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Life Table and Demographic Parameters of the Metallic Blue Ladybeetle, *Curinus coeruleus* Mulsant, Fed with the Asian Citrus Psyllid, *Diaphorina citri* Kuwayama

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ABSTRACT

Life table parameters were calculated for the blue metallic ladybeetle, *Curinus coeruleus* Mulsant, fed with the Asian citrus psyllid, *Diaphorina citri* Kuwayama, at temperatures of 26 - 28°C and 75 - 85% RH and natural photoperiod in the insectary of Faculty of Agriculture, Universiti Putra Malaysia. The development of immature stages took 19.1 ± 0.3 days; mated adult females lived for 34.2 ± 4.7 (range 24-39) days and produced a mean of 80.3 ± 13.6 progeny female^{-1} during oviposition period of 21.3 ± 1.4 days, with a sex ratio of 1:1.8 ($\male : \female$). The net reproductive rate (R_0) was 59.1 and the capacity for increase (r_c) was 0.113. The finite rate of increase (λ) was 1.29 female^{-1} day^{-1} while the intrinsic rate of natural increase (r_m) was 0.116 female^{-1} day^{-1}. Each female contributed 60.2 individuals to the population in a mean generation time (T) of 35.3 days.

Keywords: *Curinus coeruleus*, *Diaphorina citri*, life table, intrinsic rate of increase, progeny

INTRODUCTION

The metallic blue ladybeetle, *Curinus coeruleus* Mulsant (Coleoptera: Coccinellidae), originating from Mexico is an important generalist predator of many insect pests. It has been recorded feeding on the spiralling whitefly *Aleurodicus dispersus* (Waterhouse and Norris, 1989; Villacarlos and Robin, 1992; Ramani *et al.*, 2002), mealybugs and green scale (*Coccus viridis* (Green)), (Wagiman *et al.*, 1990 in Showler, 1995). It also attacks psyllid, particularly the *Leucaena* psyllid, *Heteropsylla cubana* Crawford (Funasaki, 1988 in N.F.T.A., 1990; Michaud, 2001) and the Asian citrus psyllid, *Diaphorina citri* (Michaud, 2002).

Curinus coeruleus was introduced to Hawaii in the 1920s to control mealybugs and scale insects infesting coconuts. It was also introduced in Florida in the 1950s (N.F.T.A., 1990; Showler, 1995). Beginning mid-80s to early-90s, it was introduced into Indonesia, the Philippines, Thailand, India, Myanmar, Vietnam, Guam, Papua New Guinea, Nepal and Reunion Island

for controlling the *Leucaena* psyllid. It has proven successful in the control of the *Leucaena* psyllids, besides being a biological control agent for the Asian citrus psyllid, *Diaphorina citri* (Homoptera: Psyllidae) (Michaud *et al.*, 2002). *Diaphorina citri* is a serious pest of citrus trees and is a vector of the citrus greening disease, the most serious and devastating of all the diseases affecting citrus (Ko, 1996; Hoy and Nguyen, 1998).

So far, research on *C. coeruleus* has emphasized its ability to suppress the *Leucaena* psyllid and its distribution after released. Except for information on its potential to suppress *D. citri*, little is known of its biology, particularly on its population growth. Essential information on its developmental rate, age-specific fecundity, and survival in relation to its host *D. citri* is unavailable. These data are made available in a life table that can be used to examine the demographic structure of a population.

The objectives of this study were to gather life history information on *C. coeruleus* fed on *D.*

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citri for demographic analysis, and to measure the intrinsic rate of natural increase in order to elucidate the stable age-distribution of *C. coeruleus* fed on *D. citri*.

MATERIALS AND METHODS

Predator and Prey Source

The prey *Diaphorina citri* and the predator *C. coeruleus* were obtained from glasshouse and laboratory cultures, respectively. *Diaphorina citri* was cultured on orange jusmin plants, *Murraya paniculata* (L.) Jack (Fam. Rutaceae) while *C. coeruleus* was reared on a mixed population of psyllid nymphs comprising *D. citri, H. cubana* and an identified legume psyllid. The ambient environmental conditions were 28-34°C, 60-75% RH and a natural photoperiod in the greenhouse for rearing *D. citri*, and 26-28°C, 75-85% RH and a natural photoperiod in the laboratory for rearing the predator.

Life Table Construction

In order to construct the age-specific survival/mortality life table, a cohort comprising 100 eggs of *C. coeruleus* was placed in 10 batches, 10 eggs each in a 25 cm Petri dishes. The eggs were placed on white filter paper in one row to facilitate observations. Upon hatching, the larvae were provisioned daily with fresh psyllid nymphs of mixed instars in groups of four in separate Petri dishes to avoid cannibalism. Developmental time of larval stages until adult emergence was measured as days within each stadium. Determination of instars was affirmed by the presence of exuviae. Observations on age-specific survival and mortality of eggs, larvae, pupae and adults were made daily.

Age-specific Survival and Fertility Table

To determine the age-specific fertility, an index (sex ratio) was required to compute the number of female progeny female^{-1} in the life table. Fifty newly hatched larvae were reared individually each in a 12 cm Petri dish until adult emergence. Upon adult emergence, 15 pairs of male and female were separately confined within a mating-oviposition container. Since males of many ladybird species are consistently smaller (Dixon, 2000) and lighter (Otteenheim *et al.*, 1992) than females, beetles of similar body length (0.4-<0.5 cm for males and >0.5-0.6 cm for females) were selected for the study to avoid high variability in egg production. Eggs deposited by each female were recorded daily and observed for hatching. Newly hatched larvae were individually transferred into plastic containers (15 x 12 x 10 cm) that were covered with fine mesh nylon screen for aeration. Individuals were sexed at adult emergence in order to get an index for fecundity. Observations were recorded on such parameters as development period, oviposition and reproductive periods, fertility and longevity until death.

To construct age-specific survival and fertility table of female predators, 40 pairs of adult males and females that had mated (6-7 days after emergence) were maintained separately in plastic containers (15 x 12 x 10 cm) for egg laying. The number of eggs laid and the proportion of live females were recorded daily until all had died. Standard life table parameters were calculated from daily records of mortality and fecundity of the cohort using the procedure adopted from Carey (Vegas *et al.*, 2002).

RESULTS

Age-specific Survival/Mortality Life Table

The duration of immature stages from egg to adult is shown in Table 1. The egg mortality had contributed to the drop in the survivorship (l_x) of *C. coeruleus* by the 6th day. The decrease in l_x was further recorded between day 7 and 14 due to the larval mortality (8%). Thereafter, the l_x remained stable until day 48, beyond which the survivorship sharply descended after day 54 due to the death of adults until the last individual on day 77. In general, the percentage of survival from egg to adult (88%) was relatively high.

Age-specific Survival and Fertility Table

At an ambient environment of 26-28°C, 75-85% RH and natural photoperiod, the development of immature stages of *C. coeruleus* fed *D. citri* nymphs from egg hatching to adult emergence took 19.1 ± 0.3 days. Adult females lived for 34.2 ± 4.7 days (range between 24-39 days), produced a mean of 80.3 ± 13.6 progeny female^{-1} during an oviposition period of 21.3 ± 1.4 days. That progeny consisted of 38.5 males and 51.8 females, hence the sex ratio was 1 male: 1.8 females. This sex ratio was used to compute population parameters as shown in Table 2.

The female *C. coeruleus* began laying eggs around day seven of post-emergence of the adult

TABLE 1
Life table for computing life expectancy of *C. coeruleus*
feeding on *D. citri* nymphs

Age (days), x	l_x	d_x	100_{qx}	L_x	T_x	e_x
1 Egg	100	0	0	100	5230.0	52.30
2	100	0	0	100	5130.0	51.30
3	100	0	0	100	5030.0	50.30
4	100	0	0	100	4930.0	49.30
5	100	4	4.0	98	4830.0	48.30
6 1st instar	96	2	2.08	95	4732.0	49.29
7	94	1	1.06	93.5	4637.0	49.33
8	93	0	0	93	4543.5	48.85
9 2nd instar	93	0	0	93	4450.5	47.85
10	93	0	0	93	4357.5	46.85
11	93	1	1.08	92.5	4264.5	45.85
12 3rd instar	92	0	0	92	4172.0	45.35
13	92	0	0	92	4080.0	44.35
14	92	0	0	92	3988.0	43.35
15	92	2	2.17	91	3896.0	42.35
16 4th instar	90	0	0	90	3805.0	42.28
17	90	0	0	90	3715.0	41.28
18	90	1	1.11	89.5	3625.0	40.28
19	89	0	0	89	3535.5	39.72
20 Pupa	89	0	0	89	3446.5	38.72
21	89	0	0	89	3357.5	37.72
22	89	0	0	89	3268.5	36.72
23	89	0	0	89	3179.5	35.72
24	89	1	1.12	88.5	3090.5	34.72
25 Adult	88	0	0	88	3002.0	34.11
26	88	0	0	88	2914.0	33.11
27	88	0	0	88	2826.0	32.11
28	88	0	0	88	2738.0	31.11
29	88	0	0	88	2650.0	30.11
30	88	0	0	88	2562.0	29.11
31	88	0	0	88	2474.0	28.11
32	88	0	0	88	2386.0	27.11
33	88	0	0	88	2298.0	26.11
34	88	0	0	88	2210.0	25.11
35	88	0	0	88	2122.0	24.11
36	88	0	0	88	2034.0	23.11
37	88	0	0	88	1946.0	22.11
38	88	0	0	88	1858.0	21.11
39	88	0	0	88	1770.0	20.11
40	88	0	0	88	1682.0	19.11
41	88	0	0	88	1594.0	18.11
42	88	0	0	88	1506.0	17.11
43	88	0	0	88	1418.0	16.11
44	88	0	0	88	1330.0	15.11
45	88	0	0	88	1242.0	14.11
46	88	0	0	88	1154.0	13.11
47	88	0	0	88	1066.0	12.11
48	88	4	4.55	86	978.0	11.11
49	84	4	4.76	82	892.0	10.62

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TABLE 1 (continue)

50	80	5	6.25	77.5	810.0	110.13
51	75	2	2.67	74	732.5	9.77
52	73	0	0	73	658.5	9.02
53	73	7	9.59	69.5	585.5	8.02
54	66	0	0	66	516.0	6.82
55	66	4	6.06	64	450.0	6.82
56	62	14	22.58	55	386.0	6.23
57	48	13	27.08	41.5	331.0	6.90
58	35	3	8.57	33.5	289.5	8.27
59	32	8	25.0	28	256.0	8.00
60	24	0	0	24	228.0	9.50
61	24	0	0	24	204.0	8.50
62	24	0	0	24	180.0	7.50
63	24	4	16.67	22	156.0	6.50
64	20	0	0	20	134.0	6.70
65	20	0	0	20	114.0	5.70
66	20	4	20.0	18	94.0	4.70
67	16	2	12.5	15	76.0	4.75
68	14	0	0	14	61.0	4.36
69	14	2	14.29	13	47.0	3.36
70	12	2	16.67	11	34.0	2.83
71	10	1	10.0	9.5	23.0	2.30
72	9	5	55.56	6.5	13.5	1.50
73	4	2	50.0	3	7.0	1.75
74	2	0	0	2	4.0	2.00
75	2	1	50.0	1.5	2.0	1.00
76	1	1	100.0	0.5	0.5	0.50
77	0	0	0	0		

and kept going for up to 22 days (Table 2). The average total number of eggs laid day^{-1} female^{-1} ranged from a high of 10.05 on day eight of adult emergence to a low of 0.67 eggs female^{-1} on day 22. The first female death was recorded on day 24 of adult life and increased gradually thereafter. However, the females could live for a maximum of up to 42 days after pupal emergence or 62 days of age. During the entire egg laying period, egg production female^{-1} showed a variable or an undulating pattern (*Fig. 2*), thus indicating that the reproductive output of *C. coeruleus* demonstrated a tendency toward a sharp rise and and reached the peak on the 8th day of the oviposition period. The decline in egg production (m_x) coincided with the aging and death of adult females. The survivorship curve of adult females was similar to that of all stages (*Fig. 1*), showing a Type 1 survivorship in which mortality was most heavy on the old individuals.

Reproductive and population parameters are summarised in Table 3. The intrinsic rate of natural increase (r_m) was 0.116 female^{-1} day^{-1} and a daily finite rate of increase (λ) was 1.122

females female^{-1} day^{-1}. With a mean generation time T of 35.33 days, theoretically each female would contribute 60.24 individuals. When a stable-age distribution is reached, each development stage (egg, larva, pupa and adult stages) would contribute 46.0, 43.8, 4.6 and 5.6% respectively to the population. This proportion would be considered advantageous to prey suppression since around 50% of the population comprised the nymph-preying stages, ie. larvae and adults.

DISCUSSION

Life table construction termed demography by Stilling (1992) contains such vital statistics as the probability of an individual of a certain age dying, or conversely, the average number of offspring produced by a female of a given age (Poole, 1974). The demographic parameters like the intrinsic rate, mean generation time, and population doubling time are useful indices of population growth of an insect under a given set of growing conditions (Tsai, 1998). No demography comparison study on *C. coeruleus*

TABLE 2
Life- and age-specific fecundity table of *C. coeruleus*
(1-19 days immature stages and 20-26 days preoviposition period)

Pivotal age (days) x	Proportion of surviving females l_x	No. of female progeny/female m_x	$l_x m_x$	$x l_x m_x$
27	1	1.08	1.08	29.16
28	1	1.08	1.08	30.24
29	1	1.70	1.70	49.30
30	1	1.86	1.86	55.80
31	1	1.72	1.72	53.32
32	1	4.63	4.63	148.16
33	1	4.46	4.46	147.18
34	1	6.46	6.46	219.64
35	1	5.17	5.17	180.95
36	1	3.66	3.66	131.76
37	1	4.10	4.10	151.70
38	1	3.23	3.23	122.74
39	1	2.70	2.70	105.30
40	1	2.80	2.80	112.00
41	1	3.02	3.02	123.82
42	1	2.91	2.91	122.22
43	1	2.26	2.26	97.18
44	0.950	2.59	2.46	108.26
45	0.900	1.51	1.36	61.16
46	0.850	1.11	0.94	43.40
47	0.825	1.40	1.16	54.29
48	0.750	0.43	0.32	15.48
49	0.750	0	0	0
50	0.700	0	0	0
51	0.550	0	0	0
52	0.400	0	0	0
53	0.350	0	0	0
54	0.350	0	0	0
55	0.225	0	0	0
56	0.150	0	0	0
57	0.050	0	0	0
58	0.050	0	0	0
59	0.025	0	0	0
60	0.025	0	0	0
61	0.025	0	0	0
62	0	0	0	0
Σ	24.93	59.88	59.08	2163.05

has been reported. This study noted the mean incubation period of *C. coeruleus* when fed with *D. citri* was 5 days with a viability of 96%. The hi1gh egg viability indicated that the adult female and male predators had successfully mated and the eggs produced were fertile. Several laboratory studies revealed that virgin females would lay eggs, but far fewer than mated females (Dixon, 2000). The nymphs underwent three moults with the total nymphal development period of 14 days, which was similar to the period of most predaceous ladybird beetles (Olsen, 2004). The average female longevity was 34.2 days.

The developmental time from egg to adult was 25 days with the proportion of the total time spent in the egg, larval and pupal stages being 0.21, 0.58 and 0.21, respectively (Table 4). This is approximately similar to that recorded for other species of ladybirds (0.18, 0.62 and 0.23, respectively) (Dixon, 2000). Even though the
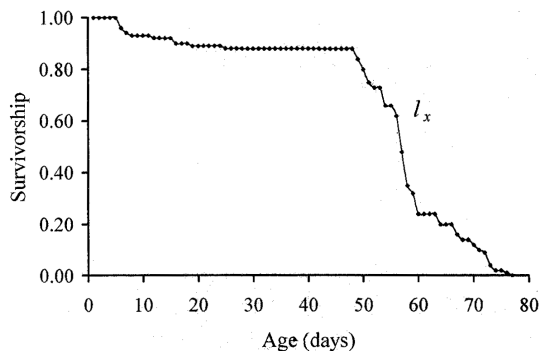
Fig. 1: *Survivorship (l_x) curve of* C. coeruleus *fed with* D. citri *nymphs*
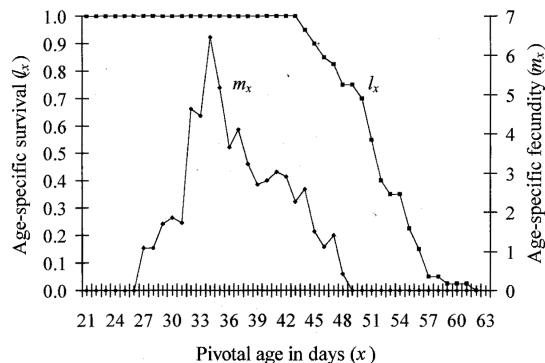

Fig. 2: *Daily age-specific survival and fecundity of* C. coeruleus *fed with* D. citri *nymphs*

developmental time is very dependent on both the temperature and food quality, the ratios of the time spent in each of the different stages do not differ. The developmental time is one of the life history parameters that can be used to measure the desirable characteristics of natural enemies (Olsen, 2004). When a predator develops slower than the prey, it is an ineffective biological agent (Hagen, 1974; Mills, 1982). According to Dixon (2000), if the developmental rate of a predator is similar or faster than that of its prey then the predator is potentially capable of dramatically reducing the abundance of its prey. In the current study, the developmental time for the four larval stages of *C. coeruleus* of 14 days (Table 7) was very comparable to that for the five nymphal stages of *D. citri* (13 days) (Tsai and Liu, 2000). The generation time and doubling time of *C. coeruleus* noted at 35.33 days and 5.97 days respectively, was relatively similar to those *of D. citri* (33.91 days and 4.28 days) (Tsai and Liu, 2000). Therefore, this predator should be considered as an important candidate of a biological control agent for the Asian citrus psyllid, *D. citri.*

Reproductive output of *C. coeruleus* was in general agreement with that of most insects; starts and reaches a maximum early in adult life and then declines with age. This situation had resulted in the sharp rise in fecundity and showed a right-angled triangular shape (*Fig. 1*). The decline in reproductive output in the triangular fecundity trend was mainly determined by the mortality (Stearn and Koella, 1986) besides due to the aging of adult females which is closely associated with a reduction in daily consumption of prey. Dixon (2000) pointed out that aging is

important in shaping the fecundity trend; the old adults are less efficient at converting biomass of prey into eggs than young adults. From *Fig. 1*, it was apparent that the female died at age of 62 days, while the maximum age could reach 77 days as presented in Table 3. This difference was presumably caused by the mated and unmated individuals whereby unmated individuals of both male and female live longer than mated individuals (Dixon, 2000).

The number of female predators produced in each generation greatly influencesd the population size of the next generation. In this study, the proportion of females was higher than males. Of the total progeny (1205) produced by 15 females, 770 progenies were females, making the sex ratio of 1 male: 1.8 females (0.36 : 0.64). Similar sex ratios were reported by Otteenheim *et al.* (1992); from 53 ladybird families, 51 had sex ratios (proportion of females) around 0.6 or slightly above while the remaining had a very low sex ratio (<0.12). The higher proportion of female predators coupled with the high egg viability and the generation time that was comparable to that of its prey could be so beneficial in terms of mass rearing and releasing that in turn could contribute in suppressing the prey population.

Since the mortality of *C. coeruleus* females heavily affected the old individuals, the survivorship curve for the females showed a Type I as in Slobodkin (in Southwood, 1978) and Stilling (1992). Overall, the survivorship curve of all stages of this predator was that of Type I., this curve was different from standard curves described by Stilling (1992) where most invertebrates often exhibits Type III survivorship

TABLE 3
Reproductive and population parameters of *C. coeruleus* feeding on *D. citri* nymphs

No.	Parameters	Values
	Reproductive parameters:	
1.	Gross reproductive rate $\sum m_x$	59.88
2.	Net reproductive rate $\sum l_x m_x$	59.08
3.	Average egg day[1] $\sum l_x m_x / \sum l_x$	2.37
4.	Mean age fecundity schedule $\sum x m_x / \sum m_x$	36.74
5.	Gross fecundity rate $\sum M_x$	93.15
6.	Net fecundity rate $\sum l_x M_x$	90.22
	Population parameters:	
7.	Approximate generation time(T_c), $\sum x l_x m_x / \sum l_x m_x$	36.61
8.	Corrected generation time (T), $\ln R_0 / r_m$	35.33
9.	Innate capacity for increase (r_c), $\ln R_0 / T_c$	0.113
10.	Intrinsic rate of natural increase (r_m), $\sum e^{-rmx} l_x m_x = 1$	0.116
11.	Finite rate of increase e^r	1.122
12.	Doubling time $\ln 2/r$	5.97
13.	Intrinsic birth rate (b), $1 / \sum e^{-rmx} l_x$	0.128
14.	Intrinsic death rate (d), $b - r_m$	0.012
15.	Stable age structure (from Table 4)	
	Eggs	46.01
	Larvae	43.79
	Pupae	4.56
	Adults	5.64

TABLE 4
Calculated stable-age distribution of *C. coeruleus* feeding on *D. citri* nymphs ($r_m = 0.116$)

Pivotal age (days), x	l_x	e^{-rmx}	$e^{-rmx} l_x$	% Distribution	
1	1.00	0.8910	0.8910	11.44	Eggs
2	1.00	0.7938	0.7938	10.19	46.01
3	1.00	0.7072	0.7072	9.08	
4	1.00	0.6301	0.6301	8.09	
5	1.00	0.5614	0.5614	7.21	
6	0.96	0.5002	0.4802	6.16	1st instar
7	0.94	0.4456	0.4189	5.38	16.28
8	0.93	0.3971	0.3693	4.74	
9	0.93	0.3538	0.3290	4.22	2nd instar
10	0.93	0.3152	0.2931	3.76	11.33
11	0.93	0.2808	0.2612	3.35	
12	0.92	0.2502	0.2302	2.95	3rd instar
13	0.92	0.2229	0.2051	2.63	10.02
14	0.92	0.1986	0.1827	2.35	
15	0.92	0.1769	0.1628	2.09	
16	0.90	0.1577	0.1419	1.82	4th instar
17	0.90	0.1405	0.1264	1.62	6.16
18	0.90	0.1251	0.1126	1.45	
19	0.89	0.1115	0.0992	1.27	
20	0.89	0.0993	0.0884	1.13	Pupae
21	0.89	0.0885	0.0788	1.01	4.56
22	0.89	0.0789	0.0702	0.90	

TABLE 4 (continue)

23	0.89	0.0703	0.0625	0.80	
24	0.89	0.0626	0.0557	0.72	
25	0.88	0.0558	0.0491	0.63	Adult
26	0.88	0.0497	0.0437	0.56	5.64
27	0.88	0.0443	0.0390	0.50	
28	0.88	0.0394	0.0347	0.45	
29	0.88	0.0351	0.0309	0.40	
30	0.88	0.0313	0.0276	0.35	
31	0.88	0.0279	0.0245	0.32	
32	0.88	0.0249	0.0219	0.28	
33	0.88	0.0221	0.0195	0.25	
34	0.88	0.0197	0.0174	0.22	
35	0.88	0.0176	0.0155	0.20	
36	0.88	0.0157	0.0138	0.18	
37	0.88	0.0140	0.0123	0.16	
38	0.88	0.0124	0.0109	0.14	
39	0.88	0.0111	0.0097	0.13	
40	0.88	0.0099	0.0087	0.11	
41	0.88	0.0088	0.0077	0.10	
42	0.88	0.0078	0.0069	0.09	
43	0.88	0.0070	0.0061	0.08	
44	0.88	0.0062	0.0055	0.07	
45	0.88	0.0055	0.0049	0.06	
46	0.88	0.0049	0.0043	0.06	
47	0.88	0.0044	0.0039	0.05	
48	0.88	0.0039	0.0034	0.04	
49	0.84	0.0035	0.0029	0.04	
50	0.80	0.0031	0.0025	0.03	
51	0.75	0.0028	0.0021	0.03	
52	0.73	0.0025	0.0018	0.02	
53	0.73	0.0022	0.0016	0.02	
54	0.66	0.0020	0.0013	0.02	
55	0.66	0.0017	0.0012	0.01	
56	0.62	0.0016	0.0010	0.01	
57	0.48	0.0014	0.0007	0.01	
58	0.35	0.0012	0.0004	0.01	
59	0.32	0.0011	0.0004	0.0	
60	0.24	0.0010	0.0002	0.0	
61	0.24	0.0009	0.0002	0.0	
62	0.24	0.0008	0.0002	0.0	
63	0.24	0.0007	0.0002	0.0	
64	0.20	0.0006	0.0001	0.0	
65	0.20	0.0006	0.0001	0.0	
66	0.20	0.0005	0.0001	0.0	
67	0.16	0.0004	0.0001	0.0	
68	0.14	0.0004	0.0001	0.0	
69	0.14	0.0003	0.0	0.0	
70	0.12	0.0003	0.0	0.0	
71	0.10	0.0003	0.0	0.0	
72	0.09	0.0002	0.0	0.0	
73	0.04	0.0002	0.0	0.0	
74	0.02	0.0002	0.0	0.0	
75	0.02	0.0002	0.0	0.0	
76	0.01	0.0002	0.0	0.0	
77	0	0.0001	0.0	0.0	
			$\sum = 7.7907$		

curve in which a large fraction of the population is lost in the juvenile stages, whilst Type I curves are often observed in higher organisms, especially vertebrates. However, similar survivorship curve with this predator was also observed for other coccinellids such as *Scymnus hoffmani* and *Coelophora mulsanti* (Sallee and Chazeau, 1985 in Dixon, 2000). The Type I curve could be advantageous in the multiplication point of view as high survival rate of the immature and young female predator will contribute to the increasing rate of its population, which in turn could increase the predation rate and hence reduce the abundance of its prey.

The overall rate of increase of predator population will depend on the survival rate of each developmental stage as well as the fecundity of the adults (Hassell, 1976). Poor survival of the immature stages can markedly reduce the rate of increase of a predator population even if the fecundity and the number of progeny produced female is high. The intrinsic rate of increase of *C. coeruleus* was slightly lower than that of *D. citri*, ie. 0.12 (Table 3) compared to 0.16 (Tsai and Liu, 2000). This might be due to the lower fecundity rate of *C. coeruleus* (90) compared to that of *D. citri* (626) although the survival of *C. coeruleus* during immature stages was higher (90% : 75%) (Tsai and Liu, 2000). Even so, *C. coeruleus* with a slightly lower r_m than its prey might be able to effectively suppress the prey population if its other attributes such as voracity, developmental time and attack rate were good. The study conducted by Jansen and Sabelis (1992) suggested that predator mites having a lower r_m than their prey could nevertheless still effectively control the abundance of their prey if they have a high voracity.

CONCLUSIONS

Based on the demographic parameters exemplifying the biological characteristics, it appears *that C. coeruleus* is a potential predator candidate for biological control of *D. citri.* With high survival rates of the immature and young female (Type I survivorship), high egg viability and mean generation time comparable to its prey, *C. coeruleus* has the desirable attributes as an effective natural enemy, hence it would be beneficial in providing an ecological framework for a biological control programme against *D. citri* in the future.

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Detecting and Quantifying Degraded Forest Land in Tanah Merah Forest District, Kelantan Using Spot-5 Image

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ABSTRACT

In sustainable forest management, information on the extent and types of degraded forest sites is essential and crucial. It enables planning of appropriate remedial strategies. This study was carried out to detect and quantify degraded forest land in Tanah Merah District, Kelantan using remotely sensed data. Spot-5 satellite data (Path/Row: 269/339) was acquired from MACRES, which covered part of three forest reserves ie. Sungai Sator, Gunung Basor and Gunung Stong. The ERDAS IMAGINE software version 8.7 was used to enhance the image for better visualization using band combination and spatial filtering techniques. This was followed by "Supervised Classification" of the image using "Maximum Likelihood Classifier" to detect and classify degraded forest features into pre-determined classes. The four classes detected were primary forest, degraded forest, gap and water bodies. Results showed that the degraded forest class constituted the largest area (57,878 ha), followed by primary forest gap (20,686 ha) and gap (3,488 ha). Degraded forest types were represented by road, agriculture, plantation areas. Based on the accuracy assessment, the overall classification accuracy obtained was 89% and showed that the remote sensing technique was able to detect and map degraded forest sites.

Keywords: Remote sensing, degraded forest detection, quantifying

INTRODUCTION

Forestry is the scientific management of forests for the continuous production of goods and services, in particular the production of timber. At the end of 2002, Malaysia had an estimated 19.93 X 106 ha. of forest covering 60.7% (32.86 X 106 ha) of its total land area. Of this total, about 14.33 X 106 ha have been designated as Permanent Forest Estate (PFE) under sustainable management, while 2.12 X 106 ha are protected by legislation for conservation purposes. Currently, forest management practices in Peninsular Malaysia are based on the Selective Management System (SMS) with the main objective of optimizing timber harvest while maintaining the sustainability of forest production. In Kelantan, the total extent of forest is 629 687 hectares including virgin forest, the logged forest and the plantation forest. In the western part of Kelantan, there are 12 permanent reserve forests, as shown in Table 1.

Based on the study by John (1991), forest degradation problems include ecological damages, especially soil erosion, climate change and nutrient degradation. The implications of forest degradation are loss in forest structures, function, species compositions, and productivities which are normally low compared to natural forest types. Technology can assist in reducing forest degradation problems especially spatial data technology such as GIS, GPS and remote sensing (Henry *et al.*, 1997).

Remote sensing is the collection of data about objects, which are not in contact with the collecting device (Sabin, 1997). It can be used for providing information on recognizable features e.g. water, vegetation and soil due to their reflectance characteristics. Geographical Information System (GIS) involves the computer-organized grouping of activities and procedures covering the input, storage and manipulation, retrieval and presentation of spatially based

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TABLE 1
The 12 permanent reserve forests (PRF) in Tanah Merah District, Kelantan

No.	PRF	Types Forest	Areas (Hectare)	Date Gazetted
1.	Balah	Forest Land	56,010	1/3/1990
2.	Berangkat	Forest Land	21,409	9/9/1941
3.	Bukit Akar	Forest Land	1,072	11/5/1989
4.	Gunong Basor	Forest Land	40,613	11/5/1989
5.	Gunong Stong Selatan	Forest Land	28,134	11/5/1989
6.	Gunong Stong Tengah	Forest Land	21,950	11/5/1989
7.	Gunong Stong Utara	Forest Land	11,044	11/5/1989
8.	Jedok	Forest Land	4,382	1/1/1957
9.	Jeli	Forest Land	3,649	6/6/1991
10.	Jentiang	Forest Land	13,673	11/5/1989
11.	Sungai Sator	Forest Land	2,777	1/4/1962
12.	Sokortaku	Forest Land	21,825	10/10/1960

reference data (John, 1991). It can be used to complement remote sensing data especially for creating operation maps. GIS has two common methods of structuring geographic data, the raster data and vector data structure. The raster data that was used is a grid. However for the vector data, a point is represented as a single x,y coordinate pair and area represented by a closed line or set of line. This study was carried out to detect and quantify the degraded forest land in Tanah Merah District using SPOT-5 imagery. SPOT-5 imagery was used due to its 20 meter spatial resolution and was the only good images available during the period of this study. GIS was used to support satellite image data collected for quantifying and mapping degraded forest areas.

MATERIALS AND METHODS

Study Area

Kelantan is one of the 13 states in Malaysia, richly endowed with resources, covers a land area of about 15 000 km², northeast of Peninsular Malaysia facing the South China Sea and is occupied by almost 60% of forest. It is situated within latitudes of 101° 20' E to 102° 40' E and longitudes of 4° 30' N to 06° 15'' N. The total land area of Kelantan is approximately 1, 493, 181 ha, of this 894,276 ha are forested areas. A total of 626 372 ha of the total forested area are forest reserves and the rest is forest state land and the National Park (Iwan, 2001). The study area was conducted in the western part of Kelantan, consist of 12 permanent forest reserves in the Tanah Merah district. The total forest area is about 184,610 ha. *Fig. 1* shows the location of the study area.

Daily temperature ranges from 21° to 32°C. There is a marked dry season in February, March and April. The geological formations of the area are mainly sedimentary in origin, accompanied by folding and metamorphism (Haryono, 1995). The soil nutrients are depleted due to the continuous leaching associated with high rainfall of the humid tropics.

