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Contents

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Morphology of *Halophila ovalis* (R.Br.) Hook. *f.* from Peninsular and East Malaysia

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ABSTRAK

Halophila ovalis *telah disampelkan dari enam lokasi yang mempunyai ciri habitat berbeza; Merambong, Johor dari Semenanjung Malaysia (beting sub-tidal), Teluk Sepinong, Sabah (muara sub-tidal), Pulau Gaya, Sabah (kawasan terumbu karang yang musnah) dan Merchang, Terengganu (inter-tidal danau air masin), Teluk Kemang, Negeri Sembilan (inter-tidal terumbu karang) dan Punang, Sarawak (pantai pasang surut). Berdasarkan pada dimensi daun (panjang dan lebar) dan bilangan pasangan urat daun, tiga jenis variasi* H. ovalis *dapat dibezakan iaitu* H. ovalis *daun besar, sederhana dan kecil.* Halophila ovalis *daun besar dijumpai di kawasan sub-tidal contohnya Merambong, Teluk Sepinong manakala* H. ovalis *daun sederhana dan kecil didapati di kawasan pasang surut yang terdedah pada udara selama 3 hingga 4 jam semasa air surut contohnya di Punang, Merchang, Teluk Kemang dan Pulau Gaya. Saliniti dan kedalaman air yang berbeza untuk setiap habitat yang menyumbang kepada perbezaan bentuk dan dimensi saiz daun. Daun* H. ovalis *menunjukkan berbagai jenis bentuk dari ovate, obovate, oblong ke spatulate. Di kawasan yang lebih dalam, air yang keruh dan tanah berlumpur, daun* H. ovalis *berbentuk lebih memanjang dan petiolnya juga panjang.* Halophila ovalis *di Malaysia mempamerkan kepelbagaian morfologi terutamanya pada daun sebagai gerak balas pada faktor persekitaran yang berbeza di pelbagai habitat.*

ABSTRACT

Halophila ovalis *plants were collected from six locations with different habitat characteristics; Merambong, Johore of Peninsular Malaysia (sub-tidal shoal), Teluk Sepinong of Sabah (sub-tidal estuary), Pulau Gaya, Sabah (sub-tidal degraded coral), Merchang, Terengganu (inter-tidal lagoon), Teluk Kemang, Negeri Sembilan (inter-tidal degraded coral platform) and Punang, Sarawak (inter-tidal beach front). Based on the leaf dimensions (length and width) and number of paired cross-veins, three* Halophila ovalis *variants can be distinguished; big, intermediate and small-leaved. The big-leaved* H. ovalis *are found at sub-tidal areas e.g. Merambong, Teluk Sepinong, while intermediate- and small-leaved variants are from inter-tidal areas that are exposed to air for 3 to 4 hours during low tides e.g. Punang, Merchang, Teluk Kemang and Pulau Gaya. The salinity and depth in a particular habitat contribute to the variability in shapes and dimension of leaf sizes.* Halophila ovalis *leaves showed diverse shapes from ovate, obovate, oblong to spathulate. At deeper depths, turbid water and muddy substrate, leaves of* H. ovalis *are elongated in shape and with longer petiole length.* Halophila ovalis *in Malaysia exhibit morphological variability particularly in the leaves in response to the different environmental factors in the various habitats.*

INTRODUCTION

Malaysia has a total of 14 species seagrasses belonging to 8 genera: *Enhalus acoroides, Halophila beccarii, H. decipiens, H. ovalis, H. minor, H. spinulosa, Thalassia hemprichii, Cymodocea rotundata, C. serrulata, Halodule uninervis, H. pinifolia, Syringodium isoetifolium, Thalassodendron ciliatum* and *Ruppia maritima* (Japar Sidik *et al.* 2001; Japar Sidik and Muta Harah 2003). Amongst these species, *H. ovalis* exhibits morphology

variability in response to different environmental factors at various habitats (den Hartog 1970; McMillan *et al.* 1983; Japar Sidik *et al.* 2001). According to den Hartog (1970), *H. ovalis* is a 'collective species' consisting of a large array of uncertain taxa. Examples of this are subspecies *australis* (Doty and Stone 1966) den Hartog of South Australia, subspecies *bullosa* (Setchell) den Hartog of Samoa, Tonga and Fiji, subspecies *hawaiiana* (Doty and Stone 1966) den Hartog of Hawaii and subspecies *linearis* (den Hartog) den Hartog of Mozambique. Den Hartog (1970) categorised *H. ovalis* into subspecies based on the leaf sizes and pairs of cross veins. *Halophila ovalis* has 12-25 pairs of cross veins, while *H. minor* has 4-12 pairs of cross veins. Observations on *H. ovalis* indicate that they have a wide range of leaf shapes and sizes, pairs of cross veins and pigmentation (Japar Sidik *et al.* 2001; Muta Harah *et al.* 2003). In addition, it was also reported that *H. ovalis* has long petioles due to heavy sedimentation and large shifts of sand by water movements to obtain maximum sunlight and big leaf blade due to less surface irradiance reaching the bottom of deeper depth, shading or water turbidity reported at Merambong, Johore (Japar Sidik *et al.* 1999; Japar Sidik *et al.* 2001). These are some responses of *H. ovalis* towards the ever changing and unstable environment. Other morphological variabilities related to environmental factors are also reported by various researchers. *Halophila ovalis* with big leaf blades are found under shaded environment (Duarte 1991), in brackish water (low salinity compared to high salinity in marine waters) (Benjamin *et al.* 1999), low light due to depth (Beer and Waisel 1982; Longstaff and Dennison 1999; Short *et al.* 2001; Ralph 1999) and muddy substrate (Young and Kirkman 1975). *Halophila ovalis* with small leaves and short petioles are found in habitats with hard carbonate substrate (coralline sand less in free nutrient, Short 1987). Changes in plant morphology and natural habits in *H. ovalis* that is locally common certainly warrant investigation. This paper reports on the distribution, habitat characteristics and morphology of *H. ovalis.*

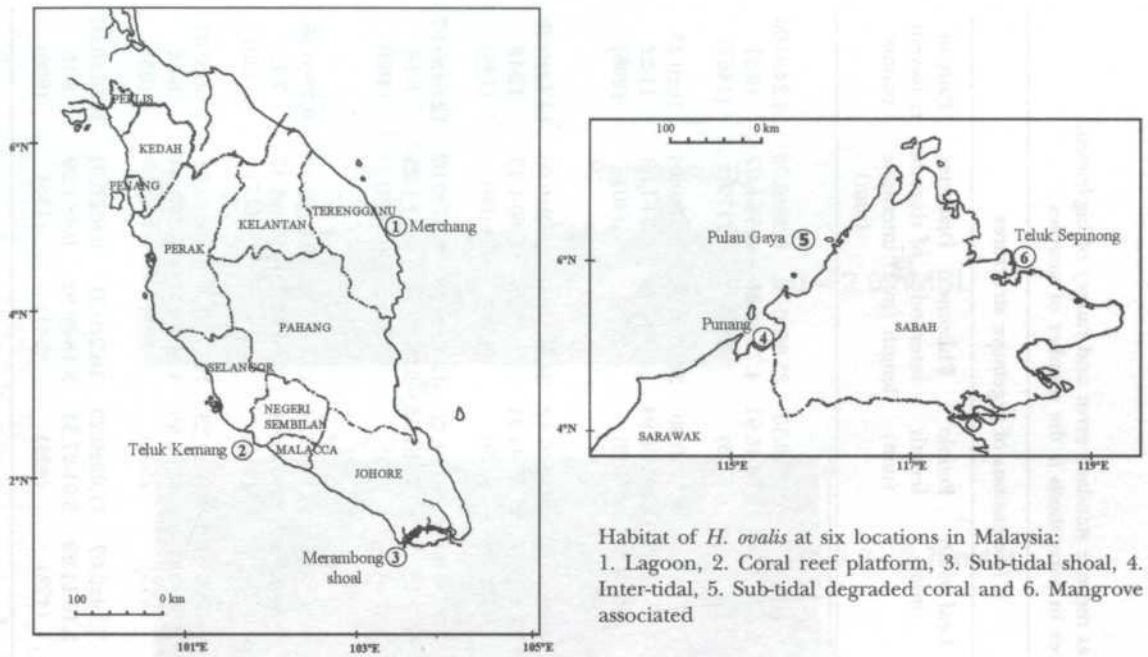
MATERIALS AND METHODS

Halophila ovalis were collected from six locations, comprising Merambong, Johore (01° 19' 00" N, 103° 36' 45" E), Merchang, Terengganu (05° 02' 15.0" N, 103° 17' 53.0" E), Teluk Kemang, Negeri Sembilan (02° 31' 00" N, 101° 47' 00" E), Teluk Sepinong, Sabah (05° 49' 3.42" N, 118° 09' 14.28" E), Punang, Sarawak (104° 54' 23.6" N, 115° 22' 36.3" E) and Pulau Gaya, Sabah (06° 00' 11.0" N, 116° 02' 30.4" E) (*Fig. 1*). At each location, the environmental parameters were recorded, e.g. substrate type, salinity, water temperature and depth. In the laboratory, the collected plants were cleaned of debris and sorted, e.g. vegetative structures comprising the length and diameter of rhizome internode, length and width of leaf blades and petiole length were measured using Mitotoyo Digital Vernier Caliper. Reproductive structures were recorded digitally and measured: male flower-pedicel length, tepal length and width, and anther length and width; female flower-style length, ovary length and width and hypanthium length and length and width of fruit were measured using UTHSCSA Image Tool for Windows software (Wilcox *et al.* 1996). The whole strand of the plants was recorded as digital images by using Nikon CoolPix. The mature leaves were recorded as digital images and pairs of cross veins were counted from the recorded images. The existing plant materials were processed for herbarium specimens as described by Menez *et al.* (1983) and some were preserved as wet specimens in 4% formalin in saline water. Leaf dimension data were analysed for various habitats and compared for differences or similarities by using Cluster Analysis using SPSS Program version 12.0.

RESULTS AND DISCUSSION

Distributions and Habitat Characteristics of H. ovalis

The distribution of *H. ovalis* is shown in *Fig. 1.* Details of the habitat characteristics of *H. ovalis* are illustrated in *Fig. 2* a - g showing; (i) inter-tidal degraded coral reef platform, e.g. Teluk Kemang (Japar Sidik and Muta Harah 2003; (ii) sub-tidal shoal e.g. Merambong; (iii) inter-tidal lagoon, e.g. Merchang; (iv) inter-tidal beach front, e.g. Punang; (v) sub-tidal degraded coral reef flat, e.g. Pulau Gaya; and (vi) sub-tidal estuary, e.g. Teluk Sepinong. In the above habitats the waters depth varied from -1.0 to -5.0 m MSL, temperatures between 25-34 °C and salinity ranging between 29-31 psu (practical salinity unit). However, at the inter-tidal lagoon of Merchang, salinity fluctuated widely between 9.41-34.41 psu. In addition, *H. ovalis* plants in Merchang can tolerate a wide fluctuation in



Habitat of *H. ovalis* at six locations in Malaysia:
1. Lagoon, 2. Coral reef platform, 3. Sub-tidal shoal, 4. Inter-tidal, 5. Sub-tidal degraded coral and 6. Mangrove associated

Fig.1: Distribution and six study locations: Peninsular Malaysia – Merchang, Terengganu; Teluk Kemang, Negeri Sembilan; Merambong shoal, Johore; Sarawak-Punang and Sabah – Pulau Gaya; Teluk Sepinong

salinity resulting from freshwater input during monsoon and dry season respectively (Japar Sidik *et al.* 1999). *Halophila ovalis* were found to grow and adapt themselves in various substrates from coralline sand, e.g. Pulau Gaya, sand covered coral, e.g. Teluk Kemang; calcareous sandy mud, e.g. Merambong; muddy loam, e.g. Teluk Sepinong to muddy sand, e.g. Merchang and Punang (Table 1). It was also noted that *H. ovalis* in certain locations e.g. at Merchang, Punang and Teluk Kemang could withstand 3-4 hours exposure to air (den Hartog 1970). *Halophila ovalis* is a eurybiontic species that is capable of surviving in very turbid and polluted waters. It was found in different places with different ecological parameters. In clear water, *H. ovalis* distribution exceeded mid-tidal level to nearly 60 m in depth on substrates ranging from soft mud to coarse rubble (den Hartog 1970; Hillman *et al.* 1995).

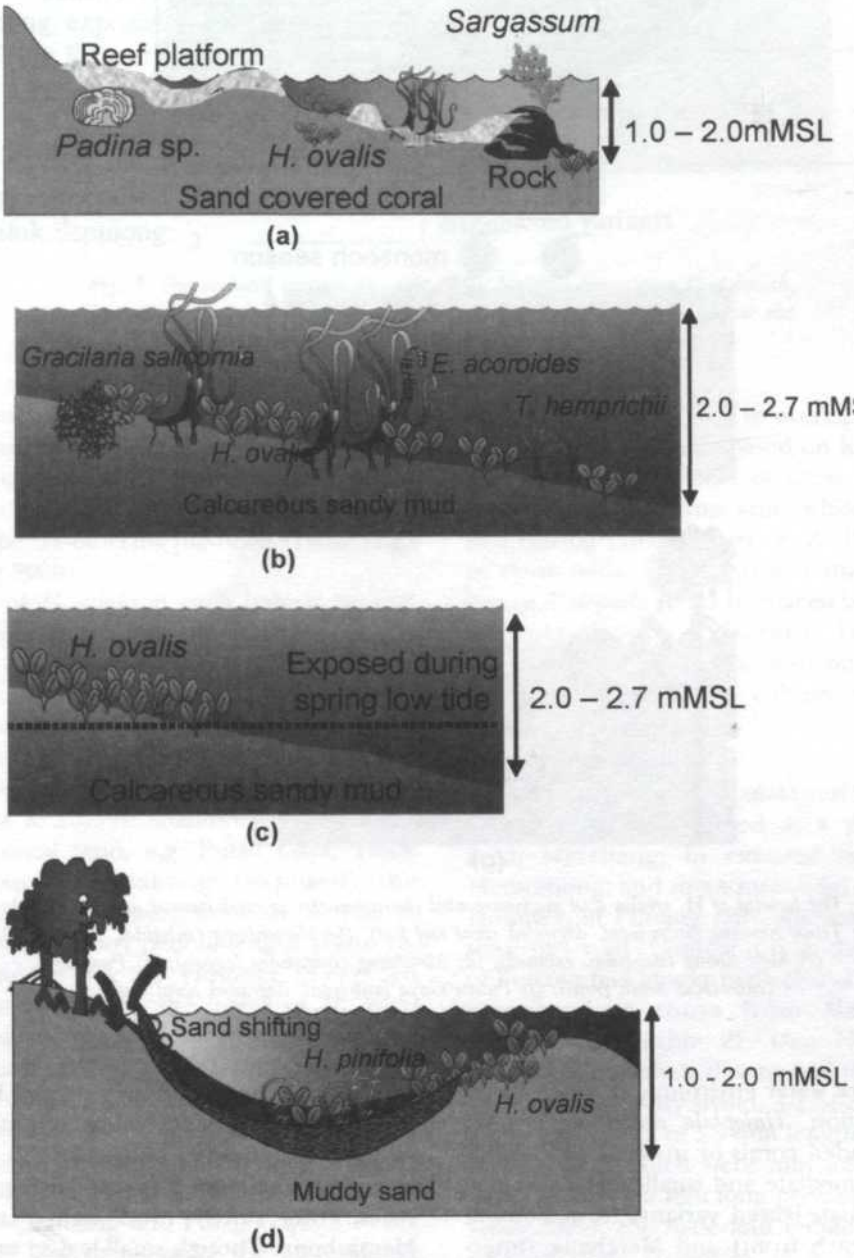
Fig. 2: The habitat of *H. ovalis* and environmental characteristics around coastal waters of Malaysia; (a) Teluk Kemang (inter-tidal, degraded coral reef flat), (b) Merambong (sub-tidal, unexposed), (c) Merambong (sub-tidal, exposed), (d) Merchang (inter-tidal lagoon), (e) Punang (inter-tidal beach front), (f) Pulau Gaya (sub-tidal, degraded coral reef) and (g) Teluk Sepinong (sub-tidal estuary).

Halophila ovalis *Variants*

Cluster analysis interpretation (*Fig. 3*) based on the leaf size dimension (leaf length and width) and pairs of cross veins distinguished *H. ovalis* into three categories: big-leaved, intermediate-leaved and small-leaved variants (Plate 1). Teluk Kemang (exposed), Pulau Gaya and Merambong (exposed) exhibit the small-leaved variants while the intermediate-leaved variants are from Merchang and Punang. The big-leaved variants are from Merambong (sub-tidal shoal, unexposed) and Teluk Sepinong (sub-tidal estuary) respectively. The details of vegetative structures such as leaf length and width, petiole length, rhizome internode length and diameter, pairs of cross veins are given in Table 1. Big-leaved *H. ovalis* plants were found mostly in a shaded environment, e.g. Merambong or in areas with light limitations, e.g. Teluk Sepinong. The big-leaved variants of Merambong were found growing under the shade of tall *Enhalus acoroides* canopy and found fully submerged even during the lowest tide level (*Fig. 2b*). The big-leaved variants from Teluk Sepinong are adapted to

TABLE 1
Habitat characteristics and vegetative structure characteristics (measurement given as mean ± standard error and range) of big-leaved, intermediate-leaved and small-leaved *H. ovalis* from the six locations. Number in parenthesis is the number of samples

	Habitat characteristics					Dimensions of vegetative structures					
	Associated habitat	Substrate type	Salinity psu	Water temperature (°C)	Depth (m MSL)	Leaf length (mm)	Leaf width (mm)	Petiole length (mm)	Rhizome internode length (mm)	Diameter of rhizome internode (mm)	Pairs of cross-vein counts
Big-leaved											
Merambong, Johore	Sub-tidal shoal, unexposed	Calcareous muddy sand	29.6-30.7	29.0-31.0	2.0-2.7	23.7±0.18 10.29-36.46 (761)	13.7±0.11 5.66-32.18 (763)	20.8±0.30 12.38-46.91 (753)	23.86±0.40 4.72-55.21 (656)	0.98±0.03 0.54-1.67 (179)	14.24±0.06 10-21 (1467)
Teluk Sepinong, Sabah	Sub-tidal estuary	Muddy loam	28.0-31.0	28.0-29.0	2.0-2.7	20.8±0.56 5.52-35.83 (129)	12.81±0.29 5.09-20.89 (126)	27.8±0.80 12.41-66.94 (129)	24.27±0.96 1.58-54.02 (127)	1.07±0.04 0.54-1.58 (40)	16±0.23 11-22 (208)
Intermediate-leaved											
Punang, Sarawak	Inter-tidal-beach front	Muddy sand	20.0-31.0	25.0-34.0	1.5-2.7	19.7±0.18 16.29-23.10 (76)	8.98±0.09 7.09-10.57 (132)	29.03±0.42 21.57-41.21 (129)	24.56±1.50 7.18-43.26 (138)	0.89±0.05 0.60-1.25 (88)	14.48±0.20 12-18 (144)
Merchang, Terengganu	Inter-tidal lagoon	Muddy sand	9.41-34.41	29.0-30.1	1.0-2.0	17.24±0.18 10.2-24.70 (255)	7.82±0.09 4.62-13.35 (260)	25.1±0.42 10.73-51.98 (293)	12.15±0.38 3.24-26.96 (199)	0.87±0.02 0.43-1.25 (73)	12.64±0.07 8-18 (460)
Small-leaved											
Merambong, Johore	Sub-tidal shoal, exposed	Calcareous sandy mud	29.6-30.7	29.0-31.0	2.0	11.36±0.09 6.68-15.89 (553)	6.54±0.05 3.56-9.65 (581)	11.28±0.14 2.31-23.44 (616)	13.79±0.23 2.6-27.00 (554)	0.60±0.019 0.16-1.47 (117)	9.78±0.06 7-15 (1150)
Pulau Gaya, Sabah	Sub-tidal degraded coral	Coralline sand	29.0-31.0	30.0-32.0	1.5-2.5	8.19±0.12 2.08-14.03 (474)	5.47±0.07 2.21-9.31 (475)	10.6±0,23 8.41-26.95 (505)	11.76±0.26 1.12-36.11 (803)	0.8±0.02 0.33-1.44 (113)	9.66±0.07 6-16 (1033)
Teluk Kemang, Negeri Sembilan	Inter-tidal; degraded reef flat	Sand covered coral	29.0-31.0	28.0-31.2	1.0-2.0	13.1±0.17 5.11-21.01 (473)	7.14±0.07 3.10-11.89 (473)	11.03±0.25 5.01-27.31 (425)	16.2±0.31 3.18-39.92 (541)	0.85±0.01 0.48-1.39 (136)	10.91±0.06 8-15 (806)


Sargassum
Reef platform
Padina sp.
H. ovalis
Rock
1.0 – 2.0mMSL
Sand covered coral
(a)
Gracilaria salicornia
E. acoroides
T. hemprichii
H. ovalis
2.0 – 2.7 mMSL
Calcareous sandy mud
(b)
H. ovalis
Exposed during spring low tide
2.0 – 2.7 mMSL
Calcareous sandy mud
(c)
Sand shifting
H. pinifolia
H. ovalis
1.0 - 2.0 mMSL
Muddy sand
(d)

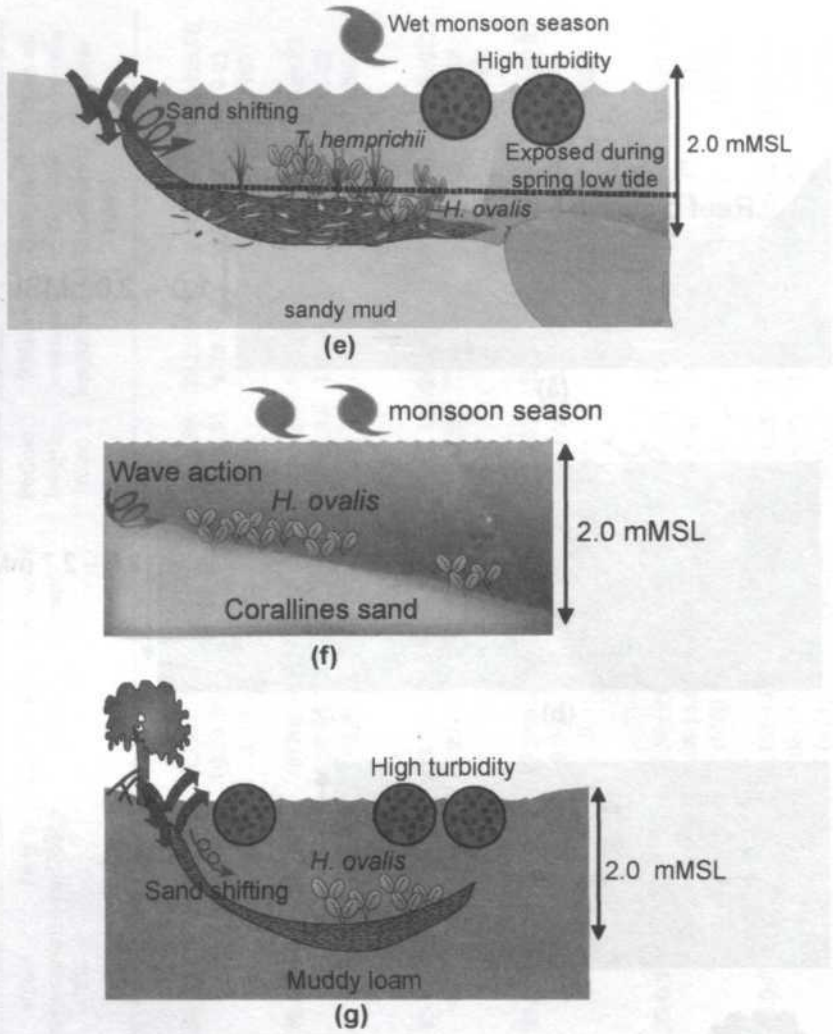


Fig. 2: The habitat of H. ovalis *and environmental characteristics around coastal waters of Malaysia: (a) Teluk Kemang (inter-tidal, degraded coral reef flat); (b) Merambong (sub-tidal, unexposed); (c) Merambong (sub-tidal, exposed); (d) Merchang (inter-tidal lagoon); (e) Punang (inter-tidal beach front); (f) Pulau Gaya (sub-tidal, degraded coral reef); and (g) Teluk Sepinong (sub-tidal estuary)*

living in a dark water environment which limits light penetration. *Halophila ovalis* plants that grow in degraded corals or in areas exposed to air have intermediate and small-leaved variants. The intermediate-leaved variants from Punang (inter-tidal beach front) and Merchang (inter-tidal lagoon) were observed to be under a wide range of salinity fluctuations. The small-leaved variants are found in degraded coral substrate, e.g. Pulau Gaya, in exposed areas e.g. Merambong or exposed degraded coral substrate, e.g. Teluk Kemang. McMillan (1983) reported that in *Halophila*, substrate types also correlated with leaf size dimension; small-leaved plants came from firm silty substrate while the large-leaved plants are from muddy calcareous sand which is always covered with water. In this study, big-leaved variants were found to be growing on the calcareous sandy mud substrate, e.g. at Merambong. Though small-leaved variants grow on the same substrate type, these plants have small leaves in response to the unfavorable environmental condition, i.e. they are exposed to air for a period of 3-4 hours during the low tide (Japar Sidik *et al.* 2001). The intermediate-leaved variants from Merchang and Punang exhibit purplish blotches or dots on the leaves (Plate 2d) and the Teluk Kemang small-leaved

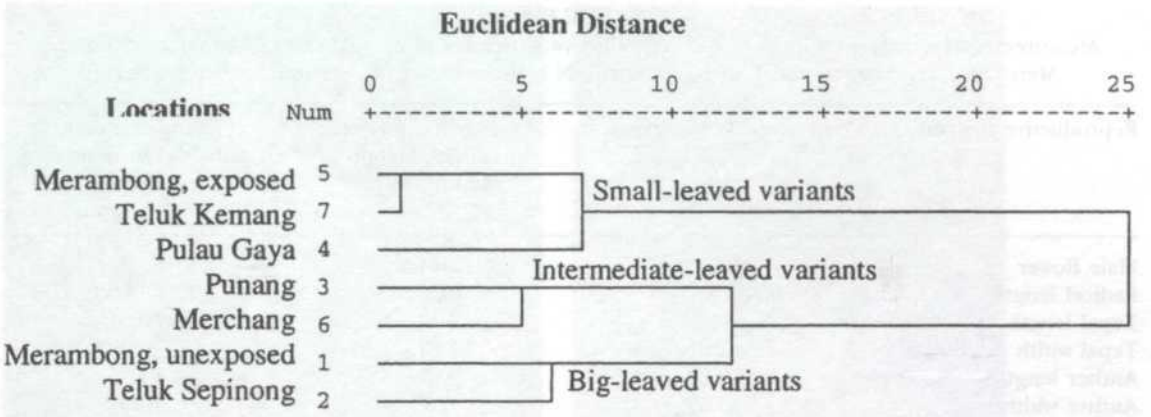


Fig. 3: Hierarchical Cluster Analysis using leaf size dimensions (leaf length, leaf width) and cross veins of H. ovalis *from six locations. The similar size leaf blades and cross veins were grouped by Euclidean Distance*

variants have pinkish pigment on the petiole near the base of the leaf (Plate 1e). These are responses to the high light conditions during exposure to air (Japar Sidik *et al.* 2001) and are believed to be UV-blocking pigments (Hemminga and Duarte 2000).

Leaves of *H. ovalis* at each habitat showed diverse shapes from ovate, obovate, oblong to spathulate. At deeper depths, turbid water and muddy substrates, e.g. Merambong (sub-tidal shoal, unexposed), Teluk Sepinong (sub-tidal estuary) the leaves of *H. ovalis* were elongated in shape and with longer petiole length (Plate 1a & 1b, Plate 2a & 2b). At shallower depths, clear water and coral sand, e.g. Pulau Gaya, Teluk Kemang, and Merambong (exposed) the *Halophila* leaves were more ovate to obovate in shape and with a short petiole length (Plate 1e, 1f & 1g, Plate 2e, 2f & 2g). However, in exposed areas and muddy sand, the leaves of *H. ovalis* were oblong and spathulated with pointed apex and with long petiole length in response to large shifts of sand from the rivers (Plate 1b & 1c, Plate 2c & 2d).

