KAMIS and NAZRE M. 2001. In *An Annotated Checklist of Flowering Plants at Wang Kelian, Perlis State Park, Perlis*, ed. I. Faridah-Hanum, K. Osman and A. Latiff, pp. 227-247. Forest Department of Perlis, Kangar, Perlis, Malaysia.

GO, R., P. LEPUN and A. BADROL. 1999. *Noteworthy Herbaceous Plants of Tasik Meranti and Bukit Pelarit, Wang Kelian, Perlis*, ed. I. Faridah Hanum, K. Osman, A.R. Yussof and A. Latiff, pp. 267—274. Forest Department of Perlis, Kangar Perlis, Malaysia.

HOLTUM, R.E. 1957. A revised *Flora of Malaya: An Illustrated Systematic Account of the Malayan Flora, including Commonly Cultivated Plant.* **Vol. I**, *Orchids of Malaya*. Botanic Garden, Singapore: Government Printing Office.

JASIN, B. and Z. HARUN. 2002. *Geomorphology and Geology of the Setul Formation in Perlis*, ed. I. Faridah-Hanum, K. Osman, A.R. Yussof and A. Latiff, pp. 68—82. Forest Department of Perlis, Kangar, Perlis, Malaysia.

KIEW, R., D.G.C. YONG, S. ANTHONYSAMY and J. ANTHONYSAMY. 1993. A floral survey of limestone hill in Perlis with management recommendation. WWF Malaysia. 57 pp.

LATIFF, A. KASIM OSMAN, I. FARIDAH-HANUM and RAHIMATSAH AMAT. 2001. *Perlis State Park at Wang Kelian: An Introduction*, ed. I. Faridah-Hanum, K. Osman and A. Latiff, pp. 19—27. Forest Department of Perlis, Kangar, Perlis, Malaysia.

MATHEW, D. S. K., SHARMA. 1993. Biodiversity in Peninsular Malaysia Transfromtier Forest: A case study at Mata Ayer Forest Reserve (FR) and Proposed Bukit Wang Mu, Perlis. 26 p.

MOHD NOOR, S. 2002. Kajian kepelbagaian flora Orchidaceae di kawasan batu kapur, Bukit Pelarit, Wang Kelian, Taman Negeri Perlis. BSc. Thesis, Universiti Putra Malaysia, Serdang, Malaysia. 159 p.

RAHIMATSAH, A. and K. OSMAN. 2002. *The Administration and Management of Perlis State Park* ed. I. Faridah-Hanum, K. Osman, A.R. Yussof and A. Latiff, pp. 23-43. Forest Department of Perlis, Kangar, Perlis, Malaysia.

RIDLEY, H.N. 1911. The flora of lower Siam. *Journal of Strait Branch Royal Asiatic Society* **59:** 27-234.

SCHUITEMAN, A. 1999. Biogeography of Malesian Orchidaceae. *Flora Malesiana Bulletin* **12(6):** 273-287.

SEIDENFADEN, G. 1968. The genus Oberonia in Mainland Asia. *Dansk Botanisk Arkiv* **32 (2):** 108-180.

SEIDENFADEN, G. 1975a. Orchid genera in Thailand 2. *Cleisostoma* Bl. *Dansk. Bot. Ark.* **29(3):** 80 p.

SEIDENFADEN, G. 1975b. Orchid genera in Thailand 3. *Coelogyne* Lindl. *Dansk. Bot. Ark.* **29(4)**: 92 p.

SEIDENFADEN, G. 1976. Orchid genera in Thailand 4. *Liparis* A. C. Rich. *Dansk. Bot. Ark.* **31(1):** 61-84.

SEIDENFADEN, G. 1977. Orchid genera in Thailand 5. Orchidoideae. *Dansk. Bot. Ark.* **31(3):** 149 p.

SEIDENFADEN, G. 1978a. Orchid genera in Thailand 6. Neottioideae. *Dansk. Bot. Ark.* **32(2):** 195 p.

SEIDENFADEN, G. 1978b. Orchid genera in Thailand 7. *Oberonia* Lindl. and *Malaxis* Sol. ex. Sw. *Dansk. Bot. Ark.* **33(1)**.

SEIDENFADEN, G. 1979. Orchid genera in Thailand 8. *Bulbophyllum* Thou. *Dansk. Bot. Ark.* **33(3):** 228.

SEIDENFADEN, G. 1980. Orchid genera in Thailand 9. *Flickingeria* hawkes and *Epigenium* gagnep. *Dansk. Bot. Ark.* **34(1):** 104.

SEIDENFADEN, G. 1982. Orchid genera in Thailand 10. *Trichotosia* Bl. and *Eria* Lindl. *Dansk. Op. Bot.* **62:** 145.

SEIDENFADEN, G. and J.J. WOOD. 1992. *The Orchids of Peninsular Malaysia and Singapore, A Revision of R.E. Holttum: Orchids of Malaya*. Fredenborg, Denmark: Olsen & Olsen.

SHARMA, D. 1992. A survey of the fauna, flora and geological features at the Mata Ayer Forest Reserve and the proposed Bukit Wang Mu Forest Reserve, Perlis. WWF Malaysia.

TEO, C.K.H. 1985. *Native Orchids of Peninsular Malaysia*. Singapore: Times Books International.

WONG, S. L. 2002. Plant life. In *Pearl of Perlis: Perlis State Park Guide*, ed. K. Osman, R. Amat and S. Suksuwan, pp. 45—59.Forest Department of Perlis, Kangar, Perlis, Malaysia.

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