Data Acquisitiozn and Digital Image Analysis

The satellite image was acquired from the Malaysian Centre for Remote Sensing (MACRES) for path and row of 269/339 dated 13th March 2005. The geocorrected data with spatial resolution of 20 m has about 10 percent cloud cover. Secondary data used in this study are topographical map (scale 1: 50 000) from the Forestry Department Headquarters, Kuala Lumpur and digital compartment map from the Kelantan State Forestry Department.

ERDAS IMAGINE software version 8.9 was used for digital image processing such as remote sensing analysis, digital photogrammetry, data visualization, image analysis, GIS and Digital Terrain Model (DTM) analysis. ERDAS IMAGINE is an integration of remote sensing and GIS, which has the ability to digitize images, process images, generate maps and analyse remotely sensed data in raster and vector formats. In this study, overlaying maps of compartments and boundaries of forest reserves were undertaken to complement analysis satellite image.
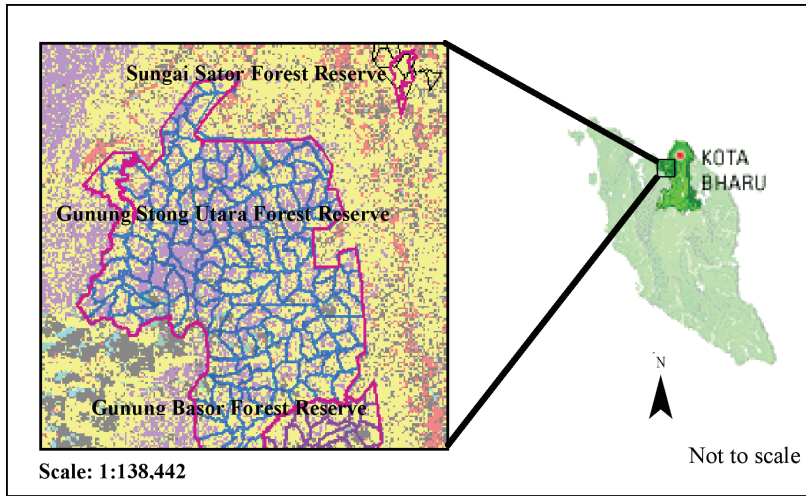
Fig. 1: A map of the Peninsular Malaysia showing a satellite image of forest compartments in the study area

Methods

Briefly, the procedure for the first step was data acquisition, digital image processing and analysis, ground verification, image classification, accuracy assessment and degraded forest land map derivation (*Fig. 2*). SPOT-5 data was corrected from geometric distortion. The image was enhanced through adjustment of the linear stretch line and increasing the image appearance by modifying the contrast level and then the image was filtered using a low pass type filter. False color composite, band 4-1-2 (R-G-B) was used since it showed much better information in land cover type discrimination (Mohd. Hasmadi and Kamaruzaman, 2004).

Classification of the land cover type and degraded forest land from SPOT-5 imagery involved both visual interpretation and computer assisted analysis (Maximum Likelihood supervised classification approach). For the purpose of this study, a total of six sample pixels for each class were selected, which are called training samples. Comparisons of spectral reflectance from these training samples were discriminate land cover features at the study area. The classes were forest, poor forest, opened areas of roads or plantation, river and water bodies.

Fig. 2: Flowchart of the study

In a remote sensing study, ground verification is an essential component to evaluate accuracy of classified satellite imagery (Story and Congalton, 1986). In order to determine the accuracy of image classification, ground verification survey was carr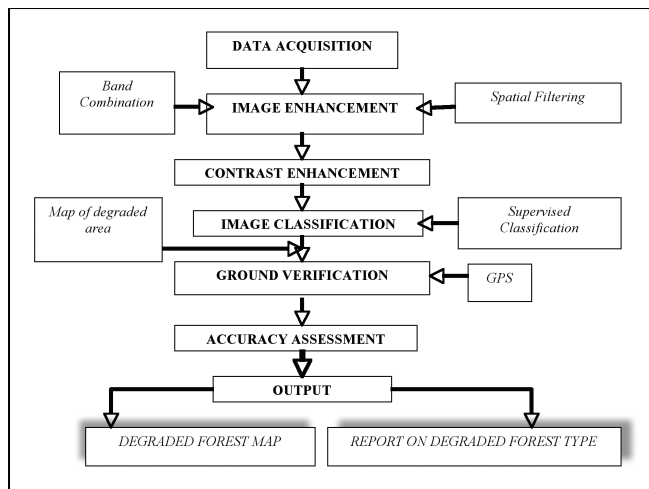ied out. A total of 61 training areas were selected randomly and visited with the support of satellite imagery, topographical map and land use map. For each visited site, photographs, locational data and type of land cover were observed and recorded in a form. The ground truth verification data were used in the maximum likelihood report as the independent data set from which the classification accuracy was compared (Kamaruzaman and Mohd.Hasmadi, 1999). The accuracy is essentially a measure of how many ground truth pixels were correctly classified.

RESULTS AND DISCUSSION

Band Combination and Image Enhancement

Selection of band combinations is one of the essential procedures for making enhanced color composite images and it is possible to visualize maximum information of the data. *Fig. 3* shows the study area image in band combinations of 4-1-2, (R-G-B). This image can clearly distinguish the pattern of land cover features in the study area such as water bodies, primary forest, secondary forest and cleared land. Low temperature areas such as the forest and water

bodies were represented in the image as green for forest and dark blue for water respectively. Areas with high temperature such as logging area, opening land, road network and plantation areas were showed as light color.

Spatial Filtering

In spatial filtering, the results showed that median filter with a low pass filter (3x3) was found to be the most suitable filter to apply on Spot-5 satellite imagery. Using median filter enabled identification of features such as opened area because of their light color. *Fig. 4* showed that the image was filtered by low pass median filter (3 x 3) and enhanced using contrast stretching. For the entire filtered image, a composite image of band 4-1-2 (R-G-B) was later used in the image classification process for delineating forest and degraded areas.
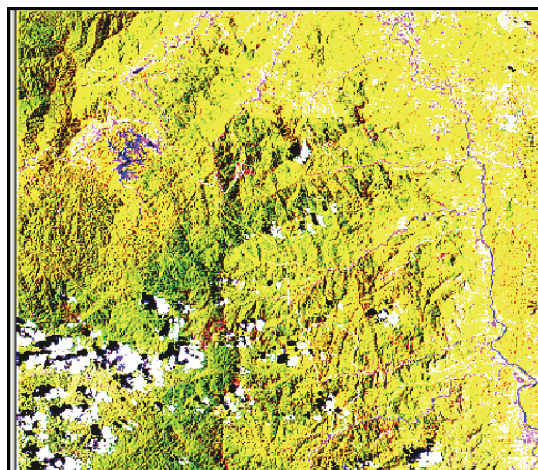
Fig. 4: Enhanced image of 4-1-2 (R-G-B) false color composite filtered using low pass median filter

Supervised Classification

Maximum Likelihood classifier (MLC) was used in the classification. This classifier produced the best results when 30 training sites were sampled, that is, each site has 50- 100 times as many pixel as there were bands in the data set which were closely homogeneous. However, this approach is slow compared to the Parallepiped Classifier and Minimum Distance classifier for image classification (Wan Zuraidi, 2000). Classification using prior information on the study site helped accuracy of classification. This implies that there are advantages for the interpreter to know the

Fig. 3: False color composite of 4-1-2 (R-G-B) band using contrast stretching enhancement technique

study area well before interpreting any satellite image. *Fig. 5* shows the results of supervised classification of the study area.

The supervised classification using MLC produced six classes and were identified as follows; forest (purple and green), gap (red), poor forest (yellow), water bodies (light blue), bamboo (light green), and plantation (pink).

Ground verification points were collected using Garmin hand held GPS (<10 m accuracy), which were marked initially on topographic and image maps. The area indicated by the GPS point represents different features on the scene. Most of the area visited on the ground can be identified and discriminated in the image. A total of 61 samples locations were chosen randomly and visited during ground verification work. During the verification process most of the verified points from Sungai Sator Forest Reserve areas represented degraded area and constituted areas of agriculture, plantations and shrubs. More than 80% of the degraded areas were within rubber plantations and roads.

Water bodies such as rivers were easily spotted due to its size and can be identified clearly in the map and image. Opened area such as road networks and human settlement can also be easily spotted in the image. Most of the bamboo areas were found in the forest, especially

along the secondary roads in Gunung Basor Forest Reserve, where the site has been previously degraded by logging.

Accuracy Assessment

Final classification categorised the area into four classes namely forest, gap, poor forest (including bamboo and plantation), and water bodies. Table 2 shows the results of confusion matrix for the four classes in the study area. The average and overall accuracy of classification results were 85 % and 89 %, respectively. Water bodies showed the highest accuracy (100%) while gap or opened area showed the lowest accuracy (65%). The accuracy of other classes such as forest and poor forest were 92% and 100%. The presence of cloud in the image slightly affected the image classification results.

The overall accuracy indicates that the remotely sensed classified image was sufficiently accurate in mapping the types of degraded forest in the study site. The high accuracy achieved was due to the selection of dominant spectral response patterns and to the researcher's familiarity with the study site. The area for each class obtained from the final classification is shown in Table 3 which shows that the degraded forest area is the largest occupying 57,878 ha, and the primary forests of Gunung Stong Utara
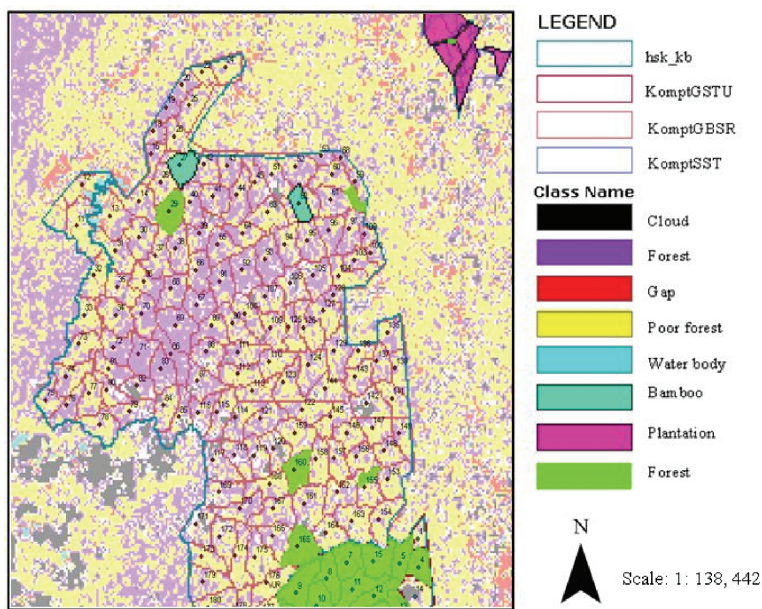

Fig. 5: Six cluster of land cover in the study area Ground Verification

TABLE 2
Confusion matrix for four classes of the study area

Referred code	Classified Data					Accuracy (%)
	1	2	3	4	Total	
1	13	1	0	0	14	66.5
2	8	15	0	0	23	92.86
3	0	0	13	0	13	100
4	0	0	0	11	11	100
Total	22	15	13	11	61	

1: Forest   Average accuracy   = 85.02%
2: Gap   Overall accuracy   = 89.52%
3: Poor forest   Mean overall accuracy = 0.21%
4: Water bodies   Kappa coefficient   = 0.81

TABLE 3
Classification of degraded forest in Tanah Merah district, Kelantan

No.	Classes	Area (ha)
1	Forest (Gunung Stong Utara Forest Reserve, Gunung Basor Forest Reserve and Sungai Sator Forest Reserve)	20, 685.93
2	Gap	3488.68
3	Degraded forest (Poor forest, bamboo and plantation)	57, 878.4

Forest Reserve, Gunung Basor Forest Reserve and Sungai Sator Forest Reserve occupying 20, 685.93 ha.

CONCLUSIONS

From the results of this study, Spot-5 satellite data based on computed- assisted analysis could serve as a useful tool to identify degraded forest land in the study area. This identification was extracted from enhanced image of 4-1-2 (R-G-B) false color composite band, and filtered with low pass 3 x 3 window. The image classification supervised classification technique of Maximum Likelihood Classifier (MLC) was capable of classifying with an accuracy of about 89% of the land cover type in the study area. However, the presence of clouds (about 7%) in the image affected the results of the accuracy assessment. The total degraded forest area classified in the study area was about 57,878.4 ha. This comprises land cover such as poor forest, plantation and bamboo areas. The outputs in the form of maps

or digital images are useful for better management and rehabilitation of degraded forest lands.

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Effect of *Exserohilum monoceras* (Drechslera) Leonard & Suggs on the Competitiveness of *Echinocloa cruss-galli* (L.) P. Beauv.

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ABSTRACT

The use of bioherbicide to reduce interference by barnyard grass in rice cropping system has been suggested but has not been reported. Against this conceptual background, a mini-plot study was conducted to simulate the efficacy of *Exserohilum monoceras* to reduce competitiveness of barnyard grass in rice using replacement series experiment. The effect of *E. monoceras* on rice was negligible, as it did not cause any infection. Severe infection was observed on barnyard grass inoculated with this fungus at all plant densities as indicated by high AUDPC values (ranges from 610.35-468.28 unit^2) and fast disease progress rates ($r_L = 0.48$ logit/day). Rice biomass in mixture with diseased weed was higher than in the presence of healthy weed, and is not significantly different from rice biomass in the non-weedy control. In the inoculated experiment, at lower weed density, competition between barnyard grass and rice was not apparent despite the fact that the weed growth was reduced. As the weed density increased, rice continued to grow, but barnyard grass was suppressed; the growth difference was bigger and more measureable. In the non-inoculated control, the interaction between barnyard grass and rice was observed at 2:2 ratio, but at 3:1 in the inoculated experiment, indicating that rice was more competitive over barnyard grass. It took three barnyard grass to equal the shoot dry weight of one rice plant. This study provides strong evidence of the ability of *E. monoceras* in reducing the competitive ability of barnyard grass and thus provides new opportunities for the future of biological weed control in Malaysia.

Keywords: Barnyard grass, *Echinocloa crus-galli*, *Exserohilum monoceras*, weed competition, biological control

INTRODUCTION

Bioherbicide has been proposed as one of the components of Integrated Weed Management (IWM), but little research has been done on this aspect. The efficacy of a potential bioherbicide needs to be established and this is normally done after the study on inoculum production and epidemiological studies (Morin *et al.*, 1990; Charudattan, 2001). The efficacy of any bioherbicide can be measured in terms of weed control, the level of disease stress or increase in crop yield resulting from reduced weed competition (Charudattan, 1988). The use of bioherbicides to reduce interference by barnyard grass in rice cropping systems has only recently started in Malaysia. However, there are several reports of successes in reducing weed interference in different cropping systems (Kennedy *et al.*, 1991; Jacobs *et al.*, 1996; Kadir *et al.*, 2000b).

Tasrif *et al.* (2003) has indicated the potential of *Exserohilum* monoceras as a bioherbicide for barnyard grass, but its control efficacy has not been determined in a rice cropping system. Therefore the objective of this study was to determine the potential of using *E. monoceras* in reducing the interference of barnyard grass on rice in the field and its role in controlling barnyard grass.

MATERIALS AND METHODS

Location

The experiment was conducted in 2003 at Field 2 of the University Research Farm, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The location of the plot was 03°00559' N, 101°42.195' E, and the daily average temperature was 30 ± 2°C with an average annual rainfall of about 2500 mm.

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Soil Preparation

Soil taken from Tanjung Karang (Malaysia) rice field estate was filled into 1 m³ fiberglass boxes until three-quarters full. The soil was flooded to 10 cm deep for 3 to 4 days so as to simulate real field conditions, and any emerging weed seedlings were killed. Thereafter, water level was reduced to 1-2 cm for planting.

Preparation of Inoculum

Inoculum of *E. monoceras* was produced using a biphasic culturing technique (Chandramohan, 2000) with modifications. Five mycelium plugs were transferred in a 250 mL flask containing 100 mL V8 broth. The inoculated flask was then shaken at 100 rpm on a rotary shaker for 2 days at 28°C, after which it was transferred into a 1L flask containing 400 mL of v8 broth. The resultant broth was blended with a Waring blender at low speed for 30-60 sec; 25 ml of the suspension was poured onto a layer of V8 agar (250 ml) in a tray (35x 26 x 2.5 cm), exposed to 24 h light at 30±2°C. The conidia was gently scraped off with sterile rubber spatula and transferred into sterile water. After filtering through two layers of cheese cloth, the spores were rinsed with sterile water. The conidial suspensions were pooled and the concentration was determined with a hemocytometer.

Plant Preparation

The substitutive or replacement series approach that was used in this study was designed basically for mini-plot trials. By this method, plant densities remain constant and the proportions of both species were varied from monoculture of one species at that given density to the monoculture of the other species (Radosevich, 1988).

The rice and barnyard grass seedlings were planted randomly in the fiberglass boxes using the approach in the proportions of 4:0, 3:1, 2:2, 1:3, and 0:4 (rice : barnyard) with a final total of 250 seedlings; a 4:0 means that 250 rice seedlings were planted as a monoculture.

Plant Inoculation

Barnyard grass and rice at three to four-leaf stage were sprayed with 15 ml of conidial suspension (6.5 x 10⁵ conidial⁻¹ mL) in a 10% oil emulsion using an AiFA pressure sprayer (Winstar Enterprise, Model 8505). The oil emulsion was used to maintain uniform conidial distribution on the leaves and to break through the protective cuticle layers for better penetration of the fungus. Each fiberglass box was therefore sprayed with 9.7 million spores. The control was sprayed with 10% oil emulsion only. Disease incidence and severity were recorded daily for seven days. The plants were harvested for dry weight biomass assessment 30 days after inoculation.

Disease Assessment

Disease incidence was assessed based on the proportion of plants affected out of the total number inoculated and expressed as the percentage of diseased plants (James, 1974; Horsfall and Cowling, 1978; Kranz, 1988). The disease severity was based on the percentage area of plant tissues showing symptoms of the disease (Kranz, 1988). The disease progress was assessed by monitoring the disease development. The disease severity was scored on a scale of 1-10 whereby 0 = healthy, 5 = 50% diseased, and 10 = plant death (Kadir *et al.,* 2000a).

Measured Variables

For the dry weight, shoots were harvested by cutting all plants just atop the soil level. The plants were oven dried for four days at 75°C and the dry biomass was determined.

Assessment for competitive interaction was carried out by the resulting models on replacement series (Radosevich, 1988). The "Relative Yield Total" was adopted to draw conclusions from the data collected; it determines the relative amounts of biomass produced by any two species by adding the relative yields (RY) of both species within each proportion used. Both variables were obtained from the equations below as explained by Willard and Shilling (1990).

RYT = RY_a + RY_b (relative yield total)

RY_a = $Biomass_{ap}/Biomass_{am}$ (relative yield)

RY_b = Relative yield of species *b*.

$Biomass_{ap}$ = Biomass production of species *a* at *a* particular proportion *p*.

$Biomass_{am}$: Biomass production of species *a* as *a* monoculture.

STATISTICAL ANALYSIS OF DATA

The field studies were carried out with two trials; one in early January and another in mid August 2003, arranged in a randomized complete block design with four replications. The average temperatures for these trial dates were 32°C and 30°C respectively. Data were pooled since the individual trials did not show significant difference. A two-way ANOVA set to 5% significant level was performed to test treatment effects and interaction between factors. When interactions between proportions were significant, each possible combination was considered as an independent treatment. Means were compared with a Fisher's Protected LSD test. Standard error bars were calculated to show differences in relative yield graphs.

RESULTS

Disease Progress

Exserohilum monoceras was very pathogenic to barnyard grass and the disease started with specks that became numerous as the disease progressed. The margins around the lesions turned grayish and eventually the areas turned necrotic. The infected leaves then turned dark green to brown and eventually shrivelled and dried.

The older lower leaves were more afflicted with larger necrotic areas lined with dark watery borders. Most of the leaf blade was blighted within 24 h. The control plants sprayed with 10% oil emulsion remained healthy and asymptomatic throughout. The disease progress could be best described by the logistic growth model (*Fig. 1*) with the overall apparent infection
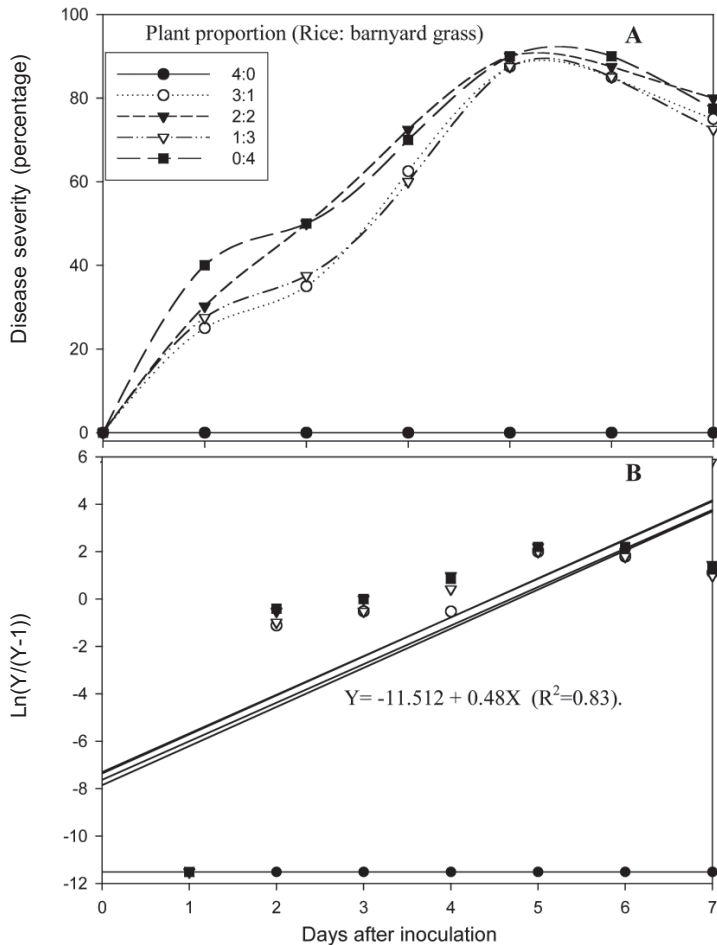

Fig. 1: *Disease progress of leaf blight by* E. monoceras *on barnyard grass seedlings; (A) Untransformed diseased severity value. (B) Regression of transformed disease severity using the logistic model ln (Y/(1-Y))*

TABLE 1
The effect of *E. monoceras* on relative yield (RY) and relative yield total
(RYT) within proportions of rice and barnyard grass

Treatment	Proportion (*O. sativa: E. crusgalli*)				
	4:0	3:1	2:2	1:3	0:4
Non Inoculated					
RY Rice	1.00a	0.85a	0.49b	0.21c	0d
RYBarnyard grass	0e	0.21d	0.51c	0.78b	1.00a
RYT	1.00a	1.05a	1.00a	0.99a	1.00a
Inoculated					
RY Rice	1.00a	0.88a	0.79a	0.78a	0c
RYBarnyard grass	0c	0.20b	0.16b	0.21b	1.00a
RYT	1.00a	1.08a	0.95a	0.99a	1.00a

Values for RY and RYT within rows followed by the same letters are not significantly different at P<0.05
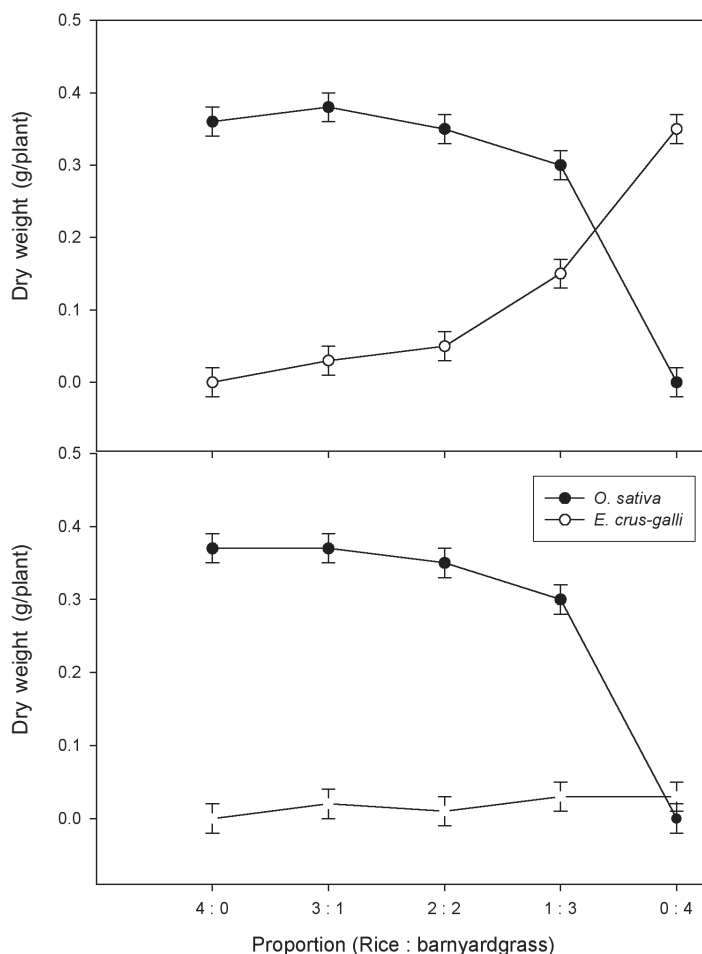according to Fisher's Protected LSD test.

Fig. 2: Effect of E. monoceras *on dry weight per plant of "replacement series-proportion" between rice and
barnyard grass; (A) uninoculated treatment and (B) inoculated treatment. Bars represent
the standard deviation of the difference between means*

rate of the two inoculated trials averaging $r_L = 0.48$ logit/day (SE = 0.001, $R^2 = 0.83$; P <0.005). Although initially *E. monoceras* caused severe infection on barnyard grass, 100% mortality by *E. monoceras* was not recorded. However, the infected plants remain stunted and never resume normal growth.

Plant Dry Weight

In the non-inoculated control, rice grown as a monoculture (4:0) and 3:1 proportion produced the highest dry weight per plant (*Fig. 2A*). Dry weights within these proportions were not significantly different. Barnyard grass as a monoculture and at 1:3 proportions produced significantly greater dry weight per plant compared to other proportions (*Fig. 2A*). In the inoculated experiment, the dry weight of rice remained consistently high for all proportions except for the 0:4 ratio (*Fig. 2B*). The dry weight

of barnyard grass was significantly reduced in all plant proportions in the inoculated experiment but the difference in dry weight according to plant proportions was not significant (*Fig. 2B*).

Derived Variables: Relative Yield (RY) and Relative Yield Total (RYT)

The RY is the yield of each species in a mixture as a percentage of its monoculture yield produced under the same growing condition. RYT is the result of adding relative yield for each species within a given proportion. Both variables are coefficients indicating species that is more competitive, without partitioning intra-specific or inter-specific effects (Radosevich, 1988; Willard and Schilling, 1990). Plant proportion significantly affected (P <0.01) RY of rice and barnyard grass (Table 1; *Fig. 2*); however, RYT did not differ statistically among proportions. At all proportions, RY of both species in mixture
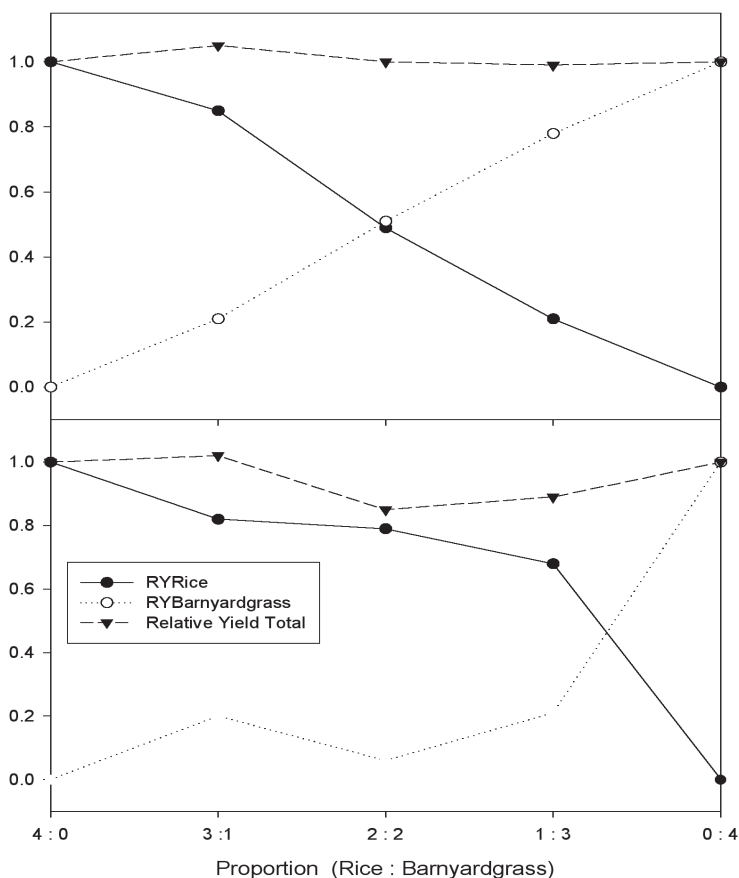

Fig. 3: *The relative yield total results of rice and barnyard grass grown in replacement series:*
(A) non inoculated control and (B) E. monoceras *inoculated treatment*

differed from monoculture of each species, except at 3:1 (Table 1; *Fig. 3*) where the RY rice was not significantly different from RY rice as a monoculture (4:0). The interaction between rice and barnyard grass was observed at 2:2 for the non-inoculated experiment (*Fig. 3*), which implied that rice and barnyard grass were both equally competitive.

In the inoculated experiment, similar results were observed. The RYT was not significantly different among proportions. The RY of barnyard was not significantly different among proportions except at 0:4; the RY barnyard grass was significantly lower compared to the RY rice at all proportion except at 0:4. The RY rice for all proportions was not significantly different from RY rice as a monoculture. The interaction point for the inoculated experiment was reduced to between 1:3 and 0:4 (*Fig. 3*). The results implied that it took one rice plant or less to equal the shoot dry weight production of three barnyard grasses.

This study revealed that the application of *E. monoceras* reduced barnyard grass growth, hence having the advantage of rendering rice more competitive. The RYT values in non inoculated mixtures were equal to the monoculture at all proportions. These results indicated that both species in a mixture produced the same dry weight that would be expected if they had been planted separately. The mutual antagonism had resulted in both species producing less than their respective monoculture biomass and the result obtained was similar to Model III of Radosevich (1988) based on the replacement series experiments.

DISCUSSION

The infection of *E. monoceras* on rice was negligible and was expected as this fungus had been previously determined to be host specific to barnyard grass (Juraimi *et al.*, 2006). Severe infection was observed on inoculated barnyard grass although seedlings mortality was negligible; however, the growth of the infected plants was retarded.

The inability of *E. monoceras* to kill barnyard grass under field conditions compared to glasshouse may be attributed to the inadequate conidial concentration used as well as the environmental factors, especially the leaf wetness (dew). Researchers working on other mycoherbicides have reported that extended dew period requirement was responsible for the poor efficacy of many weed bioherbicides in the field (Daigle *et al.*, 1990; TeBeest *et al.*, 1992; Zhang *et al.*, 1996; Kadir *et al.*, 2000a). However, the length of the dew period required by most effective bioherbicides can be reduced by appropriate timing of the application to take advantage of the humidity provided by rain, dew and irrigation in the field.

The formulation of foliar pathogens with water-retaining materials is another promising approach to make pathogens less dependent on available water to initiate infections. Recent research on formulation had shown the potential of materials such as surfactants (Zhang and Watson, 1997), invert emulsions (Amsellem *et al.*, 1991; vegetable oils (Kadir and Ng, 2004); humectants (Boyette *et al.*, 1996; Kadir *et al.*, 2000a) to overcome dew requirements, which will broaden the application strategies for bioherbicides.