The least number of pairs of cross veins (6-16) were in small-leaved variants for Teluk Kemang, Merambong and Pulau Gaya, 8-18 for Punang and Merchang, while the Merambong big-leaved plants had 10-22 pairs of cross veins. In the previous studies, the number of pairs of cross veins of *H. ovalis* big-leaved was in the range of 10-25 as reported by den Hartog (1970); 12-25, (Sachet and Fosberg 1973) and 10-25 (Kuo 2000). The range in number of paired cross veins counts for the small-leaved *Halophila* populations were found to overlap with those of *H. ovalis* and *H. minor*. Based on Kuo (2000), *H. minor* posses 7-12 pairs of cross veins and *H. ovata* 4-12 pairs of cross veins, while according to den Hartog (1970) *H. minor* (Zoll) has 5-9 pairs of cross veins. In the present study, the small-leaved *Halophila* could be placed in the category of either *H. ovalis* or *H. minor*. To confirm the taxonomic uncertainty, a study on both variants should be conducted in culture conditions.

Reproductive Structures

Reproductive structures, male and female flowers and fruits were observed at a coastal lagoon area, Merchang; in exposed sub-tidal area, Merambong; and in an inter-tidal area, Punang. However, at Punang only opened male flowers were observed. Comparatively, the male flower of Punang had longer pedicel and anther length compared to those from Merchang and Merambong (Table 2). Den Hartog (1970) reported that female flowers had three styles, 10-20 mm long, ovary ovoid, 1-1.5(-2.5) mm long, and a hypantium of 3-5 mm length. Male flowers had tepals 3 which were mm long and 2 mm wide and a 10-20 mm long pedicel. For *H. minor* (Kuo 2000), the male and female flowers were smaller than *Halophila ovalis* in Merchang, Merambong and Punang, while the styles and hypantium were long, 4.99-21.79 mm and 1.17-6.61 mm.

CONCLUSION

1. *Halophila ovalis* in Malaysia exhibits morphological variability in response to the

TABLE 2
Measurement (as ranges in mm) of the reproductive structures of *H. ovalis* from Merambong, Johore, Merchang, Terengganu and Punang, Sarawak. N is the number of samples, nd = not observed

Reproductive structure	Merchang, Terengganu.	Merambong, Johore. Inter-tidal, lagoon Muddy sand	Punang, Sarawak. Sub-tidal Inter-tidal, Calcareous sandy mud Muddy sand
Male flower	N=6	N=4	N=5
Pedicel length	0.78-14.58	0.7	1.11-14.98
Tepal length	3.46-4.7	3.16	3.7-3.91
Tepal width	1.38-2.31	1.84	1.79-2.22
Anther length	2.67-3.82	3.03	3.4-4.45
Anther width	1.29-1.69	1.6	1.38-1.66
Female flower	N=6	N=6	nd
Style length	5.23-21.79	4.99-18.51	nd
Ovary length	0.83-1.42	0.99-1.77	nd
Ovary width	1.65-5.97	0.67-1.22	nd
Hypanthium length	1.07-5.97	1.17-6.61	nd
Fruit	N=3	N=4	nd
Fruit length	1.25-3.34	(1.93-3.82)	nd
Fruit width	0.82-2.58	(1.28-2.5)	nd

different environmental factors in the various habitats. Such responses are reflected in the leaf sizes and shapes. *Halophila ovalis* can be distinguished into three categories; big-leaved, intermediate-leaved and small-leaved variants. The big-leaved variants, e.g. at Merambong (unexposed sub-tidal) and Teluk Sepinong (sub-tidal estuary) in response to shaded conditions and light limitations have longer leaf length, petiole length, higher number of paired cross veins and are elongated in shape. The intermediate-leaved *H. ovalis*, e.g. from Merchang (inter-tidal lagoon) and Punang (inter-tidal beach front) were under exposure to air during low tide and in an environment of wide salinity fluctuation. Small-leaved *H. ovalis* from Pulau Gaya (sub-tidal degraded coral), Merambong (exposed sub-tidal) and Teluk Kemang (inter-tidal degraded coral reef flat) have relatively small leaves compared to the intermediate-leaved and big-leaved variants. They are observed either from shallow areas or exposed to air for a period of 3-4 hours during low tide.

2. The paired cross veins counts for the small-leaved *Halophila* populations show ranges that overlap with those of *H. ovalis* and *H. minor*. The small-leaved *Halophila* plants can be placed in either the category of *H. ovalis* or *H. minor*. To confirm the taxonomic uncertainty, a study on both variants should be conducted in culture conditions.

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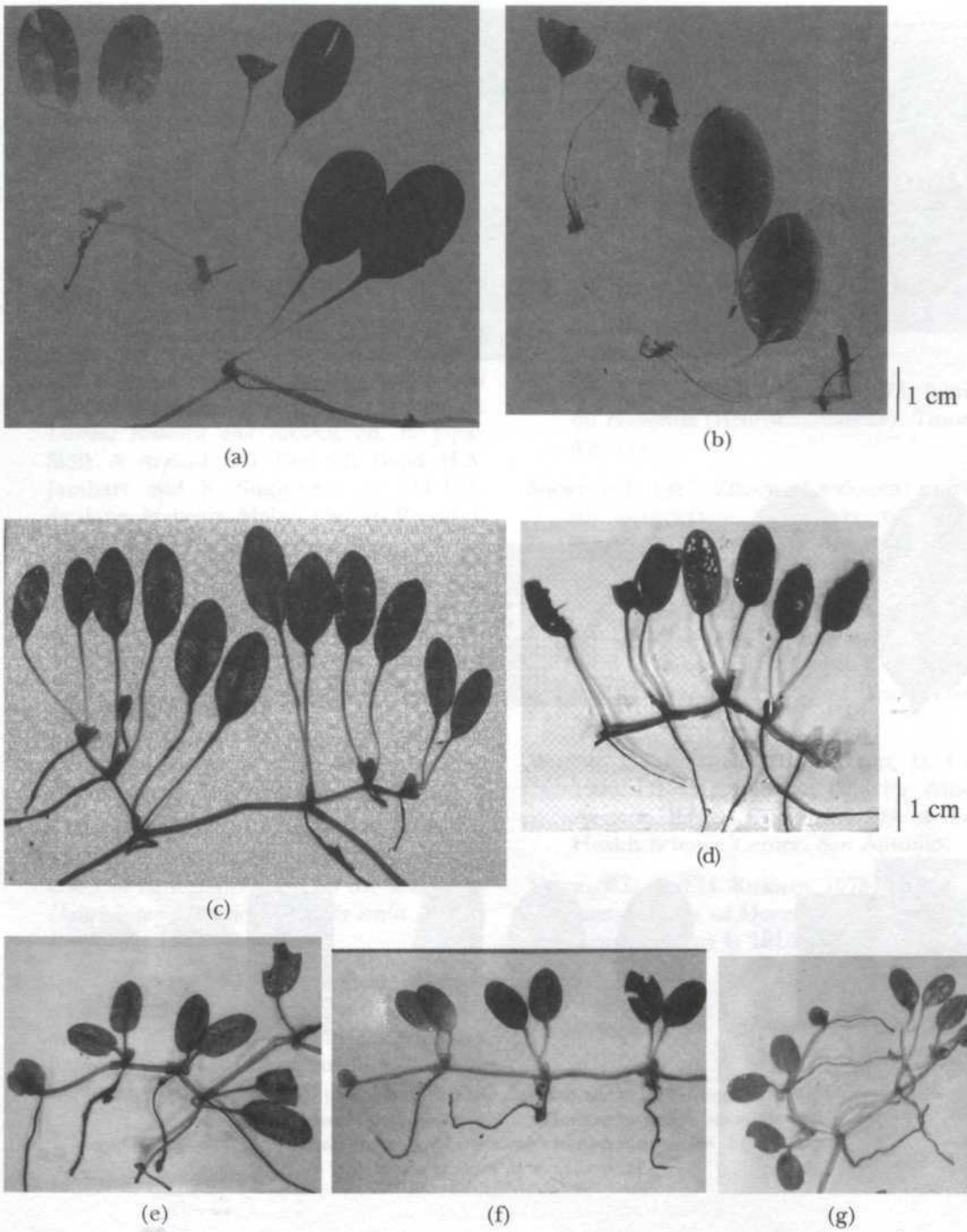

Plate 1: Cluster Analysis; a- big-leaved H. ovalis *from Merambong, unexposed sub-tidal shoal; b- big-leaved* H. ovalis *from Teluk Sepinong sub-tidal estuary; c- Intermediate-leaved* H. ovalis *from Punang, inter-tidal beach front; d- Intermediate-leaved* H. ovalis *from Merchang, inter-tidal lagoon; e, f, g- small-leaved* H. ovalis *from Teluk Kemang, inter-tidal coral reef flat, Merambong, sub-tidal exposed and Pulau Gaya, sub-tidal degraded coral reef respectively*

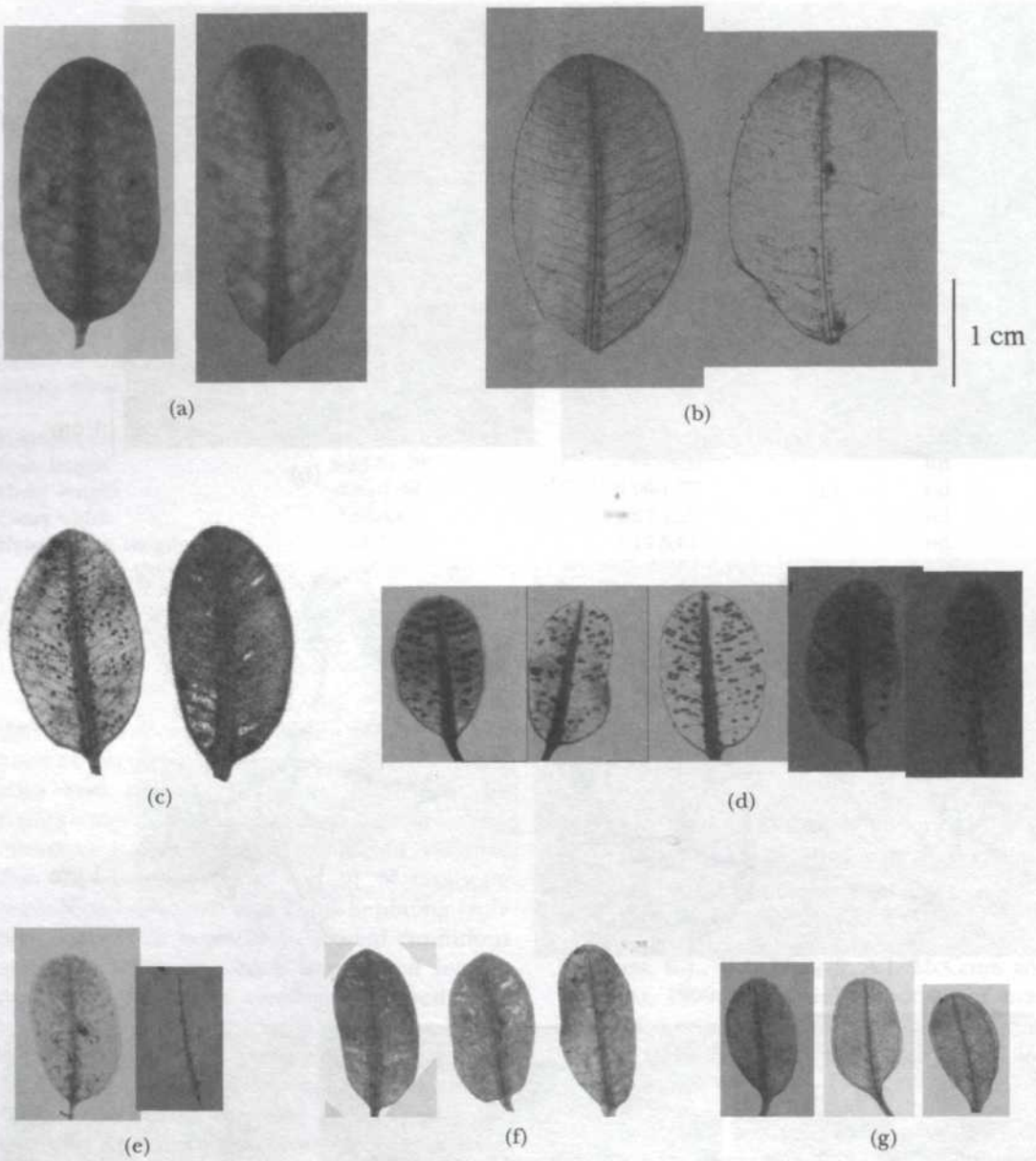

Plate 2: Category of H. ovalis *based on leaf sizes and shapes; a, b - big-leaved* H. ovalis-*ovate, oblong to spathulate; c, d - intermediate-leaved* H. ovalis-*ovate, oblong with pointed apex and e, f, g - small-leaved* H. ovalis-*round, ovate to obovate*

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Tissue Distribution of Heavy Metals (Cd, Cu, Pb and Zn) in the Green-lipped Mussel *Perna viridis* from Nenasi and Kuala Pontian, East Coast of Peninsular Malaysia

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Keywords: Heavy metals, *Perna viridis*, east coast of Peninsular Malaysia

ABSTRAK

Kajian lepas melaporkan kupang Perna viridis *adalah bertaburan secara meluas in persisiran pantai barat Semenanjung Malaysia tetapi bukan pada persisiran pantai timur Semenanjung. Dalam kajian ini, pemantauan dan pensampelan telah dilakukan dari Tumpat ke Mersing pada April 2004, tetapi kupang hanya boleh dijumpai pada Nenasi dan Kuala Pontian di persisiran pantai Pahang. Oleh itu, kajian ini memastikan bahawa kupang pada persisiran pantai timur adalah tidak bertaburan meluas seperti pada persisiran pantai barat Semenanjung. Kupang yang disampel telah dianalisis untuk kadmium (Cd), kuprum (Cu), plumbum (Pb) dan zink (Zn) dan kepekatan (µg/g berat kering) logam dalam tisu lembut keseluruhan adalah Cd: 1.89-2.13, Cu: 3.84-10.34, Pb: 7.95-8.84 dan Zn: 93.1-119.6. Keputusan menunjukkan bahawa sampel Kuala Pontian menimbun lebih tinggi kepekatan Cu (dalam tinggalan tisu lembut dan mantel) dan Zn (dalam tinggalan tisu lembut, mantel dan otot) apabila dibandingkan dengan Nenasi manakala Cd dan Pb menunjukkan tiada perbezaan yang signifikan (P> 0.05) di antara kedua-dua lokasi. Oleh sebab tiada input dan aktiviti antropogenik pada kedua-dua lokasi, perubahan kepekatan logam yang dibiotimbun di dalam tisu lembut berkemungkinan disebabkan oleh perbezaan yang signifikan dari segi saiz (panjang cangkerang dan lebar cangkerang) dan tahap kemasinan bagi kedua-dua lokasi. Faktor-faktor persekitaran yang lain juga mungkin boleh menyebabkan perbezaan dalam 'bioavailability' logam di dalam persisiran pantai yang dicerminkan oleh kepekatan berlainan di tisu lembut yang berlainan bagi kupang tersebut.*

ABSTRACT

Previous studies reported that the green-lipped mussel Perna viridis *were widely found on the west coast of Peninsular Malaysia but not on the east coast of the Peninsula. In this study, surveys and sampling were conducted from Tumpat to Mersing in April 2004, but the mussels were only found at Nenasi and Kuala Pontian in Pahang coastal waters. Therefore, from this study, we confirm that the mussels on the east coast were not as widely found as on the west coast of Peninsular Malaysia. The mussel samples collected were analysed for cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) and the metal concentrations (µg/g dry weight) were 1.89-2.13, 3.84-10.34, 7.95-8.84 and 93.1-119.6 for Cd, Cu, Pb and Zn, respectively. These results indicate that Kuala Pontian samples accumulated higher concentrations of Cu (in remaining soft tissue and mantle) and Zn (in remaining soft tissue, mantle and muscle) compared to those in Nenasi while Cd and Pb showed no significant difference (P> 0.05) between the two sample sites. Since there were no observable anthropogenic inputs or activities at the two sampling sites, the variation of metal concentrations accumulated in the soft tissues could be mostly attributable to the significant difference in size (shell length, and shell width) and salinity of the two sites. The other environmental factors could also cause differences in the metal bioavailabilities in the coastal waters that were reflected in different concentrations accumulated in the different soft tissues of the mussels.*

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INTRODUCTION

The *Perna viridis* mussels are widely distributed in the Asian Pacific coastal waters (Tanabe 2000) and they are an ecologically and economically important species. The mussel species has been proposed as a biomonitor of heavy metals (like its temperate counterpart *Mytilus*) in Hong Kong (Phillips 1985), Thailand (Sukasem and Tabucanon 1993) and in Peninsular Malaysia (Yap *et al.* 2003a). The wide distribution of *P. viridis* in coastal waters, sedentary life style, long life, easy identification and sampling (reasonably abundant and available throughout the year), tolerance of natural environmental fluctuations and pollution and enough tissue for metal analysis (Yap *et al.* 2003a) make it a good biomonitoring agent. *Perna viridis* are abundant on the west coast of Peninsular Malaysia since they are widely found in the west coastal waters (Yap *et al.* 2002; 2003b). Yap *et al.* (2003a) reported that the mussel species could only be found at Kg. Tg. Batu (east coast of Peninsular Malaysia). However, it is still not sure if *P. viridis* could be found in other sites on the east coast of the Peninsula. Therefore, in this study, surveys and sampling were conducted from Tumpat to Mersing along the east coast of Peninsular Malaysia. The objectives for this study are (1) to survey and to sample any available *P. viridis* along the coastal waters from Tumpat to Mersing of the east coast of Peninsular Malaysia, and (2) to measure the background concentrations of heavy metals in mussels collected in the east coast (Nenasi and Kuala Pontian).

METHOD AND MATERIALS

Sampling and Storage of Samples

The samples were collected during a sampling trip from Kuala Pontian and Nenasi in April 2004 (*Fig. 1*) on the east coast of Peninsular Malaysia. These two sites are known to be fishing and aquacultural sites and there were no signs of direct pollution. The collected mussels were immediately put into an ice compartment and transported to the laboratory for further analysis.

Sample Preparation

In each sampling site, twenty individual mussels were separated by gender and carefully dissected into different soft tissues (remainder, mantle, muscle, gills, gonad, foot, byssus and crystalline style). They were placed in aluminium foil and later dried in the oven for 72 hours at 60°C to a constant dry weight. Dried samples were then stored in clean plastic bags.

Metal Analysis

About 0.5g of dried soft tissues were digested in concentrated nitric acid (AnalaR grade, BDH 69%) and placed in a digestion block at 40°C for 1 hour and then fully digested at 140°C for 3 hours (Yap *et al.* 2002; 2003a). After cooling, it was diluted to a certain volume with double de-ionised water. The digested samples were then filtered through Whatman No. 1 (filter speed: medium) filter paper into acid-washed pillbox. All samples stored in acid-washed pillboxes were then analysed using an air-acetylene Perkin-Elmer™ flame atomic absorption spectrophotometer (AAS) model Analyst 800 for four heavy metals (Cd, Cu, Pb and Zn). Blank determination was carried out for calibration of the instrument. Standard solutions were prepared from 1000 mg/L stock solution provided by MERCK Titrisol for metals such as Cu, Zn, Cd and Pb (Yap *et al.* 2002) and data obtained from the AAS were presented in μg/g dry weight basis. Recoveries were done by using standard solutions as quality control samples. The percentage of recoveries for all metals was in the range of 90-110%.

RESULTS

Distributions of Cd, Cu, Pb, and Zn in the different soft tissues of *P. viridis* are shown in *Fig. 2(a)* and *2(b)*. For Cu, it was found that the crystalline style accumulated the highest level (Nenasi=33.5 μg/g, Kuala Pontian=68.4 μg/g) among all the different soft tissues in both sites followed by the byssus (Nenasi=18.3 μg/g, Kuala Pontian=22.2 μg/g) and other soft tissues.

For Zn, remainder of Nenasi accumulated the highest level (140 μg/g) followed by crystalline style (106 μg/g), muscle (80.7 μg/g), gills (79.3 μg/g), gonad (70.1 μg/g), mantle (65.7 μg/g), byssus (65.5 μg/g) and foot (62.4 μg/g). In Kuala Pontian, gills accumulated the highest level of Zn (167 μg/g) followed by remainder (140 μg/g), gonad (115 μg/g), muscle (113 μg/g), mantle (103 μg/g), byssus (94.9 μg/g), foot (72.1 μg/g) and crystalline style (59.9 μg/g).

For Cd, crystalline style for both sites accumulated the highest levels, which were 3.60 μg/g (Nenasi) and 4.14 μg/g (Kuala Pontian),

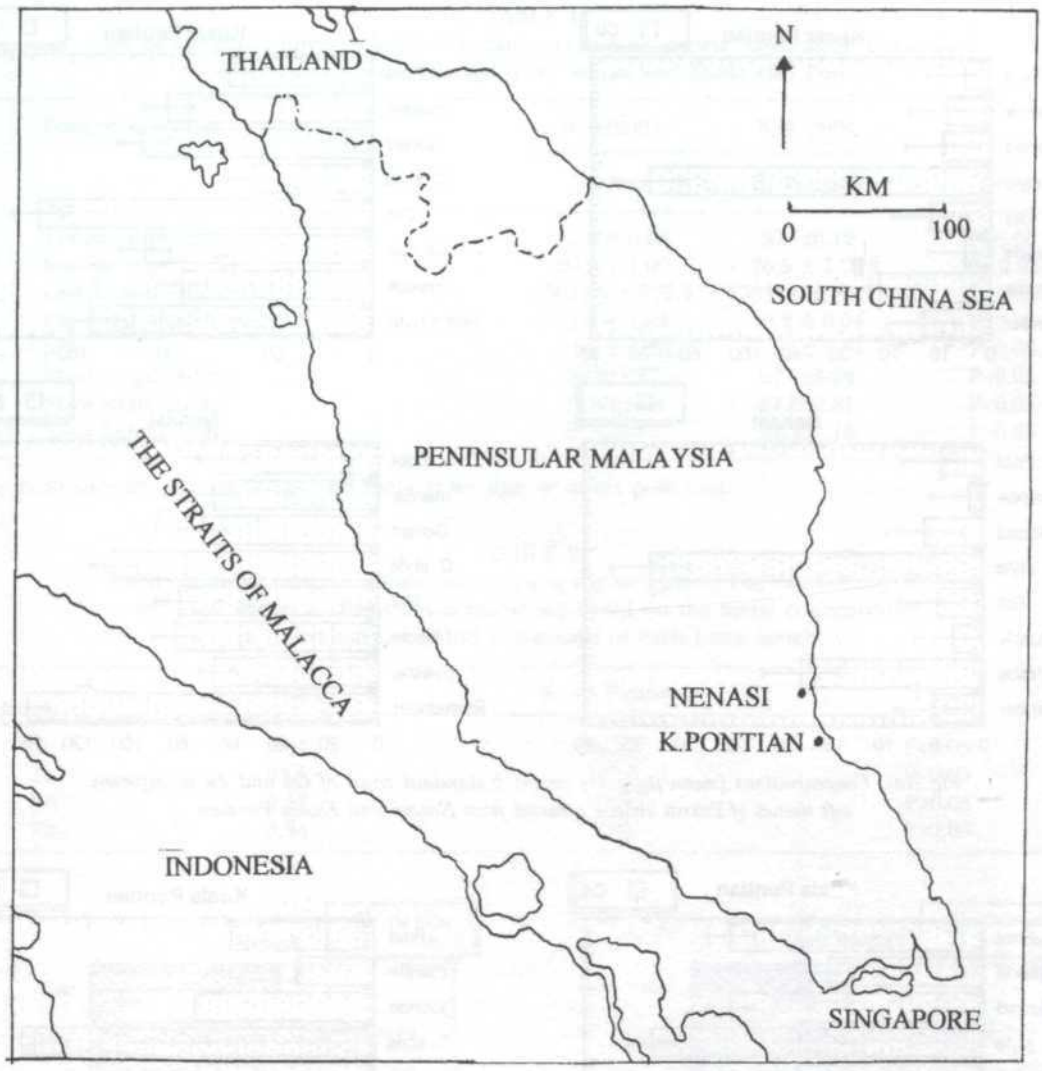


Fig. 1: Sampling sites of Kuala Pontian and Nenasi of the east coast of Peninsular Malaysia

followed by foot (Nenasi= 62.4 μg/g, Kuala Pontian= 3.16 μg/g) while the rest of the tissues showed insignificant (P> 0.05) differences among one another.

For Pb, gills (19.2 μg/g) and byssus (18.8 μg/g) accumulated the higher level when compared to the other tissues for Nenasi. In Kuala Pontian, crystalline style (30.9 μg/g) and byssus (26.3 μg/g) accumulated the highest level of Pb.

As shown in Table 1, shell length and shell width of the mussels collected from the two sampling sites show significant differences (P< 0.05) while shell height showed no significant difference (P> 0.05). The metal concentrations in total soft tissues, calculated based on metal concentration in each individual tissue and percentage of each tissue weight, are shown in Table 2. The concentrations (μg/g dry weight) of Cd, Cu, Pb and Zn ranged from 1.89-2.13, 3.84-10.34, 7.95-8.84 and 93.1-119.6, respectively.

Fig. 3 shows the comparison between metal concentrations of male and female *P. viridis*, based on T-tests carried out on the metal concentrations of remainder, mantle, muscle, gonad and foot. Most obviously, in both sites, female gonad accumulated significantly (P< 0.05) higher concentrations of Cu, Zn and Cd than in the male. However, for Pb, the male accumulated significantly (P< 0.05) higher level than the female. The rest showed no significant (P> 0.05) differences between the two genders.