The results of this research confirmed the presence of inter-specific and possibly intra-specific competition in rice-barnyard grass system affecting the plant growth and biomass production. Competition increases with increasing plant density. Application of *E. monoceras* reduces the growth of barnyard grass, in terms of biomass, and hence decreased the level of competition by the weed. At lower weed proportion, competition was not apparent despite the fact that weed growth was reduced. As the weed density increased, the rice continued to grow but the barnyard grass showed suppressed growth hence the difference was bigger and more measurable. The rice biomass in a mixture with diseased weeds was higher than in the presence of healthy weeds, and was not significantly different from rice as a monoculture.

Inoculation of *E. momoceras* caused the highest growth suppression in barnyard grass at the highest density. At the proportion of 1:3 (rice:barnyard grass), which is equivalent to approximately 50 rice plants to 200 barnyard grass plants m^{-1}, it is expected that plants experience more inter- and/or intra-specific competition; however, this phenomenon was not observed in the inoculated treatments. This result was consistent with other findings that fungal pathogens gave greater negative effects on host plants in higher plant density situations (Ditommaso and Watson, 1995; Kadir *et al.*, 1999; Jahromi *et al.*, 2001).

Paul and Ayres (1987) found that lettuce (*Lactuca scariola* L.) showed a competitive advantage when in a mixture with grounsel (*Senecio vulgaris* L.). This advantage was further exaggerated if the groundsel was infected by rust fungus (*Puccinia langenophorae* Cooke). They reported that the effect of the rust on the weed was expressed by reduction in the dry weight yield of groundsel, which also appeared to be the case with the barnyard grass-rice competition examined here. Kadir *et al.* (1999) reported that the top biomass and tuber production of purple nutsedge (*Cyperus rotundus* L.) was reduced drastically when this weed was inoculated with *Dactylaria higginsii* (Luttrel) MB Ellis, thus reducing competitiveness of this weed in tomato-nutsedge system.

Given the high genetic variability between barnyard grass populations (Juraimi *et al.*, 2006), the different responses of the weed population to the pathogen may become a major concern; however, this constraint can be circumvented by increasing the virulence of the pathogen through addition of additives in the formulation. Juraimi *et al.* (2006) reported the water-oil-water (WOW) formulation of *E. monoceras* had increased the efficacy of this fungus in the presence of biotype difference of barnyard grass. The use of a rice cultivar that is fast growing can provide a more successful competitor and therefore would enhance the effectiveness of the inoculation.

In this study *E. monoceras* did not cause 100% mortality of barnyard grass. It should be emphasized that any consideration of the efficacy of a biocontrol agent should be based on crop yield rather than injury or mortality of the weed (Paul and Ayres, 1987). The difference in relative yield of rice in the non inoculated and inoculated treatments was not apparent; however, the relative yield of barnyard grass was higher in the non inoculated compared to the inoculated experiments. Although the results of the glasshouse study did not concur with the field study in terms of plant mortality, it was concluded that *E. monoceras* was capable of suppressing the barnyard grass growth based on the strong evidence of its ability to reduce the competitive ability of the latter. This study has provided a new hope for the future of biological weed control in Malaysia.

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Major Postharvest Fungal Diseases of Papaya cv. 'Sekaki' in Selangor, Malaysia

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ABSTRACT

A total of seven fungi were identified from the surface of fully matured papaya fruits cv. 'Sekaki' collected form two different fields namely University Agriculture Park, UPM and MARDI, Selangor and a fruit exporter [Seng Chew Hup Kee (M) Sdn Bhd, Kajang, Selangor, Malaysia]. They were identified as *Botryodiplodia theobromae, Colletotrichum capsici, C. gloeosporioides, Fusarium* sp.*, Phomopsis* sp.*, Rhizopus stolonifer* and *Stemphylium* sp.. Among the diseases, the highest incidence ranged from 90 to 98% and severity of 25 to 38% were recorded for anthracnose caused by *C. gloeosporioides* for all three sources, followed by stem-end-rot caused by *Botryodiplodia theobromae.* Pathogenicity test showed that both wounded and unwounded fruits inoculated with conidial suspension of *C. gloeosporioides* developed distinct symptoms of anthracnose after three and five days of inoculation, respectively.

Keywords: Disease incidence and severity, fungal pathogens, postharvest, papaya

INTRODUCTION

Papaya (*Carica papaya* L.), a native of tropical America, is grown throughout the tropics and subtropics for its melonlike fruit (Alvarez and Nishijima, 1987). This fruit is rapidly becoming an important fruit internationally, both as fresh and processed products (Sankat and Maharaj, 1997). In Malaysia, it is a smallholders' crop and planting is widespread throughout the country. At present, sekaki is considered as a leading cultivar for export as well as domestic consumption. Papaya has an excellent potential as an export crop in Malaysia. In 2003, the export value of fresh papaya was estimated at RM 100.8 million, up from 23.6 million in 1992 (Anonymous, 2006). Greater commercial production of papaya in Malaysia has increased due to higher returns compared to other fruits. As a result, the production has jumped from 4,938 tonnes in 1980 to 6500 tonnes in 2003 (FAO, 2005). Hong Kong continued to be the major export destination of Malaysian papaya followed by Singapore, United Arab Emirates and Brunei.

Papaya fruits are very susceptible to diseases caused by many microorganisms especially fungi, as papaya fruit is high in moisture and nutrients (Sankat and Maharaj, 1997). Orchard and postharvest diseases are very important in terms of reducing yield and quality of papaya, which are primarily responsible for the losses that occur during shipment of the fruits (Couey *et al.*, 1984; Chau and Alvarez, 1983a; Alvarez, 1980). Papaya postharvest losses of 10-40% in sea shipments and of 5-30% in air shipments are not unusual and losses due to diseases ranged from 1 to 93%, depending on postharvest handling and packing procedure (Alvarez and Nishijima, 1987). Many postharvest diseases are initiated through injuries created during and following harvest. The infection process, particularly during postharvest, is greatly aided by mechanical injuries to the skin of the produce such as fingernail scratches and abrasions, insect punctures and cut (Wills *et al.*, 1989). In some cases, as for latent infections, inoculation occurs prior to harvest but the disease dose not develop until the postharvest period (Kays, 1991). Fruits

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may be infected by direct penetration of certain fungi through intact cuticle or through wounds and/or natural opening in their surfaces. For some pathogens, the synthesis of enzymes is essential for initial invasion. Furthermore, the development of fungal infection during the postharvest phase can depend upon the physiological age of the fruit, mechanical injuries, temperature and storage environment (Ilag *et al.*, 1994).

The diseases caused by fungi, virus and bacteria, as well as the damages caused by insects threaten world-wide agriculture and export policy (Albornett and Sanabria de Albarracin, 1994). For this reason, these problems must be solved to guarantee the continuous supply of healthy and fresh fruits of acceptable standards to national and international markets.

Therefore, this study was conducted to determine the status of major postharvest diseases of papaya caused by various fungi that largely affect the fruits of export, thus acquiring knowledge for the implementation of appropriate and effective controls measures for good storage and transport.

MATERIALS AND METHODS

Fruit Materials

Papaya fruits of 'Sekaki' cultivar at color stage two (green with trace yellow) were used in this experiment. Forty five healthy fruits with uniform size, shape, and maturity were collected from each of the two fields namely University Agriculture Park, UPM and MARDI, Selangor and a fruits exporter [Seng Chew Hup Kee (M) Sdn Bhd, Kajang, Selangor, Malaysia].

Isolation and Identification of Pathogenic Fungi Associated with Postharvest Decay of Papaya

Fungi were isolated from naturally infected fruits of papaya. Pieces of tissue were cut from the advancing margin of the lesion, surface sterilized in 5% sodium hypochlorite solution and washed in three changes of sterile distilled water. The tissues were then dried on sterilized filter paper and then plated on potato dextrose agar (PDA). The plates were incubated for seven days at 28±2ºC and observed regularly. After the emergence of mycelial growth, each of the fungal colonies were transferred to fresh PDA plates and incubated at room temperature for 2-4 days to obtain pure cultures. Fungal mycelium from pure cultures were examined under dissecting

and compound microscopes and identified by comparing their morphological and cultural characteristics with descriptions published in the literature (Sutton, 1992; Nelson *et al.*, 1983; Sutton, 1980; Booth, 1977; Barnett and Hunter, 1972; Ellis, 1971). Isolates of different fungi were then randomly selected and cultured from a single conidium for further purification. Cultures of fungal isolates were maintained on PDA slants for further use.

Incidence and Severity of Postharvest Diseases of Papaya

Forty five full mature papaya fruits at color stage two were collected from each of the three locations (University Agriculture Park, UPM; MARDI and an exporter, Kajang), Selangor, Malaysia. On arrival at the laboratory, the fruits were surface sterilized with 70% ethanol and air-dried. The fruits were then placed in a commercial packaging, held at room temperature (28±2ºC) for 10 days and observed regularly for the development of disease symptoms. Data on incidence and severity of different postharvest diseases were recorded when disease symptoms developed on the surface of ripened fruits. Disease incidence was calculated by the following formula:

$$\% \text{ Disease incidence} = \frac{\text{Number of infected fruits}}{\text{Total number of fruits assessed}} \times 100$$

Data on disease severity was indexed on a 0-4 scales, where, 0 = no disease symptom on the fruit surface area, 1 = 1–10% diseased area, 2 = 11-20% diseased area, 3 = 21-30% diseased area and 4 = 31% and over diseased area (Illeperuma and Jayasuriya, 2002). Percent disease index (PDI) was calculated according to Singh (1984) as follows:

$$\text{PDI} = \frac{\sum (\text{rating number x number of fruits in the rating})}{\text{Total number of fruits x highest rating}} \times 100$$

Pathogenicity of Colletotrichum gloeosporioides on Papaya Fruit

Healthy papaya fruits were surface-disinfested with 75% ethanol and air dried. Inoculations were done on both wounded and unwounded fruits and disease incidence and severity were

compared between them. Each fruit was wounded (3 mm deep and 5 mm diameter) at two different positions with a sterilized cork borer. For inoculum preparation, isolate of *C. gloeosporioides* was grown on PDA at 28±2˚C for 7 days. Spores were subsequently harvested by flooding the surface of the media with sterilized distilled water and gently agitating the plate with a bent glass rod to dislodge the spores. The resulting suspension was filtered through two layers of sterile muslin cloth. The concentration of conidia in the filtered suspension was adjusted to 5x10^5 conidia ml^{-1} with sterile distilled water using a haemacytometer. 100µl of inoculum was placed on each wounded or marked places of unwounded papaya fruit surface. All fruits were incubated for 24 h in moist chambers at room temperature (28±2˚C), then packed in a commercial packaging and held at room temperature for 7 days. The fruits were examined daily and disease incidence was recorded after lesion development caused by the test fungus. The experiment was conducted with 10 fruits per treatment. Sterilized distilled water was used on control fruits in place of inoculum.

Statistical Analysis

The experiment was arranged in a complete randomized design with three replicates. All of the percentage data were arcsine transformed before subjecting to analysis of variance (ANOVA) and the means separation was done using the Tukey's Studentized Range (HSD) Test using SAS version 8.2.

RESULTS

Isolation and Identification of Fungi

After eight days of storage at ambient temperature (28±2ºC), more than 90% of the fruits were fully ripened and disease symptoms began to develop on the surface of the fruits. In most cases a diseased fruit had more than one lesion. The diseases appeared in variable degrees of development. Fungi were isolated from rotted papayas and identified based on the morphological and cultural characteristics on PDA plates (Table 1). A total of seven fungi were isolated and identified, namely *Botryodiplodia theobromae, Colletotrichum capsici, C. gloeosporioides, Fusarium* sp.*, Phomopsis* sp.*, Rhizopus stolonifer and Stemphylium* sp.

Colletotrichum gloeosporioides was mainly isolated from lesions associated with the orange pustules. Hyphae were brown, smooth and septate. Conidia were cylindrical with obtuse ends, hyline, aseptate, uninucleated, and 10-15 µm x 3-5 µm in size. Conidia were formed on the conidiophores in the acervuli, which were round to irregular in shape. Setae were present, sparse to many, dark brown, straight to slightly curved, 2-3 septate, swollen at the base and tapering towards the apex. On the PDA, colony appeared white and gradually turned grayish salmon in color as the culture grew older. *C. capsici* was separated from *C. gloeosporioides* on the basis of lesion color and conidial shape. This fungus was isolated from lesions producing black acervuli, which bear sickle-shaped conidia. Setae were brown in color, 1-5 septate, rigid, hardly swollen at the base and slightly tapered to the apex. Conidia were hyaline, falcate with acute apex, aseptate and uninucleated.

Botryodiplodia theobromae Pat. produced a wide margin of water soaked tissues at the base of the fruit. In advanced stages, the lesion margin remains translucent as the rest of the infected tissues became wrinkled, black and dry. Numerous pycnidia appeared on the affected zone. On PDA plates, the fungal colonies were grayish with abundant mycelium. Initially the conidia were hyaline, unicellular, oblong in

TABLE 1
List of fungal pathogens associated with major postharvest diseases of papaya

Name of the Diseases	Causal Organisms
Anthracnose	*Colletotrichum gloeosporioides, C. capsici*
Stem-end rot	*Botryodiplodia theobromae, Phomopsis* sp.
Fusarium fruit rot	*Fusarium* sp.
Stemphylium rot	*Stemphylium lycopersici*
Rhizopus rot	*Rhizopus stolonifer*
Phomopsis rot	*Phomopsis* sp.

shape, thick-walled with granular content. Mature conidia were two-celled, light brown in color with longitudinal bands resembling striations.

The initial disease symptom of wet rot caused by *Phomopsis* sp. was discoloration of the infected area. Thus, the tissues of the infected area became softer, which was covered with a white mycelial carpet. Fungal colonies on PDA media showed white aerial mycelium with pycnidia scattered on the agar. Conidiophores were simple, septate, sometimes branched. A-conidia were hyaline, fusiform, unicellular, with a guttulate at each end and B-conidia were hyaline, elongated, filiform and curved at the apex.

Fusarium rot caused by *Fusarium* sp. appeared initially as a circular water-soaked lesion, which later became depressed. At the advanced stage of disease development, the soft rotted area was covered with a white mycelial mat of the fungus. Conidia were hyaline, three to four-celled and crescent shaped with sharply pointed ends, which were produced from phialides.

Rhizopus stolonifer, produced a generalized soft and watery lesion on the fruit surfaces. Black masses of fungal sporangia were observed on the surface of infected area. In the advanced stage of disease development, fluids leak out from the rotted portion of the fruit. The fungus first appeared as white cottony colonies on PDA and became heavily speckled by the presence of

sporangia and then brownish black with age. Sporangiophores were smooth walled, non-septate and light brown in color. The sporangia were globose to sub-globose with somewhat flattened base, white at first, then black with numerous spores. The sporangiospores were irregular round to oval in shape.

Early symptoms of Stemphylium fruit rot caused by S*temphylium lycopersici,* was small, round and dark brown lesions. The lesions became sunken and developed reddish-brown to purple margins as they enlarged. A velvety, dark green spore mass formed in the center of the lesion. White to gray mycelia were grown over the lesions in the advanced stage of the disease. Conidia were light brown in color, minutely warted with two or three transverse septa, where the medial septum was most prominent.

Disease Incidence and Severity

Among the fungi that caused different postharvest diseases of papaya, significantly higher incidence (90 to 98%) was recorded for anthracnose disease caused by *C. gloeosporioides*, in all three locations, which was followed by stem-end rot disease caused by *Botryodiplodia theobromae,* with disease incidence ranging from 25 to 38% (*Fig. 1*). However, the lowest disease incidence (11.7 to 16.7%) recorded was for Rhizopus soft rot caused by *Rhizopus stolonifer.*
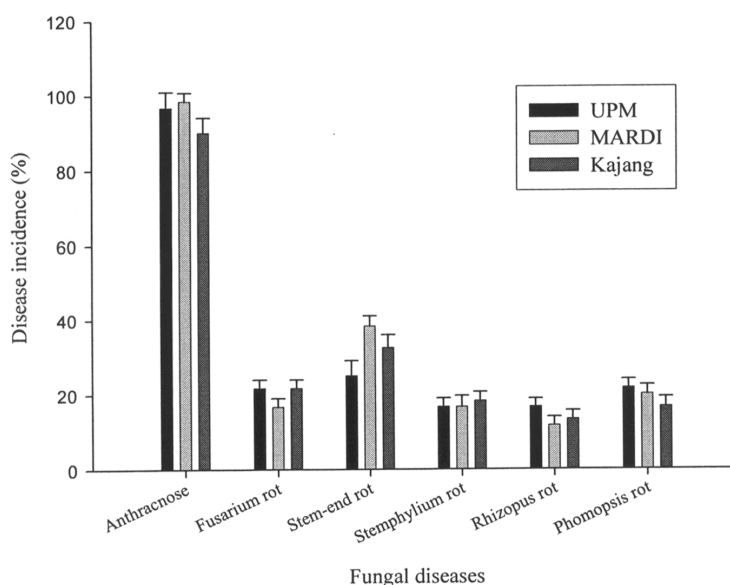

Fig. 1: Incidence of major postharvest diseases of papaya. For each location, values are the mean of three replicates with 15 fruits each. Means were separated by Tukey's Studentized Range (HSD) Test at P≤0.05. Vertical bars represent standard errors of the means

Regarding disease severity, a similar trend was also observed, for all locations, with the highest disease severity ranging from 26 to 34 % for anthracnose followed by stem-end rot, with a severity range of 11 to 16 % (*Fig. 2*).

Pathogenicity Test

Small, round water-soaked lesions were observed on wound inoculated papaya after three days of inoculation. As the infection advanced, lesions became circular and slightly sunken, and covered with dense whitish mycelial growth (*Fig. 3A*). In unwounded fruits, small water-soaked areas were observed on each inoculation site after five days of inoculation (*Fig. 3B*). At the advanced stage of disease development, a round sunken lesion with translucent, light brown margin was formed. After seven days of inoculation the fungus produced light orange spore masses in the central portion of the lesion.
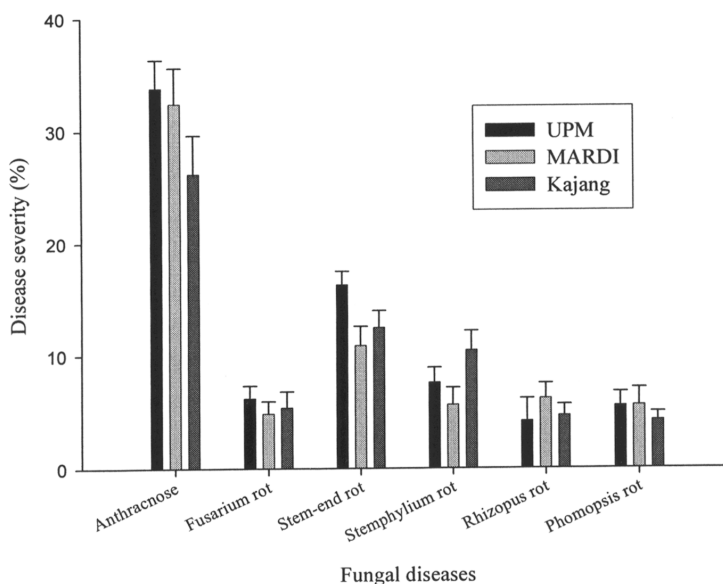

Fig. 2: Severity of major postharvest diseases of papaya. For each location, values are the mean of three replicates with 15 fruits each. Means were separated by Tukey's Studentized Range (HSD) Test at P≤0.05. Vertical bars represent standard errors of the means
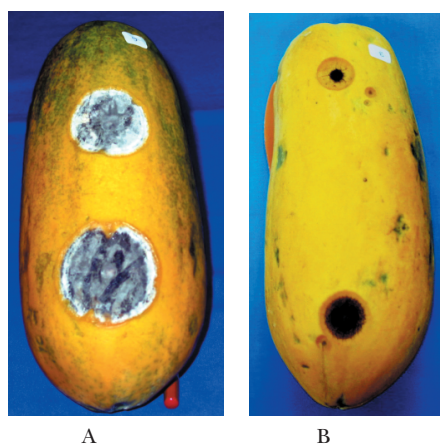

A   B

Fig. 3: Pathogenicity of C. gloeosporioides on (A) wounded and (B) unwounded papaya fruits inoculated by the spore suspension (5x 10⁵ spore ml⁻¹) of the test fungus. Inoculated fruits were incubated at room temperature (28±2°C) for seven days

DISCUSSION

Various species of pathogenic fungi such as *Colletotrichum, Phomopsis, Rhizopus, Botryodiplodia,* and *Stemphylium* are responsible for enormous losses of papaya after harvest. Most of these fungi cause rotting that spreads rapidly in the ripe fruit, thus rendering them unfit for consumption (Ilag *et al.,* 1994). There are three general types of postharvest diseases of papaya such as fruit-surface rots, stem-end rots and internal fruit infections (Alvarez and Nishijima, 1987). However, papaya fruits are subjected to several types of postharvest diseases including anthracnose and chocolate spot caused by *Colletotrichum gloeosporioides,* dry rot caused by *Mycosphaerella sp.,* wet rot caused by *Phomopsis sp.,* alternaria fruit spot caused by *Alternaria alternate,* stemphylium fruit rot caused by *Stemphylium lycopersici,* fusarium rot caused by *Fusarium solani,* guignardia spot caused by *Guignardia sp.,* stem-end rot caused by *Ascochyta* sp., *Botryodiplodia, Phomopsis* and *Fusarium,* rhizopus rot caused by *Rhizopus stolonifer* (Albornett and Sanabria de Albarracin, 1994; Ilag *et al.,* 1994; Alvarez and Nishijima, 1987). Among these diseases, anthracnose and stem-end rots continue to cause major postharvest losses of papayas during storage and shipment (de Oliveira *et al.,* 2004; Paull *et al.,* 1997; Alvarez, 1980). Anthracnose of papaya caused by *C. gloeosporioides* is considered to be the most important postharvest disease in the state of Hawaii, and it is important in many other tropical regions where papaya is grown (Snowdon, 1990; Bolkan *et al.,* 1976), including Philippines (Quimio and Quimio, 1974), Malaysia (Lim, 1980) and Sri Lanka (Gamagae *et al.,* 2004; Sivakumar *et al.,* 2002). Similarly in our study, the highest incidence and severity were recorded for anthracnose disease caused by *Colletotrichum* spp. with some degree of variations from location to location. These variations of disease reaction in three selected locations may be due to the variation in climatic conditions, cultural practices and prevalence of the pathogens during the study period. It is reported that the disease severity of sigatoka disease of banana varied with weather conditions (Mishra and Bhattacharyya, 2001).

However, Sepiah *et al.* (1991) and Sepiah (1992) reported that the most important pathogen causing anthracnose of Eksotika papaya, the current important variety in Malaysia, is *C. capsici.* Anthracnose caused by *C. gloeosporioides* was also present on this variety. Lim and Tang (1984) reported that *C. dematium* was the cause for 5% of anthracnose of papaya in Singapore. A single isolate of *C. gloeosporioides* can produce both anthracnose and chocolate spot, but little is known about why some lesions remain superficial while others advance into the fruit parenchyma (Alvarez and Nishijima, 1987).

Anthracnose becomes a problem when fruits have 25% or more skin yellowing (Alvarez and Nishijima, 1987). Infections caused by *C. gloeosporioides* are usually initiated in the field at early stage of fruit development, but the pathogen remains quiescent until the fruit reaches the climacteric phase (Dickman and Alvarez, 1983). The fungus may penetrate the fruit surface directly with an infection peg (Chau and Alvarez, 1983b). An extracellular cutinolytic enzyme is produced, enabling the pathogen to enter green, unwounded fruit. When infected fruits begin to ripen, beads of latex are exuded at the fruit surface, and small water-soaked spots appear. As the infection advances, a circular, sunken lesion with translucent, light brown margins forms. The fungus produces light orange or pink spore masses in the central portion of the lesion. Internal tissue in the infected area is firm with a grayish white discoloration that later turn brown. A layer of callus forms in the parenchyma cells, permitting the infected area to be lifted free of the fruit surface as a plug (Stanghellini and Aragaki, 1966).

Several fungi invade the cut portion of the peduncle after harvest or may enter the fruit through minute injuries and cause stem-end rot. These fungi, individually or in various combinations, cause rotting, shriveling and discoloration of the stem end. Initially the disease was attributed only to *Ascochyta* sp. Later, other genera, including *Botryodiplodia, Phomopsis,* and occasionally *Fusarium* (Hunter and Buddenhagen, 1972) were identified in diseased tissues. Several other fungi including *Alternaria alternate, Stemphylium lycopersici, C. gloeosporioides,* and *Mycosphaerella* sp. also may cause stem-end rots when inoculated alone or in various combinations (Chau and Alvarez, 1983c; Chau and Alvarez, 1979; Alvarez *et al.,* 1977). In the Philippines, the pathogen associated with the disease is *Botryodiplodia theobromae* Pat. while in Malaysia, although this fungus is also a causal pathogen, *Phomopsis carica-papayae* is more

common (Ilag *et al.*, 1994). *C. capsici* often causes stem-end rot of papaya when the fruits are kept for long period in cold storage. This disease may also be called stem end anthracnose (Ilag *et al.*, 1994).

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A Putative Proline-rich Protein of *B. napus*

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ABSTRACT

Proline-rich proteins are among the major protein components of plant cell walls. So far, two different proline-rich cell wall proteins have been described in *Brassica napus*. This paper reports a study on expression and sequence analysis of a novel class of a proline-rich putative protein, tentatively designated Ae4. The largest ORF of Ae4 encodes 166 amino acid residues without the start and stop codons. Ae4 is a partial length cDNA. The Ae4 gene expression was investigated and the results demonstrate that it accumulates in all vegetative tissues tested as well as in the embryogenic culture of *Brassica napus*. However, expression of Ae4 was undetectable in the non-embryogenic and cytokinin-treated embryogenic tissues. These results indicate that the Ae4 gene might play a role in somatic embryo formation.

Keywords: Proline-rich protein, PRP, *Brassica napus*, oilseed rape, somatic embryogenesis

INTRODUCTION

Proline-rich proteins (PRPs) are one class of structural cell wall protein members (Showalter, 1993). All the PRPs are characterised by the repeating occurrence of Pro-Pro repeats contained within a variety of larger repeat units. These proteins lack the SerPro4 repetitive element defined for extensins (Jose-Estanyol and Puigdomenech, 2000). The most extensively studied PRPs are from soybean that contains the PPVYK motif or variations (Hong *et al.*, 1987, 1989; Datta *et al.*, 1989; Wyatt *et al.*, 1992). Members of the PRP gene family were shown to be developmentally regulated and their expression tissue/organ specific (Hong *et al.*, 1989; Lindstrom and Vodkin 1991; Jose-Estanyol *et al.*, 1992). In general, PRPs are thought to have a structural role in the cell wall (Cassab and Varner, 1988). They have been also implicated in plant defence reactions (Chen and Varner, 1985; Ebener *et al.*, 1993), nodule morphogenesis (Franssen *et al.*, 1987; Wilson *et al.*, 1994), and are expressed during somatic embryogenesis (Aleith and Reichter, 1990; Gyorgyey *et al.*, 1997; Yasuda *et al.*, 2001).

To date, two different proline-rich cell wall proteins have been described in *Brassica napus* (*B. napus*). In this paper, we report the sequence and expression analysis of an additional proline-rich putative protein, tentatively designated as *Ae*4. The amino acid sequence of *Ae*4 has been deduced from the nucleic acid sequence of a copy DNA (cDNA), isolated previously from a subtracted library of *B. napus* embryogenic culture (Namasivayam *et al.*, 2006b).

MATERIALS AND METHODS

Sources of Plant Materials

Plants of *Brassica napus* ssp. *oleifera* cv. Primor were grown from seeds, in pots with soil in the Botanic Garden, Cambridge. Sources and preparation of plant materials for the pre-embryogenic, mature embryogenic and non-embryogenic *Brassica napus* ssp. *oleifera* cv. Primor culture was as described in Namasivayam *et al.* (2006a,b). The cytokinin-treated embryogenic tissue was generated from hypocotyls of embryoids grown for 20 days on MS media containing 10^{-4} M kinetin, 2% (w/v) sucrose and

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* Corresponding Author

0.8% (w/v) agar. Various organs/tissues such as young leaves, stem, buds, flowers, siliques, roots, stamens, carpels, petals and sepals from mature *B. napus* plants were harvested, immediately frozen in liquid nitrogen and stored at–80°C until isolation of total RNA.

Sequence Analysis

The *Ae*4 cDNA sequence has been submitted to the GenBank under the accession number AY570239. Sequence analysis was carried out using BLAST 2.0 (Basic Local Alignment Search Tool; Altschul *et al.*, 1997), accessible from the internet (http://www.ncbi.nlm.nih.gov/BLAST). Alignments of the protein sequence with several closely related genes was carried out using the CLUSTAL W program from the Biology Workbench version 3.2, accessible from the internet (http://biowb.sdsc.edu/CGI/BW.cgi). Other sequence analyses were performed using Biology Workbench version 3.2 to compute molecular weight (MW), hydrophobicity and isoelectric point (pI) determination.

Total RNA Isolation

Total RNA from various frozen tissues/organs of the mature plant and tissue culture materials were extracted using the acid guanidium thiocyanate-phenol-chloroform extraction method described by Chomczynski & Sacchi (1987). The concentration of RNA in each sample was determined spectrophotometrically (Sambrook *et al.*, 1989).

Northern Blot Analysis

Equal amounts of total RNA (10 (g per lane) were resolved on 1.3% (w/v) agarose-formaldehyde denaturing gel and blotted onto Hybond™–XL nylon membrane (Amersham Biosciences). Hybridisation was carried out at 65°C using standard techniques (Sambrook et al., 1989). The entire Ae4 sequence was used as probe labelled with [^{32}P-α]-dCTP using the Prime-IT® II Random Primer Labeling Kit (Stratagene). Washes were carried out at room temperature in the first wash buffer (40 mM sodium phosphate pH 7.2, 1% (w/v) SDS and 1 mM EDTA) for 10 min and followed by second wash in 40 mM sodium phosphate pH 7.2, 5% (w/v) sodium dodecyl sulphate (SDS) and 1 mM ethylenediaminetetraacetic acid (EDTA) at 65°C for 15 min. The hybridisation signals were captured by a Phosphorimager Typhoon 8600

(Amersham Pharmacia Biotech). After removal of probe, the same blot was hybridised with radiolabelled Arabidopsis *Actin*2/7 cDNA probe as a loading control.

RT-PCR

Equal amounts of DNase-treated total RNA (200 ng) from each tissue sample was added individually to a sterile 0.2 ml polymerase chain reaction (PCR) tube and the volume adjusted to 13.5 μl with DEPC-treated sterile deionised water (SDW). Oligo (dT$_{18}$) (1 μl of 20 pmoles/μl) was added to the tube and the reaction mix incubated for 10 min at 70°C. Following brief centrifugation, the following reagents were added: 4 μl 5 x first strand buffer (Promega), 0.5 μl 'RNase Out' ribonuclease inhibitor (40 U/μl) (Invitrogen), 0.5 μl 10 mM Bioline dNTPs mix and 0.5 μl MMLV-RT RNase H minus (200 U/μl) (Promega) and incubated at 37°C for 1 h. Later, the reaction mix was heat deactivated before using for PCR reactions. PCR reactions were performed in 12.5 μl reactions with the following components: 2 μl of the RT product, 1 x Bioline PCR buffer (Mg^{2+}- free), 1.5 mM MgCl$_2$, 0.4 mM dNTP mix, 2.5 pmoles of forward primer (5′ GGACTATAAATTGGTGTTGGAGGTTTCA 3′) and reverse primer (5′- TATTTATAGT CCTCCCGTAATGCCA - 3() respectively, and 1.5 U BioTaq DNA polymerase. An internal control was prepared using actin2 primers (forward primer: 5′-CCATTCTTGCTTCCCTCAG-3′ and reverse primer: 5(-GACGTAAGTAAAAACCCAG-3′) and containing all the components as above to test for equal loading of the template. Also, a negative control without template was included. Amplification was performed as follows: 95°C for 3 min; followed by 35 cycles at 94°C for 30 s, 65°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 3 min. The annealing temperature used for *Actin*2 was 60°C. Reverse transcription polymerase chain reaction (RT-PCR) products were separated on a 2% (w/v) agarose gel and the gel was photographed. The agarose gel containing the PCR amplified products was blotted (Southern, 1975) and hybridised with a labelled specific probe (*Ae*4 cDNA).