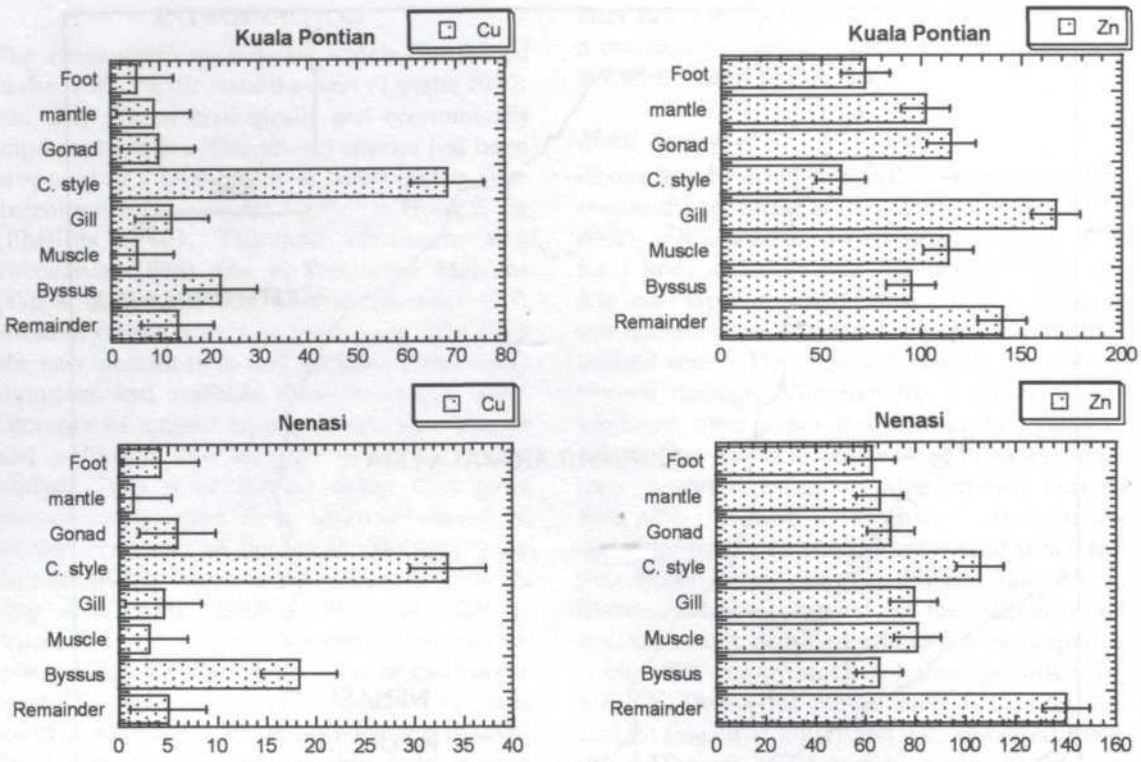


Fig.2(a): Concentrations (mean µg/g dry weight ± standard error) of Cu and Zn in different soft tissues of Perna viridis *collected from Nenasi and Kuala Pontian*

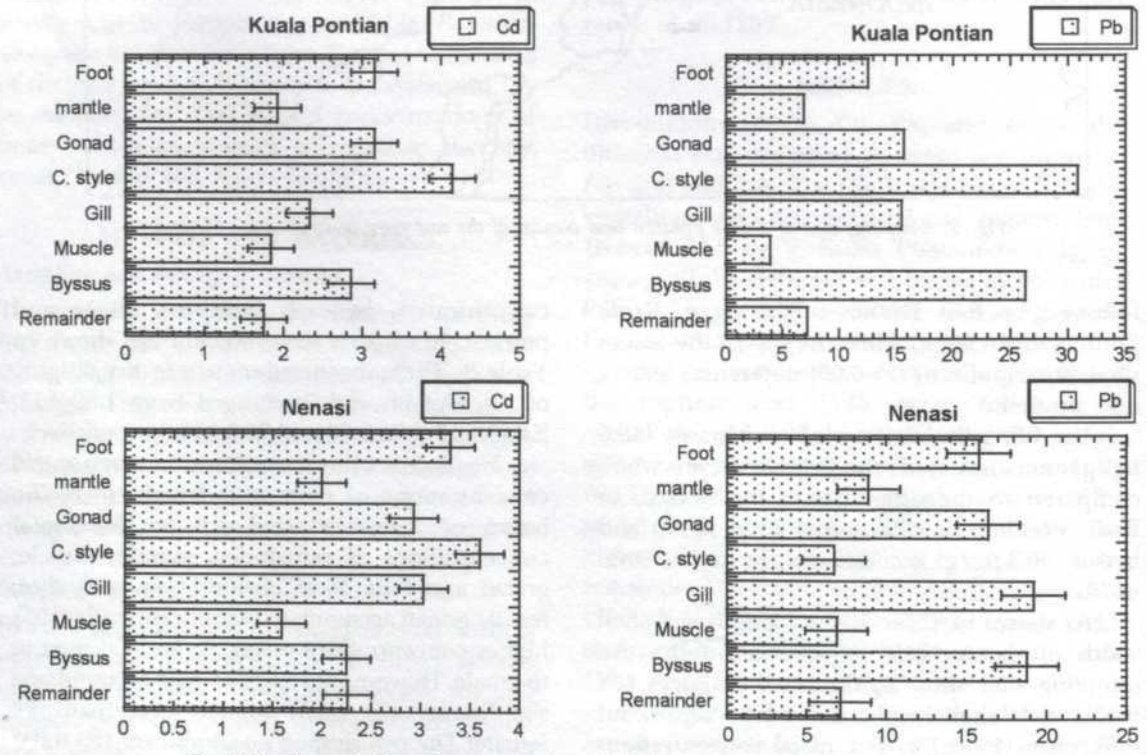


Fig. 2(b): Concentrations (mean µg/g dry weight ± standard error) of Cd and Pb in different soft tissues of Perna viridis *collected from Nenasi and Kuala Pontian*

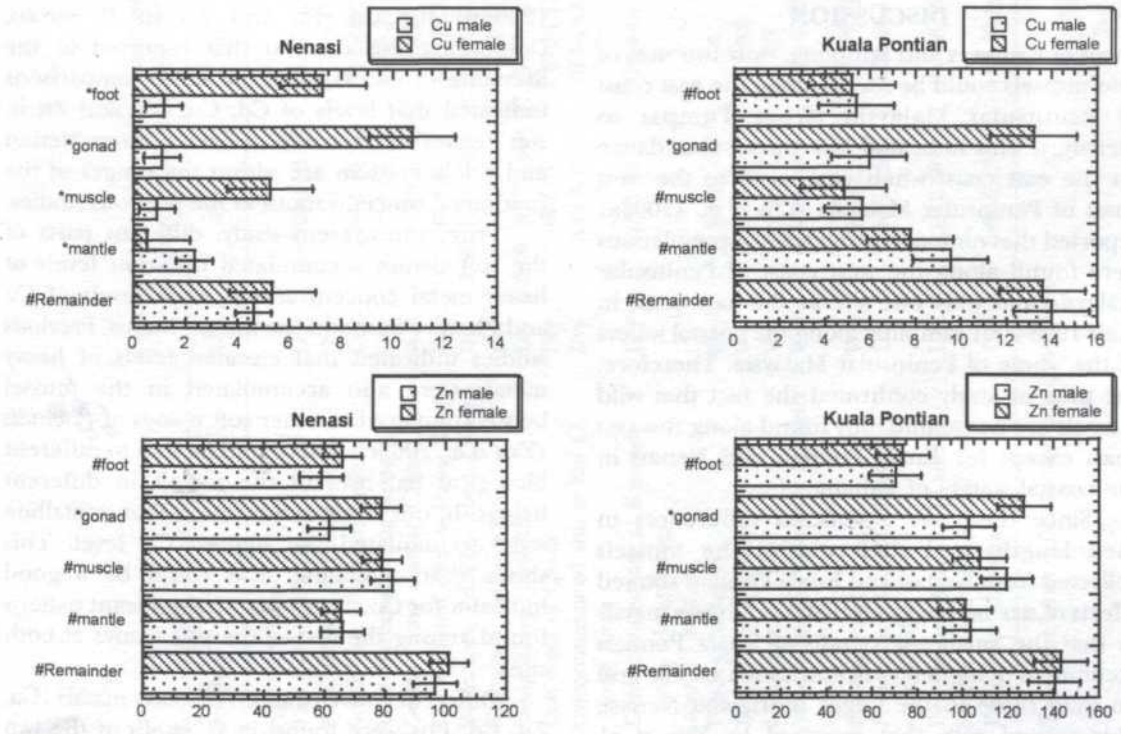
TABLE 1
Date of sampling, physico-chemical parameters and allometric data (mean ± standard error) of *Perna viridis* collected from Nenasi and Kuala (K.) Pontian

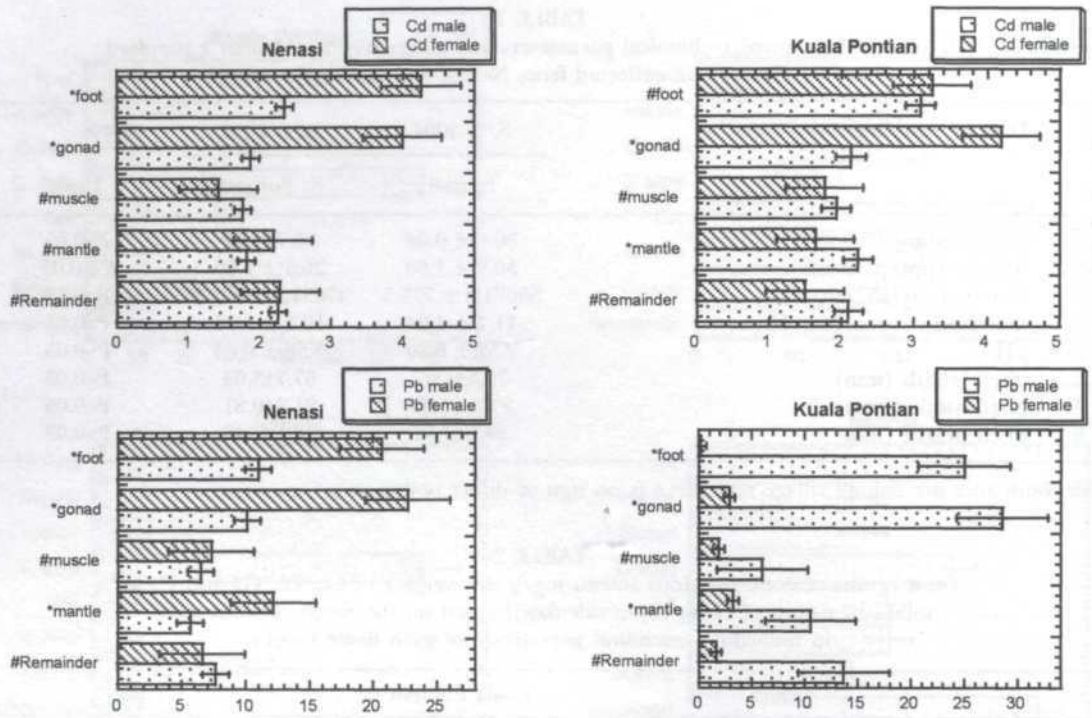
	Date of sampling	8/4/2004	8/4/2004	
		Nenasi	K. Pontian	T-test
1.	Temperature (°C)	30.6 ± 0.08	30.7±0.12	P>0.05
2.	Salinity (ppt)	30.9 ± 1.00	26.5 ± 1.00	P< 0.05
3.	Conductivity(μS/cm)	55671.5 ± 775.5	47831.5 ± 848.5	P<0.05
4.	Dissolved oxygen (mg/L)	11.4 ± 1.84	10.2 ± 0.04	P>0.05
5.	pH	7.52 ± 0.30	7.58 ± 0.04	P>0.05
6.	Shell length (mm)	76.6±1.67	67.7±3.04	P<0.05
7.	Shell width (mm)	27.3 ±1.64	21.8±0.81	P<0.05
8.	Shell height (mm)	29.5±3.52	34.1±1.16	P>0.05

Note: Both sites are fishing village and there is no sign of direct pollution.

TABLE 2
T-test results of concentrations (mean mg/g dry weight) of Cu, Zn, Cd and Pb in total soft tissues of *Perna viridis* calculated based on the metal concentration in individual tissue and percentage of each tissue weight

	Nenasi	Kuala Pontian	T-test
Cu	3.84	10.34	P<0.05
Zn	93.1	119.6	P>0.05
Cd	2.13	1.89	P>0.05
Pb	8.84	7.95	P>0.05





* Significant difference (P<0.05)
\# No significant difference (P>0.05)

Fig. 3: Heavy metal concentrations (mean µg/g dry weight ± standard error) in different soft tissues of male and female Perna viridis *in Nenasi and Kuala Pontian*

DISCUSSION

From our surveys and sampling, only two sites of wild mussels could be found along the east coast of Peninsular Malaysia (from Tumpat to Mersing). This indicated less mussel abundance on the east coast when compared to the west coast of Peninsular Malaysia. Yap *et al.* (2003a) reported that nineteen geographical populations were found along the west coast of Peninsular Malaysia and only one site at the east coast in their 1998-2001 sampling along the coastal waters of the whole of Peninsular Malaysia. Therefore, the present study confirmed the fact that wild mussels are not abundantly found along the east coast except for Kuala Pontian and Nenasi in the coastal waters of Pahang.

Since there are significant differences in shell lengths and shell widths, the mussels collected from Nenasi and Kuala Pontian showed effects of size on bioaccumulation of heavy metals in that the smaller *P. viridis* at Kuala Pontian accumulated higher concentrations of Cu and Zn than those in the larger mussels at Nenasi. This agreed with that reported by Yap *et al.* (2003b) for Cd, Pb and Zn in *P. viridis*. Comparing this data to that reported in the literature (Table 3), all of the above comparisons indicated that levels of Cd, Cu, Pb, and Zn in soft tissues of *P. viridis* collected from Nenasi and Kuala Pontian are within the ranges of the four metal concentrations of the previous studies.

From the present study, different parts of the soft tissues accumulated different levels of heavy metal concentrations. High levels of Cu and Pb were found in the mussel byssus. Previous studies indicated that elevated levels of heavy metals were also accumulated in the mussel byssus compared to other soft tissues of *P. viridis* (Yap *et al.* 2003c). This could be due to different biological half-lives of the metals in different tissues. In our study, it was found that crystalline style accumulated the highest Cu level. This shows that crystalline style could be a good indicator for Cu. There was no significant pattern found among the rest of the soft tissues at both sites.

Different concentrations of heavy metals (Cu, Zn, Cd, Pb) were found in *P. viridis* of the two

TABLE 3
A comparison of reported concentrations (μg/g) of Cd, Cu, Pb and Zn in the soft tissues of *Perna viridis* with the present results (WB: weight basis)

Location	WB	Cd	Cu	Pb	Zn	References
Regional studies						
The Gulf of Thailand	Dry	<0.02-19.1	1.50-11.3	-	25.7-79.0	Sukasem and Tabucanon (1993)
Putai coastal of Taiwan	Dry	-	1.78-5.41	-	14.4-25.7	Han *et al.* (1997)
South east coastal of India	Dry	1.59-4.40	33.6-49.2	2.48-6.92	60.4-94.1	Senthilnathan *et al.* (1998)
Tolo Harbour, Hong Kong	Dry	0.45-1.44	6.02-24	2.02-4.36	90.0-135	Wong *et al.* (2000)
Guang Dong market, China	Wet	0.38	2.05	0.18	9.9	Fang *et al.* (2001)
Fish cultured sites at Hong Kong	Dry	0.31-0.87	19.0-20.1	4.34-25.9	96.7-201	Wong *et al.* (2001)
Venezuala/Trinidad coastal waters	Dry	0.81 (0.1-3.05)	8.7 (5.1-17.2)	-	124.5 (446)	Astudillo *et al.* (2002)
Singapore coastal waters	Dry	< 0.20	28 (23-35)	5.6	280 (185-446)	Bayen *et al.* (2004)
Hong Kong (1998-2003)	Dry	0.66 (0.17-2.90)	37.15 (8.9-130)	7.65 (2.0-20.0)	116.4 (67-170)	Liu and Kueh (2005)
East coast of China	Dry	0.48-5.31	1.45-54.17	0.81-2.93	66.1-137.7	Fung *et al.* (2004)
Uncontaminated site at Kat O, Hong Kong	Dry	3.13-5.4	10.1-15.8	3.10-5.01	104-115	Nicholson and Szefer (2003)
Contaminated site at Kennedy Town, Hong Kong	Dry	1.02-1.30	16.4-18	3.76-6.98	126-152	Nicholson and Szefer (2003)
Malaysia's studies						
Penang, Malaysia	Dry	BDL	8	7	76	Sivalingam and Bhaskaran (1980)
Bau Merbok, Perak	Wet	0.05	1.93	0.24	13.8	Liong (1986)
Lekir, Perak	Wet	0.18	2.7	0.52	22.8	Devi (1986)
West coast of Peninsular Malaysia (8 sites)	Wet	0.10-1.80	1.00-3.00	0.50-5.90	10.8-30.0	Ismail (1993)
Penang waters	Wet	0.12-0.22	1.32-3.42	0.43-1.49	12.8-21.9	Din and Jamaliah (1994)
Peninsular Malaysia (20 sites; 1998-2001)	Dry	0.68-1.25	7.76-20.1	2.51-8.76	75.1-129	Yap *et al.* (2003a)
Peninsular Malaysia (10 sites)	Dry	0.10-2.88	2.09-8.55	0.20-1.69	52.12-95.43	Yusof *et al.* (2004)
Nenasi and Pontian, east coast of Peninsular Malaysia	Dry	1.89.-2.13	3.84-10.34	7.95-8.84	93.1-119.6	This study

WB=weight basis. BDL=below detection limit

sites between genders. This could be due to the gonadal condition (Lobel and Wright 1982; Karaseva 1993). Female *P. viridis* were found to accumulate more heavy metals than male and this could be due to the presence of eggs, which are not released. The weight contribution of the eggs or ovum is also larger than the sperms of the male *P. viridis* and therefore was able to store more heavy metals (Yap *et al.* 2005 In press).

Since there were no observable pollution activities found between Nenasi and Kuala Pontian, the difference in metal concentrations between the two sites could be due to the significant ($P< 0.05$) differences in shell length, shell width and salinity. The salinity of both sites showed significant difference ($P< 0.05$) while temperature, dissolved oxygen and pH showed no significant difference ($P> 0.05$). It has been reported by Mo and Neilson (1993) that salinity could affect metal availability and changes in salinity may affect several physiological processes that influence the accumulation of trace metals by bivalves.

CONCLUSION

This study confirmed that *P. viridis* is not abundantly found on the east coast of Malaysia compared to the west coast. Different soft tissues have different binding affinities to heavy metals (Cu, Zn, Cd, and Pb). The crystalline style was found to accumulate high levels of Cu and therefore could be a good indicator of Cu contamination compared to other soft tissues. Differences in heavy metal levels between genders were also found and could be due to gonadal conditions (differences of weight contribution of sperms and eggs). The different levels of heavy metals found in the soft tissues of *P. viridis* collected from Nenasi and Kuala Pontian could be due to the significant ($P< 0.05$) differences in mussels' shell length and shell width and salinity of seawater.

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Purification and Characterisation of β-1,3-glucanase from *Trichoderma harzianum* BIO 10671

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Keywords: ***Tricoderma harzianum*, β-1,3-glucanase, purification, characterisation, pH, metal ion**

ABSTRAK

Enzim β-1,3-glukanase dari cecair kultur Trichoderma harzianum *BIO 10671 telah berjaya ditulenkan melalui pemendakan dengan 80% aseton, diikuti kromatografi penukaran ion menggunakan Neobar AQ dan pemfokusan kromatografi menggunakan kolum Mono P HR 5/20. Dua β-1,3-glucanase bersaiz 32kDa dan 66kDa telah ditulenkan dan ditunjukkan pada SDS-PAGE. pH optimum bagi aktiviti enzim ini ialah pada pH 4.5 dan aktiviti maksimum dicerap pada 45°C. Manakala aktiviti enzim direncat 20-45% oleh 20mM Zn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} dan Fe^{2+}. Aktiviti hidrolisis β-1,3-glucanase tertinggi didapati pada laminarin disebabkan persamaan ikatan β-glikosidik dan diikuti masing-masing pada pustulan, glukan dan selulosa.*

ABSTRACT

β-1,3-glucanase enzyme from culture filtrate of Trichoderma harzianum *BIO 10671 was successively purified by precipitation with 80% acetone followed by anion-exchange chromatography on Neobar AQ and chromatofocusing on a Mono P HR 5/20 column. Two β-1,3-glucanases of 32kDa and 66kDa were purified to homogeneity as judged by SDS-PAGE. The pH optimum for the enzymes activity was pH 4-5 and maximum activity was obtained at 45°C. Enzyme activity was slightly inhibited by 20-45% in its activity by 20mM of Zn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{2+}. The highest β-1,3-glucanase hydrolysis activity was obtained on laminarin due to the similarity on β-glucosidic bonds and followed on pustulan, glucan and cellulose, respectively.*

INTRODUCTION

Trichoderma spp. has long been recognised as an effective biocontrol agent of plant pathogens. The antagonistic mechanisms involve are chemotropism (Chet *et al.* 1981), lectin-mediated recognition (Inbar and Chet 1994) and the formation of trapping and penetrating structures (Elad *et al.* 1983a,b). These processes are enhanced by the secretion of extracellular enzymes such as chitinases, β-glucanases and proteases (De la Cruz *et al.* 1992; Lorito *et al.* 1994; Flores *et al.* 1997).

Trichoderma harzianum produces β-glucanases which target the β–glucan chain in fungal cell walls. These enzymes are common in fungi and are classified according to their cleave type of ß-glucosidic linkage and the mechanism of substrate attack. β-1,6-glucan and α-1,3-glucan represent relatively minor components of fungal cell walls (Lora *et al.* 1995). Both β-1,3 and β-1,6-glucanase activities are secreted simultaneously in *T. harzianum* (De la Cruz *et al.* 1993).

Enzymes with ß-1,3-glucanase activity have been reported in fungi, bacteria, actinomycetes and higher plants (Bielecki and Galas 1991). β-1,3-glucanases hydrolyse the *O*-glycosidic linkages of β-1,3-glucan chains by two mechanisms. Exo-β-glucanase hydrolyses the β-glucan chain by sequentially cleaving the glucose residues sequentially from the non-reducing ends, while endo-β-glucanase cleaves β-linkages at random sites along the polysaccharide chain, thus

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releasing smaller oligosaccharides (Pitson *et al.* 1993). In most cases, multiple b-glucanases have been found rather than a single enzyme (Vazquez *et al.* 1998).

At least three endo-β-1,3-glucanases with different molecular weights which are 17kDa, 29kDa and 78kDA have been purified from *T. harzianum* (Mrsa *et al.* 1993; De la Cruz *et al.* 1995; Thrane *et al.* 1997). Purified endo-ß-1,3-glucanase isolated from *T. harzianum* inhibited the germination of encysted zoospores and the elongation of germ tubes of *Pythium ultimum in vitro* (Thane *et al.* 1997).

The role of fungal β-glucanases depends on their types and the most recognised functions of β-1,3-glucanases in *Trichoderma* is mycoparasitism (Pitson *et al.* 1993) since it can destroy the polymer fabric of the pathogen cell wall more effectively (Mauch *et al.* 1988). ß-1,3-glucanase also has other several functions such as (i) physiological role in cell wall formation and morphogenetic processes during the growth and differentiation of fungal hyphae (Wong and Maclachlan 1980); (ii) as autolytic enzymes to mobilise ß-glucan under conditions of carbon and energy source exhaustion (Papavizas 1985) and (iii) a nutritional role in saprophytes and mycoparasites (De la Cruz *et al.*, 1995). In this study, we try to purify and characterise the β-1,3-glucanase of local *T. harzianum* strain.

MATERIALS AND METHODS

Strains

T. harzianum BIO 10671 was obtained from the laboratory collection, Department of Biology and grown on Potato Dextrose Agar (PDA) for 4 days at 28°C.

Extracellular β-1,3-glucanase Purification

The spore suspensions of *Trichoderma harzianum* BIO 10671 in approximately $1x10^7$ spores/ml were added to 25 ml of Trichoderma complete medium (pH 5.5; 0.5% w/v glucose) to produce seed cultures. Seed cultures were shaken at 180rpm at 28°C for 24 hours before filtering through sterile Whatman No-1, paper then washed three times with sterile distilled water and transferred into 25 ml of Trichoderma Minimal Medium (pH 5.5; 1.0% w/v *Pleurotus sajor-caju* mycelium). Culture filtrates were harvested, filtered through Whatman no.1 filter paper, centrifuged at 6000xg for 10 minutes then dialysed against distilled water for at least 24 hours at 4°C.

The purification was carried out using anion-exchange method as described by Lima *et al.* (1997). Crude extracellular β-1,3-glucanase was precipitated with ice-cold 80% acetone and incubated at –20°C for 30 minutes. The precipitate was recovered by centrifugation at 28000xg for 10 minutes at 4°C, re-dissolved in distilled water and dialysed against distilled water for another 24 hours at 4°C.

Ninety microliter (90μl) of Buffer A (50mM Tris-HCL, pH 7.5) and 20μl of 1M Tris pH 7.5 were added to the dialysed crude culture sample and the pH was adjusted to pH 7.0. The sample was centrifuged at 12000xg for 10 min. Meanwhile, an anion exchange Neobar AQ column was washed with 10 times column volumes of buffer B (50mM Tris-HCL pH 7.5; 1M NaCl) and followed by 10 times column volumes of buffer A. The resulting supernatant was loaded onto the column and eluted at a flow rate of 1ml/min and bound protein was eluted with a 0-1mM NaCl gradient. The fractions with high b-1,3-glucanase activity were pooled before being dialysed against distilled water for 24 hours at 4°C.

Chromatofocusing was performed on a Mono P HR 5/20 column equilibrated with 25 mM Tris-CH_3COOH, pH 8. Freeze dried anion exchange samples were dissolved in 1ml of distilled water and adjusted to pH 8.0 by the addition of 25 mM Tris pH 7.5. Following this, centrifugation was done at 1200xg for 10 minutes and then the supernatant was loaded onto the column. Protein were eluted $1ml.min^{-1}$ with a pH gradient from pH 8.0 to pH 5.5 formed by Polybuffer PB 74/96. The fractions were collected and assayed for β-1,3-glucanase activity. The active fractions were pooled and dialysed against distilled water for 24 hours at 4°C.

The dialysed fraction was collected and assayed for β-1,3-glucanase activity using the b-glucanase assay method (Somogyi 1952) which involves the estimation of reducing sugars amount released from laminarin. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on collected fraction according to Laemmli (1970) using 10% acrylamide gels and stained with Coomasie R-250 brilliant blue (Sigma). Low molecular mass standard proteins were used for molecular mass determination.

The Characterisation of Purified β-1,3-glucanase

In this experiment, β-1,3-glucanase activity was assessed under standard conditions as recommended by Lorito *et al.* (1994) with three replicates.

1. Optimal temperature and temperature stability of β-1,3-glucanase activity

The optimal temperature for β-1,3-glucanase activity was determined by measuring the reducing sugars released after 30 minutes incubation at temperatures between 25°C-75°C at increments of 5°C. Meanwhile, the temperature stability for β-1,3-glucanase activity was examined by maintaining the purified β-1,3-glucanase for 1h at temperatures between 25°C-75°C at increments of 5°C before determining the β-1,3-glucanase activity.

2. Optimal pH and pH stability of β-1,3-glucanase activity

The effect of pH on enzyme activity was determined by varying the pH of the reaction mixture between pH 4-8 at increments of 1 pH unit. The pH of the mixture was adjusted to intended pH with 50mM sodium citrate buffer. pH stability was determined by incubating purified β-1,3-glucanase enzyme at pH 4-8 for 1 hours at 37°C before the pH was changed to pH 5 prior to β-1,3-glucanase activity determination.

3. Effect of metal ions on β-1,3-glucanase activity

The effect of several metal ions on the activity of b-1,3-glucanase was investigated. The metal ions used for this study were Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+}. Twenty millimolar (20mM) of metal ion solution in sodium acetate buffer pH 5.5 was prepared and added into purified β-1,3-glucanase enzyme prior to activity determination.

4. β-1,3-glucanase activity on different substrates

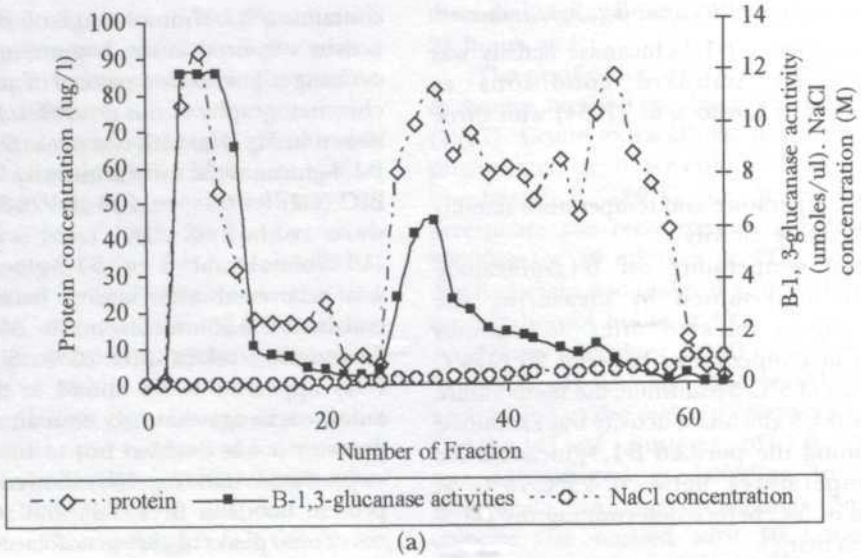
β-1,3-glucanase activity on several substrates was determined. These included pustulan (1.0% w/v), laminarin (1.0% w/v), cellulose (1.0% w/v) and glucan (1.0% w/v). Each substrate was prepared in 0.05M sodium acetate buffer pH 5.5.

RESULTS AND DISCUSSIONS

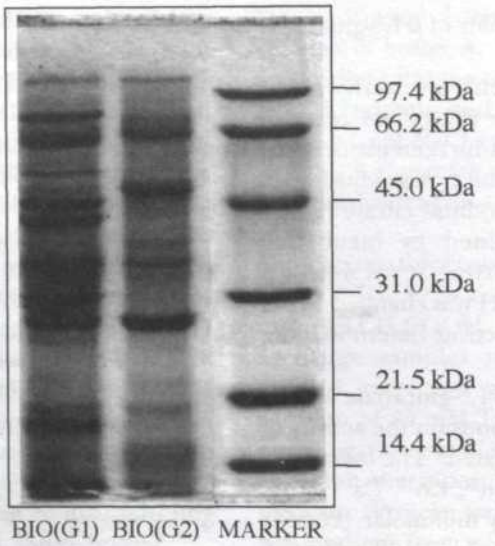
The mixture of β-1,3-glucanase was purified from *T. harzianum* using acetone precipitation. The crude enzyme from *T. harzianum* BIO 10671 containing 23.87mmoles/ml of β-1,3-glucanase activity was used at the beginning of the anion exchange. The elution pattern of anion exchange chromatography of this crude enzyme fraction is shown in *Fig. 1(a)* with two peaks for protein and β-1,3-glucanase activity arbitrarily BIO (G1) and BIO (G2) for fraction 4-10 and 28-36, respectively were obtained. In total, only 69.4% (16.57mmoles.ml^{-1}) of β-1,3-glucanase activity was recovered after anion exchange, which consist of 9.03mmoles/ml in BIO (G1) and 7.54mmoles/ml in BIO (G2). Since the BIO (G2) appeared to be bound at the middle of anion exchange that may contain contaminated proteins it was decided not to use it in further steps. SDS-PAGE (*Fig. 1(b)*) showed several major protein bands in BIO (G1) and (G2).