RESULTS

Sequence Analysis

The BNPE AE4 clone contains a cDNA insert of 678 bp, excluding a poly A tail. The longest open reading frame (ORF) encodes 166 amino

acids starting from nucleotide no. 3 and there is no start and stop codons, as shown in *Fig. 1*. Therefore, it is unlikely to be a full length clone. The predicted amino acid sequence is rich in proline (44.89 %), lysine (10.2 %), threonine (10.22 %), valine (8.89 %), serine (6.67 %), tyrosine (5.78 %) and glutamine (5.78 %). The predicted protein fragment represents a calculated molecular weight of 17.7 kDa and has a predicted pI value of 10.5. Hydropathy analysis indicated that 13 residues at the N terminal end of the predicted protein fragment are hydrophobic and the other regions are highly hydrophilic. The polypeptide is primarily composed of two repeat units: a 10-mer repeat unit (P P I/V K/M P P P V Q K/Q) and a 7-mer repeat unit (P P T P I/S/T YS). Protein database search revealed that there is no significant similarity between the predicted amino acid sequence and protein sequences deposited in

the GenBank. However, comparison of the *Ae*4 nucleotide sequence with nucleotide sequences in the GenBank showed that it is homologous (82% identical) to a genomic fragment of Arabidopsis in chromosome 2 (*At2g27380*) which encodes a putative proline-rich protein. Also, a few hits to *B. napus* seed EST sequences with a homology of 80% to 95% were found in the database. A comparison of the amino acid sequence of this clone with the Arabidopsis putative proline-rich protein and translated EST sequences is depicted in *Fig. 2*.

Expression Analysis of Ae4

The pattern of expression of *Ae*4 in organs and tissues was investigated using Northern analysis and RT-PCR. The Northern analysis on the organ/tissue specific-blot failed to detect a distinct band for *Ae*4 transcripts except for a very faint smear observed in each sample lane

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  3   caacaccaacttatagccctcctatcaaaccaccacccagtgcaaaagcctccaactccc   62
      Q  H  Q  L  I  A  L  L  S  N  H  H  P  V  Q  K  P  P  T  P

 63   acttatagtcctccgataaagccaccaccagtgcagaagcctccaacaccgacctacagt   122
      T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T  P  T  Y  S

123   ccaccagttaaaccaccaccagtgaagcctccaacacctatttatagtcctcccgtaatg   182
      P  P  V  K  P  P  P  V  K  P  P  T  P  I  Y  S  P  P  V  M

183   ccaccaccagtgcaacaacctccgacaccatcttatagtcctcctgtaaaaccaccacca   242
      P  P  P  V  Q  Q  P  P  T  P  S  Y  S  P  P  V  K  P  P  P

243   gtgcaaaaacctccaacacccacttatagtccacctgttaaaccaccacctgtgcaaaag   302
      V  Q  K  P  P  T  P  T  Y  S  P  P  V  K  P  P  P  V  Q  K

303   cctccaactccaacttacagtcctcctatcaaaccaccacccgtgcaaaaacctccaaca   362
      P  P  T  P  T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T

363   ccaacttatagccctcctatcaaaccaccacctgtgcaaaagcctccgacgcccacttat   422
      P  T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T  P  T  Y

423   agtccacctgttaaaccaccacccgtccagaagcctcccacaccaacttacagtcctcct   482
      S  P  P  V  K  P  P  P  V  Q  K  P  P  T  P  T  Y  S  P  P

483   atcaaaccacctccagtgaaacctccaacaccaatttatagtccgccagtgaaaccacca   542
      I  K  P  P  P  V  K  P  P  T  P  I  Y  S  P  P  V  K  P  P

543   cccgtgcaaaagcctccaacaccaacgtacagcccaccaattaaaccacctccagtaaaa   602
      P  V  Q  K  P  P  T  P  T  Y  S  P  P  I  K  P  P  P  V  K

603   cctccgacaccaacttatagtcctcctgtaaaaccacctccagtgcaaaagcctccgacg   662
      P  P  T  P  T  Y  S  P  P  V  K  P  P  P  V  Q  K  P  P  T

663   cccacttatagtccac   678
      P  T  Y  S  P
```

Fig. 1: Nucleotide and deduced amino acid sequence of clone Ae4 (Genbank accession no. AY570239). The ORF is underlined. This is a truncated clone without the start and stop codons.

```
CD825663 Translated       --------------------------------------------------
CD830533 Translated       --------------------------------------------------
NP_180307              1  MRVPLIDFLRFLVLILSLSGASVAADATVKQNFNKYETDSGHAHPPPIYG 50
Ae4

                          --------------------------------------------------


CD825663 Translated       --------------------------------------------------
CD830533 Translated       --------------------------------------------------
NP_180307             51  APPSYTTPPPPIYSPPIYPPPIQKPPTYSPPIYPPPIQKPPTPTYSPPIY 100
Ae4

                          --------------------------------------------------


CD825663 Translated       --------------------------------------------------
CD830533 Translated       --------------------------------------------------
NP_180307            101  PPPIQKPPTPTYSPPIYPPPIQKPPTPTYSPPIYPPPIQKPPTPSYSPPV 150
Ae4

                          --------------------------------------------------


CD825663 Translated       --------------------------------------------------
CD830533 Translated       --------------------------------------------------
NP_180307            151  KPPPVQMPPTPTYSPPIKPPPVHKPPTPTYSPPIKPPVHKPPTPIYSPPI 200
Ae4

                          -------------------------------------------------


CD825663 Translated       --------------------------------------------------
CD830533 Translated       --------------------------------------------------
NP_180307            201  KPPPVHKPPTPIYSPPIKPPPVHKPPTPTYSPPVKPPPVHKPPTPIYSPP 250
Ae4

                          --------------------------------------------------


CD825663 Translated       ------------------------------------------------P
CD830533 Translated       --------------------------------------------------
NP_180307            251  IKPPPVHKPPTPIYSPPVKPPPVQTPPTPIYSPPVKPPPVHKPPTPTYSP 300
Ae4

                       1  ----------------------QHQLIALLS------------------  9



CD825663 Translated     1  PVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYS   50

CD830533_Translated     1  --MPPPVQQPPTPSYSPPVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTYS   48

NP_180307            301  PVKSPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPIKPPPV-KPPTPIYS  350

Ae4                   10  --NHHPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPPPV-KPPTPIYS   57



CD825663 Translated    51  PPIKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPPPV-NPPAPIY   99

CD830533 Translated   100  PPIKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPP----------  139

NP_180307            351  PPVKPPPVHKPPTPIYSPPVKPPPVHKPPTPIYSPPVKPPPIQKPPTPTY  400

Ae4                   58  PPVMPPPVQQPPTPSYSPPVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTY  107



CD825663 Translated   100  SPPVKPPPVQQPPTPSYSP----------------PVKPPPVQKPPTPTY  133

CD830533 Translated   140  --------------------------------------PVQKPPTPTY  149
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```
NP_180307         401  SPPIKPPPLQKPPTPTYSPPIKLPPVKPPTPIYSPPVKPPPVHKPPTPIY 450

Ae4               108  SPPIKPPPVQKPPTPTYSP----------------PIKPPPVQKPPTPTY 141


CD825663 Translated  134  SPPVKPPPVQKPPTPTYSPPIKPPPVQ----------------KPPTPTY 167

CD830533 Translated  150  SPPIKPPPV-KPPTPTYSPPIKPPPVQ---------------KPPTPTY 183

NP_180307         451  SPPVKPPPVHKPPTPTYSPPIKPPPVKPPTPTYSPPVQPPPVQKPPTPTY 500

Ae4               142  SPPVKPPPVQKPPTPTYSPPIKPPPVKP----------------PTPIY 174


CD825663 Translated  168  SPPIKPPPVKKPPTPTYSPPVKPPPVQKPPTPTYSPPIKPPPVKTSKTNL 218

CD830533 Translated  184  SPPV--------------------------------------------- 187

BWB4372           501  SPPVKPPPIQKPPTPTYSPPIKPPPV-KPPTPTYSPPIKPPPVHKPPT-- 550

Ae4               175  SPPVKPPPVQKPPTPTYSPPIKPPPV-KPPTPTYSPPVKPPPVQKPPT-- 222


CD825663 Translated  219  PTYKATTQCNNL------------------------------------- 230

CD830533_Translated       -------------------------------------------------
NP_180307         551  PTYSPPIKPPPIHKPPTPTYSPPIKPPPVHKPPTPTYSPPIKPPPVHKPP 600

Ae4               223  PTYSP-------------------------------------------- 227


CD825663 Translated       -------------------------------------------------
CD830533 Translated       -------------------------------------------------
NP_180307         601  TPTYSPPIKPPPVHKPPTPTYSPPIKPPPVHKPPTPTYSPPIKPPPVHKP 650

Ae4                       -------------------------------------------------


CD825663 Translated       -------------------------------------------------
CD830533 Translated       -------------------------------------------------
NP_180307         651  PTPTYSPPIKPPPVQKPPTPTYSPPVKPPPVQLPPTPTYSPPVKPPPVQV 700

Ae4                       -------------------------------------------------


CD825663 Translated       -------------------------------------------------
CD830533 Translated       -------------------------------------------------
NP_180307         701  PPTPTYSPPVKPPPVQVPPTPTYSPPIKPPPVQVPPTPTTPSPPQGGYGT 750

Ae4                       -------------------------------------------------


CD825663 Translated       --------------
CD830533 Translated       --------------
NP_180307         751  PPPYAYLSHPIDIRN 761
Ae4                       --------------
```

Fig. 2: Alignment of predicted amino acid sequence of Ae4 with the Arabidopsis putative proline rich protein and translated EST sequences

Shaded sequences denote identical amino acids and gaps introduced in the alignment are marked with dashes. The amino acid sequences were obtained from GenBank: Arabidopsis At2g27380 (GenBank accession no. NP_180307), Brassica seed EST clone BN25061G23 (GenBank accession no. CD825663) and Brassica napus seed EST clone BN40045N17 (GenBank accession no. CD830533)

(*Fig. 3A,* top panel). However, hybridisation with the *Actin*2/7 cDNA probe (*Fig. 3A*, bottom panel) showed clear signals, suggesting that the RNA was not degraded. To verify these results, a RT-PCR approach was employed using *Ae*4 gene-specific primers and equal amount of cDNA from leaves, stems, buds, flowers, siliques, roots and carpels. After 30 amplification cycles, no product was visible on the ethidium bromide stained gel (*Fig. 3C*, top panel). Therefore, the gel was blotted and the RT-PCR gel blot was hybridised with a ^{32}P-labelled *Ae*4 cDNA. An autoradiograph of this blot showed the presence of an approximately 370 bp band (as expected)

in all lanes with various intensities of hybridisation (*Fig. 3C*, bottom panel), suggesting differential expression of *Ae*4 in different organs/tissues of the mature *B. napus* plant. Also, there was an additional faint band at approximately 600 bp in lane 2, suggesting the presence of another isoform of the *Ae*4 gene or possibly an unspliced *Ae*4 transcript.

Northern analysis of the tissue culture blot (*Fig. 3B*, top panel) detected expression of *Ae*4 transcripts in the embryogenic culture, both in the pre-embryogenic (lane PEC) and mature embryogenic tissue (lane MEC). Relative to the actin control, expression in both these tissues
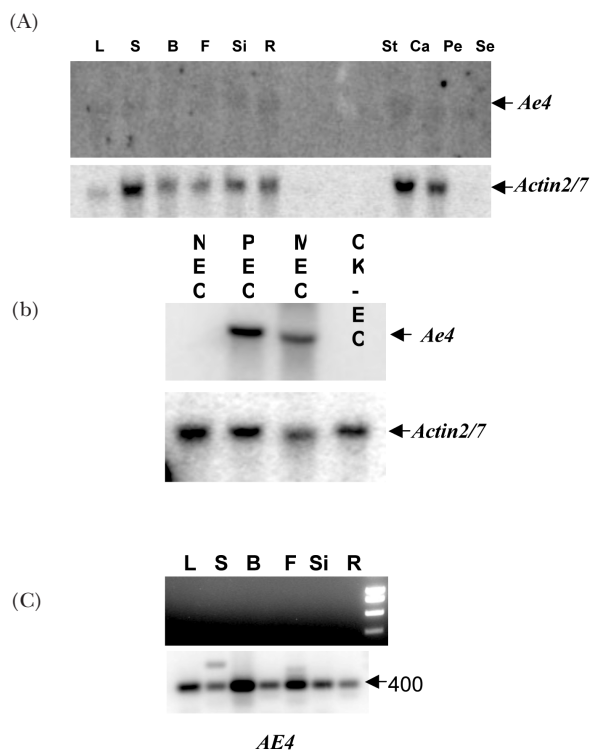
Fig. 3: Expression analysis of Ae4 in Brassica napus

(A) Tissue/organ-specific and (B) tissue culture RNA gel blots containing 10 μg of total RNA per lane were first hybridised to ^{32}P-labelled Ae4 and then to an Arabidopsis Actin2/7 cDNA (control). Lanes: L, leaves; S, stem; B, buds; F, flowers; S, siliques; R, roots; St, stamens; Ca, carpels; Pe, petals; Se, sepals; NEC, non-embryogenic tissue; PEC, pre-embryogenic tissue; MEC, mature embryogenic tissue; CK-EC, cytokinin-treated tissue.
(C) Top panel shows results of RT-PCR analysis of Ae4 gene expression. (C) Bottom panel is the RT-PCR gel blot probed with ^{32}P-labelled Ae4 cDNA. Lanes: M, 1 kb Bioline DNA ladder; L, leaves; S, stems; B, buds; F, flowers; Si, siliques; R, roots; Ca, carpels.

seems to be approximately at the same level. By contrast, *Ae*4 transcripts were not expressed at detectable levels in the non-embryogenic tissue (lane NEC) and the cytokinin-treated embryogenic tissue (lane CK-EC).

DISCUSSION

Ae4 Encodes a Partial Length Protein with Proline-rich Domain

The *Ae*4 cDNA encodes a partial length protein having a proline-rich domain. Both the amino acid composition and the presence of repeating motifs of proline are characteristics of proline-rich cell wall proteins (Jose-Estanyol and Puigdomenech, 2000). The 10-mer motif of the putative AE4 protein is unique in its sequence and belongs to a group of long repeat elements (Gyorgyey *et al.*, 1997). The repeating motifs do not correspond to any of the common motifs previously identified in proline-rich proteins and extensins (Showalter, 1993, Gyorgey *et al.*, 1997; Jose-Estanyol and Puigdomenech, 2000). Therefore, *Ae*4 may encode a novel class of proline-rich proteins (PRPs). Two PRP genes have already been isolated from *Brassica napus*, one that accumulated during pod development (Coupe *et al.*, 1993) and the other one induced by cold treatment (Goodwin *et al.*, 1996). However, the *Ae*4 sequence is not similar to either of them.

Expression Analysis of Ae4

The expression of *Ae*4 transcripts in all vegetative and floral tissues examined corresponds to the observations by Fowler *et al.* (1999) in Arabidopsis. They reported that *AtPRP2* and *AtPRP4* transcripts were most abundant in the aerial parts of the plant, namely in leaves, stems, flowers and siliques. Also, *AtPRP4* expression was detected in the early stages of lateral root formation. Since most PRPs are members of a multigene family, it is likely that the same will be true for those of *B. napus.*

Based on Northern analysis, *Ae*4 was detected in the pre-embryogenic but not non-embryogenic tissue and this suggests that the encoded protein may be associated with somatic embryogenesis. There have been a few PRP transcripts that have been shown to accumulate during somatic embryogenesis, such as in carrot (Aleith and Richter, 1990; Holk *et al.*, 1996; Yasuda *et al.*, 2001) and *Medicago sativa* (Gyorgey *et al.*, 1997).

A proline-rich protein encoded by the *DC 2.15* gene was identified as one of the genes that is differentially expressed during induction of somatic embryogenesis in carrot cell suspension culture (Aleith and Richter, 1990). The expression of this gene during somatic embryogenesis was detectable from 3 days after induction, and transcript abundance increased until the heart-shape stage (Aleith and Richter, 1990). This observation was further supported by promoter studies of the *DC 2.15* gene (Holk *et al.*, 1996). Also, Gyorgyey *et al.* (1997) reported that *MsPRP5,* a cDNA clone encoding a small proline-rich protein is preferentially expressed in alfalfa dedifferentiated callus cells. They proposed that the proline-rich protein may cause structural changes of the cell wall required for certain switches in function by plant cells.

He *et al.* (2002) reported that expression of a soybean PRP gene was inhibited by treatment with kinetin. This is consistent with our observation from the Northern analysis of the tissue culture blot that *Ae*4 transcripts could not be detected in cytokinin-treated tissue. This suggests that cytokinin treatment has suppressed *Ae*4 transcription to undetectable levels, which correlates with the suppression of secondary embryogenesis (Loh *et al.*, 1983). Alternatively, *Ae*4 transcripts could be down regulated due to the low rate of secondary embryogenesis in the cytokinin-treated tissue.

Potential Roles of Ae4 in Brassica napus

Proline-rich proteins have been thought to provide strength and rigidity in the cell wall by forming covalently cross-linked networks with cell wall components (Showalter, 1993). PRPs have a relatively high content of tyrosine and lysine residues which have been implicated as the substrate for the peroxidase-mediated insolubilisation of PRPs in soybean via isodityrosine crosslinks (Kleis-San Francisco and Tierney, 1990; Bradley *et al.*, 1992; Brisson *et al.*, 1994). Insolubilisation of PRPs in the cell wall occurs as a rapid response to wounding and treatment with fungal elicitors. PRPs are rapidly insolubilized within the cell wall in response to physical damage, treatment with fungal elicitors, and pathogen infection (Kleis-San Francisco and Tierney, 1990; Bradley *et al.*, 1992; Brisson *et al.*, 1994), indicating an active role in plant defence reactions. Thus, it was proposed that the PRPs

contribute to the cell wall structure of specific cell types based on their patterns of gene expression during plant development and induction by biotic and abiotic stresses. The enhanced expression of *Ae*4 transcripts in the pre-embryogenic and mature embryogenic tissues suggests that *Ae*4 may have a role during embryo formation. In carrot cultures, it was suggested that two PRPs encoded by No.93 and *DC 2.15* might act as extracellular signal factors during the development of somatic embryos (Yasuda *et al.*, 2001). However, there is no clear evidence for PRPs as signalling molecules that induce somatic embryogenesis.

Assuming that *Ae*4 encodes a proline-rich protein, we propose that the expression of *Ae*4 transcripts preferentially in embryogenic tissue could be possibly to provide mechanical strength to the embryonic cells that will protect the cell during later phase of embryo development in vitro. More experiments such as isolation of the full-length sequence of *Ae*4, immunolocalization and transgenic studies, especially promoter analysis, are required to provide more information on possible biological functions of the *Ae*4 gene. Also, the inducibility of the *Ae*4 gene in response to abiotic and biotic stresses in *B. napus* should be tested to explore its regulation.

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Antagonistic Potential of Selected Fungal and Bacterial Biocontrol Agents against *Colletotrichum truncatum* of Soybean Seeds

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ABSTRACT

Ten fungal and bacterial biocontrol agents (BCAs) were evaluated in vitro for their antagonistic potential against *Colletotrichum truncatum* isolated from soybean seeds. Two fungal BCAs namely *Trichoderma virens* isolate UPM23 and *Trichoderma harzianum* isolate UPM40 and a bacterial BCA namely *Pseudomonas aeruginosa* isolate UPM13B8 strongly inhibited the growth of *C. truncatum* based on high PIRG values in dual culture and culture filtrate tests. Studies on the mechanism of action using mycoparasitism technique and antibiosis observed under light microscope revealed that *T. virens* and *T. harzianum* inhibited the growth of *C. truncatum* by coiling and penetration into the hyphae. Consequently, the hyphae of *C. truncatum* became malformed and swollen. *Pseudomonas aeruginosa* also caused mycelial malformation; the mycelia turned vacuoled and swollen in or at tips of hyphae. *Pseudomonas aeruginosa* gave the highest PIRG value in the culture filtrate test, suggesting that antibiosis could be the main mechanism of antagonism. No inhibitory effect was observed on soybean seeds and seedlings when the seeds were artificially inoculated with *T. virens, T. harzianum* and *P. aeruginosa.* On the contrary, *T. virens* and *T. harzianum* were found to enhance seed germination and seedling establishment, while *P. aeruginosa* enhanced fresh and dry weights of seedlings.

Keywords: Antagonist, seed-borne fungi, *Colletotrichum truncatum*, soybean

INTRODUCTION

Colletotrichum truncatum [(Schw.) Andrus & W. D. Moore] is one of the most important seed-borne fungal pathogens that cause anthracnose of soybean. It reduces seed germination and quality (Manandhar and Hartman, 1999; Ploper and Backman 1992). Fungicidal seed treatment is used mainly to control soybean anthracnose (Hopperly, 1985). However, the growing concern against indiscriminate use of fungicides on health hazard and environmental pollution justify the exploitation of biologically based control strategies (Desai *et al.*, 2002). Recently, numerous fungal and bacterial biological control agents (BCAs) have shown the potential to augment or replace chemical pesticides (Ahmad and Baker, 1988). The most widely used BCAs in the world belong to fungal genus *Trichoderma* and bacteria *Pseudomonas* (Khetan, 2001; Tronsmo and Hjeljord, 1998). *Burkhoderia* spp. and *Serratia*

spp. have also been introduced recently in biological control programs. They are mainly patented as seed treatments to provide protection against soil-borne fungi like *Pythium* spp., *Rhizoctonia* spp., *Sclerotium* spp., and *Fusarium* spp. in many economically important crops such as tomato, melon, cotton, wheat and onion (Khetan, 2001; Tronsmo and Hjeljord, 1998; Laha *et al.*, 1996; Ordentlic *et al.*, 1988). It was reported that anthracnose of bean and cucumber caused by *Colletotrichum* spp. could be controlled by non-pathogenic rhizosphere fungi and bacteria (Dean and Kuc, 1986; Kuc, 1981). But, information regarding potential BCAs against *C. truncatum* of soybean is very limited. However, search for the suitable and superior strain of BCAs with greater biocontrol activities are necessary for alternative strategies against *C. truncatum. In vitro* screenings of antagonists have been widely used to select all groups of BCAs

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and elucidate its biocontrol mechanisms (Desai *et al.*, 2002). Therefore, an attempt was made to evaluate the biocontrol potential of BCAs against *C. truncatum* of soybean seeds.

MATERIALS AND METHODS

Fungal Isolation

The experiment was conducted in 2006 at the Plant Pathology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia (UPM) in Serdang, Selangor. Pathogenic *C. truncatum* was isolated from stored soybean seeds var. palmetto by agar plate method. The fungus was purified by hyphal tip culture method and cultured on potato dextrose agar (PDA) until subsequent studies.

Screening of Fungal and Bacterial BCAs

Seven isolates of *Trichoderma* and three isolates of bacteria obtained from the collection of Plant Pathology laboratory, Faculty of Agriculture, UPM were used in this study. These isolates were screened for their antagonistic activity against *C. truncatum* using dual culture tests based on the percent inhibition of radial growth (PIRG). A 5 mm diameter mycelial agar disc was cut from the margin of 7-day-old culture of *C. truncatum* and placed on one side of a 9 cm Petri dish containing PDA medium and incubated for 48 h. Another 5 mm mycelial agar disc from 7-day-old culture of each *Trichoderma* isolate was placed 3 cm away from the *C. truncatum* disc on the same plate. Plates were incubated at an ambient temperature of 25±1ºC for 15 days. Antagonistic activity of the *Trichoderma* isolates was assessed during incubation period by measuring the radius of the *C. truncatum* colony using the formula

Percent inhibition of radial growth (PIRG) =

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

where, R1 indicates radial growth of fungal colony in the control plate while R2 indicates radial growth of fungal colony in the dual culture plate. Data regarding the time needed to completely grow over the fungal colony by *Trichoderma* isolates were recorded. The re-growth of the fungus from the inhibition and overgrowth

zone was also determined. To test for antagonistic bacteria, a 5 mm diameter of mycelial agar disc from 7-day-old culture was placed in the centre of a 9 cm Petri dish containing nutrient agar (NA) medium. Plates were incubated at an ambient temperature of 25±1°C for 48 h. A loopful of bacteria from 48 h NA culture was then taken and streaked in a circle 3 cm away from the *C. truncatum* disc on the same plate and incubated for 10 days. Data of PIRG and inhibition category were recorded during incubation period. The test was arranged in a completely randomized design with five replications. Based on the highest PIRG values, three promising isolates, namely *Trichoderma virens* isolate UPM23, *Trichoderma harzianum* isolate UPM40 and *Pseudomonas aeruginosa* isolate UPM13B8 were selected to study their mechanism of action.

Mycoparasitism

Hyphal interaction test was used to assess the mycoparasitic activities of *T. virens* isolate UPM23, *T. harzianum* isolate UPM40 and *P. aeruginosa* isolate UPM13B8 against *C. truncatum*. Edges of parasitized fungal hyphae by *T. virens*, *T. harzianum* and *P. aeruginosa* were transferred from the inhibition zone and overgrowth zone from dual culture plate on to clean slides after seven days of incubation. Cover slips were mounted on the mycelia with a drop of lactophenol cotton blue (LCB). Hyphal interaction and morphology were examined under a light microscope.

Antibiosis

Antibiosis test was performed using culture filtrate of UPM40, UPM23 and UPM13B8 on radial growth of the *C. truncatum*. *Trichoderma virens* and *T. harzianum* were grown in potato dextrose broth (PDB) and *P. aeruginosa* in nutrient broth (NB) on an orbital shaker (100 rpm) for 14 days and five days, respectively. Cultures were then centrifuged at 10,000 rpm for 5 min before the supernatant was collected and the pellet discarded. The supernatant was then sterilized and filtered using 0.45 μm and 0.2 μm membrane filters for *Trichoderma* spp. and bacteria, respectively. The sterilized filtrate was then incorporated into sterilized double strength PDA (50ºC) in a ratio of 1:1. Twenty mL of the amended agar was then poured into

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each Petri plate and allowed to solidify. A 5 mm diameter mycelial plug of *C. truncatum* was placed centrally in each of the plate and incubated at a room temperature of 25±1°C for 14 days. Non-amended PDA was used as the control. The radial growth of *C. truncatum* was measured and transformed into PIRG in relation to the mycelial growth in the control plate. The hyphal morphology was also examined from the same culture filtrate plates. The edge of fungal mycelia was transferred carefully onto a clean slide after seven days of incubation. A cover slip was placed on the mycelia with a drop of LCB on the slide and the hyphal morphology was observed under a light microscope.

Effects of Artificial Seed Inoculation with Selected BCAs

Seeds were artificially inoculated with *T. harzianum* isolate UPM40, *T. virens* isolate UPM23 and *P. aeruginosa* isolate UPM13B8. Conidia of UPM40 and UPM23 from 7-day-old cultures were washed off separately with 1.5% sodium alginate solution in sterile distilled water. The conidial suspension obtained was adjusted to a concentration of 3.5×10^7 conidia mL^{-1} using a haemocytometer. Cell suspension of *P. aeruginosa* was adjusted to 1×10^{12} CFU mL^{-1} using a spectrophotometer (Spectronic® 20 Genesys™, USA) following the method of Mortensen (1992). To initiate the treatment, soybean seeds were surface sterilized in 5.25% sodium hypochlorite for 3 min and rinsed thrice with sterilized distilled water, and dried for 1 h in a laminar flow chamber. The Seeds were then soaked in suspensions of the respective biocontrol agents in a ratio of 1: 2 (w/v) separately in 250 mL conical flask for 1 h. The treated seeds were then surface re-dried to remove excess water in the laminar flow chamber. The number of conidia determined by a haemocytometer was 2.2×10^5 conidia seed^{-1} and 2.4×10^5 conidia seed^{-1} for UPM23 and UPM40, respectively. Similarly, *P. aeruginosa* determined by serial plating was 1.1×10^9 CFU seed^{-1}. Seeds soaked in only 1.5 % sodium alginate solution for 1 h served as the control.

Fifty seeds of each treatment were sowed at the depth of 2 cm in plastic trays (39 × 28 × 11 cm) containing sterilized soil mixture of top soil, peat grow and sand at the ratio of 3: 2: 1 (v/v/v). Trays were arranged in a completely randomized design with four replications in the glasshouse with each tray being considered as a replication. The daily temperature of the glass house ranged from 25-30°C with 85±5% relative humidity (RH). Data on germination and seedling establishment were recorded up to 14 days. The mean length, fresh and dry weights of the seedlings was also recorded based on twenty seedlings per replicate. Samples were kept for three days at 60°C before the dry weight was recorded.

Statistical Analysis

Data were subjected to one-way ANOVA (SAS, 1999) and means were subsequently compared using Tukey's Studentized Range test (HSD) of arcsine transformed values at 5% level of probability.

RESULTS

Antagonistic Activity of BCAs

Results from the dual culture test showed that all isolates of *Trichoderma* inhibited mycelial growth of *C. truncatum* however with varying efficiencies (Table 1). The PIRG values ranged from 53.85 to 80.77%, with isolates UPM23 (80.77%) and UPM40 (76.92%) being significantly the best followed by TL1 (71.15%), TV2 (67.23%), TV3 (65.24%), UPM29 (61.54%) and TK1 (53.85%). UPM23 completely overgrew the colony of *C. truncatum* within seven days, while UPM40 overgrew within eight days of co-incubation. The other five isolates did not show strong competitive effect since they were unable to colonize *C. truncatum* after 14 days of co-incubation. No distinct inhibition zone towards *C. truncatum* was discernable in any of the *Trichoderma* isolates. *Colletotrichum truncatum* was lysed and failed to re-grow when parasitized hyphae from the interaction and overgrowth zone was cultured on fresh PDA. Among the three bacterial isolates, UPM13B8 significantly exhibited the strongest antagonism against *C. truncatum* with a high PIRG value of 89.89% followed by UPM14B1 (61.80%) and UPM39B3 (35.77%) (Table 2). A distinct inhibition zone was observed when UPM13B8 and UPM14BI were used towards *C. truncatum*. However, UPM13B8 gave a significantly greater distance inhibition zone than UPM14B1 with the value of 9.25 mm and 4.13 mm after seven days of incubation, respectively. After 10 days of

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incubation, the UPM13B8 inhibited *C. truncatum* had significantly the same whereas the fungus had grown to contact with UPM14B1 and overgrown on the colony of UPM39B3. The UPM13B8 parasitized mycelia of the fungal pathogen did not recover when transferred to fresh PDA from the inhibition zone. The isolates UPM23, UPM40 and UPM13B8 clearly exhibited stronger antagonistic potential than the other seven isolates based on the high PIRG values against *C. truncatum.* As such these isolates were selected for further studies on micoparasitism and antibiosis.

Mycoparasitism

Microscopic observations revealed that UPM40 and UPM23 hyphae grew initially alongside and coiled compactly around the hyphae of *C.*

truncatum. They produced appressorium like structure as attachment to *C. truncatum* hyphae for penetration which led to cell disruption (*Fig. 1B*). Later the hyphae of *C. truncatum* was swollen, malformed and vacuoled (*Fig. 1C*). The parasitized hyphae were unable to regenerate into new colonies when inoculated onto fresh PDA. Parasitized hyphae by UPM13B8 also malformed and swelled (*Fig. 1D*), whereas normal hyphae were smooth and no swelling or vacuolation (*Fig. 1A*).

Antibiosis

Results from the culture filtrate test revealed that UPM23, UPM40 and UPM13B8 strongly inhibited the mycelial growth of *C. truncatum* with different magnitudes of inhibition (Table 3). The isolate UPM13B8 completely inhibited

TABLE 1
Antagonistic effect of *Trichoderma* isolates against *C. truncatum* in dual culture test

Code no.	Species	Antagonism (PIRG)*	Time to over grow
UPM23	*Trichoderma virens*	80.77 a	7 days
UPM40	*T. harzianum*	76.92 ab	8 days
UPM29	*T. harzianum*	61.54 cd	-
TL1	*T. longibrachiatum*	71.15 bc	-
TK1	*T. koningii*	53.85 d	-
TV3	*T. virens*	65.24 c	-
TV2	*T. virens*	67.23 bc	-

- Indicates no overgrowth after 14 days
* indicates percent inhibition of radial growth (PIRG) after five days of incubation
Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.

TABLE 2
Antagonistic effect of bacterial isolates against *C. truncatum* in dual culture test

Code no.	Species	Antagonism (PIRG)*	Inhibition category	
			After 7 days of incubation	After 10 days of incubation
UPM13 B8	*Pseudomonas aeruginosa*	89.89 a	Distance (9.25 a)	Distance (9.25 a)
UPM14 B1	*Burkholderia glumae*	61.80 b	Distance (4.13 b)	Contact (0.0 b)
UPM39 B3	*Serratia marcescens*	35.77 c	Contact (0.0 c)	Fungal Overgrowth (0.0 b)

* indicates percent inhibition of radial growth (PIRG) after 7 days of incubation.
Data in parenthesis indicates the inhibition zone between *C. truncatum* and bacteria in mm
Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.
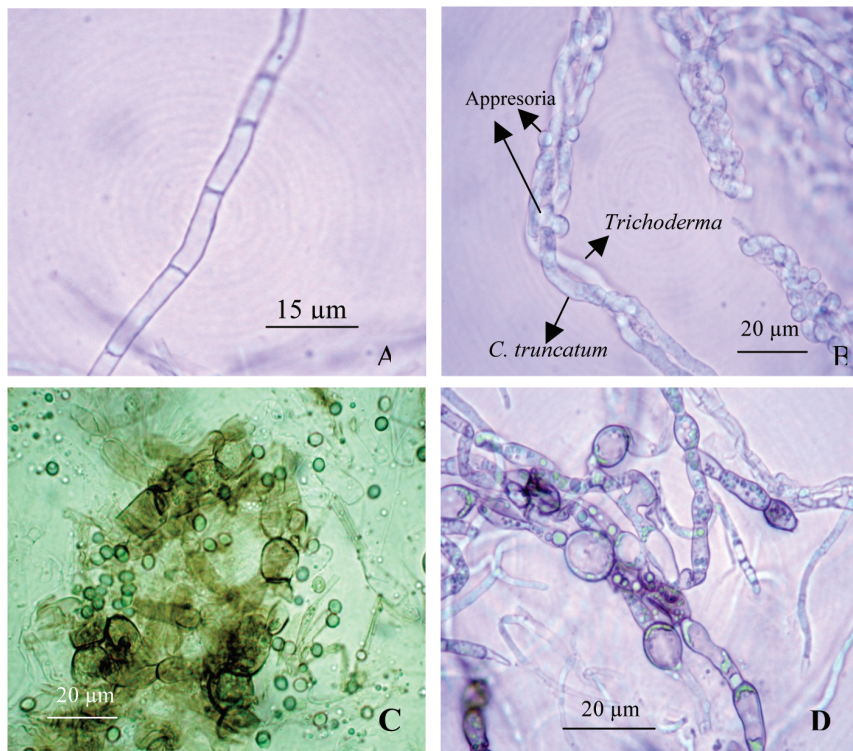
Fig. 1: Photomicrographs showing hyphal morphology of parasitized C. truncatum *by* T. harzianum *(UPM40) and* P. aeruginosa *(UPM13B8)*

A = Normal appearance of *C. truncatum* hyphae
B = Parasitized hyphae of *C. truncatum* coiled by *T. harzianum* and *T. virens*
C = Malformed and swolled hyphae of *C. truncatum* parasitized by *T. harzianum* and *T. virens*
D = Malformed and swolled hyphae of *C. truncatum* parasitized by *P. aeruginosa*

fungal growth and gave the highest PIRG value of 100% compared to UPM23 (82.47%) and UPM40 (69.23%) after seven days of incubation. After 14 days of co-incubation, the PIRG value of UPM23 and UPM40 had increased to 83.50% and 76.99%, respectively. On microscopic observation, the parasitized hyphae of *C. truncatum* by BCAs became malformed, thickened and vacuoled (*Figs. 1C, D*). Many swellings were observed in the hyphae, whereas the normal hyphal walls remained smooth (*Fig. 1A*).

Effect of BCAs on Soybean Seed Germination, Seedling Vigor and Establishment

All of the promising isolates tested did not inhibit seed germination, seedling growth and establishment based on seedling length, fresh weight and dry weight (Table 4). The highest germination was recorded in seeds treated with UPM23 (98%) and UPM40 (97%), while UPM13B8 treated seeds recorded 94% germination which was statistically similar with that of water-treated control seeds (93%). Seedling establishment was significantly higher in UPM40 (98%) treated seeds followed by UPM23 (96%), UPM13B8 (92%) and the control (93%). The maximum seedling length was recorded from seeds treated with UPM40 (50.85%) followed by UPM13B8 (46.83%), UPM23 (45.13%) and water (43.43%) treated seeds. Regarding the fresh and dry weights of seedling, the highest effect was obtained in UPM13B8 treated seeds with the values of 1.66 and 0.20 g seedling^{-1}, respectively. Statistically, similar fresh and dry weights were recorded from treated seeds with UPM23, UPM40 and the control.

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TABLE 3
Antagonistic effect of UPM23, UPM40 and UPM13B8 against *C. truncatum* in culture filtrate test

Code no.	Species	Antagonism (PIRG) After 7 days of incubation	Antagonism (PIRG) After 14 days of incubation
UPM23	*T. virens*	82.47 b	83.50 b
UPM40	*T. harzianum*	69.23 c	76.99 c
UPM13 B8	*P. aeruginosa*	100.00 a	100.00 a

Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of five replications.

TABLE 4
Effect of UPM23, UPM40 and UPM13B8 on the seed germination, seedling vigor and establishment of soybean under glass house conditions

Treatments	Germination (%)	Seedling establishment (%)	Length seedling^{-1} (cm)	Fresh weight seedling^{-1} (g)	Dry weight seedling^{-1} (g)
UPM23	97.00 a	96.00 b	45.13 bc	1.54 b	0.19 b
UPM40	98.00 a	98.00 a	50.85 a	1.58 b	0.19 b
UPM13 B8	94.00 b	92.00 c	46.83 b	1.66 a	0.20 a
Control	93.00 b	93.00 c	43.43 c	1.50 b	0.19 b

Means within the same column followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values. Values are means of four replications with fifty seeds per replication.

DISCUSSION

In the present study, seven isolates of *Trichoderma* and three isolates of bacteria were tested *in vitro* for the preliminary screening to look for potential biocontrol agents against the pathogenic fungus *C. truncatum*. A considerable variation was observed between, as well as within, the fungal and bacterial isolates with regard to the hyphal interaction and subsequent events to the inhibition in pathogen growth. Of these isolates, two isolates of *Trichoderma* namely *T. virens* isolate UPM23, *T. harzianum* isolate UPM40 and a bacterium namely *P. aeruginosa* isolate UPM13B8 demonstrated stronger antagonistic activities to inhibit the radial growth of *C. truncatum* using dual culture and culture filtrate tests. The time needed for colony overgrowth is an important parameter in the assessment of antagonistic ability to compete against the pathogen for limited nutrient resources and space (Ibrahim, 2005). The isolates UPM23 and UPM40 were able to overgrow fully the colony of *C. truncatum* within seven and eight days in the dual culture test, respectively.

In agriculture, numerous studies have been reported on the antagonistic activity against mainly soil-borne plant pathogens, but only a few studies have investigated the antagonistic activity against *Colletotrichum* spp. of different crops. *Trichoderma harzianum* was found to decrease significantly the incidence of *Glomerella* (*Colletotrichum*) *glycines* on soybean (Fernandez, 1992) and anthracnose caused by *C. lindemuthianum* on beans was controlled by *P. aeruginosa* (De Meyer *et al.*, 1999). Severity of foliar anthracnose of soybean caused by *C. dematium* was reduced significantly by fluorescent pseudomonad as stated by Tripathi *et al.* (2006).

Beside competition for resources and space, the invasive mechanism of Trichoderma includes lysis, mycoparasitism, antibiosis and local or systemic induced resistance (Harman, 2005; Howell, 2003). Similarly, in this study it was observed that the mechanisms of antagonism for UPM23 and UPM40 were through competition, lysis, mycoparasitism and antibiosis. In the dual culture test both UPM23 and UPM40 were able to compete and inhibit the mycelial growth of *C. truncatum*. They parasitized and lysed the hyphae of *C. truncatum* through coiling and subsequent penetration. They produced appressorium like structures which aided in the penetration of the host cell wall (Goldman *et al.*, 1994). A similar observation was reported on parasitized hyphae of *Sclerotium rolfsii* by *T. harzianum* (Widyastuti *et al.*, 2003), *Botryodiplodia theobromae* by *T. harzianum* (Gupta *et al.*, 1999) and *Rhizoctonia solani* by *T. virens* (Howell, 2003). Subsequent degradation of the fungal cell wall might be due to the actions of different lytic enzymes. Strong support has been given by Lorito *et al.* (1993) on the importance of cell-wall degrading enzymes secreted by *T. harzianum* and *T. virens* (syn. *G. virens*) in controlling fungal diseases. This finding strongly suggested that the inhibition of mycelial growth of *C. truncatum* in the presence of *T. harzianum* and *T. virens* were due to the effect of antifungal substances as proven by the culture filtrate test through the mechanism of antibiosis. Secreted enzymes mainly chitinase, _-1, 3 glucanase and _-1, 3 glucosidase were reported to be responsible for the degradation of the host cell wall by *T. harzianum* and *T. virens* (Howell, 2003; Khetan, 2001; Tronsmo and Hjeljord, 1998).