Three peaks of chromatofocusing containing β-1,3-glucanase activity were detected (*Fig. 2(a)*) and fractions 11-15 which comprised 60% of the total activity with 30.21μmoles/ml had been chosen for SDS-PAGE. Analysis of this pool fraction using SDS-PAGE revealed two clear protein bands at molecular mass around 66kDa and 32kDa (*Fig. 2b*) suggesting the presence of two isoforms of β-1,3-glucanase. The existence of purified β-1,3-glucanase around 33kDa to 66kDa with isoform has been reported previously. Lorito *et al.* (1994) and Noronha and Ulhoa (1996) described the characterisation of purified 32kDa β-1,3-glucanase, while De la Cruz *et al.* (1995) reported that β-1,3-glucanase from *T. harzianum* with a molecular weight of 66kDa was due to at least 4 sub-unit proteins. Meanwhile Vazquez *et al.* (1998) obtained a 39kDa b-1,3-glucanas with at least six sub unit proteins.

Several other isoform or subunit of β-1,3-glucanase with different catalytic activities, molecular weights and substrate specificities have been found in supernatants from *T. harzianum* culture and the differences in number of isoform or protein subunit for purified β-1,3-glucanase is not new because the molecular mass of β-1,3-glucanase appears to vary between species and also within species (Pitson *et al.* 1993). It is not known whether the existence of isoform for same β-1,3-glucanase molecular weight is the product of the same or separate β-1,3-glucanase gene. According to Mrsa *et al.* (1993), one of the reasons for the differences is the anomalous migration of protein in the gels rather than to the post-translation processing of the polypeptide chain; sometimes the type of growth substrate



(a)

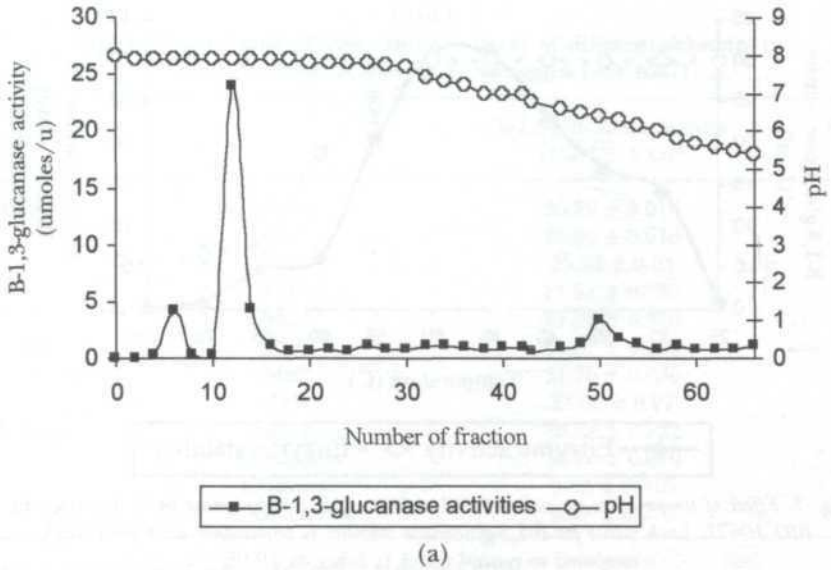


(b)

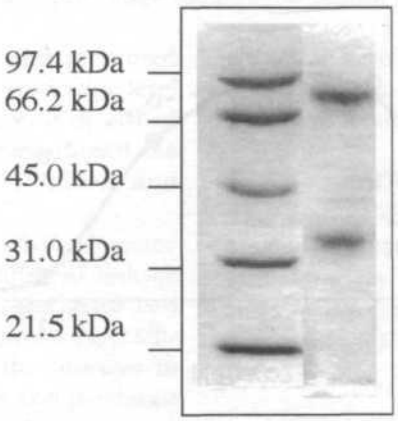
Fig. 1: Purification of b-glucanases by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5M NaCl gradient (a) Elution profile of BIO 10671 anion exchange for protein on Neobar AQ exchanger column with fraction 4-10 (BIO G1) and 28-36 (BIO G2). (b) SDS-PAGE (10%) of protein from pooled peaks and stained with Coomassie blue

used can also influence the number of bands on SDS-PAGE (Vazquez *et al.* 1998). Species, type of reaction (exo- or endo-) and method of purification may have an effect on characterisation even for the same type of purified β-glucanase (Matsuzawa *et al.* 1996).

Optimal activity for short term incubation is often seen at temperatures in the range of 30°C to 50°C and many fungal b-glucanases appear stable at temperatures up to 50°C to 60°C (Pitson *et al.* 1993). The effect of temperature on β-1,3-glucanase is shown in *Fig. 3* an optimum operation temperature at 45°C. β-1,3-glucanase activities are not affected by the temperature of incubation up to 50-55°C. In general the temperature optimum and stability for β-1,3-glucanase in this work were lower than those reported previously by Tangarone *et al.* (1989) and Matsuzawa *et al.* (1996) which had optimal temperatures of 55°C and 60°C, respectively.



(a)



(b)

Fig. 2: Chromatofocusing of BIO 10671 (G1) on mono P HR 5/20 with a 8.5 to 5.5 pH gradient. (a) Elution profile b-1,3-glucanase. (b) SDS-Polyacrylamide (10%) electrophoresis of for b-1,3-glucanase protein from pooled peaks (fraction 11-15) and stained with Coomassie blue

Very low activity was observed after the temperature exceeded 60°C and this can be concluded that even though *T. harzianum* was categorised as warm climate fungi, some of the enzymes was unable to sustain their function at the maximum level in hot condition (Danielson and Davey 1973). Many of the enzymes have optimum temperature higher than their thermal stability which indicates that they may be stabilised by their substrate (Bodenmann *et al.* 1985).

Optimal pH was determined by varying the pH of the reaction mixture at 37°C. The optimal activity of fungal β-1,3 glucanase usually appears in acidic conditions, often between pH 4.0 to 6.0. Most fungal β-glucanases have a broad pH optima, retaining their activity over 2 to 3 pH units while some have a wider range (Pitson *et al.* 1993). As shown in *Fig. 4*, there is not much difference in activities related to pH with the enzymes being stable over a pH range of approximately 4-6 and most being active at a pH of between 4-5. Theodore and Panda (1995) found that the optimum pH for β-1,3-glucanase production in *T. harzianum* in both surface and submerged culture processes was at an initial pH

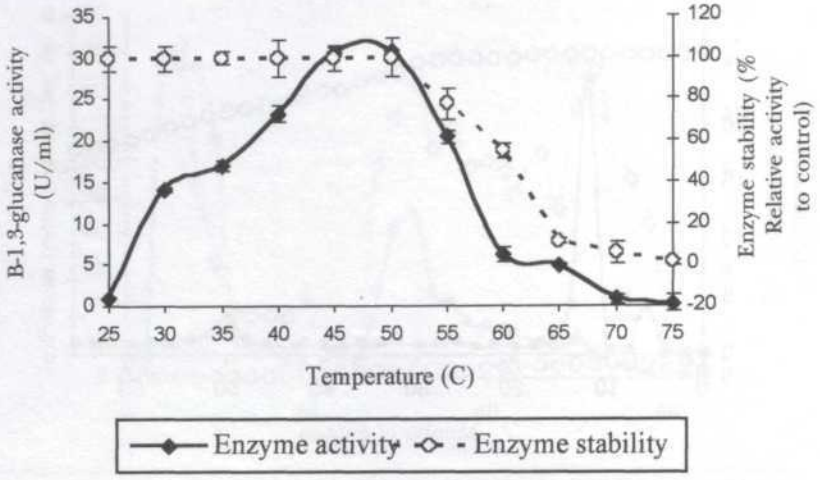


Fig. 3: Effect of temperature on activity and stability of β-1,3-glucanase in T. harzianum *BIO 10671. Each value for β-1,3-glucanase stability is represented as a percentage compared to control which is taken as 100%*

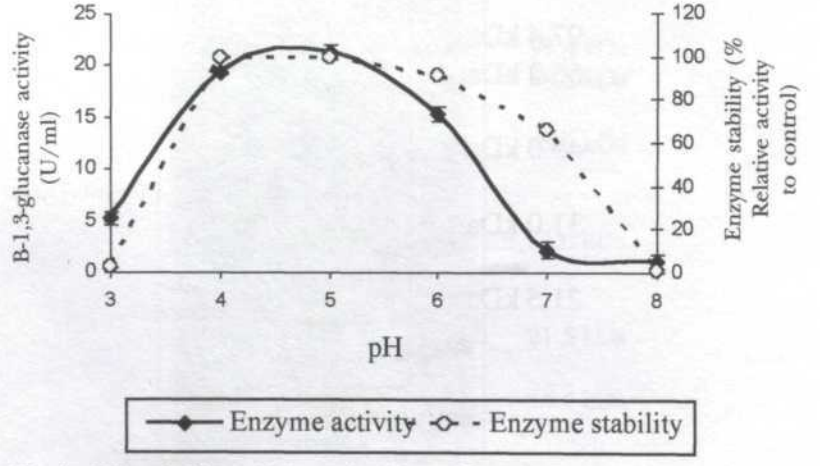


Fig. 4: Effect of pH on activity and stability of β-1,3-glucanase in T. harzianum *BIO 10671. Each value for β-1,3-glucanase stability is represented as a percentage compared to control which is taken as 100%*

of 4.7. However, it is considered low optimum compared to that has been observed for *T. harzianum* BIO 10671 in this work. The maximum activity for β-1,3-glucanase observed in the acidic conditions for this work was correlated with the observation on *Trichoderma* growth, where *Trichoderma* spp. appeared to be more prevalent in acidic soils (Gochenaur 1970). The concentration of H^+ may have a strong impact on the nutrient transport mechanism and also on the purified enzyme activity (Vazquez *et al.* 1998).

Enzyme activity was slightly inhibited (20-35%) by 1mM of Co^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} (Hiura *et al.* 1987) but significantly inhibited in the presence of 1mM $HgCl_2$, $MnCl_2$, $KmnO_4$ (Tangarone *et al.* 1989); 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol (Thrane *et al.* 1997). The effect of several known metal ion inhibitors on the activity of the purified enzymes is shown in Table 1. β-1,3-glucanase activity was inhibited by these metal ions with Cu^{2+} ion in the assay mixture resulting in the highest relative inhibition for β-1,3-glucanase (47.5%). A similar inhibition by Mg^{2+} and Cu^{2+} ions on b-1,3-glucanase activity was also obtained in this work. Metal ions affected the level of β-1,3-glucanase activity by binding to the recognition site in

TABLE 1
Effect of metal ions (20mM) and 1% (w/v) of different substrate on β-1,3-glucanase activity in *T. harzianum* BIO 10671

		B-1,3-glucanase activity ($U.ml^{-1}$) ± s.d*	Relative activity (%)**
Metal ions (20mM)	None	30.70 ± 0.016	100
	Zn^{2+}	25.26 ± 0.610	82.3
	Ca^{2+}	25.45 ± 0.03	82.9
	Co^{2+}	21.61 ± 0.280	70.4
	Mg^{2+}	21.02 ± 0.950	68.5
	Cu^{2+}	16.67 ± 0.010	54.3
	Mn^{2+}	21.76 ± 0.630	70.9
	Fe^{2+}	23.66 ± 0.97	77.1
Substrate (1% w/v)	Pustulan	26.52 ± 0.722	86.4
	Laminarin	30.70 ± 0.610	100
	Cellulose	0.33 ± 0.015	2.5
	Glucan	9.67 ± 0.775	31.5

*The results are mean values of triplicate tests ± standard error.
**Relative activity (%) is expressed as a percentage compared to control which is taken as 100%.

enzyme for substrate hydrolysis and prevented the activity from reaching the maximum level (Watanabe *et al.* 1988). Therefore, it disturbed the environmental condition, which caused the slowing down of the activity (Widden and Scattolin 1988).

Table 1 also showed the activity detected when different substrates with different linkage bonds were used. Purified β-1,3-glucanase had an ability to hydrolyse all the substrates especially on laminarin because most of the linkages in laminarin are β-1,3-linked glucan and produced a large amounts of glucose (De la Cruz *et al.* 1995). β-1,3-glucanase still can split the linkage for non specific substrates such as pustulan (β-1,6-glucan bonds) and cellulose (β-1,4-glucan bonds) but at a very low level of activity. *T. harzianum* EP-1 appeared to be a typical exo-β-1,3-glucanase but was still able to hydrolyse b-glucans containing β-1,3- and β-1,4-lingkages such as in barley glucan, or β-1,3- and β-1,6-linkage such as yeast glucan (Matsuzawa *et al.* 1996). The highest activity was obtained when the enzyme hydrolysed substrate containing the same β-glucosidic bonds specific for the enzymes and the type of linkage in the substrate influenced the determination of activity. This may due to the fact that the Cys-rich domain of *bgn13.1* (β-1,3-glucanase) may function by interacting with other components of fungal cell walls that are normally not a substrate for β-1,3-glucanase (De la Cruz *et al.* 1995). However, not all purified b-glucanase have this ability as Vazquez *et al.* (1998) found that the purified β-1,3-glucanase can only hydrolyse laminarin.

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Heavy Metals (Cd, Cu, Pb and Zn) Concentrations in *Telescopium telescopium* from Dumai Coastal Waters, Indonesia

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ABSTRAK

Kepekatan kadmium, tembaga, plumbum dan zink dalam siput laut, Telescopium telescopium *ditentukan untuk memberi maklumat tentang tahap kandungan logam di Selat Melaka di sebelah perairan Indonesia. Sampel* T. telescopium *diambil di kawasan air pasang surut di empat stesen di Dumai pada Julai 2004. Keputusan pada kajian terkini menunjukkan kepekatan logam pada tisu lembut* T. telescopium *berbeza di setiap stesen pensampelan: berat kering masing-masing 0.33 - 069 µg/g; 9.38 - 52.29 µg/g; 1.73 - 10.78 µg/g; 14.69 - 69.87 µg/g; untuk kadmium, tembaga, plumbum dan zink. Sampel dari stesen Sungai Dumai mengumpulkan lebih banyak kandungan logam apabila dibandingkan dengan stesen lain.* T. telescopium *yang kecil mengumpulkan lebih banyak logam daripada* T. telescopium *yang besar. Korelasi negatif di antara kepekatan logam dan saiz (panjang dan berat) diperhatikan (kecuali sample Cd dan Cu dari Sungai Mesjid) dengan sesetengah variasi dalam pekali kerelasi yang menunjukkan pengawalan logam oleh* T. telescopium. *Sungai Dumai menunjukkan Indeks Pencemaran Logam (MPI) yang paling tinggi iaitu 12.57 dan Indeks Beban Pencemaran Tomlinson (PLI) sebanyak 34.35 dengan Lubuk Gaung mencatatkan indeks yang rendah bagi kedua-duanya iaitu 3.26 dan 8.89. Secara umumnya, nilai MPI dan PLI menunjukkan pencemaran logam di perairan pinggir laut Dumai belum lagi merupakan satu ancaman yang serius dan tiada langkah pembaikan drastik diperlukan. Walau bagaimanapun, sebagai salah satu kawasan yang paling membangun di Sumatera, kajian lanjut adalah perlu untuk menaksir variasi masa dalam logam dan kepekatan untuk spesies ini dan organisma-organisma penunjuk lain yang mungkin selain dalam sedimen dari kawasan-kawasan sekeliling dalam usaha untuk mendapatkan lebih kefahaman tentang status pencemaran di perairan pinggir laut Dumai.*

ABSTRACT

Concentrations of cadmium, copper, lead and zinc in the marine gastropod, Telescopium telescopium, *were determined to provide background information on heavy metal levels in the Straits of Malacca in the Indonesian side. Samples of* T. telescopium *were collected from intertidal Dumai coastal waters at four stations in July 2004. The results of the present study showed that metal concentrations in the soft tissue of* T. telescopium *varied at different sampling stations: 0.33 – 0.69 µg/g; 9.38 – 52.29 µg/g; 1.73 – 10.78 µg/g; 14.69 – 69.87 µg/g dry weight for cadmium, copper, lead and zinc, respectively. Samples from Sungai Dumai station accumulated more heavy metals when compared to other stations. Smaller* T. telescopium *accumulate more metals than larger ones. Negative correlations between metal concentrations and size (length and weight) were observed (except Cd and Cu in samples from Sungai Mesjid) with some variation in the correlation coefficients which indicated metal regulation by the* T. telescopium. *Sungai Dumai showed the highest Metal Pollution Index (MPI) of 12.57 and Tomlinson Pollution Load Index (PLI) of 34.35 with Lubuk Gaung showing the lowest with 3.26 and 8.89, respectively. In general, the MPI and PLI values indicate that metal pollution in Dumai coastal waters is not a serious threat yet and no drastic rectification measures are needed. However, as one of the most developing regions in Sumatera, further studies are necessary to assess temporal variation in metal accumulation and*

concentration for this species and other possible indicator organisms as well as in sediment from the surrounding areas in order to gain a better understanding of pollution status in Dumai coastal waters.

INTRODUCTION

Increased population and rapid economic and industrial development can cause many ecological problems to marine and coastal areas. Like many other developing regions, the Dumai coastline is subjected to negative impacts of industrial development and anthropogenic activities. It is also likely to receive impacts from the Straits of Malacca which is an international waterway and is known as one of the busiest shipping lanes in the world. These days, there are many industries and major oil and commercial ports operating in Dumai. Other possible sources of heavy metal pollution in this area includes urbanization activities, land-based inputs such as deforestation, disposal of industrial wastes, sewage and solid waste disposal, mangrove swamp conversion and land reclamation and sea-based inputs from shipping, dumping and fishing.

Gastropods are common inhabitants of mangrove ecosystems and are suitable organisms for monitoring environmental contamination and metal bioavailability studies (Peerzada *et al.* 1990; Leung and Furness 1999; de Wolf *et al.* 2000; Blackmore 2001; Cubadda *et al.* 2001; Conti and Cecchetti 2003; Liang *et al.* 2004). Elevated concentrations of heavy metals in Dumai waters have been previously detected such as in surface water and sediment (Amin and Zulkifli 1997); mudskipper (Amin 2000); barnacles (Efriyeldi and Amin 2001) and mangrove (Amin 2001). The present study aims to provide background information concerning heavy metal concentrations in *T. telescopium* from Dumai coastal waters.

MATERIALS AND METHODS

A total of ninety-six specimens of *T. telescopium* were collected in July 2004 from four locations in the mangrove area of Dumai coastal waters (*Fig. 1*). Three different size groups of small (38 – 55 mm), medium (57 – 75 mm) and large (75 – 90 mm) were selected and brought back to the laboratory. They were then cleaned with distilled water and the soft tissues were removed from the shells. All the soft tissues were oven dried to constant weight at 105°C (Mo and Neilson 1994). The dried soft tissues were then digested following the procedures outlined by Yap *et al.* (2003). The dried soft tissues were digested in concentrated nitric acid (AnalaR grade, BDH 69%) and placed in a hot-block digester at low temperature (40° C) for 1 hour and fully digested at high temperature (140° C) for at least 3 hours. The digested samples were then diluted to 40 ml with double distilled water. After filtration, heavy metals were determined by an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model 3110. The data are presented in $\mu g/g$ dry weight. All glassware and equipments used were acid-washed to avoid possible contamination and the accuracy of the analyses was checked against blanks and by the standard addition testing procedure. Percentages of recoveries for heavy metal analysis were 103.6 %, 97.7 %, 96.6 % and 98.3 % for Cd, Cu, Pb and Zn respectively. Procedural blanks and quality control samples made from the standard solutions for Cd, Cu, Pb and Zn, prepared from 1000 mg/l stock solution (BDH Spectrosol) of each metal, were analyzed every five specimens in order to check for sample accuracy.

In order to compare the total concentrations of metals at different sampling sites, a metal pollution index (MPI) was calculated based on an equation used by Usero *et al.* (1996; 1997) and Giusti *et al.* (1999). A Tomlinson pollution load index (PLI) was also measured because it can be used as an index of bioavailability of heavy metal for molluscs in coastal waters (Tomlinson *et al.* 1980; Angula 1996).

RESULTS AND DISCUSSION

The results of the present study showed that the mean metal concentrations in the soft tissue of *T. telescopium* were varied at different sampling stations : 0.33 – 0.69 $\mu g/g$; 9.38 – 52.29 $\mu g/g$; 1.73 – 10.78 $\mu g/g$; 14.69 – 69.87 $\mu g/g$ dry weight for cadmium, copper, lead and zinc respectively. Higher metal concentrations were found in samples from Sungai Dumai followed by Tanjung Medang, Sungai Mesjid and Lubuk Gaung (Table 1 and *Fig. 2*). Amin and Zulkifli (1997) and Amin (2000) reported that metal concentrations in sediment and mudskippers from Sungai Dumai were higher when compared to other locations in Dumai and Rupat waters.

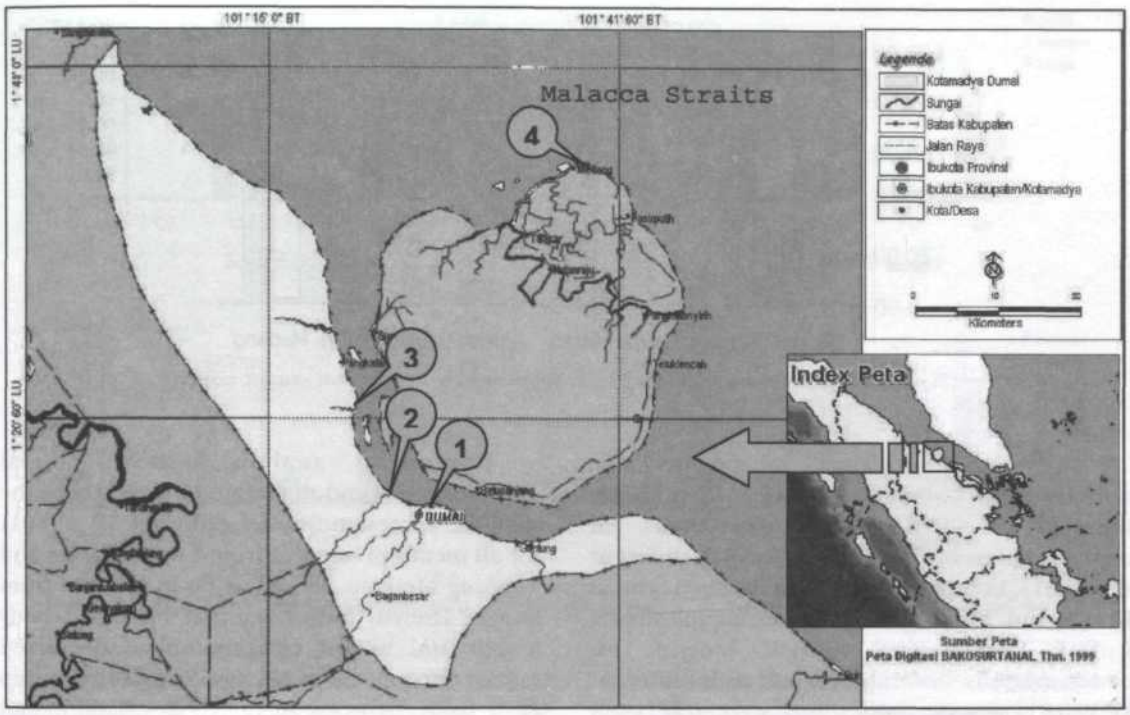

Fig. 1: Map of Dumai and sampling locations in Sungai Dumai (1), Sungai Mesjid (2), Lubuk Gaung (3) and Tanjung Medang (4)

TABLE 1
Heavy metal concentrations in *T. telescopium* from Dumai coastal waters (Means ± SD)

Station	Size class	Length (mm)	Weight (g)	Concentration (mg/g d.w)			
				Cd	Cu	Pb	Zn
Sg. Dumai	Small	51.13 ± 5.38	1.00 ± 0.48	0.83 ± 0.50	57.75 ± 26.11	14.88 ± 10.21	85.74 ± 46.28
	Medium	64.50 ± 5.93	1.42 ± 0.36	0.66 ± 0.34	58.56 ± 25.97	12.37 ± 4.03	79.60 ± 30.76
	Large	83.13 ± 5.96	3.64 ± 0.66	0.41 ± 0.14	40.56 ± 16.55	5.10 ± 1.76	44.25 ± 5.25
	Average	66.25 ± 14.49	2.02 ± 1.28	0.63 ± 0.39	52.29 ± 23.83	10.78 ± 7.45	69.87 ± 36.01
Sg. Mesjid	Small	51.75 ± 5.06	1.33 ± 0.39	0.56 ± 0.17	15.33 ± 7.20	8.69 ± 11.34	64.41 ± 32.15
	Medium	65.25 ± 4.77	2.03 ± 0.13	0.75 ± 0.20	21.24 ± 10.07	8.54 ± 1.86	49.13 ± 25.36
	Large	77.5 ± 3.78	2.99 ± 0.56	0.75 ± 0.35	23.01 ± 15.26	6.94 ± 2.19	45.69 ± 14.42
	Average	64.83 ± 11.60	2.12 ± 0.79	0.69 ± 0.26	19.86 ± 11.35	8.06 ± 2.00	53.98 ± 24.82
Lubuk Gaung	Small	43.50 ± 4.34	0.68 ± 0.35	0.61 ± 0.28	18.12 ± 7.53	4.50 ± 1.91	25.48 ± 4.95
	Medium	59.5 ± 2.62	1.52 ± 0.22	0.25 ± 0.09	6.65 ± 2.44	2.02 ± 0.91	11.85 ± 5.21
	Large	79.88 ± 2.90	2.79 ± 0.19	0.14 ± 0.08	3.36 ± 2.02	0.81 ± 0.64	6.73 ± 3.07
	Average	60.96 ± 15.54	1.66 ± 0.92	0.33 ± 0.27	9.38 ± 7.88	2.44 ± 1.99	14.69 ± 9.16
Tj. Medang	Small	57.25 ± 4.43	1.80 ± 0.16	0.91 ± 0.35	58.57 ± 9.59	2.57 ± 1.08	75.18 ± 29.05
	Medium	74.5 ± 9.09	3.11 ± 1.26	0.70 ± 0.31	53.97 ± 17.87	1.75 ± 1.25	52.425 ± 23.09
	Large	82.63 ± 6.31	5.43 ± 1.37	0.40 ± 0.25	32.99 ± 22.07	0.88 ± 0.35	30.93 ± 10.21
	Average	71.64 ± 16.64	3.44 ± 1.85	0.67 ± 0.36	48.51 ± 20.07	1.73 ± 1.17	52.85 ± 28.131

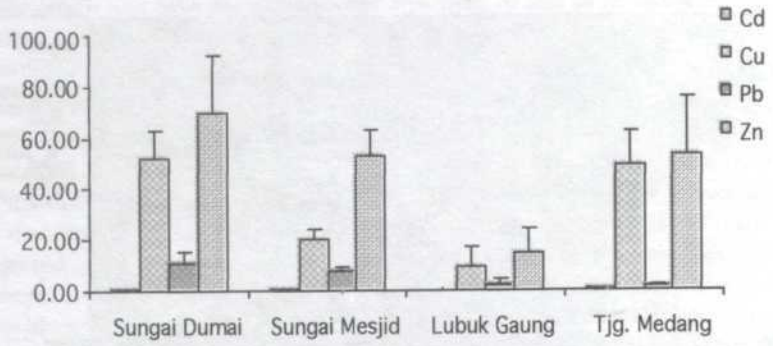

Fig. 2: Heavy metal concentrations in T. telescopium *from Dumai coastal waters*

The ANOVA test showed that Cd and Zn in samples from Lubuk Gaung were significantly different ($P < 0.01$) from other stations. All concentrations of Cu were significantly different ($P < 0.01$), except between samples from Sungai Dumai and Tanjung Medang and samples from Lubuk Gaung and Sungai Mesjid. Pb concentrations were also significantly different ($P < 0.01$) among the stations, except between Sungai Dumai and Sungai Mesjid as well as between Lubuk Gaung and Tanjung Medang.

Sungai Dumai estuary is located in the city centre and the sampling area received anthropogenic wastes from densely populated sorrounding areas carried by the river. This area is also very close to an oil refinery, dock yard and international tankers activities. Heavy metal concentrations in *T. telescopium* was in the order of Zn > Cu > Pb > Cd. In general the concentrations of these metals were considered to be low when compared with data on the same species from Australia as reported by Peerzada *et al.* (1990).

The present results showed that smaller *T. telescopium* accumulate more metals than larger ones (*Fig. 3*) and thus size is an important variable to consider for metal accumulation. Concentrations of all metals in a smaller sample size from Lubuk Gaung and Tanjung Medang as well as Pb in samples from Sungai Dumai were significantly higher ($P < 0.05$) from the larger size. However, although they showed a similar trend, metal concentrations in samples from Sungai Mesjid and Sungai Dumai (except Pb), did not differ significantly ($P > 0.05$). A study on snails by Williamson (1980) found that higher levels of metals were found in smaller specimens suggesting that increases in metabolic rates in relation to different body sizes might affect heavy metal uptake and elimination.