Pseudomonas aeruginosa inhibited the radial growth by establishing a clear inhibition zone in a dual culture test; no further growth of *C. truncatum* was observed when the PDA was amended in a culture filtrate from this strain. Moreover, mycelial malformation and vacuolation occurred frequently with parasitized mycelia as revealed in both tests. Several mechanisms are responsible for suppression of pathogens by bacteria, including competition, antibiotic and metabolite production and also induction of systemic resistance (Compant *et al.*, 2005; Whipps, 2001). The inhibition of radial growth by forming inhibition zone against fungal pathogen is considered as antibiosis, whereby the antibiotic metabolites may penetrate the cell and inhibit its activity by chemical toxicity. The mycelial malformation observed was probably due to the toxic effect of antibiotic substances interfering with normal growth processes (Sariah, 1994). *Pseudomonas aeruginosa* is known to produce metabolites such as pyoverdin, pyochelin and salicylic acid which are effective against various pathogens (De Meyer and Hofte, 1997; Buysens, 1996). The vacuolar appearance of the mycelium might be due to the antibiotic metabolites produced by the bacterium, which may penetrate and cause protoplasmic dissolution and disintegration (Rahman *et al.*, 2007). *Pseudomonas aeruginosa* produced a higher PIRG value than *T. harzianum* and *T. virens* in the cultural filtrate test thus indicating that more antibiotic substances were produced by *P. aeruginosa*.

All selected BCAs did not show any adverse effect on seed germination and seedling growth performance under glass house conditions. *Trichoderma virens* and *T. harzianum* seemed to enhance seed germination, seedling stand and length, but did not provide any positive effect on the fresh and dry weights of seedlings. However, *P. aeruginosa* improved fresh and dry weights of seedlings from treated seeds. Enhancement of plant growth is well documented by *Trichoderma* spp. (Harman, 2000; Yedidia *et al.*, 1999) and *P. aeruginosa* (Hofte *et al.*, 1991). But, the effect on seed germination and seedling growth promotion seemed to be inconsistent among antagonists. This contradictory effect in growth promotion may be dependent on the antagonistic performance to survive and develop actively in the rhizosphere (Devliegher *et al.*, 1995; Kleifeld and Chet, 1992). Thus, activities of *T.* virens, *T. harzianum* and *P. aeruginosa* in this study suggested that all the three antagonists could be utilized as BCAs against C. *truncatum*. The use of these biocontrol agents could be an economically feasible alternative to chemical biocides and environmental friendly in suppressing the anthracnose disease in biological control programs of soybean.

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Growth, Mortality and Yield-Per-Recruit of Sergestid Shrimp, *Acetes intermedius* Omori, 1975 (Decapoda: Sergestidae) from Length Frequency Analysis in the Coastal Waters of Malacca, Peninsular Malaysia

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ABSTRACT

Estimates of growth, mortality and relative yield per recruit of the sergestid shrimp, *A. intermedius* in the coastal waters of Malacca, Peninsular Malaysia were obtained from the monthly length-frequency data. The von Bertalanffy growth function (VBGF) estimates were: $L\infty$ = 34.65 mm total length; K = 1.5 yr^{-1} and t_0 = -0.1004 years. Natural mortality rate (M) was 1.5 yr^{-1}. Total mortality coefficient (Z) was estimated as 4.15 yr^{-1} and the exploitation ratio (E = F/Z) was 0.43. The recruitment pattern was continuous throughout the year with one major peak. The relative yield per recruit analysis predicted the maximum allowable limit of exploitation (E_{max}) = 0.65. The current exploitation rate E is less than the predicted E_{max}. Thus, the stock of *A. intermedius* was found to be below optimum fishing pressure (E < 0.50) in the coastal waters of Malacca, Peninsular Malaysia.

Keywords: Growth, mortality, recruitment, *Acetes intermedius*, Malaysia

INTRODUCTION

The sergestid shrimps of the genus *Acetes*, family Sergestidae, are a minor planktonic crustacean group represented by a small number of species, but are one of the economically important organisms in Asian and African waters (Omori, 1975). They occur widely in the west coast of Malay Peninsula (Pathansali, 1966; Omori, 1975; Johnson, 1976) and are locally familiar as udang geragau. The world-wide geographical distribution of *Acetes* has been summarized by Omori (1975) and Holthuis (1980).

The species *Acetes intermedius* occurs in the shallow coastal waters of the Malacca strait, Peninsular Malaysia (Arshad *et al.*, 2007). It is one of the most important commercial shrimp resources and is also an important component of the marine ecosystem in the coastal waters of south-western Taiwan (Chiou *et al.*, 2000). Annual catch of this species was greater than 2,700 tons in south-western Taiwan, and was valued at more than US\$ 2,027,680 in 2000 (Chiou, 2002). It

migrates from estuaries to offshore waters and performs a diel vertical migration in the coastal waters of south-western Taiwan during the period from June to October (Chiou *et al.*, 2000). Their diel vertical migrations coincide with the time of sunrise and sunset. Further to this, they perform nocturnal upward migration depending on the lunar phase. Such diel vertical migration allows them to avoid diurnal visual predators and may also deter nocturnal predators utilizing the moonlight (Chiou *et al.*, 2003). The feeding activity of this species mainly occurred at night and performs a nocturnal vertical migration to avoid predators and allow for safe feeding (Chiou *et al.*, 2005).

The shrimp of the genus *Acetes* plays a substantial role in the food webs of coastal waters, acting as predators, feeding on a variety of foods ranging from diatoms, copepods and larvae of decapods to detritus and in turn as prey for many fishes and other predators (Xiao and Greenwood, 1993). It appears in very large

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swarms in the shallow inshore coastal waters, which is brackish with a salinity of 30 ppt or less, during certain seasons of the year (Pathansali, 1966). Only a very small proportion of the catch is disposed off as fresh shrimp but the greater part is sun dried and sold as dried shrimp or processed into a paste known locally as 'Belachan' or pickled whole to give a product known as 'Chinchalok' (Pathansali, 1966). The annual landing of *Acetes* in Malaysia was 7,528 tons during 2004 (DOF, 2004).

Spectacular school or swarms of *Acetes*, particularly in coastal Asia, are the bases of important commercial fishes for consumption by humans and domestic animals (Mistakidis, 1973; Omori, 1974, 1975, 1978; Malley and Ho, 1978; Chullasorn and Martosubroto, 1986). The commercial importance also derives from the use and potential of *Acetes* as a food organism for aquaculture industry (Kungvankij *et al.*, 1986; Ung and Itoh, 1989). These combined features make *Acetes* excellent candidates for population dynamics studies. In spite of greater abundance and importance of the genus *Acetes* in the fishery of Asian countries, very little information is available on the population parameters like growth and mortalities so far except the studies carried out by Zafar *et al.* (1997, 1998); Zafar and Amin (2002) and Oh and Jeong (2003).

Knowledge of various population parameters like the asymptotic length (L∞) and growth coefficient (K), motilities (natural and fishing) rate and exploitation level (E) are necessary for planning and management of *Acetes* resources. Lack of knowledge of population structure and proper evaluation of the exploitation of this resource emphasized the importance of a detailed study to facilitate better management of the resource. There are many tools for assessing exploitation level and status of stock. Of these, FiSAT (FAO-ICLARM Stock Assessment Tools) has been most frequently used for estimating population parameters of shrimps (Jayawardane *et al.*, 2002, 2003; Papaconstantinou and Kapiris, 2001; Etim and Sankare, 1998; Enin *et al.*, 1996) because primarily it requires only length-frequency data. The objectives of the present study were to estimate the key population parameters and exploitation rate (E) of *A. intermedius* in order to assess the stock position of the species around the coast waters of Malacca.

MATERIALS AND METHODS

Collection of Data

Monthly samples of the *A. intermedius* were collected from Klebang Besar (N 02°13.009/ & E 102° 11.921/) in the Malacca coastal waters, Straits of Malacca (*Fig. 1*) between February 2005 and January 2006. Triangular shape push net (mesh size 3.2 cm at anterior section, 0.75 cm at middle and 0.5 cm at cod end) were used to collect the samples of *Acetes*. The fishing effort was one man per hour and towing length was approximately 1000 m along the coast of Klebang Besar, Malacca. After collection, samples were fixed in 10% formalin solution in the field and analyzed after 2-3 days of preservation. In the laboratory, *A. intermedius* was identified using a Nikon dissecting microscope. The works of Omori (1975) were followed during the identification of *A. intermedius*. Total length (TL) of 995 individuals was measured from the tip of the rostrum to the tip of the telson to the nearest 0.1 mm using vernier calipers.
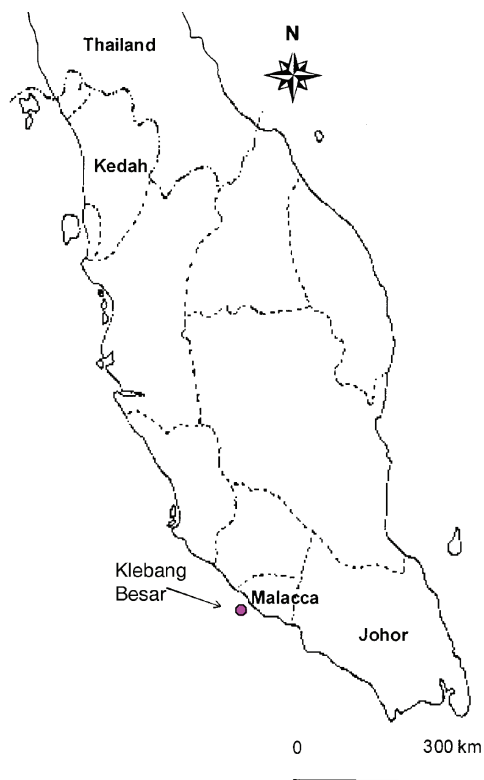

Fig. 1: Sampling location (dot) in the coastal waters of Malacca, Malaysia

Data Analysis

Size-frequency distributions of *A. intermedius* were plotted for each month from February 2005 to January 2006. Bhattacharya's method, implemented from the package FiSAT (Gayanilo *et al.*, 1996), was used to identify the modes in the polymodal length-frequency distributions of *A. intermedius*. All the identified size/age groups were derived from at least three consecutive points and selection of the best results was based on the following criteria: (a) the values of separation index (SI) for the different age groups; (b) the number of the identified age groups and (c) the standard deviation (SD) (Gayanilo *et al.*, 1989).

Monthly length-frequency distributions of *A. intermedius* were analyzed using the FiSAT computer programme (Gayanilo *et al.*, 1996). The parameters of the von Bertalanffy growth function (VBGF), asymptotic length (L∞) and growth co-efficient (K) were estimated using ELEFAN-I routing (Pauly and David, 1981) incorporated into the FiSAT software. K scan routine was conducted to assess a reliable estimate of the K value. The theoretical age at length zero (t_o) was obtained from Pauly's (1979) equation:

$$\text{Log } (-t_0) = -0.392 - 0.275 \log L_\alpha - 1.038 \log K$$

Potential longevity (t_{max}) of the species was calculated from the Pauly (1984) formula: $t_{max} = 3/K$. The estimated L_α and K were used to calculate the growth performance index (φ') (Pauly and Munro, 1984) of *A. intermedius* from the equation: $\varphi' = 2 \log_{10}L_\alpha + \log_{10}K$

Total mortality coefficient (Z) was estimated by using the length converted catch curve (Pauly, 1984) and the method of Jones and van Zaling (1981). Natural mortality rate (M) was estimated using an empirical relationship of Pauly (1980):

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

where M is the natural mortality, L∞ the asymptotic length, K the growth co-efficient of the VBGF and T the mean annual habitat water temperature ^{0}C. Once Z and M were obtained, fishing mortality (F) was estimated using the relationship:

$$F = Z - M$$

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

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

The ascending left arm of the length-converted catch curve was used to analysis the probability of capture of each length class according to the method of Pauly (1987). By plotting the cumulative probability of capture against mid-length, we obtained a resultant curve from which the length at first capture L_c was taken as corresponding to the cumulative probability at 50%.

The recruitment pattern of the stock was determined by backward projection on the length axis of the set of available length frequency data as described in FiSAT. This routine reconstructs the recruitment pulse from a time series of length-frequency data to determine the number of pulses per year and the relative strength of each pulse. Input parameters were L∞, K and t_0 ($t_0 = 0$). Normal distribution of the recruitment pattern was determined by NORMSEP (Pauly and Caddy, 1985) in FiSAT.

The estimated length structured virtual population analysis (VPA) and cohort analysis was carried out from the FiSAT routine. The values of L_α, K, M, F, a (constant) and b (exponent) were used as inputs to a VPA analysis. The t_0 value was taken as zero. The method was published by Fry (1949) and subsequently modified by many authors. Practical reviews of VPA methods were, among others given by Pauly (1984) and Jones (1984).

The relative yield-per-recruit (Y/R) and relative biomass-per-recruit (B/R) were estimated by using the model of Beverton and Holt (1966) as modified by Pauly & Soriano (1986) and incorporated in FiSAT software package. The input requirements in the procedure were the values of $L_c/L_\alpha = 0.44$ and M/K = 1.57. From the analysis, the maximum allowable limit of exploitation (E_{max}) giving maximum relative yield-per-recruit was estimated. Also $E_{0.1}$, the exploitation rate at which the marginal increase in relative yield-per-recruit is 10% of its value at E = 0, and $E_{0.5}$, the exploitation rate corresponding to 50% of the unexploited relative biomass-per-recruit (B/R), were estimated.

RESULTS

Size Frequency Distribution

Monthly length frequency distributions identified the modal lengths with cohorts in different months (*Fig. 2*). The length frequency distribution of different months suggested that the population consisted of maximum two age groups, with means of 19.03 mm and 27.40 mm of total length. The application of Bhattacharya's method through FiSAT determined model lengths of *A. intermedius* ranging from 17.12 mm (in January) to 31.22 mm (in March), with satisfactory separation index (Table 1). The two dominant modal groups of *A. intermedius* were identified reflecting two different annual cohorts. Therefore, the monthly size frequency distributions suggested that the population consisted of two age groups, with modes at approximately 19 mm and ≥ 26 mm total length.

Growth Parameters

The observed extreme length and the predicted extreme length (L_{max}) were found to be 33.00 mm and 35.62 mm respectively (*Fig. 3*). The range at 95% confidence interval for extreme length was calculated as 30.39 - 40.84 mm. This initial extreme length value was used in ELEFAN-I, included in FiSAT package (Gayanilo *et al.*, 1996) producing the optimum growth curve. The best value of VBGF growth constant (K) was estimated as 1.5 yr^1 by ELEFAN-I (*Fig. 4*). The response surface (Rn) was calculated as 0.479 which selected the best combination of growth parameters as: L_{α} = 34.65 mm and K = 1.50 yr^1. The optimized growth curve was superimposed on the restructured length-frequency histograms (*Fig. 5*). The calculated value for the growth performance index (φ') of *A. intermedius* during the present investigation was 3.26. This value
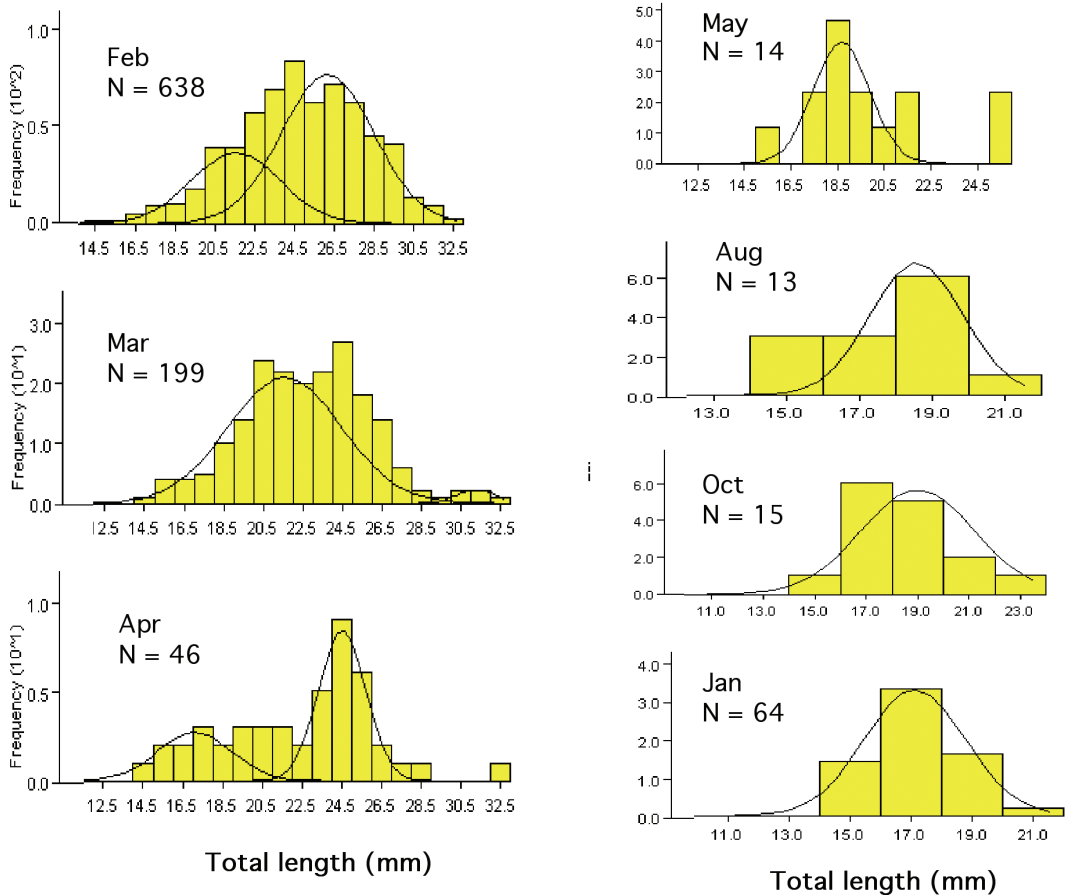
Fig. 2: Monthly length frequency distributions of A. intermedius *caught between February 2005 and January 2006 in the coastal waters of Malacca*

TABLE 1

Identified age groups from the length-frequency analysis of *A. intermedius* during the monthly sampling (Feb 05 - Jan 06), using Bhattacharya's method

Months	Mean TL (mm) of age group	SD (mm)	SI
Febuary-05	19.11	1.79	-
	25.02	2.62	2.14
March	21.92	2.73	-
	30.93	1.26	2.41
April	18.00	2.80	-
	24.83	1.99	2.20
May	19.06	2.40	-
August	19.03	1.27	-
October	19.00	2.21	-
January-06	17.12	1.65	-
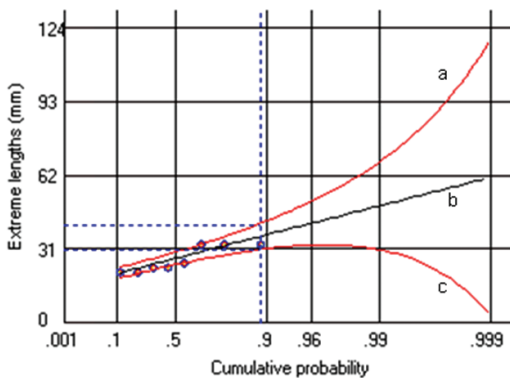
Fig. 3: Predicted maximum length of A. intermedius *based on extreme value theory (Formacion et al., 1991). The predicted maximum length value and the 95% confidence interval is obtained from the intersection of overall maximum length with the line b and a, c respectively*
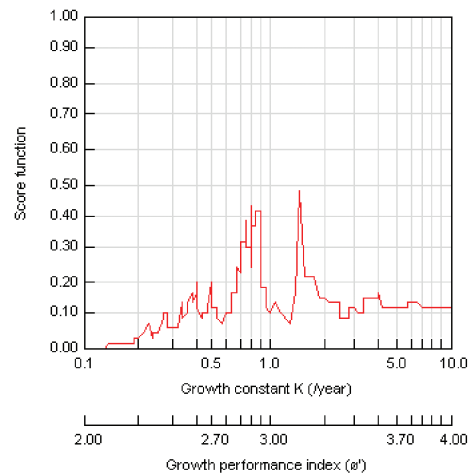
Fig. 4: Estimation of K for A. intermedius *by employing ELEFAN-I*

was close to the (φ'-values recorded in the literature (Zafar *et al.*, 1997; Zafar and Amin, 2002) but slightly higher than the value recorded by Oh and Jeong (2003). Using the estimated value of the growth coefficient (K= 1.5 yr^{-1}), the longevity (t_{max} = 3/K) was calculated as 2 years.

Mortality and Exploitation Rate

Total mortality coefficient (Z) was estimated as 4.15 yr^{-1} using length converted catch curve (*Fig. 6a*) while the Jones and van Zalinge method (*Fig. 6b*) gave a value of Z = 3.50 yr^{-1}. Natural mortality (M) was estimated at 2.35 yr^{-1}. Base on Z from length converted catch curve, fishing mortality (F) was found to be 1.81 yr^{-1} (Table 2). From these figures, an exploitation rate (E) of

0.43 which was obtained for the *A. intermedius* fishery in the coastal waters of Malacca, Peninsular Malaysia seemed to be below the optimum level of exploitation (E = 0.50).

Length at First Capture

The length at first capture (the length at which 50% of the shrimp becomes vulnerable to the gear) was calculated as a component of the length converted catch curve analysis (*Fig. 7*). The value obtained was $L_{50\%}$ = 15.29 mm from the analysis of probability of capture. The length at which 25% and 75% of the shrimps are retained in the gear was estimated as $L_{25\%}$ = 13.19 mm and $L_{75\%}$ = 17.31 mm.

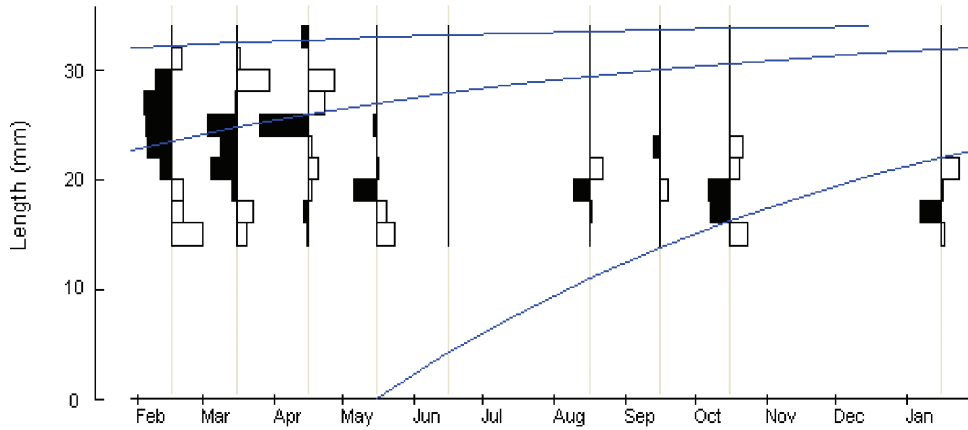Fig. 5: von Bertalanffy growth curves (L_α = 34.65 mm and K = 1.50 yr^1) for A. intermedius superimposed on the restructured length-frequency histograms. The black and white bars are positive and negative deviation from the "weighted" moving average of three length classes and they represent psedo-cohorts
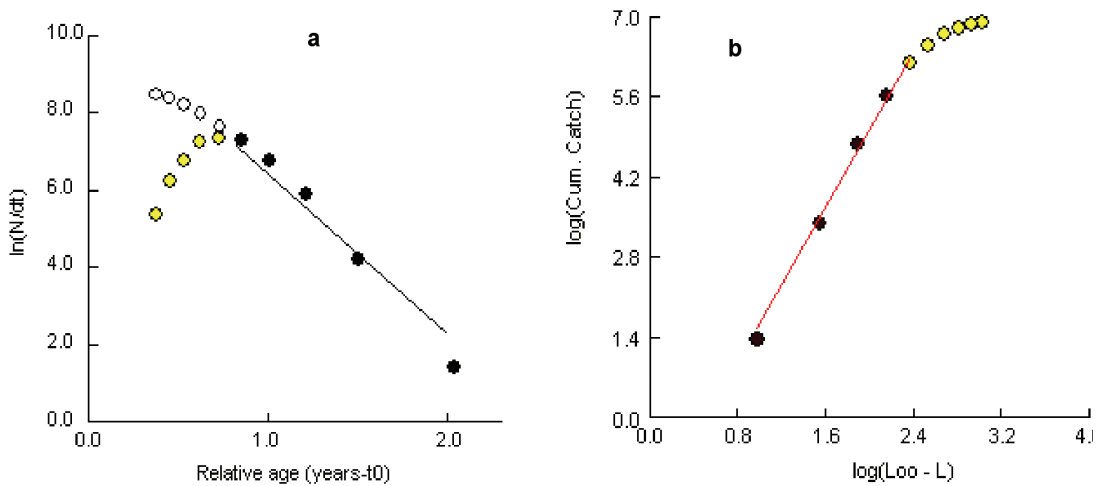

Fig. 6 (a & b): Length converted catch curve (a), the darkened full dots represent the points used in calculating through least square linear regression and the open dots represent the point either not fully recruited or nearing to L_α ; Jones and van Zalinge plot (b) for the estimation of total mortality (Z) of A. intermedius

Recruitment Pattern

The recruitment pattern of *A. intermedius* was continuous throughout the year with one major peak (*Fig. 8*). The percent recruitment varied from 0.73% to 19.01% during the study period. The highest recruitment peak occurred between June and August. The highest and lowest percent recruitment was observed in the months of July and February respectively (*Fig. 8*).

Virtual Population Analysis

Virtual population analysis (VPA) performed on *A. intermedius* indicated that (*Fig. 9*) the minimum and maximum fishing mortalities were recorded for the mid lengths 0.06 yr^{-1} and 2.79 y^{r-1} respectively. The fishing mortality (F) was comparatively high over the mid lengths between 23 mm and 31 mm. This increase is a reflection of recruitment over this length range rather

TABLE 2

Population parameters of *A. intermedius* in the coastal waters of Malacca, Malaysia

Population parameters	*A. intermedius*
Asymptotic length (L_α) in mm	34.65
Asymptotic weight (W_α) in mg	211.21
Growth co-efficient (K yr^{-1})	1.50
Growth performance index (φ')	3.25
Natural mortality (M yr^{-1})	2.35
Fishing mortality (F yr^{-1})	1.81
Total mortality (Z yr^{-1})	4.15
Exploitation level (E)	0.43
Allowable limit of exploitation (E_{max})	0.65
Sample number (N)	995
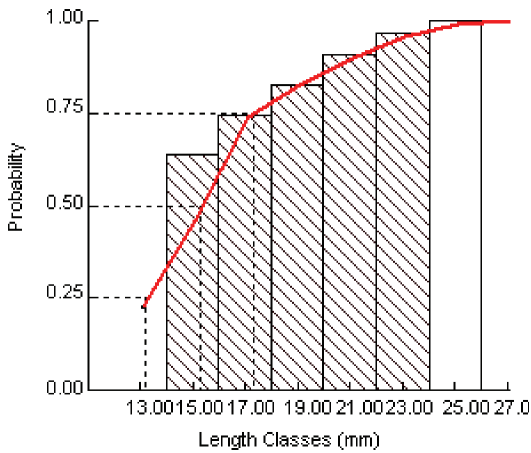
Fig. 7: Probability of capture of each length class of the A. intermedius *(L25% = 13.19 mm, L50% or Lc = 15.29 mm and L75% = 17.31 mm)*
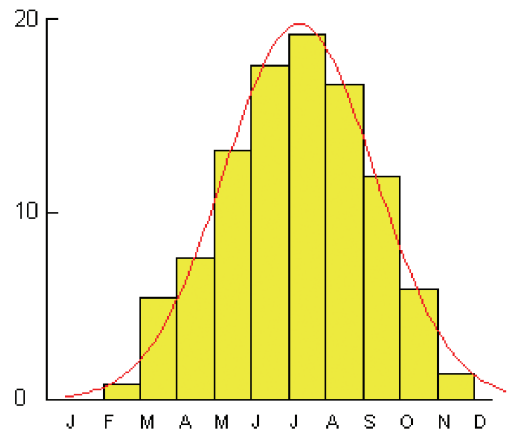
Fig. 8: Recruitment pattern of A. intermedius *in the coastal waters of Malacca, indicating one major peak pulse per year*
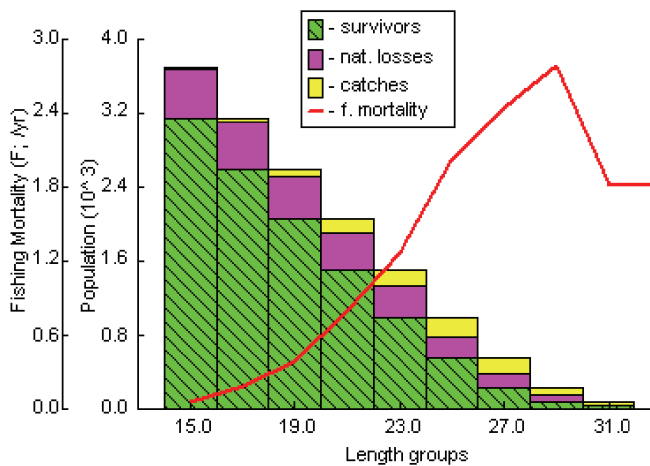
Fig. 9: Length based virtual population analysis of A. intermedius *in the coastal waters of Malacca, Peninsular Malaysia*

TABLE 3

Growth parameters (L∞ and K) and computed growth performance index (φ') of the genus *Acetes* from different tropical countries

Location	Species	L_α (mm)	K yr⁻¹	φ'	E	T (^{0}c)	Source
Malaysia	*A. intermedius*	34.65 TL	1.50	3.25	0.43	310C	Present study
Bangladesh	*A. indicus*	31.00 TL	1.70	3.22	0.22	280C	Zafar *et al.* (1997)
Bangladesh	*A. erythraeus*	37.00 TL	1.20	3.21	0.24	280C	Zafar *et al.* (2002)
Bangladesh	*A. chinensis*	40.00 TL	1.60	3.40	0.21	280C	Zafar *et al.* (1998)
Korea	*A. chinensis* (F)	13.51 CL	0.69	2.10	-	-	Oh and Jeong (2003)
Korea	*A. chinensis* (M)	10.48 CL	0.84	1.97	-	-	Oh and Jeong (2003)
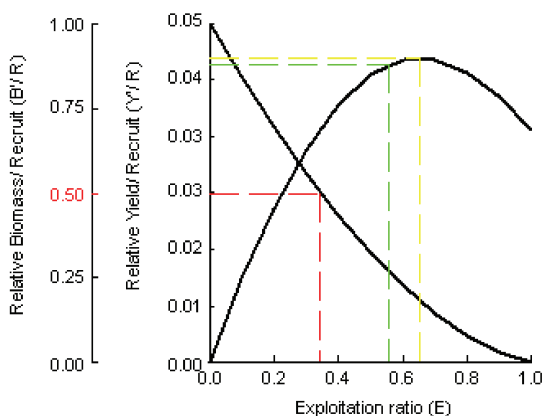
Fig.10: Relative Y/R and B/R of A. intermedius *using knife-edge procedure in the coastal waters of Malacca, Peninsular Malaysia*
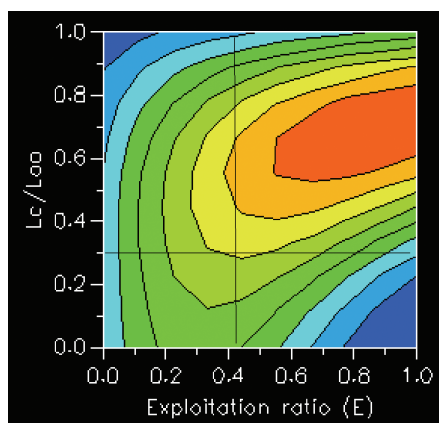
Fig. 11: Yield isopleths for the A. intermedius *in the coastal waters of Malacca, Peninsular Malaysia*

than increased efficiency of the gear with length. F reached a maximum of 2.79 yr⁻¹ at 29 mm with an average value of 1.35 yr⁻¹.

Relative Yield Per Recruit and Biomass Per Recruit

The relative Y/R and B/R analysis of *A. intermedius* were computed using knife-edge procedure assumes. The maximum allowable limit of exploitation level (E_{max}) that gives the maximum relative yield-per-recruit was estimated at 0.65 (*Fig. 10*). $E_{0.1}$, the level of exploitation at which the marginal increase in relative yield per recruit is 10% of the marginal increase computed at a very low value at E, was 0.56. The exploitation level ($E_{0.5}$) which corresponds to 50% of the relative biomass per recruit of the unexploited stock was 0.34. The response of the yield per recruit of the *A. intermedius* in the coastal waters of Malacca was demonstrated using yield isopleths (*Fig. 11*) to both variation in $L_{50\%}$ and fishing pressure as indicated by the exploitation rate E over a wide range of both parameters.

DISCUSSION

The estimated asymptotic length (L_α) is 34.65 mm and VBGF growth co-efficient (K) is 1.50 yr⁻¹ for the present study of *A. intermedius*. Comparisons with population parameters obtained in other studies (Table 3) show that differences exist for different species of the genus *Acetes* from different areas in the world. The highest value of L_α (40.0 mm) for *A. chinensis* (Zafar *et al.*, 1998) and the lowest value (31.0 mm) for *A. indicus* (Zafar *et al.*, 1997) are reported from Bangladesh. The highest (1.70yr⁻¹) value of K is observed in Bangladesh (Zafar *et al.*, 1997) and lowest value of K (0.69 yr⁻¹) is observed in Korean waters (Oh and Jeong, 2003) for *A. indicus*. It is observed that the present K value of *A. intermedius* is very close to *A. chinensis* of Bangladesh waters (Table 3). The

index of phi prime by Munro and Pauly (1983) is suitable for comparing and computing the overall growth performance of different species of fish/shrimps stock. The phi prime for this species with the present estimates of L_α and K is 3.25 whereas the phi prime values were 3.22 and 3.21 for *A. indicus* (Zafar *et al.*, 1997) and *A. erythraeus* (Zafar *et al.*, 2002) respectively. Though phi prime is supported to be more or less constant for a family or for similar taxa, the range here (Table 3) is low except the report of Oh and Jeong (2003). The estimated longevity (t_{max}) for *A. intermedius* is almost 2 years of age, indicating that it is short-lived.

Total mortality (Z) estimated by length converted catch curve here for *A. intermedius* (4.15 yr^{-1}) in the coastal waters of Malacca is close to the value (3.93 yr^{-1}) obtained by Oh and Jeong (2003) in the western coast of Korea but it is much lower than the value estimated (6.07 yr^{-1}) from Bangladesh coast (Zafar *et al.*, 1997). Higher natural mortality (2.35 yr^{-1}) verses the fishing mortality (1.81 yr^{-1}) observed for *A. intermedius* in the present study (Table 2) indicate the unbalanced position in the stock. Exploitation level (E) was computed as 0.43 indicating that the fishery of *A. intermedius* in the coastal waters of Malacca is under exploited. This is based on the assumption that a stock is optimally exploited when fishing mortality (F) equals natural mortality (M), or E = (F/Z) = 0.5 (Gulland, 1971).

This study indicated that the recruitment pattern of *A. intermedius* is a continuous with one main recruitment event per year (*Fig. 8*), i.e. one major cohort is produced per year. But Oh and Jeong (2003) and Zafar *et al.* (1997) reported two recruitment peaks per year for the *A. chinensis*. There is no published report on recruitment of *A. intermedius* in Malaysia. However, it has been reported that the *Acetes* spawns throughout the year in the tropics and subtropics, spawning peaks can be recognized and these almost always lie in the warmer months (Nataraj, 1947). Spawning patterns in these areas (tropical and subtropical) are probably related to monsoonal influences on precipitation and wind direction (Omori, 1974). In this study, it is observed that the major spawning occurs in the months of May-June (*Fig. 5*) in the coastal waters of Malacca which follow the southwest monsoon (June -July-August).

The length at first capture is an important input in the computation of relative yield-per-recruit and relative biomass-per-recruit. The maximum allowable limit of exploitation rate (E_{max}) giving maximum relative yield-per-recruit (Y/R), was estimated as 0.65, compares well with the exploitation rate (E) of 0.43 established for *A. intermedius* in this study, and approximates to the 0.50 optimum level of exploitation reported by Gulland (1971). This is a further indication that the fishery is below optimal exploitation. However, the exploitation rate of the fishery (0.43) is also below the more conservative yield concept ($E_{0.1}$ = 0.55), where the marginal increase in relative yield-per-recruit is 10% of its value at E = 0. This reveals that the fishery is probably being under exploited in terms of relative yield-per-recruit. Results from the analysis of the exploitation rate (E) based on the mortality estimates, and from the relative yield-per-recruit (Y/R), indicate that the fishery is below the level of optimum based on the $E_{0.1}$ principle. Thus, the fishing pressure on the stock is not excessive. More yields could be obtained by a reasonable increase in the effort (*Fig. 11*) without necessarily leading to over exploitation.

Based on the critical size ratio (L_c/L_α) (which is a proxy to mesh size) and current exploitation ratio (E) (which is a proxy to effort), Pauly and Soriano (1986) have shown that the relative yield isopleths could be grouped into four categories (or quadrants) each with its distinct properties. With L_c/L_α = 0.44 and E = 0.43, our yield isopleths of *Fig. 11* falls into quadrant B. This means that in terms of relative yield-per-recruit, the fishing regime for *A. intermedius* in the coastal waters of Malacca is eumetric, at a developing stage and the small shrimps are caught at a low effort. Everything remaining the same, this situation does not warrant management intervention. However, since open-access fisheries, as in the coast of Malacca, stand the risk of over-capitalization (or over-exploitation), then the critical size ratio (L_c/L_α) should be increased whenever it is necessary to increase the effort.

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Malaysian Fruit Bats Phylogeny Inferred Using Ribosomal RNA

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ABSTRACT

Fourteen species of the Malaysian fruit bats (Pteropodidae) were used in this DNA taxonomy using 1,334bp of the 12S ribosomal RNA (rRNA), transfer RNA (tRNA) valine and 16S rRNA gene segments. Previous studies using DNA found contradictions between morphology and molecular data in inferring the phylogeny of the fruit bats proposed by Andersen (1912). Our phylogenetic analysis using the neighbor-joining and the maximum parsimony methods did not support the monophyly of the subfamily Macroglossinae and the cynopterine group of the subfamily Pteropodinae as proposed by classical taxonomists. This is congruent with previous molecular studies. Here, we provide the first registered 12S rRNA, tRNA valine and 16S rRNA sequence records for *Dyacopterus spadiceus* in the GenBank database. This study represents the first attempt to infer the phylogenetic relationship of fruit bats from Malaysia using molecular methods.

Keywords: DNA phylogenetic relationship, pteropodids, ribosomal and transfer RNAs

INTRODUCTION

The suborder of Megachiroptera consists of only one family, the Pteropodidae, containing 42 genera and 166 species recorded worldwide (Corbet and Hill, 1992; Nowak 1994; Wilson and Reeder, 2005). They live in subtropical and tropical areas of Africa, through southern Asia to Australia and on the islands in the Indian and western Pacific Oceans (Mickleburgh *et al.*, 1992). Juste *et al.* (1999) recognised the Malaysian-Indonesian rainforest along with the African rainforest belt across the Congo basin as the two areas with the highest diversity of fruit bats. In Borneo, there are 11 genera of pteropodids (Payne *et al.*, 1985) while Peninsular Malaysia has 17 species (Kingston *et al.*, 2006), making it the fourth highest country in terms of their worldwide diversity.

Pteropodids are relatively small to very large bats with the forearm length ranging from 4 cm to 22 cm. They feed on plant products such as fruits, flowers, nectar and pollens. They become active in the late evening and at night when they may fly long distances in search of food. Pteropodids consist of all flying foxes and Old World fruit bats which are further divided into four subfamilies, namely, (i) the diverse subfamily Pteropodinae, (ii) subfamily Macroglossinae which consists of six genera of blossom bats, dawn bats, long-tongued fruit bats, and relatives, (iii) the aberrant subfamily Harpyionycterinae and (iv) the subfamily Nyctimeninae (Corbet and Hill, 1992). The Malaysian pteropodids consist of 18 species from two subfamilies, the Pteropodinae, which are specialised fruit and flower eating bats and the Macroglossinae, which

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contains the genera that are principally adapted to feed on nectar and pollen (Corbet and Hill, 1992).

There remains considerable uncertainty in both the subordinal and the superfamilial classification within bats (Teeling *et al.*, 2005) and many traditionally recognised groups are not monophyletic (Simmons, 2005), particularly in the pteropodids. Recent molecular data have indicated the requirement for substantial revisions of the phylogeny of pteropodids based on the morphological characters (Kirsch *et al.*, 1995; Juste *et al.*, 1999; Romangnoli and Springer, 2000; Colgan and da Costa, 2002). However, no complete classification of bat families based on molecular data yet exists and the present classifications are based on morphology that is not at all congruent with the new data (Simmons, 2005).

The classical taxonomy by Andersen (1912) categorised the subfamily Pteropodinae into three sections (or tribes) of rousettine (consisting the genera *Rousettus, Pteropus* and *Dobsonia*), epomophorine (African fruit bats) and cynopterine (genera *Myonycteris, Balionycteris, Nyctimene, Sphaerias* and *Cynopterus*); and in the subfamily Macroglossinae are the eonycterine and notopterine bats. Cladistic re-analysis of Andersen's (1912) characters supported a Macroglossinae monophyly, but monophyly of rousettine, cynopterine and epomorphorine was less clear (Springer *et al.*, 1995).

Nevertheless, the classifications of pteropodids by Andersen (1912) remain the most comprehensive of the evolutionary framework reference for the relationships among approximately 200 species of pteropodids described (Koopman, 1994; Hollar and Springer, 1997). However, relationships among pteropodid genera are not yet fully resolved and the positioning of several Southeast Asian endemic genera is still problematic (Simmons, 2005). Due to much contradiction between the morphological and genetic data, the current taxonomic status and phylogenetic relationship of the Malaysian pteropodids remains unclear. In this study, we attempt to infer the phylogenetic relationship and to reconstruct the taxonomic relationships among the pteropodids of Malaysia using the 12S ribosomal RNA (rRNA), transfer RNA (tRNA) valine and 16S rRNA gene segments.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

A list of the species of fruit bats, their voucher numbers and collection localities are shown in Table 1. A total of 14 pteropodids (out of 18 species existing in Malaysia) samples were used in this study which were representatives of the different regions in Peninsular Malaysia, Sabah and Sarawak. The samples were preserved in either vials containing 95% ethanol or stored at -20°C for fresh samples prior to analysis. Bats were captured using standard mist nets set at under storey level, and across forest trails and over water bodies (Hall *et al.*, 2004). Some samples were acquired from the museums at the Department of Wildlife and National Parks of Peninsular Malaysia (Kuala Lumpur) and Sabah Parks and were re-identified following Payne *et al.* (1985). Four species were not included in this study due to their scarcity: *Megaerops wetmorei, Rousettus spinalatus, Eonycteris major,* and *Pteropus hypomelanus. Pteropus dasymallus, Rhinolophus pumilus* and *Mystacina tuberculata* (AB042770, NC005434 and AY960981, respectively) were used in this study as a comparison to determine the position of the mitochondrial DNA (mtDNA) sequences of the fruit bats studied.

Total genomic DNA was extracted from muscle tissues from both fresh and ethanol samples following a modified cetyltrimethylammonium bromide (CTAB) procedure as described by Grewe *et al.* (1993) with the presence of Proteinase K. The quality and approximate yield was determined by electrophoresis of 2 µL of genomic DNA on a 1% agarose gel in 50 ml of 1X TAE buffer containing ethidium bromide at 90V for 30 min. Isolated genomic DNA was used for further mtDNA analysis.

Polymerase Chain Reaction and DNA Sequencing

Approximately 1,400-base pairs (bp) of the 12S rRNA, tRNA valine and 16S rRNA regions were amplified using the standard polymerase chain reaction (PCR) procedures. A set of 12S and 16S rRNA primers were used: 5'-ATG TTT TTG ATA AAC AGG CG-3' known as 16SA-H (Palumbi *et al.,* 1991) and 5'- AAA CTG GGA TTA GAT ACC CCA CTA T-3' known as 12SA-L (Palumbi *et al.*, 1991). Thermal cycle amplification was performed in a 25 µL reaction volume containing

TABLE 1
Species names, specimen voucher and localities of the pteropodids used in this study

SubFamily	Species	Specimen Voucher	Park/Museum Collection	Localities/Sources	GenBank Accession No.
Pterpodinae	Aethalops alecto	TK004	SP	Kinabalu Park, Sabah	DQ002939
	Balionycteris maculata	NNP135	UNIMAS ZM	Niah National Park, Sarawak	DQ002940
	Chironax melanocephalus	MTA96041	UNIMAS ZM	Rayu River, Kubah, Sarawak	DQ002941
	Cynopterus brachyotis	WK1	DWNP ZM	Wang Kelian, Perlis	DQ002942
	C. horsfieldi	MTA96318	UNIMAS ZM	Rayu River, Kubah, Sarawak	DQ002943
	C. sphinx	CS29	DWNP ZM	Taiping, Perak	DQ002944
	Dyacopterus spadiceus	DS10	UNIMAS ZM	Balambangan Island, Sabah	DQ002945
	Megaerops ecaudatus	BD013	UNIMAS ZM	Lelang Dam, Bario, Sarawak	DQ002949
	Penthetor lucasi	NNP084	UNIMAS ZM	Niah National Park, Sarawak	DQ002950
	Pteropus dasymallus	-	-	GenBank (Nikaido et al., 2000)	AB042770
	P. vampyrus	P. V. 1	UNIMAS ZM	Serian, Sarawak	DQ002952
	Rousettus amplexicaudatus	1017	UNIMAS ZM	Balambangan Island, Sabah	DQ002951
	Eonycteris spelaea	P015	UNIMAS ZM	Pueh Mountain, Sematan, Sarawak	SDQ002946
Macroglossinae	Macroglossus minimus	UMS/Bf/00104	UNIMAS ZM	Niah, Miri, Sarawak	DQ002947
	M. sobrinus	MTA96376	UNIMAS ZM	Kuala Gandan, Pahang	DQ002948
OUTGROUP	Rhinolophus pumilus	-	-	GenBank (Nikaido et al., 2001)	NC_005434
	Mystacina tuberculata	-	-	GenBank (Sandbrook et al., unpublished)	AY960981

INGROUP

SP: Sabah Park; UNIMAS ZM: Universiti Malaysia Sarawak Zoological Museum; DWNP ZM: Department of Wildlife and National Parks Zoological Museum

15.5 µL of sterilised distilled water, 0.5 µL of *Taq* DNA polymerase (Promega), 2.5 µL of 10X reaction buffer (Promega), 0.5 µL of dNTP (10mM), 1.5 µL of magnesium chloride (25 mM), 1.25 µL of each primer (10 µM) and 2.0 µL of the genomic DNA. The cycle profile was 5 min at 96°C for initial denaturation, followed by 35 cycles of 45 sec at 95°C for denaturation, 1 min 30 sec at 56°C for annealing and 1 min 30 sec at 72°C for elongation, and finally 7 min at 72°C for final elongation. The amplified products were later visualised on 1% agarose gel containing ethidium bromide, ran for approximately 30 min at 90 V and photographed under UV trans-illuminator (Bio-Rad). 1 kb DNA ladder (Promega) was used as a standard size marker to quantify the size of the PCR products. The amplified DNA products were purified using a commercial kit (Fermentas) and subsequently sent for sequencing. Sequencing of each sample was carried out on both the forward and reverse strands by using the same primers as for the PCR amplification on the ABI PRISM® 377 DNA Sequencer in a private laboratory (First BASE Laboratories Sdn. Bhd.).

Sequence Alignment and Phylogenetic Analyses
Multiple alignments of the nucleotide sequences were done using the program CLUSTAL X 1.81 (Thompson *et al.*, 1997) and subsequently aligned by eye. Pairwise distance calculations were conducted using the two-parameter model of Kimura (1980) to estimate genetic distances among the species of pteropodids under study using *MEGA* (version 2.1, Kumar *et al.*, 2001). Nucleotide compositions (% of A, C, T and G bases) were also estimated for each species using *MEGA*. Phylogenetic trees were reconstructed using the neighbour joining (NJ) and unweighted maximum parsimony (MP) as well as the maximum likelihood (ML) methods implemented in PAUP (version 4.0b 10; Swofford, 1998). The NJ clustering was performed using the two-parameter model of Kimura (1980) while the ML analysis corresponded to the HKY85 evolutionary model (Hasegawa *et al.*, 1985). All trees were rooted with two Microchiroptera sequences from GenBank: *R. pumilus* (NC_005434) and *M. tuberculata* (AY960981) as outgroups. Phylogenetic confidence was estimated by bootstrapping (Felsenstien, 1985) with 1000 replicate data sets for the NJ and MP methods while for the ML method there were 100 replicate data sets. All the sequences were submitted to GenBank with Accession Numbers: DQ002939-DQ002952.

According to Miyamoto and Boyle (1989) and Irwin *et al.* (1991), transversion substitutions in mammals showed a linear relationship with time. Similar to the calculations done by Bastian *et al.* (2001), we calculated the estimate of divergence between the pteropodids and the outgroup species using a constant transversion rate of 0.2% per mya (million years ago) (Miyamoto and Boyle, 1989; Irwin *et al.*, 1991).

RESULTS AND DISCUSSION

Sequence Analysis and Estimate of Divergence
Partial sequences with the length of 1334-bp comprising the 12S rRNA, tRNA valine and 16S rRNA gene segments from 14 species of Malaysian pteropodids were successfully sequenced and aligned (including gaps). By comparing with the complete mitochondrion genome (using *R. pumilus, M. tuberculata* and *P. dasymallus* taken from GenBank with accession number, NC_005434, AY960981 and AB042770, respectively), our sequences begin at the 547-bp until the 1880-bp of the complete mitochondrial sequence. The base composition showed an anti-G bias (data not shown), which is characteristic for the mitochondrial gene (Cantatore *et al.*, 1994; Briolay *et al.*, 1998; Ryan and Esa, 2006). From the 1334-bp sequence, 519 (38.9%) variable or polymorphic (segregating) sites were observed. In addition, among the 519 variable sites, 373 (71.9%) were parsimoniously informative sites.

The pairwise genetic distances (number of nucleotide substitutions per site) calculated by using the Kimura two-parameter model (Kimura 1980) are shown in Table 2. Pairwise comparisons among all the sequences range from 0.2% to 24.5% of differences. Within the subfamily Pteropodinae, the distances range from 0.2% to 18.1% of differences, with the least differences observed between *Cynopterus brachyotis* and *C. sphinx* within the genus *Cynopterus*. The differences between the subfamilies ranged from 11.8% to 17.9%. Within the subfamily Macroglossinae the difference between *Macroglossus minimus* and *M. sobrinus* was 1.6% while *Eonycteris spelaea* was 15.7% and 15.0% different from *M. minimus* and *M. sobrinus*, respectively. All the pteropodid sequences were

TABLE 2

Pairwise distances with all sites considered (below the diagonal; including gaps) and transversion pairwise distances (above the diagonal) in percentage using the two-parameter model of Kimura (1980) among the pteropodids species used in this study

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.	*Aethalops alecto*		4.0	3.4	4.6	4.6	4.6	5.0	5.6	2.5	5.0	4.9	5.1	4.5	6.9	6.7	8.4	9.4
2.	*Balionycteris maculata*	10.8		5.1	6.0	6.0	6.0	6.6	7.6	4.5	7.1	7.0	6.8	6.4	7.8	7.6	8.9	11.2
3.	*Chironax melanocephalus*	9.9	14.4		5.4	5.5	5.4	5.7	6.6	2.7	5.4	5.3	5.8	5.2	7.1	6.7	9.1	10.0
4.	*Cynopterus brachyotis*	12.3	16.1	14.4		0.2	0	5.4	2.4	4.3	3.9	4.0	4.4	3.6	5.5	5.3	7.6	8.9
5.	*Cynopterus horsfieldi*	11.9	15.8	14.1	3.3		0.2	5.4	2.4	4.3	4.1	4.2	4.4	3.6	5.3	5.1	7.6	8.9
6.	*Cynopterus sphinx*	12.2	15.9	14.4	0.2	3.3		5.4	2.4	4.3	3.9	4.0	4.4	3.6	5.5	5.3	7.6	8.9
7.	*Dyacopterus spadiceus*	11.5	15.5	13.0	12.9	12.9	12.7		6.2		5.7	5.6	5.8	5.4	6.4	6.4	8.6	10.4
8.	*Megaerops ecaudatus*	12.2	15.4	14.5	7.5	7.3	7.4	6.2		5.5	4.9	5.0	5.2	4.2	6.5	6.5	7.7	10.1
9.	*Penthetor lucasi*	8.1	12.6	9.4	11.7	11.7	11.6	13.2	11.6		4.6	5.0	4.4	4.3	5.8	5.5	8.0	9.2
10.	*Pteropus dasymallus*	12.7	17.6	14.6	13.2	12.9	13.3	12.9	13.5	12.7		0.1	4.2	3.2	5.5	5.3	7.0	10.6
11.	*Pteropus vampyrus*	12.0	17.4	14.9	12.6	12.7	12.7	12.4	12.7	12.0	2.3		4.3	3.3	5.5	5.4	7.1	10.5
12.	*Rousettus amplexicaudatus*	12.7	16.7	14.0	12.3	12.3	12.1	13.2	12.0	11.4	13.0	12.7		2.8	6.1	5.7	8.1	10.5
13.	*Eonycteris spelaea*	12.8	18.1	14.5	12.7	12.5	12.5	13.1	13.0	13.8	12.0	12.3	11.8		5.5	5.3	7.2	10.2
14.	*Macroglossus minimus*	14.8	17.9	15.8	15.6	14.6	15.6	14.0	16.0	15.5	13.0	12.4	15.2	15.7		0.3	8.5	11.2
15.	*Macroglossus sobrinus*	14.8	17.8	15.6	15.9	14.5	15.7	13.9	16.1	15.2	12.7	12.1	15.3	15.0	1.6		8.1	11.2
16.	*Rhinolophus pumilus*	19.6	22.6	21.8	22.0	21.2	22.0	21.3	20.9	20.4	19.6	19.3	20.9	21.0	22.1	22.0		11.3
17.	*Mystacina tuberculata*	20.3	24.5	21.3	21.4	20.6	21.3	22.4	20.8	21.7	23.7	23.2	22.1	23.1	21.9	22.3	21.8	

distantly related to both the outgroup sequences with an average distance value of 21.6% (data not shown).

Our estimation on the divergence time between the outgroups (Microchiroptera) and the pteropodids dates back to around 45-mya ± 4.75-mya. This estimation is very close to the earliest fossil record of bats back in the Early Eocene period (about 50 to 55-mya) and is also comparable to the estimation by Bastian *et al.* (2001), which suggests that the megachiropterans diverge from the microchiropterans at 50.2-mya. We estimated that the speciation of *Cynopterus* occurred 12-mya ± 2.5-mya, which is comparable with the divergence time of *Cynopterus* from *Ptenochirus* as calculated by Bastian *et al.* (2001) (divergence time of 12.3-mya). *Dyacopterus spadiceus* which is an endemic species of Borneo, diverged out from the other cynopterine group (*Aethalops*, *Balionycteris*, *Chironax* and *Penthetor*) around 27-mya ± 3.5-mya. The divergence time for the Malaysian fruit bats predates the Pleistocene epoch glaciations during the Quaternary Period (about 2 million to 10,000 years ago) in the Sunda Shelf. The divergence time between the subfamily Pteropodinae and Macroglossinae was however unclear.

Phylogenetic Tree Analyses

The topologies of the tree reconstructions are similar among all the three methods with high confidence levels (based on 1000 bootstrap replicates for NJ and MP and 100 replicates for ML). The NJ tree is presented in *Fig. 1* while MP and ML produced matching topologies which are combined and presented in *Fig. 2*. The relationship of the pteropodids as proposed by Andersen (1912) was also shown in both figures. Megachiroptera is monophyletic with bootstrap values between 93 to 100% for the NJ, MP and ML methods.

The NJ method formed three major groups (*Fig. 1*). The first group was formed by the genera *Macroglossus* and *Pteropus*, the second group consisted of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus* while the genera *Cynopterus*, *Megaerops*, *Eonycteris* and *Rousettus* formed the third group.

Using the MP method, where all the characters were weighted equally, the tree length was 1465 with consistency index (CI) of 0.5468 and retention index (RI) of 0.4943. The ML tree (-ln likelihood = 8595.50874) produced a similar topology with the MP tree with only minor differences. The tree topology separated the pteropodids into five clades with a 100% bootstrap value (*Fig. 2*). Members of the family Pteropodinae formed two major groups. The first group consisted of the *Cynopterus* genera (*C. brachyotis*, *C. horsfieldi* and *C. sphinx*) and the genus *Megaerops* and the second group consisted of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus*. Interestingly, the members of the family Macroglossinae (*Eonycteris* and *Macroglossus*) were not grouped together and did not form a monophyletic clade as proposed earlier by morphological studies. The genus *Pteropus* was grouped with the species *M.*
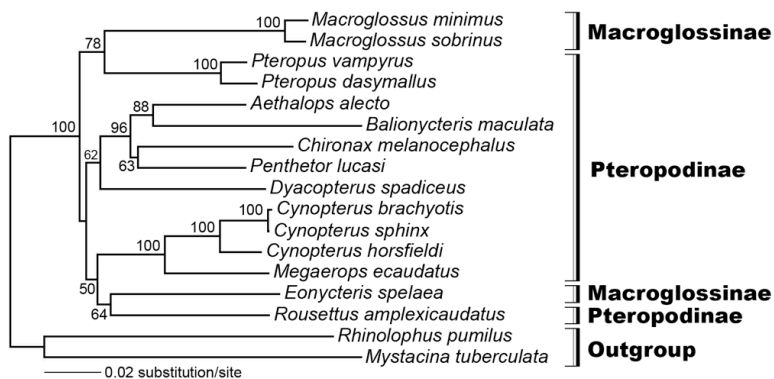

Fig. 1: NJ tree generated using the 12S rRNA, tRNA valine, and 16S rRNA gene segments of the pteropodid species used in this study (only bootstrap values >50% are shown). Values on the branches represent NJ bootstrap estimates, based on 1000 replicates.
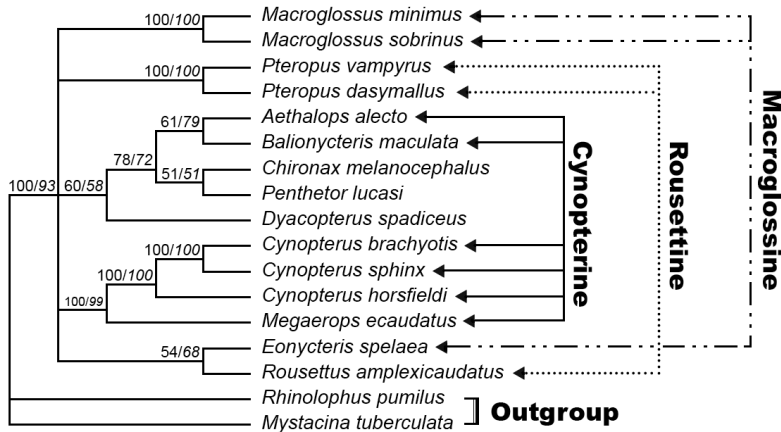
Fig. 2: Combined phylogenetic tree of unweighted MP (tree length=1465; CI=0.5468; RI=0.4943) and ML (-ln likelihood = 8595.50874) tree generated using the 12S rRNA, tRNA valine, and 16S rRNA of the megachiropteran species used in this study (only bootstrap values >50% are shown). Regular font values on the branches represent MP and italic font values represent ML bootstrap estimates, based on 1000 replicate for MP and 100 replicate for ML.

minimus and *Rousettus amplexicaudatus* was seen grouped with the species *E. spelaea*.

Considering all three phylogenetic trees, the clade consisting of the genera *Aethalops*, *Balionycteris*, *Chironax*, *Penthetor* and *Dyacopterus*, and *Cynopterus* group (all three species of *Cynopterus*) and the single species of *Megaerops ecaudatus*, was the most consistent. The NJ tree grouped together the genera *Macroglossus* and *Pteropus* into a clade with moderate support (78% bootstrap value) while MP and ML did not support the groupings. Additionally, even though *E. spelaea* and *R. amplexicaudatus* was placed together in one clade for all the three methods used, their relationship was poorly supported with low bootstrap values (64%, 54% and 68% for NJ, MP and ML, respectively).

Andersen's (1912) monograph remains the most comprehensive treatment of the pteropodids. However, recent studies using molecular approaches challenged his morphological classification and proposed a re-organisation on their taxonomic status. The contradictions between classical and molecular data on the phylogenetic relationships of the pteropodids are well documented. A study of 19 genera based on single-copy (sc) DNA hybridisation contradicted the monophyly of the cynopterine section (Kirsch *et al.*, 1995). The authors also suggested that the rousettine section and the subfamily Macroglossinae respectively are not monophyletic groupings. Colgan and

Flannery (1995) used 23 informative Restriction Fragment Length Polymorphism (RFLP) markers for their analysis and included *Eonycteris* within a paraphyletic cynopterine section, thus challenging both cynopterine and macroglossine monophyly. Hollar and Springer (1997) used 12S rRNA and tRNA valine gene sequences and their results agree with the scDNA hybridisation work by Kirsch *et al.* (1995) in contradicting both rousettine and macroglossine monophyly. Romangnoli and Springer (2000) later used additional 16S rRNA gene segment apart from the one used by Hollar and Springer (1997) and further confirmed the non-monophyletic state of the macroglossine section. Next, Colgan and da Costa (2002) studied the evolution of the African pteropodid clade using 12S rDNA and *c*-mos DNA sequences. Their results confirmed the non-monophyletic state of Macroglossinae and weakly supported the cynopterine section as a monophyletic group. Similarly, Juste *et al.* (1999) conducted a study on the phylogenetic relationships among the African pteropodids using a combined 16S rRNA and cytochrome *b* (cyt *b*) gene region. Their results contradicted the classical morphology-based subdivisions of the pteropodids. Furthermore, they discovered that the Asian cynopterine group (*Cynopterus*, *Megaerops*, *Aethalops*, *Balionycteris*) did not form a monophyletic group, which also contradicted with the traditional classification (Andersen, 1912; Mickleburgh *et al.*, 1992). In the Southeast

Asia region, a study by Bastian *et al.* (2001) analysed five species of pteropodids in the Philippines using the complete sequence of the cyt *b* gene and found that the genetic divergence between *R. amplexicaudatus*, *E. spelaea*, and *C. brachyotis* was small. Recent studies by Abdullah *et al.* (2000), Abdullah (2003) and Campbell *et al.* (2004) revealed at least two cryptic species within the *C. brachyotis* complex. In this study, some aspects of the positioning of the pteropodids at the suprageneric level was clarified (e.g. grouping of the cynopterine group) but failed to elucidate at the subfamilial categories (i.e. relationships between Pteropodinae and Macroglossinae).

The Malaysian pteropodids consist of two subfamilies of the Pteropodinae and Macroglossinae with 18 species in 11 genera which are widespread in both Borneo and Peninsular Malaysia on the Asian mainland. Only one species, *E. major* is distributed in Borneo (Payne *et al.,* 1985). According to Andersen (1912), the genus *Rousettus* and *Pteropus* are assembled within the group rousettines. Rousettines has a mixed diet that includes soft fruits and / or fruit juices as well as nectar (Nowak, 1994). However, from our phylogenetic trees, both genera did not from a cluster together, which is in concordance with Ahmad (2005), Bastian *et al.* (2001), Juste *et al.* (1999), and Hollar and Springer (1997). Instead, *Rousettus* clustered with *Eonycteris* while *Pteropus* clustered with *Macroglossus*, where both *Eonycteris* and *Macroglossus* are from the subfamily Macroglossinae. The clustering of *Rousettus* and *Eonycteris* were in agreement with the findings by Rickart *et al.* (1989) who observed a close similarity of the morphological features and identical chromosome number, $2n = 36$ between *Rousettus* and *Eonycteris*. In conclusion, our phylogenetic analyses reject the sister-group relationship of *Rousettus* and *Pteropus* within the rousettine section.

Within the subfamily Pteropodinae, the clustering of the three species of *Cynopterus* with *Megaerops* and between *Aethalops* and *Balionycteris* in our study is similar with the results of the analysis done by Juste *et al.* (1999). According to traditional classification (Rickart *et al.*, 1989; Mickleburgh *et al.*, 1992), the cynopterine group (consisting of the genera *Aethalops*, *Balionycteris*, *Cynopterus* and *Megaerops*) are grouped in a monophyletic clade. However, our phylogenetic trees divided these genera into two well-supported clades (*Figs. 1* and *2*) which appeared to be non-monophyletic.

CONCLUSIONS

Overall, the phylogenetic analysis in this study was able to clarify some confusion on the relationships among the pteropodids. Our results reconfirmed some of the findings by several authors (using molecular approaches) particularly about the obscure monophyletic status of *Pteropus* and *Rousettus*, and also the monophyletic status of the cynopterine group. Here, we provide the first registered 12S rRNA, tRNA valine and 16S rRNA sequence records for *D. spadiceus* in the GenBank database. Finally, the 12S rRNA, tRNA valine and 16S rRNA genes are highly conserved and unable to discriminate the evolutionary relationships among the Malaysian pteropodids at the species level. Further studies should explore using fast evolving genes (e.g. control region and cytochrome *b*) and to include all of the pterodid species found in Malaysia and the rest of the Sunda region. Also, each species should be represented by more than one sample which is the major weakness of this study.

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Distribution of Ni and Zn in the Surface Sediments Collected from Drainages and Intertidal Areas in Selangor

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ABSTRACT

Surface sediments were collected from 11 sampling sites in selected intertidals and drainages of Selangor. The sediment samples were analysed for Ni and Zn. The metal concentrations ranged from 15.1 to 121 µg/g dry weight for Ni and 50.2 to 336 µg/g dry weight for Zn. The highest total (Ni and Zn) concentrations in sediments were found at an industrial site in Serdang. The Ni and Zn ranges resulting from this study were wider and higher than those reported previously in Malaysia. Generally, the 'oxidisable-organic' fraction contributed the largest percentage of metals among the other three anthropogenic-related fractions. This study shows that the non-resistant fraction dominated the total Zn based on sequential extraction technique. Some sites had higher percentage (>50%) of non-resistant fraction of Ni and Zn, indicating anthropogenic sources of these metals. Therefore, it is suggested that continuous monitoring of the study areas be implemented especially at industrial areas in Serdang. Perhaps, the industrial waste must be treated before draining to the waterways.

Keywords: Heavy metal, surface sediments, drainages, Selangor, Malaysia

INTRODUCTION

Many anthropogenic activities such as shipping, industry, agriculture and urbanization are based on the west coast of Peninsular Malaysia (Abdullah *et al.*, 1999) and mainly concentrated in the state of Selangor. Industrialization in Selangor has prompted the economic development as well as population expansion. From an ecotoxicological point of view, this is very interesting to know if those industrial activities impacted our natural resources in the coastal area. This study focused on 2 essential elements which are Ni and Zn (Boyle and Robinson, 1988; Astorga España *et al.*, 2007). Though essential, excessive occurrence of these two metals will cause toxicity to organisms in the environment. The toxic responses to Ni and Zn involve interference with Fe metabolism in the organism which causes anemic effects (Magee and Matrone, 1960; Stokes, 1988). Therefore, any risk assessment of the potential effects of Ni and Zn on organisms must take into account

local environmental conditions. Previously, Yap *et al.* (2002a, 2002b, 2003b) has reported the concentrations of Cd, Cu, Pb and Zn in sediments collected from the offshore and intertidal area of the west coast of Peninsular Malaysia. However, studies on the Ni and Zn levels in the area of Selangor is still lacking in the literature. In order to estimate such a possible environmental problem, the background concentrations of heavy metals in the sediment samples collected from the aquatic ecosystems should be known. Therefore, studies monitoring heavy metal pollution are very significant and important.

The use of sediments is advantageous to assess human impacts on the aquatic environment. This is because, sediments play a major role in the transport and storage of metals and are also frequently used to identify sources of pollutants spatially and temporally and to locate the main sinks for heavy metals and the heavy metals that are persistent in the marine environment (Yap *et al.*, 2002b). Takarina (2004)

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† Abbreviations: dw=dry weight; *et al.*= and all; Jln = Jalan; Peng= Pengkalan; Sg= Sungai; Tjg= Tanjung; Tmn= Taman; DDW= Double Deionized Water.

reported that analysis of the speciation of the various heavy metals allowed for identification of potential pollution sources that would have otherwise been missed if only total metal content was known.

Since there is no current information in the concentrations and speciation of Ni and Zn in the surface sediments of Selangor, the objective of this study was to provide such information which mainly focused on the surface sediment samples collected from 11 sampling sites in Selangor including intertidal areas and drainages to which metal industrial effluents are deposited.

MATERIALS AND METHODS

Sampling of surface sediments was conducted in 6 intertidal sites and 5 urban drainages or rivers, in Selangor. The top 3 to 5 cm of surface sediments were collected from each site on 25th April 2005 (*Fig. 1*). The longitude, latitude and site descriptions for each sampling sites are given in Table 1. Each sediment sample was placed in an acid-washed polyethylene bag and deep frozen prior to analysis and brought back to the laboratory (Yap *et al.*, 2002a).

In the laboratory, the surface sediment samples were dried at 60ºC for at least 16 hrs until a constant dry weight. Then the samples were sieved through a 63μm stainless steel sieve and shaken vigorously to produce homogeneity.

Total Metal Concentration

Direct aqua-regia method was used for the analyses of total Ni and Zn concentrations in sediment samples (Yap *et al.*, 2002a). About one gram of each dried sample was weighed and digested in a combination of concentrated nitric acid (AnalaR grade, BDH 69%) and perchloric acid (AnalaR grade, BDH 60%) in the ratio of 4:1, first at low temperature (40 ºC) for 1 h and then the temperature was increased to 140ºC for at least 3 h. Double distilled water (DDW) was used to dilute the digested samples to 40 ml and the samples then filtered through Whatman No.1 filter paper and the filtrate stored until metal determination (Yap *et al.*, 2002b).

Speciations of Ni and Zn of Sediments Samples

Geochemical fractions of Ni and Zn in the sediments were obtained by using the modified sequential extraction technique (Badri and Aston, 1983; Yap *et al.*, 2002a). The four fractions considered, the extraction procedures and the conditions employed were:-

i. Easy, freely, leachable or exchangeable (EFLE): About 10 g of sample was continuously shaken for 3 hrs with 50 ml 1.0 M ammonium acetate (NH$_4$CH$_3$COO), pH 7.0 at room temperature.

ii. 'Acid-reducible': The residue was continuously shaken for 3 hrs with 50 ml

TABLE 1
Longitude, latitude and descriptions of sampling sites for surface sediment samples in some locations of Selangor

No	Locations	Longitude	Latitude	Site description
1.	JP Metal, Serdang	05º20.072' N	100º26.080' E	Drainage at the industrial area
2.	Subang Utama Industry	03º02.665' N	101º32.512' E	An industrial area
3.	Jln Renggam Urban	03º03.683' N	101º31.173' E	A riverside near the Fire Station
4.	Tmn Rashna Urban	03º03.684' N	101º30.347' E	A river beside a residential area
5.	Sultan Suleiman Urban	03º01.151' N	101º22.421' E	A drainage beside a residential area
6.	Peng Nelayan Intertidal	03º01.120' N	101º22.453' E	A jetty with fishing activities and
	abundant mangroves.			
7.	Tjg Harapan Intertidal	03º005.96' N	101º21.637' E	A rocky beach with shipping activities
	around.			
8.	Sg Kapar Intertidal	03º00.141' N	101º21.823' E	A riverside near a main highway
9.	Sg Janggut Intertidal	03º08.161' N	101º22.511' E	A small river near the dam and
				agriculture area
10.	Pantai Jeram Intertidal	03º10.403' N	101º18.819' E	An estuary with fishing activities, 'Ikan
	Bakar' Stalls nearby.			
11.	Sungai Buloh Intertidal	03º15.467' N	101º18.245' E	A fishing village with '*dried prawn*'
				industry area

Sampling Stations:
1   JP Metal
2   Subang Utama
3   Jln Renggam
4   Tmn Rashna
5   Sultan Suleiman
6   Pg. Nelayan
7   Tjg Harapan
8   Sg. Kapar
9   Sg. Janggut
10 Pantai Jeram
11 Sg. Buloh

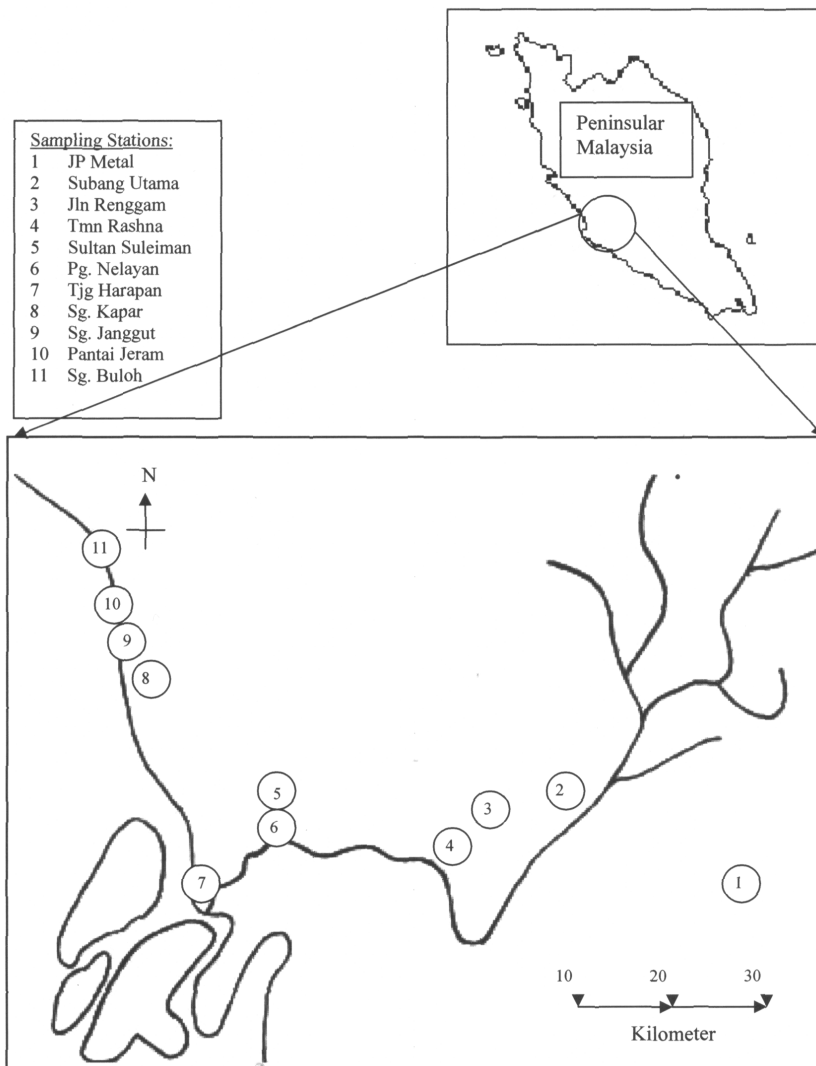*Fig. 1: Map showing sampling sites of surface sediment samples from some places in Selangor*

0.25 M hydroxylammonium chloride ($NH_2OH.HCL$) acidified to pH 2 with HCl, at room temperature.

iii. 'Oxidisable-organic': The residue was first oxidized with 30% H_2O_2 in a water bath at 90-95°C. After cooling, the metal released from the organic complexes was continuously shaken for 3 h with 1.0 M ammonium acetate (NH_4CH_3COO) acidified to pH 2.0 with HCL, at room temperature.

iv. 'Resistant': The residue from (iii) was digested in a combination of concentrated nitric acid (69%) and perchloric acid (60%) as in the direct aqua-regia method.

The residue used for each fraction was weighed before the next fractionation was carried out. The residue was washed with 20 mL DDW. It was then filtered through Whatman No. 1 filter paper and the filtrate was stored until metal determination. For each fraction of the sequential extraction procedure, a blank was employed using the same procedure to ensure that the samples were free of contaminants.

Analysis of Ni, and Zn

The prepared samples were determined for Ni and Zn by using an atomic absorption spectrophotometer (AAS) Perkin Elmer Model

A Analyst 800 and the data were presented in μg/g of sample dry weight (dw).