Regression analysis between metal concentrations and the length and weight of the samples were significantly different ($P < 0.01$) for all metals in samples from Lubuk Gaung and Tanjung Medang, as well as Pb in samples from Sungai Dumai, indicating that increased body length and weight corresponds to decreased metal concentrations. No significant relationship ($P > 0.05$) between metal concentrations and body length and weight in samples from Sungai Mesjid as well as for Cd, Cu and Zn in samples from Sungai Dumai was found.

Negative correlations between metal concentrations and size (length and weight) were observed (except Cd and Cu in samples from Sungai Mesjid) with some variation in the correlation coefficients which gave an indication of metal regulation by the *T. telescopium*. The decrease in heavy metal concentrations with an increase in body size of the organisms have also been found in other molluscan species and their possible causes were mainly related to the metabolic activities at different ages of the organisms (Boyden, 1977; Olafsson, 1986; Jones *et al.* 1992; Swaileh and Adelung, 1994; Kraak *et al.* 1994; Bilos *et al.* 1998).

The MPI values based on cadmium, copper, lead and zinc in *T. telescopium* for each sampling site as well as the PLI values which were calculated based on the minimum concentrations measured in the present study (as the baseline) are shown in Table 2. Sungai Dumai showed the highest Metal Pollution Index (MPI) of 12.57 and Tomlinson Pollution Load index (PLI) of 34.35 and Lubuk Gaung being the lowest with 3.26 and 8.89 respectively. Giusti *et al.* (1999) reported that MPI values based on nine metals of *Mytilus edulis* from the Northeast England coast were in the range of 10.50 – 25.10 and Chiu *et al.* (2000)

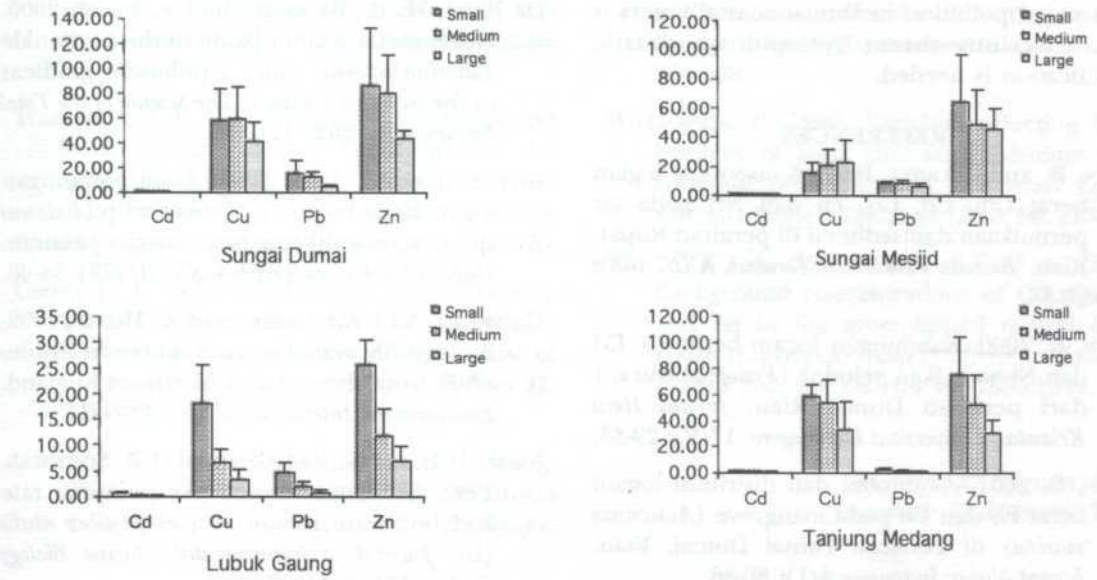

Fig. 3: Mean heavy metal concentration in different size groups of T. telescopium from Dumai coastal waters

reported MPI values of between 5.00 to 9.23 based on five metals of *Perna viridis* from Hong Kong waters; whilst Yap *et al.* (2003) reported MPI values of 4.35 – 11.70 in *P. viridis* from Peninsular Malaysia which was based on four metals.

According to Angula (1996), PLI is able to give an estimate of the metal contamination status and the necessary action that should be taken. A PLI value of ≥ 100 indicates an immediate intervention to ameliorate pollution; a PLI value of ≥ 50 indicates a more detailed study is needed to monitor the site, whilst a value of <50 indicates that drastic rectification measures are not needed.

The MPI and PLI values indicated that metal pollution in Dumai coastal waters is not a serious threat yet and no drastic rectification measures are needed. However, as one of the most developing regions in Sumatera, further studies are required to assess temporal variation in metal accumulation and concentration for this species and other possible indicator organisms as well as in sediment from the surrounding areas in order to gain a better understanding of the pollution status in Dumai coastal waters.

CONCLUSION

The present study showed that *T. telescopium* from Sungai Dumai accumulates more heavy metals as compared to other stations. Smaller *T. telescopium* accumulate more metals than larger ones and thus size is an important variable to consider for metal accumulation. Simple linear regression analyses indicated negative correlations between metal concentrations and size (except for Cd and Cu in Sungai Mesjid) with some variation in their correlation coefficients which give an indication of metal regulation by the *T. telescopium*. Sungai Dumai showed the highest MPI and PLI values and Lubuk Gaung the lowest, but these values indicate

TABLE 2
MPI and PLI values for *T. telescopium* from Dumai coastal waters

Station	MPI	PLI
Sungai Dumai	12.57	34.35
Sungai Mesjid	8.74	3.26
Lubuk Gaung	7.39	23.88
Tanjung Medang	8.89	20.21

that metal pollution in Dumai coastal waters is not a serious threat yet and no drastic rectification is needed.

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Cadmium, Copper, Lead and Zinc Levels in the Green-Lipped Mussel *Perna viridis* (L.) from the West Coast of Peninsular Malaysia: Safe as Food?

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ABSTRAK

Kupang Perna viridis *telah disampel di antara tahun 2002-2004, daripada 10 lokasi di pantai barat Semenanjung Malaysia. Sampel-sampel tersebut dianalisis untuk kadmium (Cd), kuprum (Cu), plumbum (Pb) dan zink (Zn). Kepekatan logam (µg/g berat kering) adalah di antara 0.11-5.55 bagi Cd, 3.49-31.1 bagi Cu, 1.16-18.62 bagi Pb dan 60.51-119.5 µg/g bagi Zn. Dari segi kesihatan pandangan umum, kepekatan logam adalah di bawah tahap maksimum yang dibenarkan oleh Peraturan-peraturan Makanan Malaysia (1985) dan juga di bawah standard antarabangsa median yang dianggap berbahaya mengikut FAO oleh Bangsa-bangsa Bersatu. 'Non-carcinogenic hazard indices' bagi logam di dalam kupang menunjukkan pemakanan kupang-kupang di dalam kajian ini adalah tidak berbahaya dan risiko pemakanan adalah bergantung pada jumlah kupang yang dimakan dan tempat di mana mereka diambil.*

ABSTRACT

Green-lipped mussels, Perna viridis, *were collected between 2002-2004, from 10 locations on the west coast of Peninsular Malaysia. The samples were analysed for cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn). The metal concentrations (µg/g dry weight) ranged from 0.11 to 5.55 for Cd, 3.49 to 31.1 for Cu, 1.16 to 18.62 for Pb and 60.51 to 119.5 for Zn. From the public health point of view, these metal concentrations were below the maximum permissible levels set by the Malaysian Food Regulations (1985) and were also below the levels regarded as harmful according to the median international standards for metals in mollusks compiled by the FAO of the United Nations. Non-carcinogenic hazard indices of the metals in the mussels in this present study showed that the consumption of the mussels was not risky and any risk is dependent on the amount of mussels consumed and the sites they were collected from.*

INTRODUCTION

The west coast of Peninsular Malaysia has always been an interesting area for investigation especially for chemical pollution since the area is likely to receive impacts from man-induced activities such as urbanisation, industrialisation and other land-based activities (Hamzah 1997; Yap *et al.* 2002). To monitor the sustainable natural resources from the Straits of Malacca, an easy and practical way to fulfill such a purpose is to develop a biomonitoring agent for the west coast of Peninsular Malaysia. Ismail *et al.* (2000) suggested the green-lipped mussel *Perna viridis* as a potential biomonitor of heavy metals for the area. The idea of using marine mussels as a biomonitoring agent for Malaysian coastal waters is prompted by the many studies conducted using the blue mussel *Mytilus edulis*. This is due to the practice of using mussels as biomonitoring agents of heavy metals for coastal waters developed by Goldberg in 1975 and used until today (Nicholson and Szefer 2003; Yap *et al.* 2003; 2004). One of the attributes that has led to the use of marine mussels as a biomonitoring agent for heavy metals is that they are commercially important seafood species

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worldwide (Goldberg 1975; Phillips and Rainbow 1993). Other attributes as to why mussels are often chosen for biomonitoring studies are that they are sedentary organisms, long-living, easily identified and sampled, reasonably abundant and available throughout the year, and tolerant of natural environmental fluctuations and pollution. Besides, they have good net accumulation capacities and they are important ecologically. In this region, reports on the use of the mussel *P. viridis* as a biomonitoring agent for heavy metals had been published from Thailand (Sukasem and Tabucanon 1993), Indonesia (Hutagalung 1989), India (Senthilnathan *et al.* 1998) and Hong Kong (Wong *et al.* 2000). In Malaysia, studies on heavy metals in *P. viridis* have been conducted by Sivalingam and Bhaskaran (1980), Sivalingam (1985), Devi (1986), Liong (1986), Ismail (1993), Ismail *et al.* (2000) and Yap *et al.* (2003; 2004).

This paper reports the concentrations of cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) in the total soft tissues of *P. viridis* collected from 10 sites on the west coast of Peninsular Malaysia in order to determine the safety of the edible mussels from the human health point of view. The determination was based on comparison with the permissible levels set by the Malaysian Food Regulations (1985) and median international standards for metals in mollusks compiled by the Food and Agricultural Organization (FAO) of the United Nations (California EPA 2005) besides other food safety guidelines. In addition, the metal concentrations in the soft tissues of mussels were particularly assessed based on the non-carcinogenic hazard index established by Wong *et al.* (2000), since they were consumed by human.

MATERIALS AND METHODS

The sampling locations are shown in *Fig. 1*. The sampling was conducted between 2002 and 2004, from Bagan Tiang (in the northern part) to Kuala Belungkor (in the southern part) of Peninsular Malaysia. The samples were stored in clean plastic bags and brought back to the laboratory in an ice compartment. In the laboratory, the samples were kept at –10°C until analysis. Before dissection, the mussel samples were thawed at room temperature (27°C) and 20 relatively similar sized mussels from each site were selected and analysed for Cd, Cu, Pb and Zn. The soft tissues from the mussels were dissected by removing the byssus and the shell. Soft tissues were dried in an oven at 105°C until constant dry weight (Mo and Neilson 1994). The dried soft mussel tissues were digested in concentrated HNO_3 (AnalaR grade, BDH 69%). They were placed in a hot-block digester first at low temperature for one hour and then fully digested at high temperature (140°C) for at least 3 hours. The digested samples were then diluted to a certain volume with double distilled water (DDW). After filtration, the prepared samples were determined for heavy metals by an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model 4100. The data are presented in μg/g dry weight. To avoid possible contamination, all glassware and equipment used were acid-washed. Procedural blanks and quality control samples made from the standard solutions for Cd, Cu, Pb and Zn were analysed once for every five samples in order to check for accuracy. Percentages of recoveries for the analysis were 110% for Cd, 96% for Cu, 92.5% for Pb and 92% for Zn. The dry weight basis was converted into the wet weight basis by using a conversion factor of 0.17 (Yap 1999).

Calculation of Non-Carcinogenic Risk

For the public hazard assessment of mussel consumption, the non-carcinogenic hazard index was determined. The non-carcinogenic hazard index is expressed in terms of the ratio of an individual's exposure to the defined maximum level of exposure. The mean concentrations of heavy metals in all mussel populations were determined and were used to calculate the non-carcinogenic hazard index, according to Wong *et al.* (2000). The formula is given as:

$$\text{Non-carcinogenic} = \frac{\text{chronic daily intake}}{\text{reference dose}}$$

Hazard Index

The reference doses for Cd, Cu, Pb and Zn used in this study were 20, 2000, 100 and 10,000 μg, according to Wong *et al.* (2000).

RESULTS AND DISCUSSION

The concentrations of Cd, Cu, Pb and Zn are presented in Table 1. The metal concentrations ranged from 0.11 to 5.55 μg/g dry weight (0.02 to 0.94 μg/g wet weight) for Cd, 3.49 to 31.1

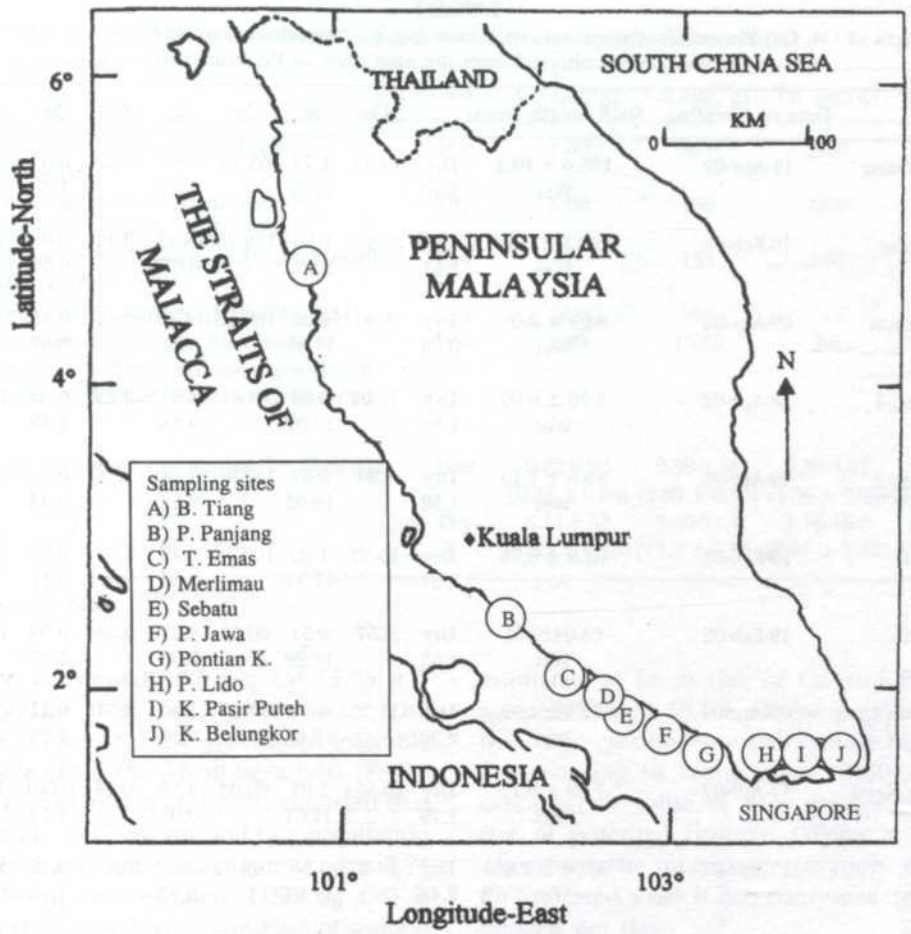

Fig. 1: Sampling sites of Perna viridis *in the coastal waters of Peninsular Malaysia*

μg/g dry weight (0.59 to 5.28 μg/g wet weight) for Cu, 1.16 to 18.62 μg/g dry weight (0.20 to 3.17 μg/g wet weight) for Pb and 60.51 to 119.5 μg/g dry weight (10.29 to 20.32 μg/g wet weight) for Zn (Table 1).

In the present results, the metal concentrations in the mussel samples from the west coast were within the ranges reported by Ismail *et al.* (2000) for mussels. The data in Table 1 were compared to the results from the same locations in the previous collections done in 1998-2001 (Yap *et al.* 2003). It was found that the concentrations of the investigated metals in the mussels from the present study were generally higher than those reported in our previous study. Sukasem and Tabucanon (1993) suggested that the difference in metal levels from two periods of sampling could be explained by the time of sampling. The samples of 1998 were collected during the rainy season (September to December) while samples taken in 2000 were obtained just at the end of the rainy season (January-April). However, little local information on heavy metal variations due to rainy season is available. Therefore, this conclusion can only be confirmed by further studies.

After conversion of the dry weight data into the wet weight basis, the metal levels in *P. viridis* found in this study were lower than those of the maximum permissible levels of heavy metals in food set by the Malaysian Food Regulations (1985) (Table 1). Our mean values (μg/g wet weight) of these metals from all the populations were lower than the limits for Cd (1.00 μg/g wet weight), Cu (30.0 μg/g wet weight), Pb (2.00 μg/g wet weight) and Zn (100 μg/g wet weight).

The metal levels were also lower than the recommended guidelines for Cd, Cu, Pb and Zn set by the Ministry of Public Health of Thailand (MPHT 1986), the Australian Legal Requirement for food safety (NHMRC 1987) and the limits established by the Brazilian Ministry of Health

TABLE 1
Ranges of Cd, Cu, Pb and Zn concentrations (mean [μg/g] ± standard error [SE]) in the total soft tissues of *Perna viridis* collected from the west coast of Peninsular Malaysia

		Date of sampling	Shell length (mm)		Cu	SE	Zn	SE	Cd	SE	Pb	SE
A.	Bagan Tiang	11-Apr-02	135.0 ± 10.2	Dry	14.14	1.77	65.75	4.88	0.32	0.09	10.32	3.73
				Wet	2.40	11.18		0.05		1.75		
B.	P. Panjang	19-Feb-04	82.15 ± 0.99	Dry	8.89	0.97	119.50	18.45	0.44	0.08	1.16	0.02
				Wet	1.51	20.32		0.07		0.20		
C.	Telok Emas	09-Apr-04	84.7 ± 0.69	Dry	3.49	0.00	102.13	14.68	1.47	0.43	3.92	1.20
				Wet	0.59	17.36		0.25		0.67		
D.	Merlimau-1	18-Apr-02	6.79 ± 0.09	Dry	11.07	0.95	88.64	8.64	2.24	0.67	13.99	2.80
				Wet	1.88	15.07		0.38		2.38		
	Merlimau-2	09-Apr-04	82.5 ± 1.15	Dry	8.94	0.67	111.86	2.14	1.46	0.14	2.53	0.99
				Wet	1.52	19.02		0.25		0.43		
E.	Sebatu-1	19-Feb-02	62.9 ± 0.78	Dry	15.72	1.99	105.38	6.19	1.95	0.56	18.62	2.17
				Wet	2.67	17.92		0.33		3.17		
	Sebatu-2	19-Feb-02	63.04±0.81	Dry	9.57	0.51	60.51	4.27	0.35	0.04	11.01	0.00
				Wet	1.63	10.29		0.06		1.87		
F.	Parit Jawa	09-Apr-04	77.18±4.59	Dry	11.75	0.18	97.97	1.40	2.31	0.11	7.26	4.59
				Wet	2.00	16.65		0.39		1.23		
G.	Pontian Kecil	17-Apr-02	7.89 ± 0.13	Dry	10.554	1.05	68.03	3.53	0.14	0.001	6.56	0.85
				Wet	1.79	11.61		0.02		1.12		
H.	P. Lido	17-Apr-02	89.08 ± 2.60	Dry	14.27	1.58	105.52	23.21	0.43	0.14	6.09	1.71
				Wet	2.43	17.94		0.07		1.03		
I.	K.Pasir Puteh	17-Apr-02	92.84 ± 3.21	Dry	31.09	2.48	69.89	5.07	5.55	1.05	11.85	1.24
				Wet	5.28	11.88		0.94		2.01		
J.	K. Belungkor	18-Apr-02	63.4 ± 1.10	Dry	7.96	0.98	86.26	16.77	0.11	0.02	2.78	1.32
				Wet	1.35	14.66		0.02		0.47		

Note: Alphabets follows those indicated in *Fig. 1.*

(ABIA 1991) (Table 2). When compared to median international standards for metals in mollusks compiled by FAO of the United Nations, again our ranges of Cd, Cu, Pb and Zn are lower than the FAO limits and therefore our mussels are safe for human consumption.

However, the potential hazards of metals transferred to humans are probably dependent on the amount (g wet weight) of mussels consumed by an individual. For example, an adult who consumed 2.50 g/day of *P. viridis* daily from Sebatu-1 would take in approximately 7.93 μg (3.17 μg/g × 2.50 g) of Pb per day. If the consumer were to take the mussel for 7 days, then he would have consumed 55.5 μg Pb (7.93 μg × 7 days). This is slightly higher than the recommended limit for the provisional tolerable weekly intake of Pb (50.0 μg/adult) (FAO/WHO 1984). Tukimat *et al.* (2002) reported that the daily intake of Pb in seafood by a population from Kuala Kemaman, Terengganu (east coast of Peninsular Malaysia), was 2.82 μg/day. The estimate of Pb intake from the present study (55.5 μg Pb) is higher than a week's consumption of seafood by a person from Kemaman (2.82 μg/day × 7 days= 20.0 μg Pb).

Similarly, if an adult roughly consumed 2.50 g of mussel per day, then the person who consumed mussels collected from Kg. Pasir Puteh would consume approximately 2.35 μg (0.94 μg/g × 2.50 g) of Cd each day. If the consumer takes mussels for 7 consecutive days, then he

TABLE 2
Guidelines on heavy metals for food safety set by different countries

Location	WB	Cd (μg/g)	Cu (μg/g)	Pb (μg/g)	Zn (μg/g)
Permissible limits set by Malaysian Food Regulations (1985)	Wet	1.00	30.0	2.00	100
Maximum permissible levels established by Brazilian Ministry of Health (ABIA 1991)	Dry	5.00	150	10.0	250
Permissible limit set by Ministry of Public Health, Thailand (MPHT 1986)	Dry	-	133	6.67	667
Australian Legal Requirements (NHMRC 1987)	Dry	10.0	350	-	750
Median international standards for metals in mollusks compiled by the Food and Agricultural Organization of the United Nations (California EPA 2005)	Wet	2.00	10-30	1-6	40-100
Metal levels of *P. viridis* from Peninsular Malaysia (This study)	Wet	0.02-0.94 (0.24 ± 0.08)	0.29-5.28 (2.09 ± 0.33)	0.20-3.17 (1.36 ± 0.26)	10.3-20.3 (15.33 ± 0.98)
	Dry	0.11-5.55 (1.39 ± 0.45)	3.49-31.1 (12.3 ± 1.95)	1.16-18.6 (8.01 ± 1.52)	60.5-119.5 (90.1 ± 5.77)

Note: WB= weight basis.

would have consumed 16.5 mg Cd (2.35 × 7 days). Again, this is higher than the recommended limit for the provisional tolerable weekly intake of Cd (6.70-8.30 μg/adult) (FAO/WHO 1984). Tukimat *et al.* (2002) reported that the daily intake of Cd in seafood by a population from Kuala Kemaman, Terengganu, was 0.74 μg/day. The present estimate (16.5 μg Cd) is also higher than a week's consumption of seafood from Kemaman (0.74 μg/day × 7 days= 5.18 μg Cd). Therefore the above calculations show that the risk of metal toxicity could be dependent on the amount of mussels consumed.

For the calculation of the non-carcinogenic risk of all mussels collected, the mean concentrations of Cd, Cu, Pb and Zn in the mussels are summarized in Table 3. For a person who takes one mussel per day (13.5 g wet weight/day), all the indices are below 1 (Table 3a), indicating that there is no non-carcinogenic risk. However, for a person who takes five mussels per day (67.3 g wet weight /day) (Table 3), the indices for the concentrations of Cd in mussels collected from Merlimau-1, Parit Jawa and Kg. Pasir Puteh are above 1, whereas the indices for Pb concentrations in mussels collected from Bagan Tiang, Merlimau-1, Sebatu-1, Sebatu-2 and Kg. Pasir Puteh are above 1. The same phenomenon of metal risk can be seen if an individual consumed 10 mussels per day in which a greater proportion of the populations is estimated to be at risk of Cd and Pb toxicity since 50% and 58.3% of the population have their non-carcinogenic risk indices higher than 1. According to Wong *et al.* (2000), the non-carcinogenic value of 1 or greater indicates a risk of potential concern (Wong *et al.* 2000). Therefore, the mean concentrations of Cd and Pb indicated a risk if one consumes five or more mussels per day.

CONCLUSION

Although the present data indicate that the possibility of the occurrence of acute toxicities of Cd, Cu, Pb and Zn is unlikely, low-level and chronic toxicities to consumers may still cause health problems in human beings. The latter is poorly understood although this would be expected based on the information found in the literature. By using *P. viridis* as a biomonitoring agent, the contamination of Cd, Cu, Pb and Zn in the west coast of Peninsular Malaysia was found to be not serious. The heavy metal concentrations in the mussels from the west coast of Peninsular Malaysia could be attributed to natural or anthropogenic metal sources affecting their habitats. From the human public health point of view, these results seem to show no possibility of acute toxicities of Cd, Cu, Pb and Zn if edible mussels are consumed. However, the risk is dependent on the amount of mussels consumed and the sites from where the mussels are collected.

TABLE 3
Non-carcinogenic hazard index (CDI/RfD) for oral intake of 1 day, 5 and 10 consecutive days

	Sites	Cu			Zn			Cd			Pb		
		Day(s)			Day(s)			Day(s)			Day(s)		
		1	5	10	1	5	10	1	5	10	1	5	10
1.	Bagan Tiang	0.02	0.08	0.16	0.02	0.08	0.16	0.03	0.17	0.34	0.24	**1.18**	**2.36**
2.	P. Panjang	0.01	0.05	0.10	0.03	0.14	0.24	0.05	0.24	0.48	0.03	0.14	0.28
3.	Telok Emas	0.00	0.02	0.04	0.02	0.12	0.24	0.17	0.84	**1.68**	0.09	0.45	0.90
4.	Merlimau-1	0.01	0.06	0.12	0.02	0.10	0.20	0.26	**1.28**	**2.48**	0.32	**1.61**	**3.22**
	Merlimau-2	0.01	0.05	0.10	0.03	0.13	0.26	0.17	0.84	**1.68**	0.06	0.29	0.58
5.	Sebatu-1	0.02	0.09	0.18	0.02	0.12	0.24	0.22	1.11	**2.22**	0.43	**2.14**	**4.28**
	Sebatu-2	0.01	0.06	0.12	0.01	0.07	0.14	0.04	0.20	0.40	0.25	**1.26**	**2.52**
6.	Parit Jawa	0.01	0.07	0.14	0.02	0.11	0.22	0.26	**1.32**	**2.64**	0.17	0.83	**1.66**
7.	Pontian Kecil	0.01	0.06	0.12	0.02	0.08	0.16	0.01	0.07	0.14	0.15	0.75	**1.50**
8.	P. Lido	0.02	0.08	0.16	0.02	0.12	0.24	0.05	0.24	0.48	0.14	0.70	0.14
9.	K. Pasir Puteh	0.04	0.18	0.36	0.02	0.08	0.16	0.63	**3.17**	**6.34**	0.27	**1.36**	**2.72**
10.	K. Belungkor	0.01	0.05	0.10	0.02	0.10	0.20	0.01	0.07	0.14	0.06	0.32	0.64

Note: Values in bold indicate a risk of potential concern (Wong *et al.* 2000) since they are > 1.0.

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Distribution of Cocoa Pod Borer (CPB) *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae) Egg Population with Respect to the Pod Phenology

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Keywords: *Conopomorpha cramerella*, **egg population, pod phenology**

ABSTRAK

Pada tahun 2000 dan 2001, satu kajian mendalam ke atas pensampelan ulat pengorek buah koko (UPBK) telah dijalankan selama 12 bulan di Lembaga Koko Hilir Perak, Sg. Sumun, Perak. Beberapa komponen meliputi penentuan bentuk taburan UPBK berasaskan fenologi buah dan penentuan saiz sampel optimum UPBK menggunakan tiga kaedah pensampelan rawak, sistematik dan strata. Data dianalisis menggunakan dua model pengelompokan, Hukum Kuasa Taylor (pekali b) dan Indeks Pengelompokan Iwao (pekali β). UPBK ditemui berkelompok. Bagi penentuan saiz sampel optimum, dua parameter dihitung, pekali b bagi model Green dan pekali β bagi model Kuno. Model Green didapati paling sesuai bagi menghasilkan pensampelan paling efisien pada kepadatan populasi rendah dan tinggi UPBK bagi kesemua kaedah pensampelan yang digunakan.

ABSTRACT

At the Malaysian Cocoa Board Research Station at Hilir Perak, Sg. Sumun, Perak, an in-depth investigation on sampling of the cocoa pod borer (CPB) was conducted over a period of 12 months from 2000 to 2001. Several components were examined including determining distribution of CPB with respect to pod phenology and determining optimum sample size to produce sampling parameters for CPB using three different sampling techniques, random, systematic and stratified. Data were analysed using two aggregation models; Taylor's Power Law (b coefficient) and Iwao's mean crowding (β coefficient). CPB's egg were found to be highly aggregated. In determining optimum sample sizes, two parameters were calculated; b coefficient of Green's model and β coefficient for Kuno's model. The Green model was suitable to produce the most efficient sampling sizes during low and high population densities for all sampling techniques.

INTRODUCTION

Cocoa is the third most important primary crop in Malaysia providing RM 0.66 billion revenue to the country (MCB 2001). The Malaysian cocoa industry has developed rapidly since the late 1970s with production reaching 240, 000 tons in 1990. It is the most productive crop in the world with a yield of 1 tons/ha/year (MCB 2001). However, in the 1980s the industry was beset with problems of prolonged depressed price and infestation of a serious pest, the cocoa pod borer, *Conopomorpha cramerella* (Snellen) (CPB).

The CPB lays eggs on the pod surface, mostly in the furrows or in pod's morphological depressions. The larvae hatch and bore directly into the pods to feed within the placenta resulting in the formation of malformed beans. These problems if left uncontrolled may result in severe economic loss. Several methods are recommended to control CPB including frequent harvesting, bagging or sleeving, chemical treatment, natural enemies and pheromones. Currently, insecticides are widely used by the growers at biweekly prophylactic applications (Tay *et al.* 1989), often causing additional productivity costs through unnecessary spraying without knowing the exact information on the pest status. Hence, sampling may alleviate this problem through proper estimation of CPB population status which facilitates treatment decisions.

A sampling strategy is the adoption of sequential sampling methodology that will reduce time and labour cost. The objective of this study, was to determine the distribution of CPB eggs population.

MATERIALS AND METHODS

The study was conducted from 2000 to 2001 over a period of 12 months at the Cocoa Research Center, Malaysian Cocoa Board, Hilir Perak, approximately 25 km northwest to Teluk Intan. Three blocks having three different levels of CPB egg densities-high, medium and low- were used. Approximately, 60 trees in each block were randomly selected for sampling pods. Sampling was carried out by recording the number of eggs on pods, time taken for egg counting and the length and diameter of the pod. Three sampling techniques were compared including random, systematic and stratified.

Statistical Analysis

Distribution data of eggs for all sampled trees were used to calculated the means, variances, negative binomial parameter k (Long and Theroux 1979) and index mean crowding (Llyod 1967). Frequency histogram and simple linear regression (Woolf 1968) were used to indicate relationship between statistical and observed mean densities.

Distribution Indices

Taylor's Power Law relates variance (s^2) to mean densities (x) at $\log s^2 = \log a + b \log x$. The parameter a is the intercept which is considered as the factor related to sample size while the parameter b is an index value of aggregation, which is a constant species. When the b value < 1, it describes uniform distribution, b=1 for random distribution and $b > 1$ for aggregated distribution (Taylor 1961).

Iwao's model (1968) showed a regression of mean crowding $\overset{*}{x}$ on the mean ($\bar{x}$), where $\overset{*}{x} = x + [(s^2/x) - 1]$ (Lloyd 1967) and the linear regression model is $\overset{*}{x} = A + Bx$. Intercept A is the basic contagion while B represents the individual distribution. B<1 shows uniform distribution, B=1 shows random distribution and B>1 shows aggregated distribution pattern.

RESULTS AND DISCUSSION

A frequency histogram based on the sampling technique describes CPBs egg population throughout the sampling program (*Figs. 1* to *3*). The proportion of pods without eggs in each block was inversely related to the overall egg population density in the block. The levels of CPB egg density were very low, where trees were not attacked randomly but certain trees were more likely to be infested than the others. The results from this finding are similar to the finding of Azhar and Long (1993). Since the number of trees examined was large, and therefore, the degree of freedom was high, a small number of trees with high CPB egg density was sufficient to reject the hypothesis of randomness. One of the reasons why aggregated spatial distribution describes CPB egg population is that the adults

TABLE 1
Distribution parameter obtained using TPL based on different sampling techniques.

	Log a	SE	B	SE	r^2	t	p
Random	2.391	0.166	1.716	0.092	0.902	18.748	0.005
Systematic	2.072	0.174	1.678	0.091	0.900	18.49	0.005
Stratified	2.099	0.222	1.684	0.118	0.842	14.236	0.005

TABLE 2
Distribution parameter obtained using Iwao's based on different sampling techniques.

	A	SE	ß	SE	r2	t	p
Random	0.731	0.934	2.897	1.433	0.097	2.023	0.005
Systematic	0.323	0.418	8.623	0.831	0.739	10.388	0.005
Stratified	0.667	0.568	8.637	1.341	0.522	6.439	0.005

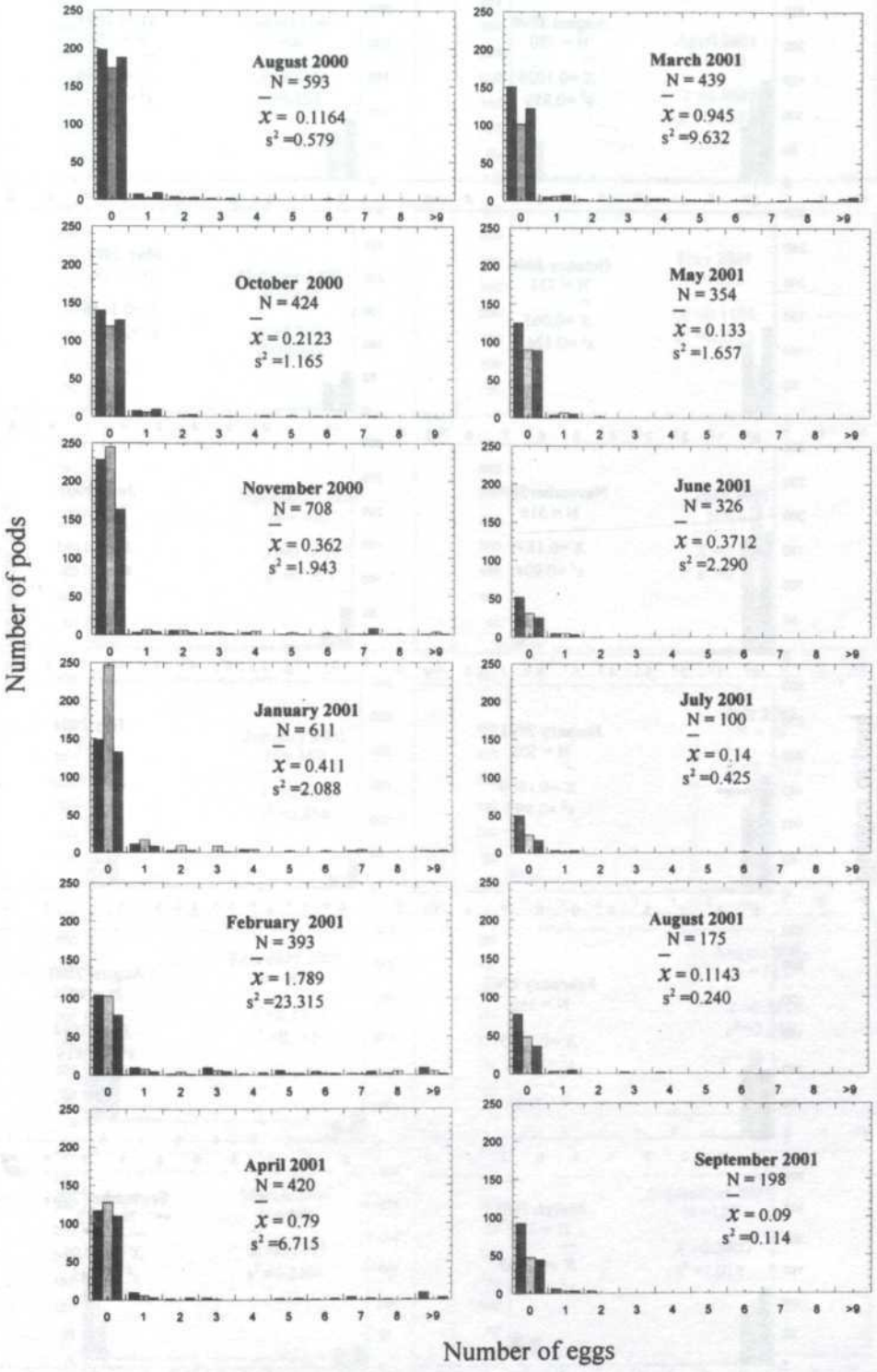

Fig. 1: Frequency of pods with CPB eggs sampled using random, systematic and stratified sampling techniques at block 12D

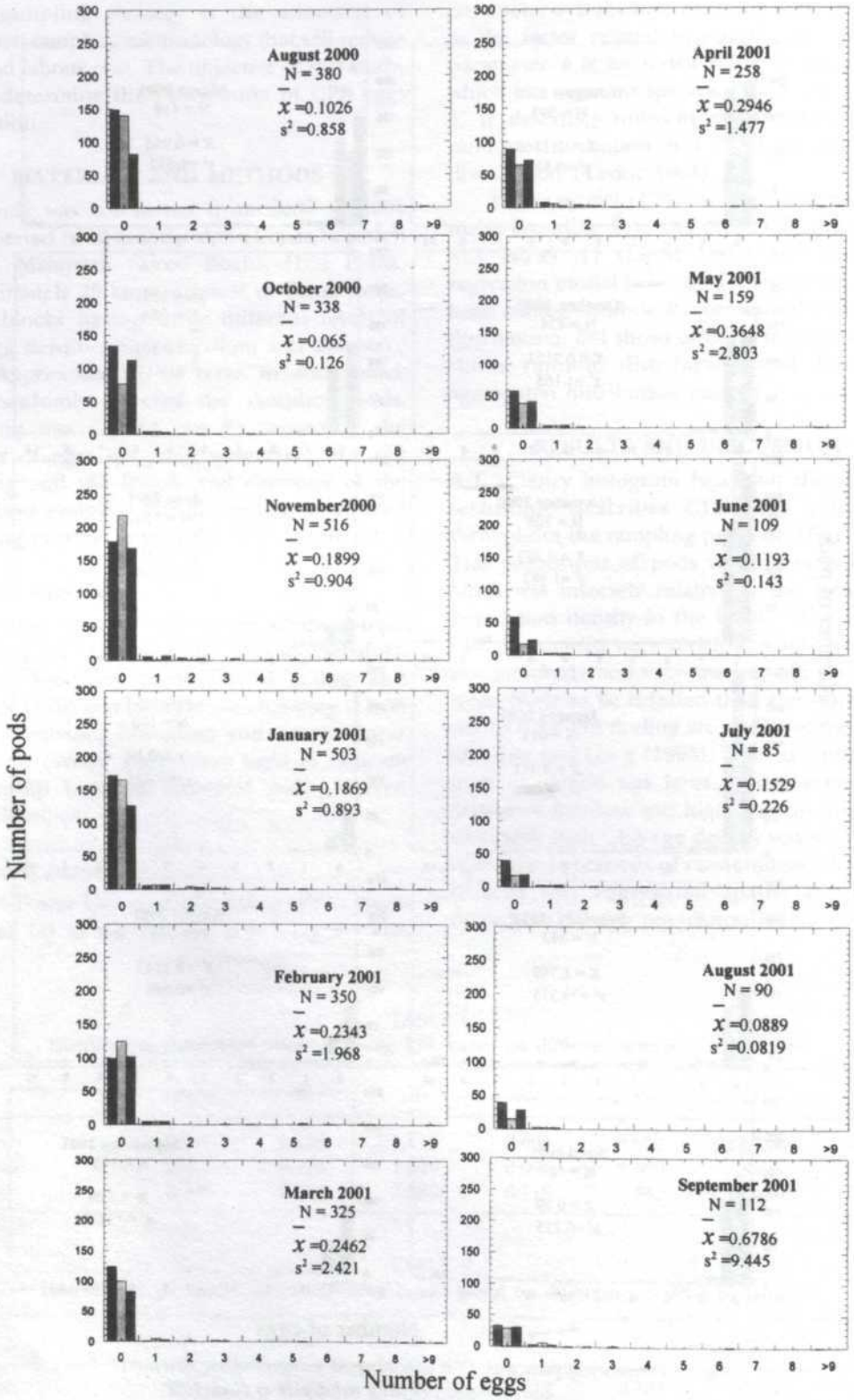

Fig. 2: Frequency of pods with CPB eggs sampled using random, systematic and stratified sampling techniques at block 10A

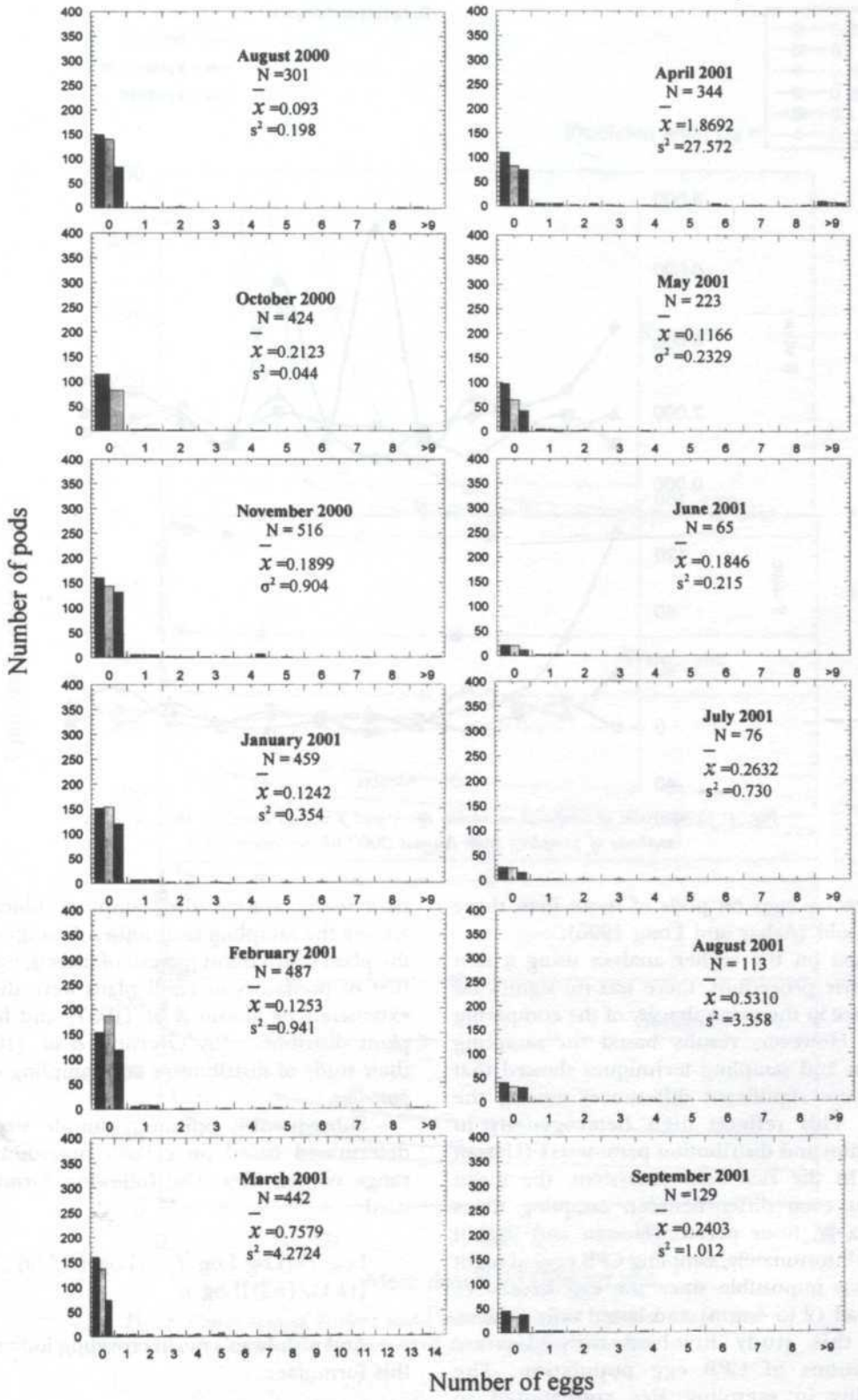

Fig. 3: Frequency of pods with CPB eggs sampled using random, systematic and stratified sampling techniques at block 3A

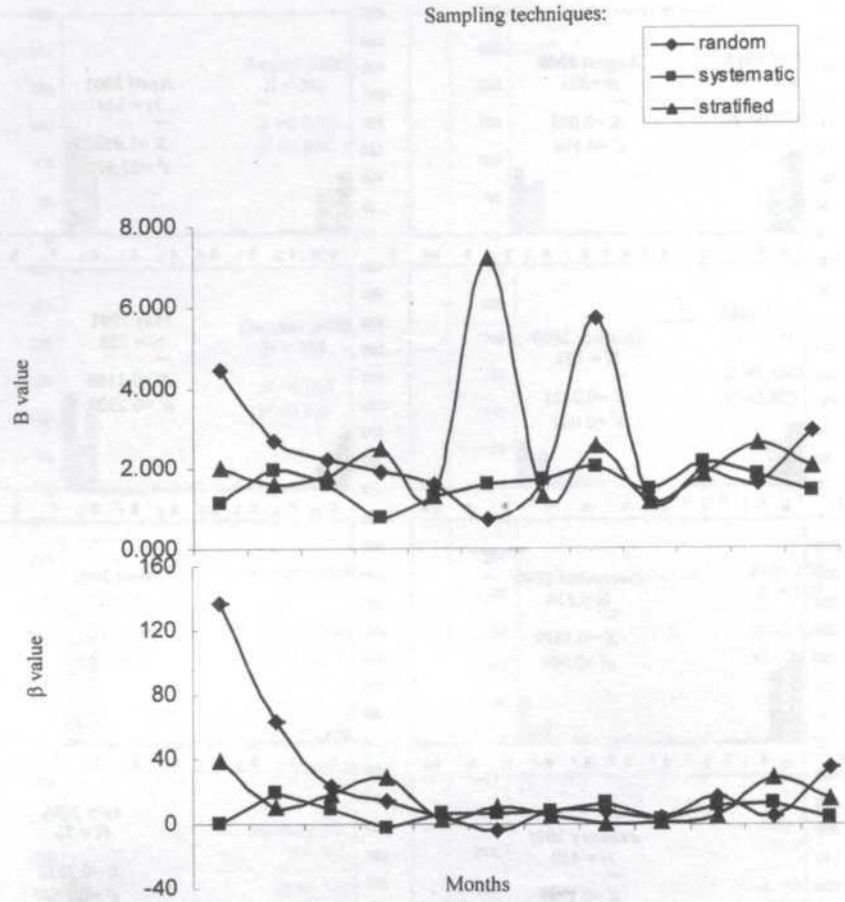

Fig. 4: Comparison of temporal variation of b and β values based on three different methods of sampling from August 2000 till September 2001

prefer to lay eggs on pods of more than three months old (Azhar and Long 1996).

Based on the earlier analysis using a non parametric procedure, there was no significant difference in the mean density of the comparing blocks. However, results based on sampling duration and sampling techniques showed that there were significant differences among the means. This reflects high heterogeneity in population and distribution parameters (Hassan 1996). In the rice field ecosystem, the mean densities even differ between sampling times within a 24 hour period (Hassan and Rashid 1997). Unfortunately, sampling CPB eggs at night times was impossible since the egg sizes were very small (2 to 4 mm) and lasted only 4 days. Thus, this study involved only daytime observations of CPB egg population. The variability in sampling size contributed to variation of the b and ß values. Consequently, there might have been differences in distribution parameters among the sampling blocks and among the sampling techniques. Changes within the plant distribution pattern of insects, especially that of predators in chilli plant were discussed extensively by Maisin *et al.* (1997) and between plant distribution by Overholt *et al.* (1994) in their study of distribution and sampling of *Chilo partellus.*

Subsequently, optimum sample sizes were determined based on certain precisions for a range of densities. The following formula was used:

$$\text{Log } T=[\text{Log Log } T = [\text{Log } (D_0^2/a) \ / \ b\text{-}2] + [(b\text{-}1)/(b\text{-}2)]\text{Log } n$$

And with Iwao's mean crowding indices using this formulae:

$$T = (A+1)/ \ [\ D_0^2\text{-} \ (B\text{-}1)/n).$$

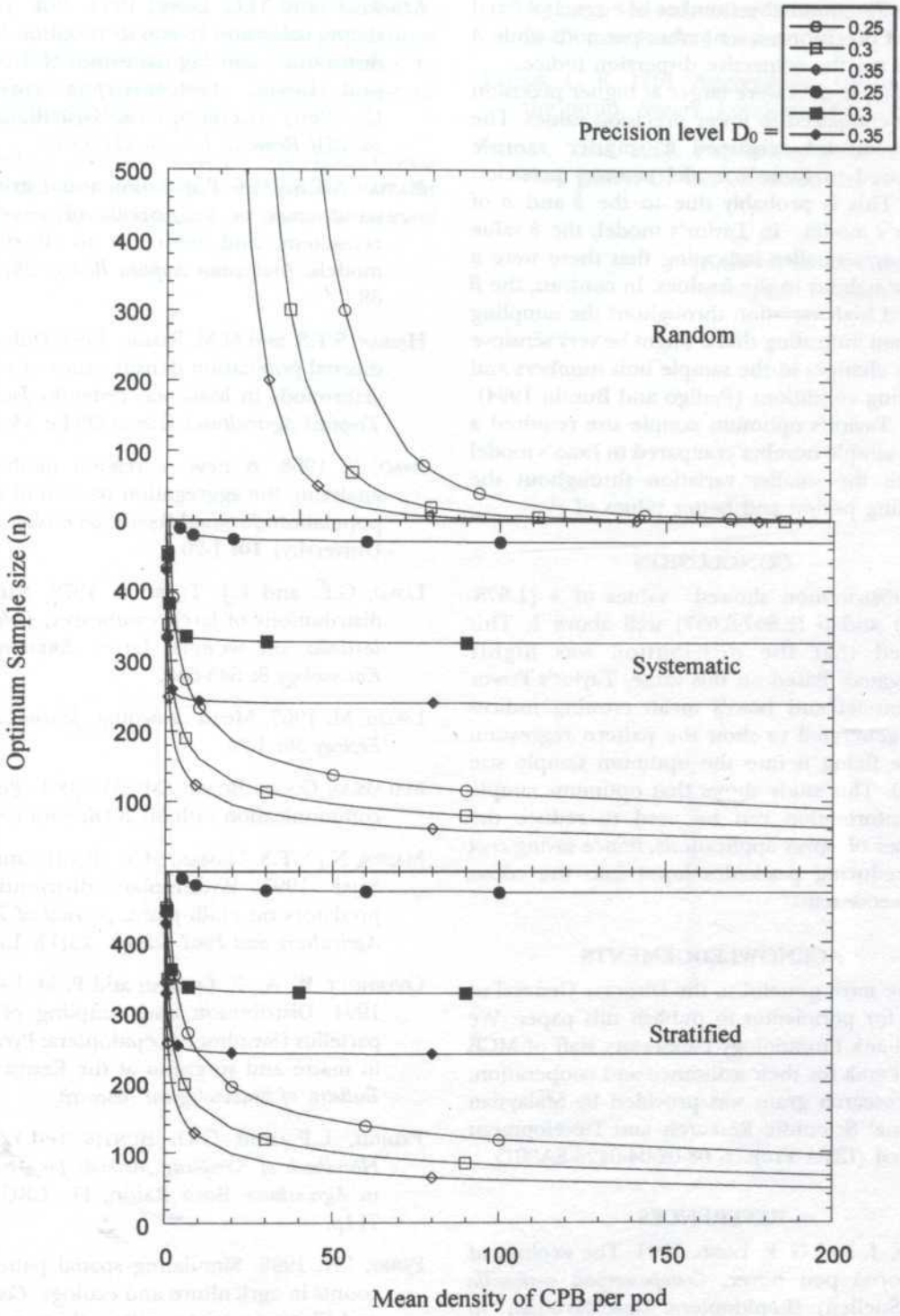

Fig. 5: Comparison of Taylor's model and Iwao's model optimum sample size at various precision levels for three different method

where T= cumulative number of eggs, D_0= fixed level of precisions, n = number per pods while A and B are the respective dispersion indices.

Sample sizes were larger at higher precision values compared to lower precision values. The green model required a smaller sample compared to Kuno's model at every presicion level. This is probably due to the b and a of Taylor's model. In Taylor's model, the b value range was smaller indicating that there were a higher stability in the b values. In contrast, the β showed high variation throughout the sampling program indicating that it might be very sensitive to any changes in the sample unit numbers and sampling conditions (Pedigo and Buntin 1994). Thus, Taylor's optimum sample size required a lower sample number compared to Iwao's model due to the smaller variation throughout the sampling period and better values of r^2.

CONCLUSION

CPB distribution showed values of b (1.678-1.716) and β (2.897-8.637) well above 1. This showed that the distribution was highly aggregated. Based on this value, Taylor's Power Law model and Iwao's mean crowing indices were generated to show the pattern regression before fixing it into the optimum sample size model. This study shows that optimum sample size information can be used to reduce the number of spray applications, hence saving cost and reducing pesticides input into the cocoa agro ecosystem.

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Genetic Relationship and Allozyme Expression of Insecticide Susceptible and Resistant *Helopeltis theivora* Populations from Peninsular Malaysia

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Keywords: Allozyme, *H. theivora*, PAGE, insecticide resistance

ABSTRAK

Helopeltis theivora *dikenali sebagai perosak bagi koko dan teh di Malaysia. Beberapa mekanisme bagi kerintangan serangga telah dicadangkan, contohnya penurunan kadar sensitiviti bagi kawasan sasaran, detoksifikasi metabolik pestisid dan pengurangan kadar penembusan atau translokasi bagi racun serangga. Elektroforesis gel poliakrilamida (PAGE) digunakan untuk mengenal pasti enzim metabolik yang terlibat dalam perkembangan kerintangan bagi* Helopeltis theivora. *Serangga dewasa* Helopeltis theivora *diperoleh daripada tiga populasi yang berlainan iaitu Bukit Cheeding (Banting, Selangor), Sg. Palas (Cameron Highlands) dan MARDI (Cameron Highlands, Pahang). Dua puluh lima enzim telah diuji untuk menentukan polimorfisme dan 8 enzim telah dikenal pasti terdapat di dalam* Helopeltis theivora. *Dendogram yang dihasilkan daripada analisis kluster mengumpulkan populasi Banting dan MARDI dalam satu kumpulan manakala populasi Sg. Palas dikluster dalam kumpulan tersendiri.*

ABSTRACT

Helopeltis theivora *is a known pest of cocoa and tea in Malaysia. Several mechanisms of insecticide resistance have been proposed such as reduction in the sensitivity of target sites, metabolic detoxification of pesticides and decreased penetration or translocation of pesticides. Polyacrylamide gel electrophoresis (PAGE) was used to determine the metabolic enzymes involved in the development of resistance in* Helopeltis theivora. *Adults of* Helopeltis theivora *were collected from three geographical locations namely Bukit Cheeding (Banting, Selangor), Sg. Palas (Cameron Highlands) and MARDI (Cameron Highlands, Pahang). In total, 25 enzymes were screened for polymorphisms and 8 enzymes were detectable in* H. theivora. *The dendogram resulting from the cluster analysis grouped the infrequently sprayed Banting and the MARDI populations together while the intensively sprayed Sg. Palas population clustered by itself.*

INTRODUCTION

Helopeltis theivora is a known pest of cocoa (Entwistle 1972) and tea (Wilson 1999) in Malaysia. Insecticides have been used for the control of the mirids as cocoa is grown widely in this country. Dzolkhifli *et al.* (1998) reported the development of resistance in the Sungai Tekam, Pahang and Serdang populations of this insect to γHCH, deltamethrin and cypermethrin. Several mechanisms of insecticide resistance have been proposed such as reduction in the sensitivity of target sites, metabolic detoxification of pesticides and decreased penetration or translocation of pesticides. Among them, metabolic detoxification was shown to play a major role in insecticide resistance (Sun 1992). The metabolic enzymes involved are those with roles in oxidation (mixed function oxidase), reduction (gluthatione s- transferase) and hydrolysis (esterase) (Matsumura 1985). Organophosphate and carbamate resistances are associated with increased esterase activity in a variety of insects including aphids (Takada and Murakami 1988; Abdel-Aal *et al.* 1992), Colorado potato beetle (Anspaugh *et al.* 1995), cockroaches (Siegfried *et al.* 1990; Prabhakaran and Kamble 1993), and mosquitoes (Pasteur *et al.* 1984; Raymond *et al.* 1987). Insects use specific or

general esterases to accomplish detoxification and sequestration of these groups of insecticides and electrophoresis can play a major role in identifying the enzymes involved (Devonshire and Moore 1982). Based on allozyme electrophoresis, esterase was found to be involved in insecticide resistance in the fruit fly, *Drosophilla buzzati*, from two different populations in Australia (Barker 1994). Therefore the objective of this study was to determine the metabolic enzymes involved in the development of resistance in *H. theivora* by using polyacrylamide gel electrophoresis (PAGE).

MATERIALS AND METHODS

Adults of *Helopeltis theivora* were collected from three geographical locations namely Bukit Cheeding (Banting, Selangor), Sg. Palas (Cameron Highlands) and MARDI (Cameron Highlands, Pahang) (*Fig. 1*) in April 2002. The samples were kept at - 70°C and twenty individuals from each population were used for electrophoresis. Individuals of *H. theivora* were homogenized with 50ml distilled water. 7% polyacrylamide gel electrophoresis (PAGE) was carried out with an initial current of not more than 50mA at 230V for 3 hours until the bromophenol blue dye reached the anodal end of the gel. In total, 25 enzymes were screened for polymorphisms. The gels were stained for α-esterase (α-EST, EC 3.1.1.1) and β- esterase (β-EST, EC 3.1.1.2), octanol dehydrogenase (ODH, EC 1.1.1.37), acid phosphatase (ACP, EC 3.1.3.2), malate dehydrogenease (MDH, EC 1.1.1.37), sorbitol dehydrogenase (SDH, EC 1.1.1.14), glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.44), leucine aminopeptidase (LAP, EC 3.4.11.1), xanthine dehydrogenase (XDH, EC 1.2.1.37), alpha-glycererophosphate dehydrogenase (α-GPDH, EC 1.1.1.8), hexokinase (HK, EC 2.7.1.1), aldehyde oxidase (ALDOX, EC 1.2.3.1), phosphoglucose isomerase (PGI, EC 5.3.1.9), peptidase (PEP, EC 3.4.1.1), isocitrate dehydrogenase (IDH-NADP, EC 1.1.1.42), superoxide dismutase (SOD, EC 1.15.1.1), glutamate oxaloacetate transaminase(GOT, EC 2.6.1.1), acid phosphatase acid (ACP, EC 3.1.3.2), malic enzyme (ME, EC 1.1.1.40), alcohol dehydrogenase (ADH, EC 1.1.1.1), pyruvate kinase (PK, EC 2.7.1.40), lactate dehydrogenase (LDH, E.C 1.1.1.27), 3-Hydroxybutyrate dehydrogenase (HBDH, EC 3.1.1.31), alkaline phosphatase (AKP, EC 3.1.3.1) and adenylate kinase (AK, EC 2.7.4.3) activities separately by

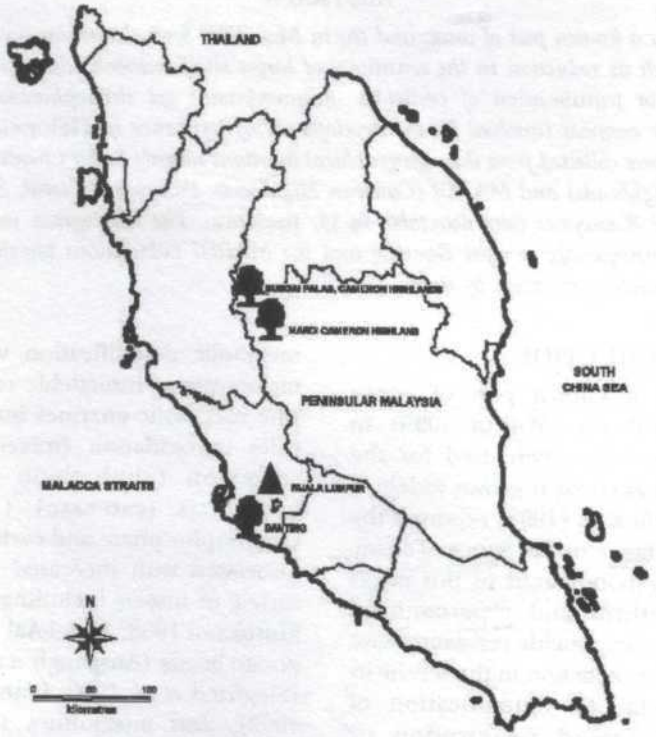

Fig. 1: Map showing sampling sites

the procedures of Shaw and Prasad (1970) with some modification. The POPGENE Version 1.31 computer package of Yeh and Boyle (1999) was used to calculate allelic frequencies, genetic distance (D) and F-statistics (Nei 1978). Based on the phenotypes observed for these eight enzymes in 20 samples from each of the three populations, alleleic frequencies were obtained which were in turn used to calculate the Nei's (1978) genetic distance coefficients among the three populations. The genetic distance coefficients were then used to cluster the populations by using UPGMA (unweighted pair group method with arithmetic averaging).

RESULTS AND DISCUSSION

The results showed that of the 25 enzymes screened, 8 enzymes were detectable in *H. theivora*. The 8 enzymes were α- and β- esterase (EST), xanthine dehydrogenase (XDH), leucine aminopeptidase (LAP), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), glucose 6-phosphate dehydrogenase (G6PDH), aldehyde oxidase (ALDOX) and alkaline phosphatase (ALKP) (Table 1). Allelic frequency data for the 11 loci that could be scored in *H. theivora* are presented in Table 2, except for β-EST-2 that was monomorphic in all populations. The esterase loci showed different allelic frequencies in three populations. α-EST-1 showed a polymorphism in the Banting population. α-EST-2, and β-EST-1 were polymorphic in all populations, while α-EST-3 was monomorphic in all populations. XDH was polymorphic only in the Banting population but was monomorphic in the Sg. Palas and MARDI, Cameron Highlands populations. LAP and SOD were monomorphic in all populations while G6PDH and ALDOX were polymorphic in all populations. GOT was polymorphic in the Banting population but was monomorphic in the Sungai Palas and MARDI, Cameron Highlands population. AKP was polymorphic in the Banting and Sungai Palas, Cameron Highlands population but monomorphic in the MARDI, Cameron Highlands population. Allelic frequencies for the polymorphic loci ranged from 0.0625 to 0.9375 (Table 2). Values of *F*-statistics for *Helopeltis theivora* are presented in Table 3. The mean F_{is}, F_{it} and F_{st} values of 0.0493, 0.4822 and 0.4554 respectively, indicate the differentiation among the populations. The loci showing the highest F_{st} values had low values for gene flow (Table 3). The dendogram resulting from the cluster analysis (*Fig. 2*) grouped the Banting, Selangor and MARDI, Cameron Highlands populations together while the Sg. Palas, Cameron Highlands population clustered by itself. This clustering pattern could be due to the frequent spraying of insecticides at Sg. Palas, Cameron Highlands over the past 5 years (Philip Bauer, unpublished). For the Banting, Selangor and MARDI, Cameron Highlands populations, insecticides were not frequently sprayed when compared with the Sg. Palas, Cameron Highlands population. Based on the Nei's genetic distance value, the populations from Banting and MARDI, Cameron Highlands are closely related. In insects, esterases are known to be involved in important physiological processes, including the catabolism of juvenile hormone (Shanmugavelu *et al.* 2000), insecticide resistance (Prevec *et al.* 1992; Morton 1993; Mutero *et al.* 1994), digestion (Argentina and James 1995) and reproduction (Karotam and Oakeshott 1993). Insect esterase genes have shown high rates of intraspecific and interspecific

TABLE 1
Enzyme names, abbreviations, enzyme codes (E.C.), number of loci, enzymes structures and electrophoretic buffer systems

Enzyme	E.C. Number	Number of loci	Buffer system used
Alpha-esterase (α-EST)	3.1.1.1	3	Poulik
Beta-esterase (β-EST)	3.1.1.2	2	Poulik
Aldehyde oxidase (ALDOX)	1.2.3.1	1	Poulik
Superoxide dismutase (SOD)	1.15.1.1	1	Poulik
Glucose 6-phosphate dehydrogenase (G6PDH)	1.1.1.44	1	Poulik
Xanthine dehydrogenase (XDH)	1.2.1.37	1	TEB
Alkaline phosphatase (AKP)	3.1.3.1	1	Poulik
Glutamate oxaloacetate transaminase (GOT)	2.6.1.1	1	TEMM
Leucine aminopeptidase (LAP)	3.4.11.1	1	TEMM

TABLE 2
Allele frequencies and sample sizes (N) for 11 loci in *Helopeltis theivora* from three different populations

Locus	Allele	Banting	Sg. Palas, C.H	MARDI, C.H
α-EST-1	(N)	40	40	36
	110	0.5000	0.0000	0.0000
	100	0.5000	0.0000	0.0000
α-EST-2	(N)	40	40	22
	110	0.1000	0.2500	0.0000
	100	0.7000	0.7500	1.0000
α-EST-3	(N)	40	40	4
	10	0.0000	0.0000	0.5000
	100	1.0000	1.0000	0.5000
β-EST-1	(N)	40	32	40
	105	0.4000	0.2500	0.0000
	100	0.6000	0.7500	1.0000
XDH	(N)	40	40	10
	102	0.3000	1.0000	1.0000
	100	0.7000	0.0000	0.0000
LAP	(N)	14	38	10
	102	1.0000	0.0000	1.0000
	100	0.0000	1.0000	0.0000
GOT	(N)	40	40	36
	102	0.8750	0.3333	0.0000
	100	0.1250	0.6667	1.0000
SOD	(N)	32	12	18
	105	1.0000	0.1389	1.0000
	100	0.0000	0.8611	0.0000
	(N)	32	6	20
G6PDH	102	0.0625	1.0000	0.2000
	100	0.9375	0.0000	0.8000
ALDOX	(N)	40	30	32
	102	1.0000	0.2727	1.0000
	100	0.0000	0.7273	0.0000
	(N)	30	22	34
AKP	102	0.8667	0.2750	1.0000
	100	0.1333	0.7250	0.0000

*C.H= Cameron Highlands

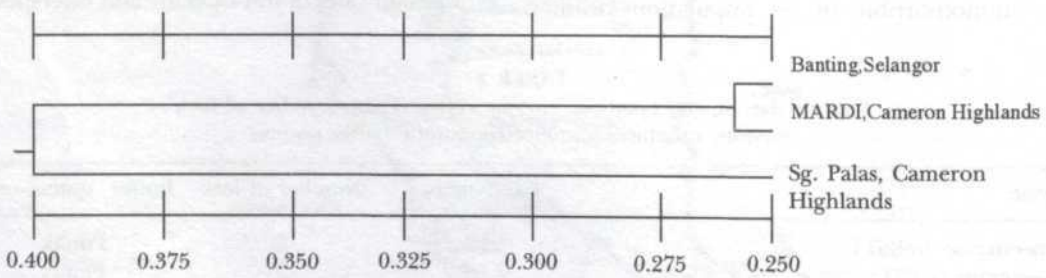

Fig. 2: Dendogram constructed by using UPGMA clustering of three populations of H. theivora *based on Nei's (1978) genetic distance coefficients*

TABLE 3
F-statistics values for the 12 loci of 3 populations of *Helopeltis theivora*

Locus	F_{is}	F_{it}	F_{st}	N_m
XDH	1.0000	1.0000	0.6087	0.1607
LAP	******	1.0000	1.0000	0.0000
GOT	-0.1429	0.7983	0.8235	0.0536
SOD	1.0000	1.0000	0.5714	0.1875
G6PDH	0.4045	0.4208	0.0273	8.9073
ALDOX	******	*****	1.0000	*****
AKP	0.5752	0.7827	0.4885	0.2617
_-EST-1	-0.8231	-0.7391	0.0460	5.1806
_-EST-2	0.6407	0.6825	0.1164	1.8977
_-EST-3	-1.0000	-0.2000	0.4000	0.3750
_-EST-1	-0.5205	-0.2766	0.1604	1.3087
_-EST-2	******	1.0000	1.0000	0.0000
Mean	0.0493	0.4822	0.4554	0.2990

*N_m =Gene flow estimated from $F_{st} = 0.25(1-F_{st})/F_{st}$

TABLE 4
Nei's Unbiased Measures of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) (1978) for three different populations of *H. theivora*

Population	Banting	Sg.Palas, C.H	Mardi, C.H
Banting	******	0.6381	0.7732
Sg. Palas, C.H	0.4493	******	0.7097
Mardi, C.H	0.2573	0.3428	******

variation. Gene duplication followed by divergence of duplicated genes seem to be involved in the origin of at least part of this variability (Brady and Richmond 1992). The stability of insecticide resistance was found to be the result of modifications of the insect genome (Guillemaud *et al.* 1998; Raymond *et al.* 2001). In the sheep blowfly, *Lucilia cuprina*, there is a fitness cost associated with an allele coding for resistance to diazinon. But, following the intensive use of diazinon, a modifier gene had been selected in the field which completely supressed this cost. As a result, resistant insects carrying the modifier gene are equally fit as the susceptible insects.

CONCLUSION

Our results showed that the genetic structure of the population at Sg. Palas could have been affected by the intensive applications of insecticide when compared to the infrequently sprayed populations of Banting and MARDI, Cameron Highlands.

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Mendelian Inheritance of Microsatellite Markers in Southeast Asian River Catfish, *Mystus nemurus*

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ABSTRAK

Lapan primer polimorfik yang telah direka, Mnc434a, Mnc65b, Mnc441, MnBp5-1-2b, MnBp5-1-30b, MnBp5-2-2b, MnBp8-4-43a, dan MnBp8-4-43b, digunakan untuk menguji corak pengasingan dalam uji kaji famili Mystus nemurus. *Induk yang dipilih secara rawak dari populasi Terengganu digunakan untuk menghasilkan populasi F_1. DNA telah diekstrak daripada sampel tisu induk, manakala DNA progeni telah diekstrak daripada keseluruhan ikan yang berumur satu bulan. Analisis Chi-kuasa dua telah dijalankan untuk memastikan kesignifikanan keputusan yang didapati. Keputusan menunjukkan bahawa kelapan-lapan lokus mikrosatelit yang diuji terasing hampir dengan nisbah pewarisan Mendel, yang berguna untuk menghasilkan satu peta genetik bagi spesies ini.*

ABSTRACT

Eight previously designed polymorphic microsatellite loci, namely, Mnc434a, Mnc65b, Mnc441, MnBp5-1-02b, MnBp5-1-30b, MnBp5-2-02b, MnBp8-4-43a, and MnBp8-4-43b, were used to examine their modes of segregation in Mystus nemurus *family study. Randomly selected broodstocks from Terengganu population were used to generate F_1 population. DNA was isolated from tissue samples of the parents, whilst the whole one-month-old F_1 progeny was used to isolate the DNA. Chi-square analysis was performed to test the significance of the results obtained. It was shown that all the eight microsatellite loci examined segregated close to Mendelian inheritance ratios, which would be useful in generating a genetic linkage map for this species.*

INTRODUCTION

The Southeast Asian River Catfish (*Mystus nemurus*), locally known as "ikan baung," is an important food fish cultured in Southeast Asian countries, especially in Malaysia and Thailand. Therefore, it is of interest to obtain genetic information on the local species in order to construct an informative genetic linkage map for efficient use in any future breeding program. However, knowledge of Mendelian ratios must be available before any linkage map can be constructed. Mendelian inheritance deals with hereditary transmission of genes or genetic markers from one generation to the next. One key principle is segregation in which the two alleles in an individual separate during the formation of gametes so that each gamete is equally likely to contain either member of the pair. Earlier studies had been done based on various types of markers, particularly on the dominant markers (Siraj *et al.* 1998; Chong *et al.* 1999; Leesa-Nga *et al.* 2000; Hoh *et al.* 2003; Usmani *et al.* 2003). Nonetheless, very limited information was available for these markers to characterize the species and to construct a genetic linkage map. Therefore, there is a need for a more powerful marker.

Microsatellite, a single locus codominant, and highly polymorphic marker, has been the marker of choice in past studies. This marker has been found in all eukaryotic organisms studied to date and is widely applied in the construction of genetic linkage maps in various organisms.

In this study, eight polymorphic microsatellite markers, which were isolated by using the

enrichment techniques-Random Amplified Hybridizing Microsatellites (RAHMS); Hoh *et al.* 2004), and 5' anchored Polymerase Chain Reaction (PCR) (unpublished), were examined for their modes of segregation in a fish family, which contained 100 offsprings. The results obtained would determine the usefulness of these loci in future studies of genetic linkage mapping in this species.

MATERIALS AND METHODS

Samples

A family of *M. nemurus* with a total of 100 F_1 progenies was bred from a wild population in Terengganu. Broodstocks with body weight ranging from 550g to 650g were randomly selected. Each female was injected twice with ovaprim (Syndel International Cooperation, Canada) at 0.1ml kg^{-1} body weight for the first injection, and 0.4ml kg^{-1} body weight for the second injection after eight hours interval, while each male was injected once with ovaprim at 0.4ml kg^{-1} body weight. The males and females were kept separately in one-tonne capacity fiberglass tanks prior to stripping. After eight hours, eggs from female and sperm from male were stripped into a porcelain bowl for dry fertilization and spread into 2 one tonne-fibre tanks. Hatching occurred around 24 to 48 hours after fertilization. Cultured *Artemia* was fed to the progenies twice a day for two weeks and then changed to red worms (commercially available) for the following weeks. F_1 progenies were harvested at the end of the fourth week and samples of tissue from the parents were collected. DNA extraction was done using QiaAmp DNA minikit (Qiagen, USA) according to the manufacturer's instruction.

Microsatellite Analysis

Eight primer pairs, specifically designed for this species using the 5' anchored PCR method and Random Amplified Hybridizing Microsatellites (RAHMs) technique, were screened against both the parents and the F_1 progenies (Table 1). Amplifications were performed using the MJ Research PTC200 thermal cycler (USA). The reaction mixture contained about 30ng DNA template, 1mM of each nucleotide, 1 unit of *Taq* polymerase (Promega, USA), 1 X PCR reaction buffer, 1mM Mg^{2+}, and 10pmol of each microsatellite primer pairs in a total volume of 10μl. The amplification conditions included 4 min of initial denaturation at 94°C, then followed by 35 cycles of 30s denaturation at 94°C, 30s of appropriate annealing temperature (as shown in Table 1), 30s extension at 72°C. A final extension of 5 min at 72°C was also included. Amplicons were examined against 20bp ladder (BMA, USA) on either 4% metaphor gels and then later validated with 8% non-denaturing polyacrylamide gels, and then photographed using Alpha Imager gel documentation system (Siber Hegner, Germany) after ethidium bromide staining.

Data Analysis

Segregation of microsatellite loci were expected to be in a 1:1 ratio for markers heterozygous in either one of the parents; 1:2:1 and 1:1:1:1 ratios for multiallelic segregating among two parents. Goodness of fit to the Mendelian inheritance ratio was determined by χ^2 analysis (Chong *et al.* 1999). The null hypothesis of this study was that the alleles segregating do not deviate from Mendelian inheritance ratio at a significance level of P= 0.05.

RESULTS

All the eight markers examined showed variations between the parents. Six primer pairs segregated according to Mendelian expectations at a significance level of P=0.05. Four of these loci were expected to show Mendelian ratio of 1:1; while three were expected to show a ratio of 1:2:1. The last one, which had three alleles in the parents were expected to segregate in the ratio of 1:1:1:1 (Table 2). Locus MnBp5-1-30b was observed to have a segregation ratio of 2.125:1 (*Fig. 1*); while MnBp5-1-05b segregated in the ratio of 1:1.19:2.25:1.81, slightly deviated from the expected ratio.

DISCUSSION AND CONCLUSIONS

There have been very few reports on the study on Mendelian inheritance of microsatellite markers (Usmani 2002; Chan 2003). Therefore, information on the Mendelian inheritance ratio available is limited. Besides, examination of these studies showed relatively low variation on their family sample (Usmani 2002; Chan 2003).

The codominant nature of microsatellite markers enables the identification of both homozygous and heterozygous at a particular loci. Genotypes and phenotypes of parents and offsprings could be determined directly. Consequently this feature makes them very

TABLE 1
Markers tested for Mendelian inheritance ratio, annealing temperature and the accession numbers

No	Locus	Primer sequence	Repeat motif	Annealing temp (°C)	GenBank accession
1.	Mnc434a	F: TCAGCATGCGACTAAAACA R: TGGTTTTTCAGCAGTATTGG	$(TAT)_3N_5(CA)_{10}$	55	AF346466
2.	Mnc65b	F: CCTGGTTTTTCAGCAGTATT R: GGATCAGCATGCAACTAAA	$(GT)_{10}N_4(ATA)_3$	55	AF346467
3.	Mnc441	F: CAGGTGGAACATTTTGGAT R: TTTAGAGCTATTCCCTTGGA	$(AAAT)_4AAT$ $(TGG)_3$		55 AF346470
4.	MnBp5-1-02b	F: TCAAAGTGAGGAGATGGA R: TTTTGTCACTACAGAGCTGCAT	$(TG)_{10}$	60	AY205992
5.	MnBp5-2-02b	F: ACACCAAAGAGATGACCATT R: TCTCTGTGAAACGCTTCTTT	$(GA)_{12}N_5(GA)_5$ $(CA)_{10}$ $(A)_{11}N_5(A)_8(GA)_5$	55	AF205994
6.	MnBp5-1-30b	F: TTTGGCTACTAGAGACTGACTT R: GGATTATTAGGCAAAACGTG	$(TG)_4$	55	AY852259
7.	MnBp8-4-43a	F: GTTATTTTCGTTGTTGTTG R: GACCGAAGAACATAAACTAT	$(GTT)_5(GT)_2$	55	AY806612
8.	MnBp8-4-43b	F: CACGTGTGTAAGATAAATAG R: GCACTGAGAAATGTGAGAAA	$(AAGG)_5(GA)_{12}GAAAA(GAAA)_2$		55 AY806612

TABLE 2
The observed and expected microsatellites Mendelian inheritance ratios among F_1 progenies and their χ^2 values

Locus	Parent (♂x♀)	Obs. F_1 genotype	Exp. ratio	χ^2	Probability, P
Mnc434a	DD X CD	45:55	CD:DD 50:50	1.00	0.317
Mnc65b	EE X DE	48:52	DE:EE 50:50	0.16	0.689
Mnc441	CG X CG	27:49:24	CC:CG:GG 25:50:25	0.54	0.763
MnBp5-2-02b	JK X JK	30:50:20	JJ:JK:KK 25:50:25	2.00	0.368
MnBp8-4-43a	AA X AB	44:56	AA:AB 50:50	1.44	0.230
MnBp8-4-43b	NP X NP	21:52:27	NN:NP:PP 25:50:25	0.88	0.644
MnBp5-1-30b	GG X GH	68:32	GG:GH 50:50	12.96	0.000*
MnBp5-1-02b	FI X GI	17:19:35:29	FG:FI:GI:II 25:25:25:25	10.16	0.017*

*significant deviation at P<0.05.

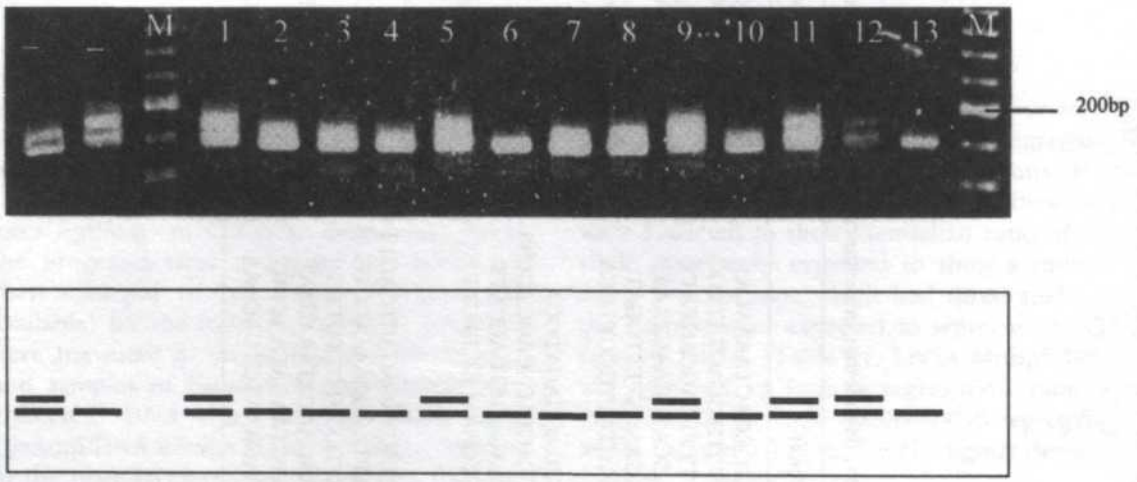

Fig. 1: Profile of primer MnBp5-1-30b for family study. Lane 1, Female; Lane2, Male; Lane 3- 13, progenies F_1; Lane M, 20bp ladder

attractive markers. In contrast, the genotypes of parents studied previously could only be inferred by phenotypic ratio observed in the progenies. While it was demonstrated that the RAPD and AFLP studied showed inheritance in a dominant Mendelian fashion (Chong *et al.* 1999), direct determination of heterozygosity is only possible using backcross.

The unusual segregation ratio shown by MnBp5-1-30b was probably due to the meiotic drive, in which two alleles did not show Mendelian segregation from the heterozygous genotype. This could be due to biological mechanisms that would favor the maintenance of a large number of alleles in the natural populations, which is not uncommon in mammals, insects, fungi and other organisms (Hartl 2000).

Locus MnBp8-4-43a and MnBp8-4-43b are syntenic. They are physically close to each other as these markers are isolated from the same insert. χ^2 analysis showed that there was significant linkage between these loci (χ^2= 11.742, P=0.03). Though it is not suitable to use both of

these loci together in the population studies, the linkage of these two loci may have provided us with valuable information for the Quantitative Trait Loci (QTL) and evolutionary studies. Nonetheless, not all syntenic loci are linked. Findings are often influenced by the sample sizes, interference of the other loci, and different recombination rates among the individuals. There are advantages of studying Mendelian inheritance of a species. First, the information obtained will be used for the construction of a genetic linkage map. Secondly, this study ensures the presence of bands that are inherited in the Mendelian fashion, as this is important for monitoring the effectiveness of selective programs and QTLs (Quantitative Trait Loci). Thirdly, it could be used for the application of pedigree tracing of broodstocks in the breeding programs conducted in any hatchery. Thus, microsatellite markers have proven to be markers of choice for genetic characterization, population structure, and molecular breeding programs.

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Rapid and Non-radioactive Detection Method of Microsatellites in *Mystus nemurus*: A Refined Technique

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Keywords: Microsatellites, *Mystus nemurus*, cloning, polymerase chain reaction (PCR), random amplified microsatellites (RAMs)

ABSTRAK

Satu kaedah mudah dan cepat untuk pemencilan mikrosatelit DNA berdasarkan teknik PCR 'Random Amplified Microsatellites' (RAMs) telah digunakan dalam kajian ini. Kajian ini adalah sebahagian usaha yang berterusan untuk memperbaiki dan menyempurnakan teknik tersebut supaya lebih cepat, efektif dan mencapai produktiviti optimum dalam membangunkan penanda mikrosatelit lokus tunggal bagi ikan Baung, Mystus nemurus. *Protokol pemencilan mikrosatelit yang telah diperbaiki ini berkebolehan mengesan sebanyak 135 bahagian mikrosatelit yang menghasilkan 42 jujukan genom unik yang telah diserahkan ke GenBank. Teknik yang diperbaiki ini dapat mengurangkan jumlah masa yang diperlukan dari pengklonan PCR hingga penjujukan bahagian mikrosatelit yang spesifik kepada kurang dari tiga setengah bulan.*

ABSTRACT

A simple and rapid method of DNA microsatellite isolation based on the Random Amplified Microsatellites (RAMs) PCR technique was used in this study. The work presented here is part of a continuous effort in refining and perfecting the technique for more rapid, effective and optimum productivity in single locus microsatellite marker development for the River catfish, Mystus nemurus. *The current refined protocol for microsatellite isolation was able to detect a total of 135 microsatellite regions resulting in 42 unique genomic sequences being submitted to GenBank. This refined technique is able to reduce the total time required from PCR cloning till sequencing specific microsatellite regions to less than three and a half months.*

INTRODUCTION

The importance of microsatellites in genetic studies has been greatly acknowledged over the years (Chambers and MacAvoy 2000). This is due to microsatellite markers being a codominant marker system which is more informative than dominant markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Direct Amplification of Length Polymorphisms (DALP). Another added advantage of microsatellite markers is that they amplify regions of repetitive elements with simple repeat motifs of one to six nucleotides which show high levels of allelic variations in the number of repeat units. All these make microsatellites a popular and effective marker system that is useful for various genetic studies such as population, linkage and phylogenetic studies and also for quantitative traits loci (QTL) studies.

However the conventional method used for microsatellite detection is far from being cost effective as it is a laborious and time-consuming process (Tan 2002). In recent years, researchers around the world especially in developing countries had developed different methods for detecting microsatellites. The main objectives were targeted on saving cost and time, increasing effectiveness and productivity. Generally, most methods use the library-enrichment approach which can later be divided into several categories such as probe hybridisation, streps-avidin capture of microsatellites and conversion of multi-locus

microsatellite banding patterns to single locus microsatellite markers. In this paper, the method described was used to achieve the last of the categories previously mentioned. This approach is the most direct and straightforward method, giving rapid results. The concept behind this method is the use of a multi-locus microsatellite marker system namely Random Amplified Microsatellites (RAMs) with the amplified microsatellite bands later being converted to a marker amplifying a single microsatellite locus through a series of steps.

MATERIALS AND METHODS

DNA Extraction and PCR Amplification by RAMs Primers

Genomic DNA was extracted from a single *Mystus nemurus* blood sample following the method of Taggart *et al.* (1992) for microsatellite isolation. A total of 8 RAMs primers (Table 1) were selected for PCR amplification which were cloned in the later part of this work. Amplification was done in a total reaction volume of 10 μL containing 1.75 mM of magnesium chloride, 1X of Promega reaction buffer, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 3 units of Promega *Taq* polymerase, 50 ρmol of RAMs primer and 0.3 μL of template DNA ($\approx$ 50 ng). The general PCR profile consisted of 3 minutes of pre-denaturation at 95°C and 35 cycles each consisting of 20 seconds of denaturation, 20 seconds of annealing at an optimised temperature (Table 1), 20 seconds for elongation at 68°C and final elongation at 68°C for 5 minutes. This was followed by electrophoresis of the PCR product on a 2% agarose gel to detect the presence of bands when viewed under transilluminator after ethidium bromide staining.

Cloning of RAMs Primer

Fresh PCR amplicon of RAMs primer were cloned into pCR®2.1-TOPO® vector using TOPO TA Cloning Kit (Invitrogen, USA) following the manufacturer's instructions. Positive clones that contained inserted PCR amplicon were identified as white colonies in LB agar (Pronasida, USA) plates containing ampicillin (50 μg/mL) and X-gal (40 mg/mL). All the positive clones were selected and grown in another LB agar plate.

Storage of Clones Containing Inserts of Microsatellite Sequences

All the identified positive clones were cultured separately in LB medium (5 mL) containing ampicillin (50 μg/mL) in a 15 mL Falcon tube. The culture was incubated overnight at 37°C with vigorous shaking at a speed of 250 rpm. In the morning, approximately 0.85 mL of each culture was mixed with 0.15 mL of sterile glycerol and transferred into a 1.5 mL microcentrifuge tube. The mixture was vortexed for a few seconds and stored at -80°C. The stored clones could be retrieved at any time in the future for further analysis.

Preparation of Crude Plasmid DNA

The remaining culture left in the Falcon tube (approximately 4.15 mL) in the previous section was centrifuged at 15,300 x g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 100 μL of de-ionised distilled water by vortexing. The mixture was then transferred

TABLE 1
List of the 7 RAMs primers used in this study

No.	Name	Sequences	Targeted repeat motifs	Annealing temperature (°C)
1	SC1	KRKRDKDKDKDK$(CA)_6$	CA	52
2	SC2	N_5MMHYHYHYH$(GA)_6$	GA	52
3	SC6	N_7MMBRBRB$(GA)_{10}$	GA	50
4	BP3	N_3YYHMHMHMH$(TG)_6$	TG	58
5	BP9	WWWVYVYVY$(AG)_8$	AG	58
6	BP10	KKDRDRD$(TC)_{10}$	TC	58
7	BP13	KKBSBSBSB$(CT)_6$	CT	58

(*Note:* B=C/G/T, D=A/G/T, H=A/C/T, K=G/T, M=A/C, N=A/C/G/T, R=A/G, S=C/G, V=A/C/G, W=A/T, and Y=T/C)

into a 0.5 mL PCR tube followed by heating at 99°C by using a thermocycler for 20 minutes. This was followed by centrifugation at 14,000 rpm for 5 minutes to pellet down the bacterial cell debris. The supernatant containing crude plasmid DNA was ready for the next step of the PCR work.

M13 PCR Amplification

This step was to analyse and estimate the insertion size of all the positive clones stored in glycerol by using M13 Forward (-20) and M13 Reverse primer. The PCR amplification was done in a reaction mixture containing 2.0 mM of magnesium chloride, 1X of Promega reaction buffer, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of Promega *Taq* polymerase, 50 rmol of each M13 Forward (-20) and M13 Reverse primer and 1.0 μL of crude plasmid DNA. De-ionised distilled water was added to a final volume of 10 μL. The PCR profile consisted of a pre-denaturation step of 95°C for 3 minutes followed by 35 cycles at 91°C for 20 seconds, at 55°C for 20 seconds and at 72°C for 30 seconds with a final elongation step at 72°C for 5 minutes. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualised under UV light (*Fig. 1*).

Identifying Positive Clones with Different Insert Size for DNA Sequencing

After the M13 PCR amplification, the single band that appeared on the 2% agarose gel for each of the positive clones showed that the insertion size of the RAMs amplicon were successfully cloned. A band that appeared in the region around 200 bp indicated that no PCR amplicon was successfully cloned into the vector. This is because the region flanking the PCR inserted by the M13 Forward (-20) to M13 Reverse primer is 202 bp in length. Any length of PCR amplicon inserted in between the flanking region after cloning will add to the 202 bp resulting in a band which appears in the gel with a molecular weight of more than 202 bp. This step is essential to allow the researchers to select the 'real' positive clones and clones that contained different PCR insertion sizes to be sent for sequencing. This is crucial to ensure that not a single RAMs amplicons cloned is left out of the sequencing process. In this study, three positive clones having the same insertion size were selected for plasmid extraction followed by sequencing. The rest of the positive clones of the same insertion size were considered as a redundancy of the same PCR insert.

Plasmids DNA Extraction and Automated DNA Sequencing

Once the positive clones with the estimated PCR insertion size were identified for sequencing, the desired clones were re-grown in LB agar (Pronasida, USA) plates retrieved from the previously stored clones. The plasmid DNA was extracted according to Sambrook *et al.* (1989). Automated sequencing of the plasmid DNA was carried out at the Institute of Bioscience (IBS), Universiti Putra Malaysia by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA).

Submission of DNA Sequences to GenBank

Unique DNA sequences containing microsatellites were submitted online to GenBank. This was done by using the online tool BankIT provided by the GenBank website, *http://www.ncbi.nlm.nih.gov/*.

RESULTS AND DISCUSSION

The method described in this study was able to detect a total of 135 microsatellite regions by utilising only seven RAMs primers within three and a half months.

The novel idea of introducing the M13 PCR amplification step in this refined technique for rapid and enhanced efficiency in detecting microsatellites was stressed in this study. The aim of this refined technique is to solve some of the limitations encountered by the previously published protocols (Teh *et al.* 2003; Kumar *et al.* 2002a; Kumar *et al.* 2002b) using the RAMs method.

The limitation of using the RAMs method in the previously described protocols is the inability to handle the large number of positive clones produced after the cloning process. In studies reported by Teh *et al.* (2003), Kumar *et al.* (2002a) and Kumar *et al.* (2002b), no protocol was suggested for screening the large numbers of clones produced. The studies, instead, used the approach of randomly picking up clones for sequencing. The shortcoming of this approach is that not all the PCR amplicons with different sizes that had been cloned are sequenced. Without guidance from the M13 amplification analysis, the probability of maximising the

potential of the RAMs technique for detecting more microsatellites is greatly reduced. The M13 PCR amplification enhanced the method's efficiency by allowing researchers to determine the size of the PCR insert of each positive clone thus helping in choosing the right clones for sequencing. Another advantage is the ability to distinguish between a false positive clone (one without a PCR insert in the vector) from a 'real' positive clone. This advantage served as a safer alternative by avoiding the use of radioactive components (Chenuil *et al.* 2003; Watanabe *et al.* 2001; Kawai *et al.* 2001; Miller *et al.* 2001) and the toxic chemicals of fluorescein-labeled oligonucleotides (Wimberger *et al.* 1999) for screening of 'real' positive clones.

More researchers are encouraged to be involved in isolation of microsatellites efforts for the species of their interest since microsatellites is becoming the popular choice of marker systems. The perception that isolating single locus microsatellite markers is time consuming, technically demanding and cost ineffective should be reconsidered with an open mind given the availability of the approaches used in this study.

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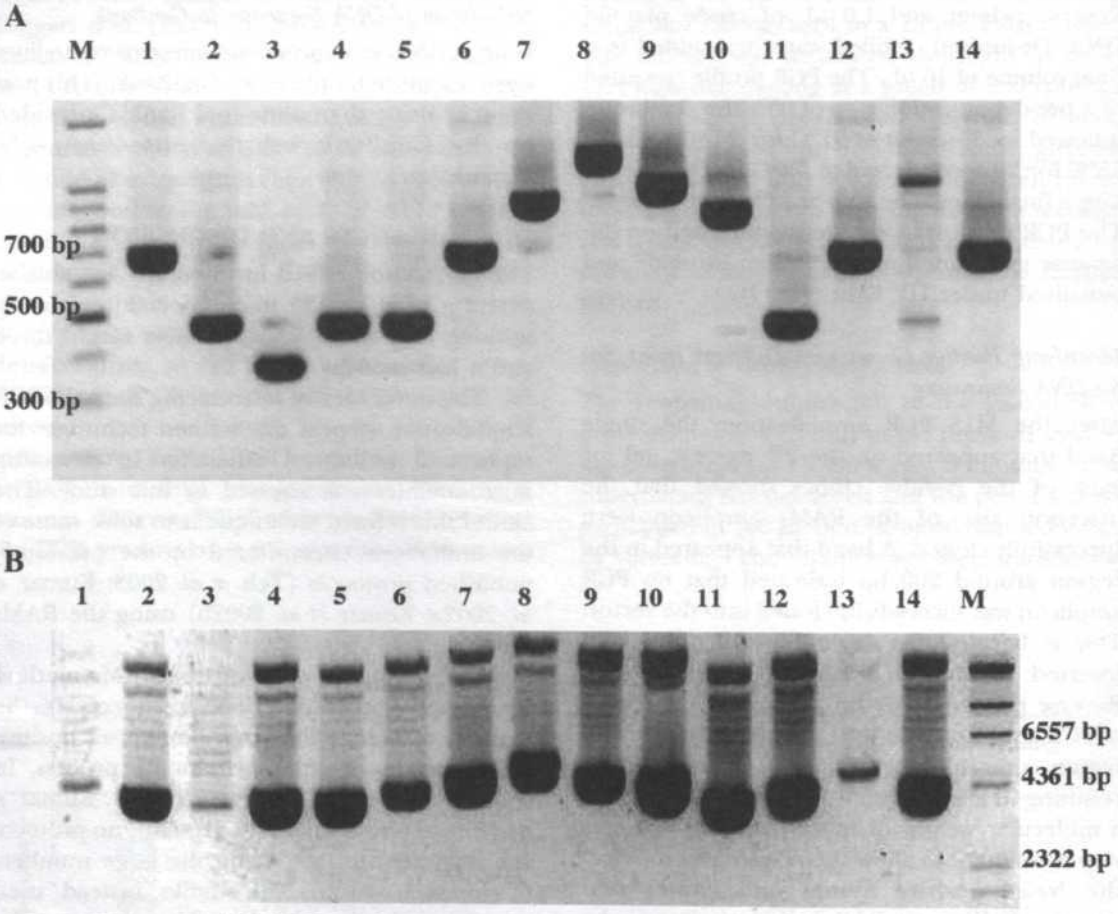

Fig. 1. Comparison of M13 PCR amplification banding profiles corresponding to the banding profile of the extracted plasmid DNA for RAMs primer BP3. (A) Banding profile of M13 PCR amplification for positive clones of BP3. Lane M: 100 bp ladder; lanes 1-14: band profile of positive clones 1-14. Amplified PCR products were electrophoresed on a 2% agarose gel in 1X TBE buffer at 70 V (B) Banding profiles of plasmid DNA for positive clones of BP3. Lanes 1-14: banding profile of plasmid DNA for positive clones 1-14; lane M: Lambda Hind III marker. Plasmids DNA were electrophoresed on a 1% agarose gel in 1X TBE buffer at 70 V

TABLE 2
List of GenBank accession number for the unique genomic sequences submitted and the number of microsatellite regions detected by using RAMs primers

No.	RAMs primer	Total no. of micro-satellite regions isolated	Total no. of unique genomic sequences produced	GenBank accession no.
1	SC1	14	4	AY845085-AY845088
2	SC2	31	7	AY845089-AY845095
3	SC6	26	6	AY845096-AY845101
4	BP3	14	9	AY845102-AY845110
5	BP9	21	7	AY845111-AY845117
6	BP10	15	4	AY845118-AY845121
7	BP13	14	5	AY845122-AY845126

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Isolation of DNA Microsatellite Markers in the Green-lipped Mussel, *Perna viridis*

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ABSTRAK

Sejumlah 21 mikrosatelit DNA telah dipencilkan daripada Perna viridis *dengan menggunakan teknik rantaian tindak balas '5' anchored polymerase '. Primer-primer telah direka untuk tujuh lokus mikrosatelit dan amplifikasi PCR bagi ketujuh-tujuh lokus mikrosatelit ini menunjukkan bahawa lima adalah polimorfik dengan jumlah alel per lokus menjulat daripada 2 hingga 4. Ini merupakan penanda set mikrosatelit pertama yang telah dibangunkan untuk spesies ini. Penanda-penanda ini amat berguna sebagai alat untuk kajian terperinci latar belakang genetik* P. viridis *di negara kita memandangkan spesies ini dikultur sebagai sumber protein murah bagi manusia dan telah dikenal pasti sebagai agen biomonitor yang berpotensi untuk pencemaran logam berat di Pantai Barat Semenanjung Malaysia.*

ABSTRACT

A total of 21 DNA microsatellites were isolated from Perna viridis *by using a 5' anchored polymerase chain reaction technique. Primers were designed for seven microsatellite loci and the PCR amplifications of these seven microsatellite loci showed that five were polymorphic with the number of alleles per locus ranging from 2 to 4. These are the first set of microsatellite markers developed for this species. These markers are useful as tools for more detailed studies of the genetic backgrounds of the green-lipped mussel,* P. viridis, *in our country as this species is being cultured as a cheap source of protein for human consumption and has been identified as a potential biomonitoring agent for heavy metal pollution in the west coast of Peninsular Malaysia.*

INTRODUCTION

The green-lipped mussel, *Perna viridis*, is widely distributed in the Indo-Pacific region (Siddall 1980). This mussel is important ecologically because of its widespread distribution and biological filtration activity, and also economically because of its value as a cheap source of animal protein for human consumption. It was once regarded as a nuisance by oyster farmers in the Philippines and today it is being extensively cultured in many Asian countries including Malaysia (Rosell 1991; Monirith *et al.* 2003). Recently, this species has been used as a biomonitor for a wide range of contaminants such as metals, organochlorines, polycyclic aromatic hydrocarbons and organotins throughout the Indo-Pacific region and has considerable potential as a pollution monitoring agent throughout its geographical range (Nicholson and Lam 2005).

In Malaysia, *P. viridis* is widely distributed along the west coast of Peninsular Malaysia and to a lesser extent, in certain parts of Sabah and the east coast of Peninsular Malaysia. It is a local seafood delicacy and is one of the few local species that is successfully cultured in the Straits of Malacca. Furthermore, its sessile lifestyle and widespread distribution in the Straits has prompted its use as a bioindicator of heavy metal contamination in the Straits of Malacca; which is one of the busiest shipping lanes in the world (Ismail *et al.* 2002). Therefore, information

on the population genetic structure of *P. viridis* along the Straits of Malacca is undoubtedly very important since different species may accumulate metals at different rates (Gyllensten and Ryman 1985). Besides, this information can be applied to improve the broodstocks of this commercially important seafood delicacy and hence increase the productivity of mussel farms.

In the last few years, microsatellites have become one of the most popular molecular markers used in various fields of study. Being codominant, PCR-based, highly polymorphic and easy to score contribute to the popularity of microsatellites as a marker of choice. One of the major drawbacks of this DNA marker is that it needs to be isolated *de novo* from species that are being examined for the first time (Zane *et al.* 2002). Although microsatellite isolation is a tedious, expensive and laborious procedure, this does not seem to be a strong deterrent factor. The conventional method of isolating microsatellites is like searching for a needle in a haystack. It involves the creation of a size-fragmented genomic library, cloning of bands and subsequently screening thousands of colonies for the presence of microsatellite repeats through hybridization. The success rate usually ranges from 12% to less than 0.04% (Zane *et al.* 2002). Here we report on the development of microsatellites from *P. viridis* using a modification technique of the 5' anchored PCR technique of Fisher *et al.* (1996).

MATERIALS AND METHODS

Sample Collection

Mussels were collected from five different locations along the west coast of Peninsular Malaysia. The five collection sites were Tanjung Rhu (Pulau Langkawi, Kedah), Bagan Tiang (Perak), Pulau Ketam (Selangor), Muar (Johor) and Kampung Pasir Puteh (Johor) with a sample size of 20 individuals per location. The adductor muscle was excised from the mussel and kept at -80°C prior to DNA analysis.

DNA Extraction

DNA was extracted from approximately 20-30 mg of the adductor muscle by using a CTAB based protocol described by Winnepennincks *et al.* (1993) with minor modifications.

5' Anchored PCR Amplification

Two degenerate RAMs primers, VJ1 and BP2, were used to produce a genomic library enriched for microsatellites (Table 1).

A polymerase chain reaction (PCR) was carried out in a total volume of 10 μL containing 25 ng of genomic DNA, 1X PCR buffer, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 15 pmol of either primer VJ1 or BP2, 2mM $MgCl_2$, and 1.5 U of *Taq* DNA polymerase (Promega, USA). Amplifications were performed in a Peltier Thermal Cycler PTC-220 (MJ Research, USA) with an initial 3 min of predenaturation at 96°C, followed by 40 cycles of denaturation at 96°C for 15 seconds, an appropriate annealing temperature for 15 seconds (as shown in Table 1) and extension at 72°C for 30 seconds. A final extension step at 72°C for 7 min was included for the attachment of a dATP at the 3' terminal for the cloning reaction. The PCR product was run on a 2% agarose gel and visualised by ethidium bromide staining to confirm the presence of bands. After performing the PCR reaction, the resultant products were then ready for cloning.

TABLE 1
Degenerate RAMs primers used to produce a genomic library enriched for microsatellites and the annealing temperatures for the PCR amplifications

Primer	Sequence 5' to 3'	Annealing temperature (°C)
VJ1	NNN NNN NKK VRV RV $(CT)_{10}$	56
BP2	NNN NNK KYW $(BD)_3B(CA)_{10}$	55

Note: K = G/T, N = A/C/G/T, V = G/C/A, R = G/A, Y = T/C, B = C/G/T, D =A/G/T (IUB code)

Cloning of the 5' Anchored PCR Products into pCR 2.1-TOPO Vector

The PCR products were then cloned into the TOPO TA cloning vector according to the manufacturer's instructions (TOPO TA Kit, Invitrogen). Five recombinant clones from each primer were randomly selected for plasmid extraction.

Plasmid Extraction

The plasmid extractions were performed according to the protocol of Sambrook *et al.* (1989) and the clones were sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied biosystems, USA) on the ABI PRISM 377 DNA sequencer at Institute of Biosciences, Universiti Putra Malaysia.

Primer Design

Primer pairs flanking each of the microsatellite regions were designed by using a free online primer designing software, PRIMER 3 (Rozen and Skaletsky 1997) provided at http://www.genome.wi.mit.edu/genome_software/other/ primer3.html.

Amplifying Microsatellites

The primer pairs so designed were then used to screen for polymorphisms in *P. viridis*. PCR amplifications were performed in a 10 μL volume containing 25 ng of genomic DNA, 1X PCR buffer, 0.2 mM each of dNTPs, 0.15 μM of each reverse and forward primers, 1 – 3 mM $MgCl_2$, and 0.5 – 1 U of *Taq* DNA polymerase (Promega, USA). The PCR amplifications were performed in a Peltier Thermal Cycler PTC-220 (MJ Research, USA) with the following temperatures: a predenaturation for 3 min at 95°C, followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at appropriate temperatures (as shown in Table 2), 30 seconds extension at 68°C and concluded with a 5 min final extension at 68°C. The PCR products were run on a 4% (w/v) horizontal MetaPhor gel (BMA, USA) and visualized over UV after ethidium bromide staining. A 20 bp DNA ladder (BioWhittaker Molecular Applications) was used as the molecular weight standard. Twenty samples from each of the following *P. viridis* populations: Tanjung Rhu, Bagan Tiang, Pulau Ketam, Muar and Kampung Pasir Puteh were typed for each of the five microsatellite loci. The population data were analysed by using the POPGENE (version 1.32) computer software (Yeh & Boyle, 1997). An Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed based on Nei's (1978) genetic distance estimates by using the NTSYS software (Rohf 1990).

RESULTS AND DISCCUSION

5' anchored PCR is a technique that involves the use of arbitrary primers, which are specifically designed to incorporate an anchor and a microsatellite repeat motif to produce a library, which is highly enriched for microsatellites. This technique offers a number of advantages. One of the advantages is that the amplification of genomic DNA yields PCR products that contain a microsatellite region at both the 5' and the 3' terminal ends. This means every band sequenced would theoretically contain at least two microsatellites. Another advantage of this technique is its ability to target a specific repeat motif that is required, which means we can produce a library that is highly enriched for the specific repeat motif that we want (Fisher *et al.* 1996).

In this study, a total of ten clones (five from each of the degenerate RAMs primer, VJ1 and BP2) were randomly selected and sent for sequencing. The ten clones sequenced revealed a total of 21 microsatellite regions. Of the 21 microsatellite regions, ten were perfect microsatellites while eight were imperfect or interrupted microsatellite and the rest were compound microsatellites. Five of the clones had one or more additional microsatellites in the internal sequence. The presence of additional internal microsatellites was an unexpected bonus, implying that there may be clustering of microsatellites in some genomic regions (Fisher *et al.* 1996).

Primer pairs were designed for seven microsatellite loci. These are the first set of microsatellite markers that have been identified and characterized in *P. viridis*. Out of these seven loci, five were polymorphic with the number of alleles per locus ranging from 2 to 4 with an average of 3.2. The observed heterozygosity ranged from 0.0104-0.3776 (Table 2) and was similar with those reported using allozyme studies (Yap *et al.* 2002).

Cluster analysis revealed two major groups. Tanjung Rhu, Pulau Ketam, Bagan Tiang and

TABLE 2
Microsatellite variation in five populations of *Perna viridis*

Locus	Repeat motif	Primer sequence (5'-3')	T_a (°C)	Allele size range (bp)	No. of alleles	H_O	H_E	GenBank Accession no.
VJ1-18-1	$(CT)_n$	F: GTAGCGGCTCTCTCTCTCT R: GCGTGACACTCTTTTTCTTT	55	260-290	4	0.3776	0.7105	AY850126
VJ1-12-2	$(AG)_n(GACA)_N$, $(AG)_N$	F: ATAGGATAGAGTCACGTTAG R: TAAGACCTCTCTCTCTCTC	40	200-210	2	0.1579	0.1462	AY850124
VJ1-23-2	$(GAAA)_N$, $(AG)_n$	F: CAGGACTCCCGCTGGGTAAA R: TCCACTGGCCGGCTCTCT	44	205-220	2	0.0104	0.0104	AY850125
BP2-49-1	$(CA)_n$, $(CAAC)_N$	F: GGTACTTTTCTCACTTCACA R: GGAGTGAACCTCTTCGAC	44	165-230	4	0.2418	0.2541	AY850129
BP2-49-2	$(TG)_N$	F: GTTAAACAACCAACCAACG R: GTCTTTTTGTCATTGCACAC	44	180-260	4	0.2366	0.2471	AY850129

Repeat motif: N, pure; n, interrupted. T_a = annealing temperature, H_O = observed heterozygosity, H_E = expected heterozygosity

Muar were clustered together while Kampung Pasir Puteh was clustered by itself (*Fig. 3*). The Kampung Pasir Puteh population was collected from a highly polluted environment and this finding is in line with the results obtained by Yap *et al.* (2004) based on codominant allozyme markers but contradicted those of Chua *et al.* (2003) which were based on the dominant RAPD and RAMs markers. No diagnostic alleles that could differentiate between samples from polluted and non-polluted environments were found in this study.

This is the first report that microsatellite markers were isolated and characterized for *P. viridis*. Seven microsatellite primer pairs were designed based on the *P. viridis* DNA sequences obtained from a modified 5' anchored PCR technique (Fisher *et al.* 1996). This study is currently ongoing and the primers described will be used for more detailed studies on the population genetic structure of the green-lipped mussel, *P. viridis* from along the west coast of Peninsular Malaysia.

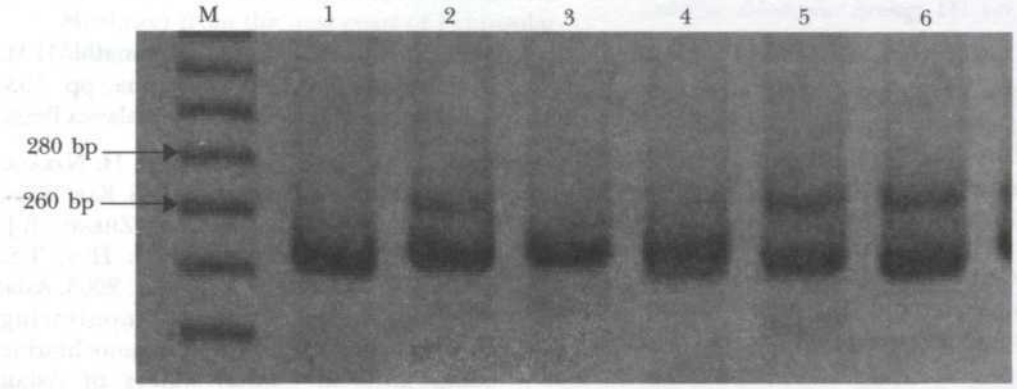

Fig. 1: Microsatellite profile of samples from the Bagan Tiang population generated by primer pairs VJ1-18-1. Lane M: 20 bp marker; lanes 1-6: microsatellite profiles of samples from Bagan Tiang

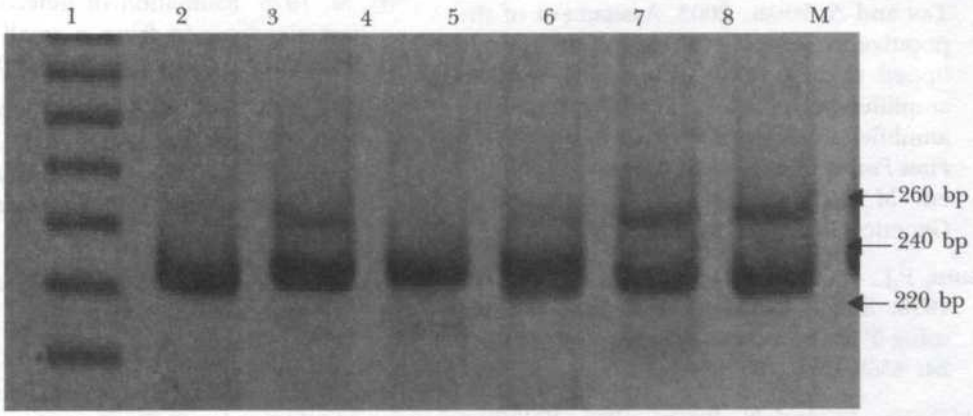

Fig. 2: Microsatellite profile of samples from the Muar population generated by primer pairs BP2-49-1. Lane M: 20 bp marker; lanes 1-8: microsatellite profiles of samples from Muar

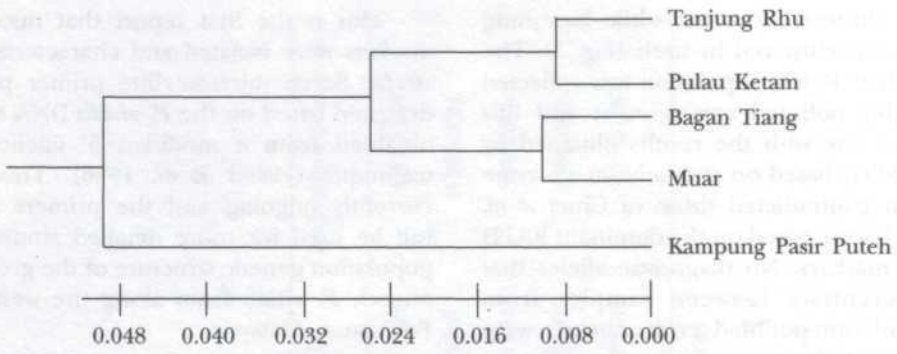

Fig. 3: UPGMA dendrogram of genetic relationships among five populations of Perna viridis based on Nei's (1978) genetic distance

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03
9 771511 370234