Quality control samples of known concentrations made from standard solutions for each metal were routinely run through during the period of metal analysis. To avoid possible contamination, all glassware and equipment used were acid-washed. The metal percentages of recoveries were between 90-110%. The quality of the method used was checked with a Certified Reference Material (CRM) for Soil (International Atomic Energy Agency, Soil-5, Vienna, Austria). The agreement between the analytical results for the reference material and its certified values for each metal was satisfactory with recoveries of Zn: 87.8% and Ni: 124.6% as shown in Table 2.

In order to check the accuracy of this method, the sum of all extraction steps for each metal was compared with that found by using the direct digestion with the aqua-regia method. Our method was acceptable since satisfactory recoveries (90-105%) for Ni and Zn were found in the analytical results by using the SET when compared to those of the direct aqua-regia method and they correlated significantly (P< 0.05) with each other.

Data Analysis

The data recorded from the analysis of heavy metal were statistically analyzed using the Statistical Analysis System (SAS) for Windows, version 6.12. Microsoft Excel was used for Spearman' correlation analysis to ascertain the strength of the correlation coefficients among the samples. The analyzed data obtained were depicted as graphs using Kaleida Graphs, version 3.08, November 1996.

RESULTS AND DISCUSSION

Total Metal Concentrations

Based on the 11 sampling sites in Selangor (*Fig. 2*), the total metal concentrations based on direct aqua-regia method ranged from 15.1 to 121 μg/g dw for Ni and 50.2 to 336 μg/g dw for Zn. From *Fig. 2*, JP Metal site in Serdang was found to have the highest concentrations of Ni and Zn. On the other hand, Tanjung Harapan recorded the lowest concentrations of both the metals.

Both metal concentrations in the sediments obtained in this study were higher when compared to values from other regional and Malaysian studies (Table 3). This indicated

TABLE 2
A comparison of the measured results (μg/g dry weight) of the CRM for soil with its certified concentrations for Ni and Zn

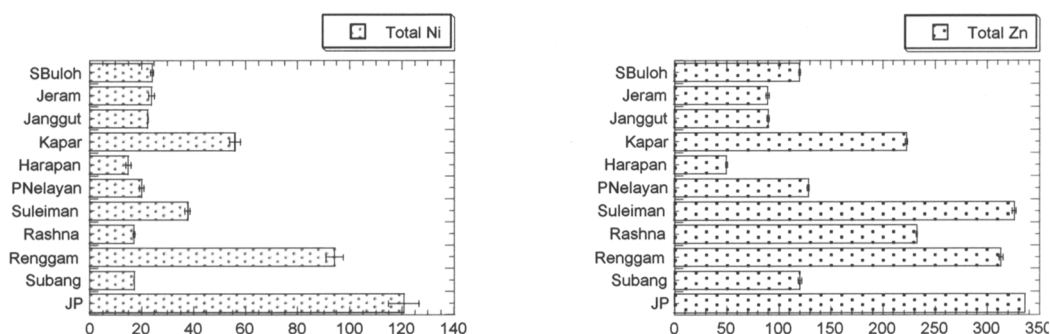| Metal	Certified value (C)	Measured value (M)	Percentage of recovery (M/C)
Zn	368	323.24	87.8
Ni	1.3	1.62	124.6

Fig. 2: Total concentrations (mean mg/g ± SE dry weight) of Ni and Zn in surface sediments collected from Tanjung Harapan to Sungai Buloh, based on direct aqua-regia method

TABLE 3
Comparison of the study data with concentrations (µg/g dry weight) of Ni and
Zn reported from this region and Malaysia

Location	Ni	Zn	References
Regional studies			
Manila Bay, Philipines	10-19.0	60-329	Prudente *et al.* (1994)
Pitchavaram, India	21-58	25-60	Kurokawa & Tatsukawa (1990)
Cochin estuary, India	-	1266	Balachandran *et al.* (2005)
Central Java Coast, Indonesia	17.8 – 36.1	84 – 259	Takarina *et al.* (2004)
Singapore estuary	-	100 – 550	Sin *et al.* (1991)
Singapore coral reefs	-	58 – 95	Flammang *et al.* (1997)
Jurujuba Sound, Brazil	-	158	Baptista *et al.* (2000)
Java Sea, Indonesia	-	33 – 192	Evaraarts (1989)
Mai Po, Hong Kong	65.3 – 66.0	277.2 – 321.2	Ong Che (1999)
Deep bay, Hong Kong	30	240	Tam and Wong (2000)
South China Sea	-	12.5 – 49.9	Shazili *et al.* (1987)
Malaysian Studies			
Port Klang, Selangor	-	11.0 – 66	Ismail *et al.* (1989)
West coast Peninsular Malaysia	-	50 – 1400	Ismail *et al.* (1993)
Langat River -	71.0 – 374		Sarmani (1989)
Sepang Besar River	-	4.0 – 550	Ismail and Rosniza (1997)
Urban Lake of Kelana Jaya	-	34.3 – 529	Ismail *et al.* (2004)
Offshore of west coast Malaysia	-	4.00 – 79.05	Yap *et al.* (2003)
Intertidal of west coast Malaysia	-	3.12 – 306.20	Yap *et al.* (2003)
Sediments in Selangor (11 sites)	15.1 to 121	50.2 to 336	This study

anthropogenic sources from the effluents of the metal factory in the vicinity.

The overall concentrations of four geochemical fractions of Ni and Zn in the surface sediments are shown in Table 4. For the four geochemical fractions, the abundance of metal concentrations follow Zn> Ni. This disagrees with the fact that Ni is ranked 23rd [with an average concentration of 75 µg/g] while Zn as 24th [with an average concentration of 70 µg/g] most abundant element in the earth's crust (James, 1991). However, based on the present finding the concentrations of Zn is significantly higher than Ni, indicating anthropogenic input of Zn into the aquatic environment of the study sites.

Geochemical Fractions of Heavy Metal Concentrations in Sediments

Comparisons of the metal concentrations in the EFLE, acid-reducible, oxidisable-organic and resistant fractions among the sampling sites are shown in *Figs. 3* and *4*. The percentages of all fractions for each site are shown in Table 5.

Fig. 3 shows the concentrations of the Ni and Zn released in EFLE fraction and 'acid-reducible' fraction. The EFLE fraction contributed only a small portion (0–12.18%) of the total Ni and Zn in the sediments of the study areas. This clear pattern shows that JP Metal had the highest concentrations of EFLE Ni (3.59 µg/g dw) and EFLE Zn (59.96 µg/g dw). 'Acid-reducible' fraction contributed about 0.4 – 12.1% of the total concentrations of Ni and Zn in the sediments of all the sampling sites. A high concentration of Ni in the acid-reducible fraction was recorded in Jln Renggam (11.55 µg/g dw) and a high Zn concentrations (70.66 µg/g dw) was found at Sg. Kapar.

Fig. 4 shows the concentrations of the Ni and Zn released in 'oxidisable-organic' and 'resistant' fractions. 'Oxidisable-organic' fraction contributed 3.4 – 65.7% and covers the metals which are organically bound, and which are released when oxidised by, for example, peroxides. A clear pattern was shown by the Ni concentrations in which JP Metal site was found to have the highest 'oxidisable-organic' Ni with 51.70 µg/g dw. However, there was no clear pattern for Zn concentrations in the 'oxidisable-organic fraction'. The 'resistant' fraction contributed the largest ranges (0.6 –73.5%) of Ni and Zn in the sediments of the study areas and
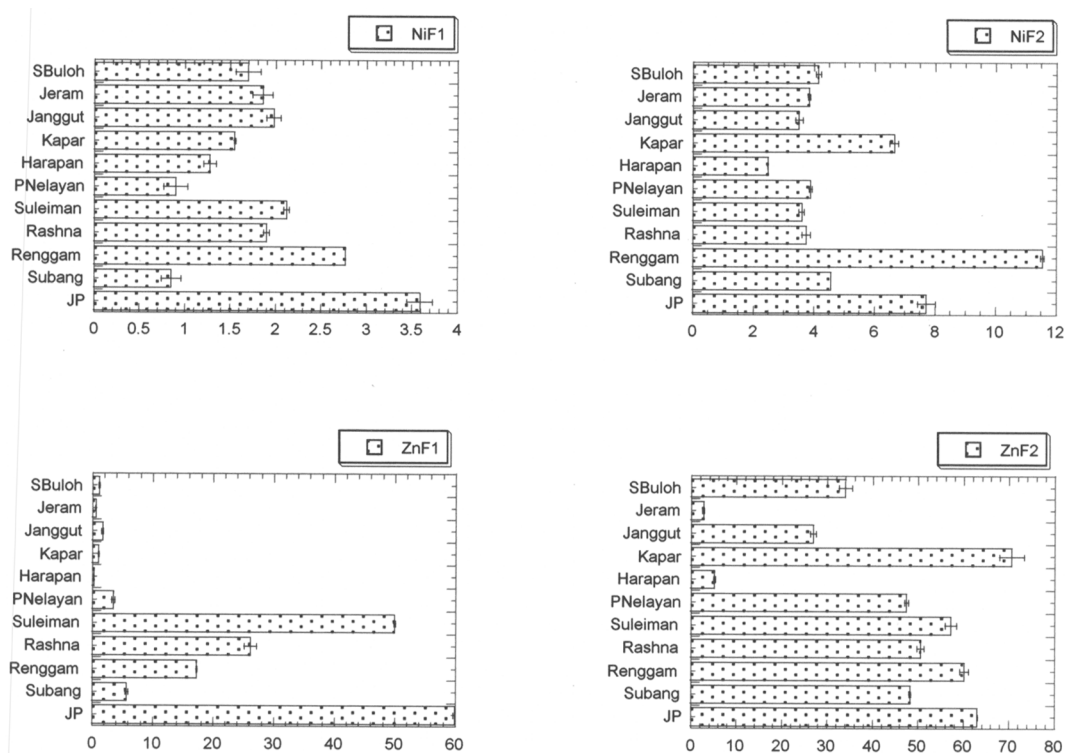
Fig. 3: Ni and Zn concentrations (mean mg/g ± SE dry weight) of the EFLE (F1) and acid-reducible (F2) fractions in the surface sediments collected from Tanjung Harapan to Sungai Buloh, based on sequential extraction technique
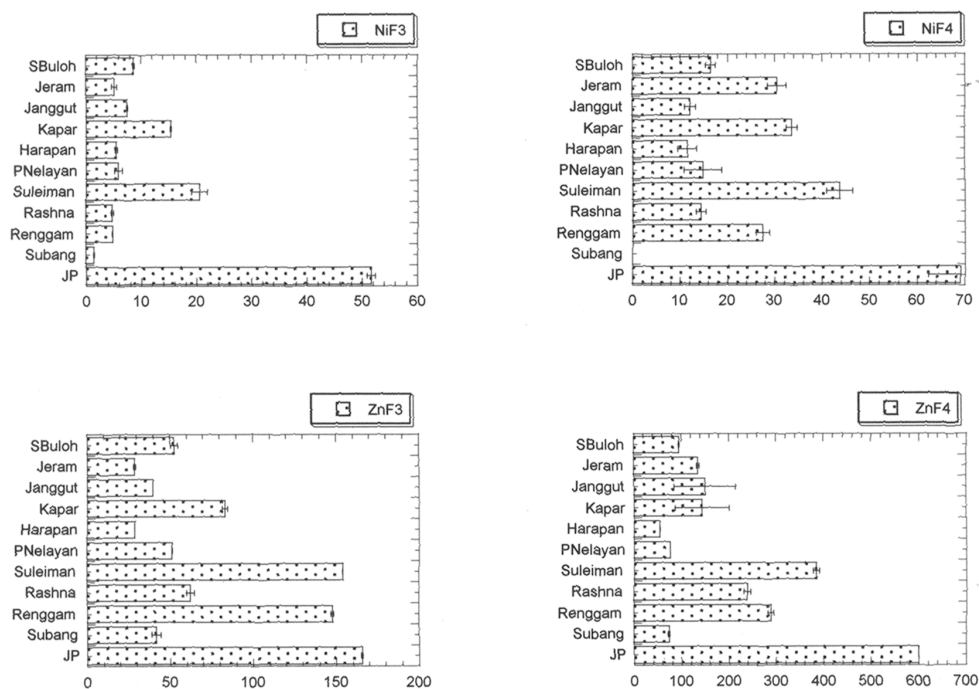

Fig. 4: Ni and Zn concentrations (mean mg/g ± SE dry weight) of the oxidisable-organic fraction (F3) and resistant (F4) fractions in the surface sediments collected from Tanjung Harapan to Sg Buloh, based on sequential extraction technique

TABLE 4

Overall concentrations (μg/g dry weight) of Ni and Zn in the surface sediments collected
from urban drainages and intertidal areas of Selangor

		Minimum	Maximum	Mean	Std Error
Ni	Total	15.09	120.85	40.88	10.70
	F1	0.85	3.59	1.86	0.24
	F2	2.49	11.55	5.07	0.79
	F3	1.52	51.70	11.97	4.30
	F4	0.04	69.12	24.95	5.76
Zn	Total	59.89	344.39	197.62	30.85
	F1	0.26	59.96	15.21	6.45
	F2	2.75	70.66	42.41	6.85
	F3	28.68	165.73	77.83	15.88
	F4	29.68	793.71	165.99	67.31

F1= EFLE, F2= acid-reducible, F3= oxidisable-organic and F4= resistant. Total= based direct aqua-regia method. (N = 11)

TABLE 5

Percentages of four geochemical fractions of Zn and Ni in the sediments collected
from Selangor

No	Ni%	F1	F2	F3	F4
1.	JP	2.7	5.9	39.3	52.1
2.	Subang	12.1	65.7	21.7	0.6
3.	Renggam	6.0	24.8	10.5	58.8
4.	Rashna	7.7	15.1	19.4	57.8
5.	Suleiman	3.1	5.2	29.4	62.4
6.	PNelayan	4.0	16.5	23.6	55.9
7.	Harapan	6.2	12.0	26.8	55.0
8.	Kapar	2.7	11.6	26.9	58.8
9.	Janggut	7.9	14.1	29.7	48.4
10.	Jeram	4.5	9.3	12.7	73.5
11.	SBuloh	5.5	13.4	28.1	53.0

No	Zn%	F1	F2	F3	F4
1.	JP	5.5	5.8	15.3	73.3
2.	Subang	3.8	32.6	27.8	35.7
3.	Renggam	4.1	14.2	34.9	46.9
4.	Rashna	8.4	16.2	20.0	55.4
5.	Suleiman	9.3	10.6	28.7	51.4
6.	PNelayan	2.4	32.1	34.8	30.7
7.	Harapan	0.4	8.2	45.0	46.5
8.	Kapar	0.4	26.3	30.9	42.4
9.	Janggut	1.5	23.6	34.7	40.3
10.	Jeram	0.6	3.4	35.2	60.7
11.	SBuloh	0.9	25.9	39.9	33.3

Note: F1= EFLE, F2= acid-reducible, F3= oxidisable-organic and F4= resistant

presumably there were those strongly trapped within the silicate minerals (Badri and Aston, 1983). In this fraction, JP Metal site showed the highest Ni and Zn concentrations which were 69.1 μg/g dw and 343.0 μg/g dw, respectively.

The Spearman's rank correlation coefficients among the four SET fractions and total concentrations of each metal are shown in Tables 6 and 7. For Ni (Table 6), all the 15 pairwises were significantly correlated (R = 0.62-0.98,

TABLE 6

Spearman's correlation coefficients among the geochemical fractions of Ni in the
sediments from some places in Selangor

	F1	F2	F3	F4	Sum	Total
F1	1.00	0.17^{ns}	0.39^{ns}	0.62	0.66	0.66
F2		1.00	0.05^{ns}	0.42^{ns}	0.46^{ns}	0.68
F3			1.00	0.70	0.74	0.61
F4				1.00	0.98	0.86
Sum					1.00	0.92
Total						1.00

Note: F1= EFLE, F2= acid-reducible, F3= oxidisable-organic, F4= resistant and ns=not significant ($P>0.05$). Total= based direct aqua-regia method. SUM= summation of all the 4 fractions based on sequential extraction technique.

TABLE 7

Spearman's correlation coefficients among the geochemical fractions of Zn in
the sediments from some places in Selangor

	F1	F2	F3	F4	Sum	Total
F1	1.00	0.63	0.77	0.68	0.86	0.88
F2		1.00	0.90	0.68	0.86	0.88
F3			1.00	0.83	0.97	0.96
F4				1.00	0.86	0.84
Sum					1.00	0.99
Total						1.00

Note: F1= EFLE, F2= acid-reducible, F3= oxidisable-organic, F4= resistant and ns=not significant ($P>0.05$). Total= based direct aqua-regia method. SUM= summation of all the 4 fractions based on sequential extraction technique.

$P<0.05$) except for 5 pairwises ($R = 0.05$-0.46, $P>0.05$). For Zn (Table 7), all the pairwises were significantly correlated ($R = 0.63$-0.99, $P<0.05$).

Only small portions of Ni and Zn from the total concentrations in the sediments from all the sampling sites were contributed by the EFLE fraction. This fraction was the most available fraction since it could be released from the soil even at pH 7. The low EFLE fraction indicated that heavy metals in the sediments were not easily leached out by water. This fraction might be a model for "bioavailability" to sediment ingesting animals (Yap *et al.*, 2002a). However, the high concentrations of Ni and Zn found in the EFLE fractions at some sites indicated the potential harmful effects that can be posed directly to the living organisms.

As for the 'acid-reducible' fraction, its contribution was the second lowest after the EFLE fraction in the sediment of the sampling sites. This fraction which may include metals associated with manganese and iron dioxides and hydroxides and possibly also with carbonates (Yap *et al.*, 2002b), had been proven to be sensitive to anthropogenic inputs (Modak *et al.*, 1992; Singh *et al.*, 2005). The low percentages of these fractions indicated that the affinities for this fraction in the sediment of the study areas were not high.

The 'oxidisable-organic' fraction usually contributed the highest percentage among the three anthropogenic fractions. The final fraction; the resistant fraction usually contributed the largest portion of the total concentrations of metals among the fractions. Metals in this form are not soluble under experimental conditions and may therefore be considered as being tightly bound and are highly associated with natural origins (Badri and Aston, 1983).

The high percentage in the nonresistant fraction of the total concentration of Ni in the sediment of Subang Utama (99.43%) was recorded while only 51.37% of the total Ni was accumulated in the nonresistant fraction of the

TABLE 8

Comparison of Ni and Zn concentrations (mg/g dry weight) in intertidal
sediments with established Sediment Quality Criteria

Source		Ni	Zn	References
1. Hong Kong Sediment Quality Criteria.	Action level	40	200	Lau Wong and Rootham, 1993
2. Interim Sediment Quality Values (ISQVs) for Hong Kong.	ISQVs-low	40	200	Chapman *et al.*, 1999
3. Interim Sediment Quality Values (ISQVs) for Hong Kong.	ISQVs-high	NA	410	Chapman *et al.*, 1999
4. Interim freshwater sediment quality guidelines for Canada.	-	NA	123	CCME, 2002
Aquatic sediments in Selangor, Peninsular Malaysia (11 sites)		15.1 to 121	50.2 to 336	This study

sediment of Sg Janggut. The high percentage of Ni in the nonresistant fraction of Subang Utama may be due to the effluents of factories along the river bank of the Klang River. Sari and Cagatay (2001) reported that the high Ni values in the northwest of the Gulf of Saros were due to the industrial discharges delivered by the Meric River.

Nine out of the eleven stations of the study areas had high percentages of Zn in the nonresistant fraction. Peng. Nelayan, for example, showed a percentage of 70.2% from the total concentration of Zn in the sediment.

The clear pattern in the distribution of Ni and Zn in the various fractions at the 11 sampling sites could be because some (6 sites) were intertidal sediments while others (5 sites) were drainage sediments.

Since the Malaysian Interim Sediment Quality Guideline is not available, comparison of the present data with the established Sediment Quality Criteria found in the literature is presented in Table 8. The highest concentrations of Ni and Zn (at JP Metal) were found to be higher than the Action Levels of these metals established by the Hong Kong Sediment Quality Criteria (Lau Wong and Rootham, 1993) and the Interim Sediment Quality Values-low (ISQVs-low) for Hong Kong (Chapman *et al.*, 1999). In addition, comparisons with the Interim Freshwater Sediment Quality Guidelines for Canada (CCME, 2002), showed that there are 6 sampling sites (*Fig. 2*) with Zn concentrations higher than the established Zn value. All of the above comparisons indicated that these elevated metal concentrations are most likely resulting from contributions by nearby industrial activities especially at the JP Metal site.

CONCLUSIONS

The highest total concentrations of Ni and Zn in sediments were found at the JP Metal site. It was found to have the highest Ni concentrations with 120.85 µg/g dry weight which could potentially pollute the nearby river, Sg. Kuyoh. All the metal concentrations in the sediments obtained from this study were mostly higher when compared to values from previous studies. From the geochemical study of heavy metals, the results show that the non-resistant fraction dominated the total Zn. These metal fractions contributed more than 50% of the total Zn concentration in most of the study areas. It is suggested that a treatment plant should be established especially at JP Metal site in the Seri Serdang Industrial area.

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The Nucleocapsid Protein of Newcastle Disease Virus Promotes Solubility of the VP2 Hypervariable Region of Infectious Bursal Disease Virus in *Escherichia coli*

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ABSTRACT

The hypervariable region (HVR) of VP2 protein of infectious bursal disease virus (IBDV) elicits neutralising antibodies, but it is highly hydrophobic and tends to form inclusion bodies when expressed in *Escherichia coli*. To improve its solubility, the VP2(HVR) was fused to the C-terminal end of Newcastle disease virus (NDV) nucleocapsid (NP) protein and expressed in *E. coli* TOP 10 cells under the control of *trc* promoter. However, the fusion protein, NP-VP2(HVR)-trc, aggregated into insoluble inclusion bodies in the host cells. Therefore the coding region of NP-VP2(HVR) was sub-cloned into expression vectors containing the T7 promoter. The solubility of the NP-VP2(HVR)-$_{T7}$ fusion proteins improved dramatically in *E. coli* BL21 (DE3), BL21 (SI) and Origami B cells.

Keywords: Newcastle disease virus, infectious bursal disease virus, VP2 solubility, hypervariable region

INTRODUCTION

The nucleocapsid (NP) protein of Newcastle disease virus (NDV) is the most abundant protein in the viral structure (Yusoff and Tan, 2001). It has a calculated molecular mass of approximately 53 kDa (Kho *et al.*, 2001) and it interacts with the viral large (L) and phospho. (P) proteins as well as the viral RNA to form a herringbone-like structure (Compans and Choppin, 1967). The recombinant NP protein in the absence of other viral proteins also assembles into a herringbone-like structure when expressed in *E. coli* (Kho *et al.,* 2001) and baculovirus (Errington and Emmerson, 1997) systems. An NP fusion protein harbouring the *myc* epitope and six histidine residues at its C-terminal end were shown to assemble into ring-like and herringbone-like particles with these extra sequences exposed on the surface of the ring-like particles (Kho *et al.*,

2001). This suggests that the NP protein can be used as a carrier for presenting foreign epitopes (Kho *et al.*, 2001; Yusoff and Tan, 2001).

Infectious bursal disease virus (IBDV) is the etiological agent for infectious bursal disease (IBD). It is a member of the genus *Avibirnavirus* of the family *Birnaviridae*, which causes an immunosuppression in young chickens (Müller *et al.*, 2003). IBDV has five proteins, VP1, VP2, VP3, VP4 and VP5 (Fahey *et al.*, 1985). The VP2 protein is the major immunodominant protein which is responsible for the induction of virus-neutralizing antibodies (Azad *et al.*, 1987; Becht *et al.*, 1988). The neutralizing monoclonal antibodies (Mabs) produced against VP2 proteins have been shown to bind to the conformational dependent epitopes (Bayliss *et al.*, 1990; Fahey *et al.*, 1989) within residues 206-350 (Azad *et al.*, 1987; Heine *et al.*, 1991) in a region known as

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the VP2 hypervariable region (HVR). Moreover, the VP2(HVR) has also been demonstrated to contain amino acids important for virulence (Boot *et al.*, 2000; Brandt *et al.*, 2001) and antigenicity (Heine *et al.*, 1991). Therefore, it has the potential to be used in the development of a recombinant vaccine. Nevertheless, the expression of full length VP2 has been particularly difficult because of the highly hydrophobic protective epitope within the VP2(HVR) (Öppling, 1991) and its tendency to form inclusion bodies in *E. coli* (Azad *et al.*, 1987).

The fusion system is a common approach to address the solubility problems of recombinant proteins by covalently attaching the target protein to a highly soluble carrier protein (Sorensen and Mortensen, 2005). Peptide carriers or fusion partners such as thioredoxin (Trx) (Pryor and Leiting, 1997), glutathione S-transferase (GST) (Nygren *et al.*, 1994), protein A (Samuelsson *et al.*, 1994), disulfide oxidoreductase (DsbA) (Collins-Racie *et al.*, 1995), maltose-binding protein (MBP) (Pryor and Leiting, 1997), calmodulin-binding protein (Zheng *et al.*, 1997) and transcription anti-termination factor (Nus•A) (Makrides, 1996) have been successfully developed for producing soluble heterologous proteins in *E. coli*. To investigate the potential of NDV NP protein as a fusion partner, the VP2(HVR) of IBDV was fused to the C-terminal end of the NP protein. The yield and solubility of the fusion proteins were studied.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacteria strains used were *E. coli* TOP 10 [F-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac* X74*rec*A1*deo*R*ara*D139Δ (*ara-leu*)7697*gal*U*gal* K*rsp*L(Str^R) *end*A1*nup*G] (Invitrogen, USA) harbouring plasmid pTrcHis2-NP which directs the synthesis of the NP protein was as described (Kho *et al.*, 2001), BL21 (DE3) [F-*omp*T*hsd*S$_s$(r$_B^-$ m$_b^-$) *galdcm*(DE3)] (Novagen, USA), BL21 (SI) [F-*omp*T*hsd*S$_s$(r$_{s-}$ r$_{ns-}$) *galdcm*] (Invitrogen, USA) and Origami B [F-ompT*hsd*S$_B$(r$_B$-m$_B$-) *galdcmlacY1ahpC gor5 22*::Tn*10*(Tc^R)*trxB*::kan] (Novagen, USA). Plasmid pCR 2.1-VP2 containing 1.35 kb VP2 gene of very virulent (vv) IBDV strain UPM 97/61 was obtained from the Department of Veterinary Pathology and Microbiology, Universiti Putra Malaysia. Plasmids pTrcHis2, pRSETA, and pET-43.1(a), containing

the *trc* and T7 promoters, were supplied by Invitrogen (USA) and Novagen (USA).

Cloning and Construction of Recombinant Plasmids Containing VP2(HVR) of IBDV

The coding regions of VP2(HVR) and NP-VP2(HVR) were amplified by polymerase chain reaction (PCR). Two oligonucleotides used in PCR amplification of VP2(HVR) were designed based on the published nucleotide sequence of the VP2 gene of IBDV strains UPM 97/61 (GenBank accession no. AF247006): FVP2fl 5'-GGGCTCGACCCCA<u>GAATTC</u>GTAGCAACA-3' and RVP2fl 5'GAAGTTGCTCACCCCTACG<u>TACGTA</u> AC-3'. Primers used to amplify NP-VP2(HVR) coding region were designed: FNP 5'-TC<u>TGGATCC</u>ATGTCTTCCGTATTCGATG-3' and RVP2A 5'ATGATG<u>AAGCTT</u>GACCTAG GCGCTATT-3'. The underlined nucleotides represent the restriction sites of *Eco*RI (in FVP2fl), *Sna*BI (in RVP2fl), *Bam*HI (in FNP) and *Hin*dIII (in RVP2A) respectively.

Synthesis of the first strand cDNA was carried out in a reaction mixture (50 μl) containing each of the primers (1 μM), deoxynucleoside triphosphate (0.2 mM; Promega, USA), Pfu DNA polymerase (1.25 U; Fermentas, USA) and 1 x reaction buffer [200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM KCl, 100 mM (NH$_4$)$_2$SO$_4$, 20 mM MgSO$_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA]. The mixture was incubated at 94°C/ 1 min followed by 30 cycles of denaturation (94°C/1 min), annealing [57°C/45 s for VP2(HVR); 58°C/1 min for NP-VP2(HVR)] and extension (72°C/1 min) followed by a final extension step of 72°C/7 min. The PCR product VP2(HVR) and vector pTrcHis2-NP were purified from agarose gels, ligated to yield the recombinant plasmid pTrcHis2-NP-VP2(HVR) (~6.4 kb), and introduced into *E. coli* TOP 10. The amplified NP-VP2(HVR) was then sub-cloned into pRSETA and pET-43.1(a). The resulting recombinant plasmids encoding the NP-VP2(HVR) (~2 kb) were designated pRSETA-NP-VP2(HVR) (~4.9 kb) and pET-43.1(a)-NP-VP2(HVR) (~9.3 kb). The former was introduced into either *E. coli* strains BL21 (DE3), BL21 (SI), and the latter was introduced into Origami B cells. The entire NP gene fused with the VP2(HVR) so confirmed by PCR was then sequenced with CEQ DTCS kit and CEQ™ 8000 DNA sequencer (Beckman Coulter, USA).

Analysis of Protein Expression

The transformants carrying the recombinant plasmids were cultured in LB medium supplemented with ampicillin (50 µg/ml) at 30 or 37°C. When the cells reached OD_{600} of 0.6 to 0.8, the cultures were added with IPTG (1 mM) or NaCl (0.3 M). The cells pellets were subjected to SDS-PAGE. The proteins on the gels were then electrotransferred to nitrocellulose membranes and blocked with skim milk diluent blocking buffer (1:10 dilution in dH_2O; KPL, USA) for 1 h. Anti-NDV serum (1:5,000 dilution), anti-VP2 Mab/IBDV 3 (1:80,000 dilution), anti-IBDV serum (1:80,000 dilution), anti-*myc* Mab (1:2,000 dilution; Invitrogen, USA) or anti-His Mab (1:2,500 dilution; Invitrogen, USA) was added to the membrane and shaken for 1 h. After washing, alkaline phosphatase conjugated anti-chicken or anti-rabbit secondary antibody was added and left shaking for another 1 h. Finally, colour development was obtained using the chromogenic substrate mixture BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium) (Promega, USA). The appearance of the protein bands was compared with the appropriate protein markers.

Solubility Analysis of the VP2(HVR) Fusion Proteins

The overnight culture (2 ml) was added into prewarmed fresh LB broth (50 µl) containing ampicillin (50 µg/ml). After 5 h post-induction, the cells (10 ml culture) were pelleted by centrifugation at 3,000 xg for 5 min at 4°C and then resuspended in TEN buffer [0.1 M Tris-HCl (pH 8), 2 mM EDTA and 0.1 M NaCl; 0.5 ml]. The cells were lysed with lysozyme (5 mg/ml) and followed with sonication. The unlysed cells were removed by centrifugation at 3,000 xg for 5 min at 4°C. The protein was clarified and subjected to SDS-PAGE and Western blotting. Percentage of soluble VP2(HVR) fusion proteins were measured with the Quantity One® Quantitation software (BioRad, USA) as described in Tan *et al.* (2004).

RESULTS

Fig. 1 illustrates the VP2(HVR) fusion proteins encoded by recombinant plasmids pTrcHis2-NP-VP2(HVR), pRSETA-NP-VP2(HVR) and pET-43.1(a)-NP-VP2(HVR). Production of the fusion protein, NP-VP2(HVR)-$_{trc,}$ by plasmid pTrcHis2-NP-VP2(HVR) is directed by *trc* promoter. The NP-VP2(HVR)-$_{trc}$ fusion protein was expressed in *E. coli* TOP 10 as ~75 kDa. The Western blot analysis with chicken anti-NDV (*Fig. 2a,* lane 3) and rabbit anti-IBDV sera (*Fig. 2b,* lane 4) gave a positive signal at protein band of 75 kDa. The extra bands could be due to non-specific binding of these polyclonal antibodies to bacterial proteins (*Fig. 2a* and *2b*). The NP protein used as positive control gave rise to the expected band of ~57 kDa (*Fig. 2a* and 2b, lane 2). The protein bands with molecular masses smaller than the NP-VP2(HVR)-$_{trc}$ fusion protein and its derivative were also observed in bacterial lysates (*Fig. 2a,* lane 3; *Fig. 2b,* lane 4) which could be

a. NP-VP2(HVR)-$_{trc}$

NP	KGF	VP2(HVR)	V	*myc*	NSAVD	His

b. NP-VP2(HVR)-$_{T7}$

His	GMASMTGGQQMGR	Xpress™ Epitope	DRWGS	NP	KGF	VP2(HVR)

c. Nus-NP-VP2(HVR)-$_{T7}$

Nus	TSGS	His	SAG	S	PPPTGLVPRGSAGSGTIDDDDKSPGARGS	NP	KGF	VP2(HVR)	GRTAVYTC	HSV.Tag	SRA	His

Fig. 1: Schematic representation of the VP2(HVR) fusion proteins. (a) The NP-VP2(HVR)-$_{trc}$ fusion protein expressed in E. coli *strain TOP 10 under the control of the trc promoter; (b) The NP-VP2(HVR)-$_{T7}$ fusion protein produced in* E. coli *strains BL21 (DE3) and BL21 (SI) under the control of T7 promoter; (c) The Nus-NP-VP2(HVR)-$_{T7}$ fusion protein produced in* E. coli *strain Origami B under the control of T7 promoter. The amino acid sequences of the linkers are shown*

a
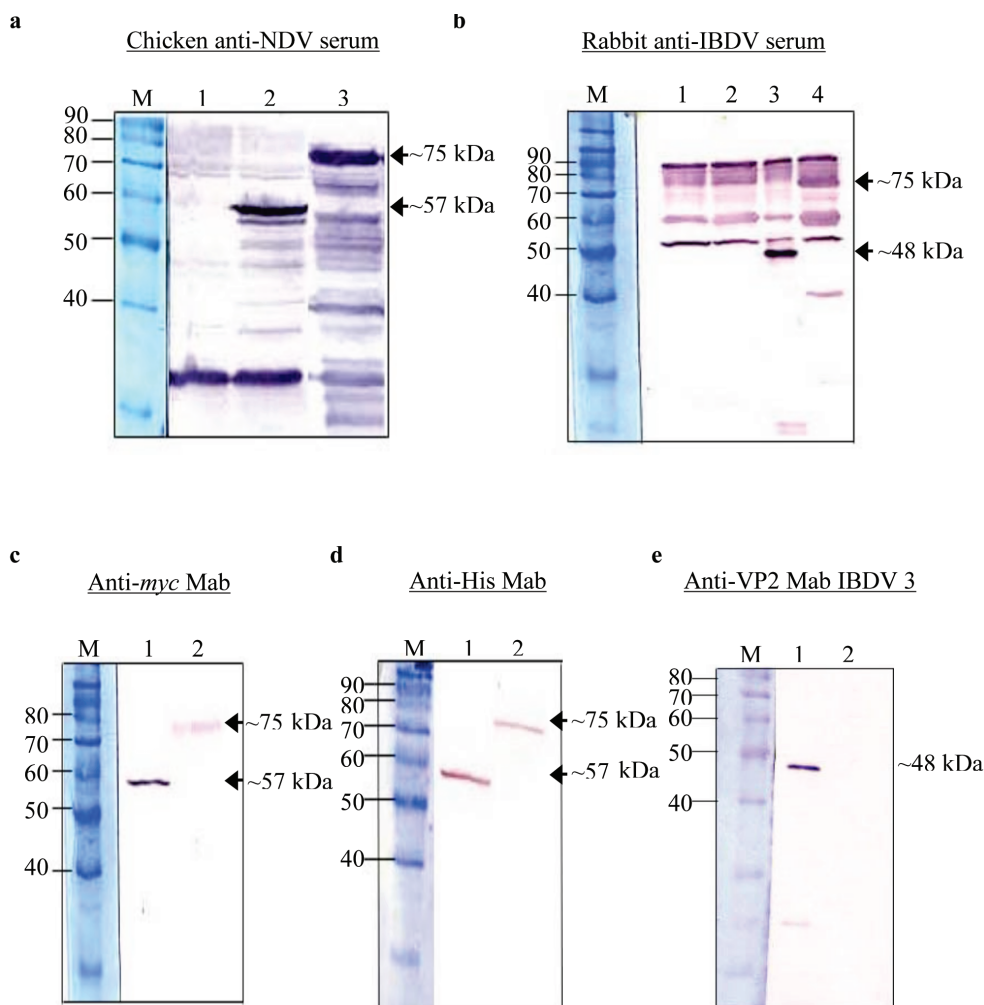Chicken anti-NDV serum

b
Rabbit anti-IBDV serum
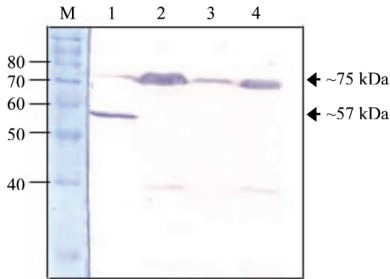

Fig. 2: Western blots of NP-VP2(HVR)-$_{trc}$ fusion protein in E. coli TOP 10 cells harbouring plasmid pTrcHis2-NP-VP2(HVR). The proteins were fractionated on 12% SDS-PAGE and Western blotted against the respective serum and Mabs. (a) Lanes: M, molecular weight markers in kDa; 1, negative control [E. coli TOP 10 cells]; 2, NP protein; 3, VP2(HVR) fusion protein. (b) Lanes: 1, E. coli TOP 10 cells; 2, NP protein; 3, VP2 protein; 4, VP2(HVR) fusion protein. (c) Lanes: 1, NP protein; 2, VP2(HVR) fusion protein. (d) Lanes: 1, NP protein; 2, VP2(HVR) fusion protein. (e) Lanes: 1, VP2 protein; 2, VP2(HVR) fusion protein

due to partially degraded fusion proteins. A single band of NP-VP2(HVR)-$_{trc}$ fusion protein was also detected by the anti-*myc* (*Fig. 2c*, lane 2) and anti-His (*Fig. 2d*, lane 2) Mabs. This result shows that the monoclonal antibodies could specifically detect the *myc* and His epitopes fused to the C-terminus of the NP-VP2(HVR)-$_{trc}$ fusion protein. It demonstrates that the coding region of the VP2(HVR) was cloned in-frame with the *myc* and His-tag fusion in the recombinant plasmid. However, the anti-VP2 Mab IBDV 3 failed to react

with the expressed NP-VP2(HVR)-$_{trc}$ fusion protein (*Fig. 2e*, lane 2). This indicates that the antibody recognizes a conformational epitope (Egbert M., pers. comm., 2003).

Most of the NP-VP2(HVR)-$_{trc}$ fusion proteins produced in *E. coli* TOP 10 cells were found to be insoluble (90%) and accumulated as inclusion bodies (*Fig. 3*, lane 4). The predicted solubility of the NP-VP2(HVR)-$_{trc}$ fusion protein with the revised Wilkinson-Harrison solubility model (Davis *et al.*, 1999) is given in Table 1. In general,

Fig. 3: *Solubility analysis of the NP-VP2(HVR)-$_{trc}$ fusion protein in* E. coli *TOP 10 cells harbouring plasmid pTrcHis2-NP-VP2(HVR). The cells were grown to OD_{600} of 0.8 and induced with IPTG (1 mM). Cell cultures were collected after 5 hour of induction and the cell lysates were analysed by SDS-12% PAGE, electrotransferred to a nitrocellulose membrane and probed with the anti-myc Mab. Lanes: M, molecular weight markers in kDa; 1, NP protein; 2, total protein fraction; 3, soluble protein fraction; 4, insoluble protein fraction.*

Fig. 4: *Western blots of NP-VP2(HVR)-$_{T7}$ fusion protein in* E. coli *BL21 (DE3), BL21 (SI) and Nus-NP-VP2(HVR)-$_{T7}$ fusion protein in Origami B cells. The proteins were separated on 12% SDS-PAGE, electrotransferred to a nitrocellulose membrane and probed with chicken anti-NDV serum. (a) Lanes: M, molecular weight markers in kDa; 1, NP protein; 2, VP2(HVR) fusion protein in* E. coli *TOP 10 cells; 3, VP2(HVR) fusion protein in* E. coli *BL21 (DE3) cells; 4, VP2(HVR) fusion protein in* E. coli *BL21 (SI) cells. (b) Lanes: 1,* E. coli *Origami B cells; 2, NP protein; 3, negative control [VP2 protein]; 4, pET-43.1(a) in* E. coli *Origami B cells; 5, Nus-NP-VP2(HVR)-$_{T7}$ fusion protein in* E. coli *Origami B cells. Arrows indicate the expected protein bands*

the theoretical and experimental data showed that most of the fusion proteins produced in *E. coli* TOP 10 cells were insoluble.

In order to improve the solubility of the NP-VP2(HVR) fusion protein, the NP-VP2(HVR) DNA region was subsequently sub-cloned into plasmids pRSETA and pET-43.1(a). The PCR product of NP-VP2(HVR) (~2 kb) contains a *Bam*HI and *Hind*III cleavage sites at the 5' and 3' ends respectively. Plasmid pRSETA-NP-VP2(HVR) contains a T7 promoter which controls the synthesis of transcript for NP-VP2(HVR)-$_{T7}$ (*Fig. 1*). Plasmid pET-43.1(a)-NP-VP2(HVR) encodes the Nus and NP proteins at the N-terminal end of VP2(HVR) protein, namely Nus-NP-VP2(HVR)-$_{T7}$ (*Fig. 1*), also under the control of a T7 promoter. The NP-VP2(HVR)-$_{T7}$.fusion protein was expressed to its expected sizes of ~79 kDa in *E. coli* BL21 (DE3) and BL21 (SI) (*Fig. 4a*, lanes 3 and 4). Nus-NP-VP2(HVR)-$_{T7}$ fusion protein gave rise to a band of ~137 kDa in *E. coli* Origami B cells (*Fig. 4b*, lane 5).

The solubility of the NP-VP2(HVR)-$_{T7}$ fusion protein produced in *E. coli* strains BL21 (DE3) and BL21 (SI) under the control of the T7 promoter was about 80% (*Fig. 5a*, lanes 3 and 6). Almost all of the fusion protein, Nus-NP-VP2(HVR)-$_{T7}$, produced in Origami B cells was soluble (97%, *Fig. 5b*, lane 2). The result shows that the Nus and NP protein improved the solubility of VP2(HVR) remarkably. However,

the amount of NP-VP2(HVR)-$_{T7}$ and Nus-NP-VP2(HVR)-$_{T7}$ fusion proteins produced in these *E. coli* strains remained the same.

The solubility of the fusion proteins predicted by the Wilkinson-Harrison solubility model (Davis *et al.*, 1999) and that determined experimentally is summarized in Table 1. The predicted results for NP-VP2(HVR)-$_{trc}$ (*in E. coli* TOP 10) and Nus-NP-VP2(HVR)-$_{T7}$ (in E. coli Origami B) correlate well with the experimental data. However, the NP-VP2(HVR)-$_{T7}$ fusion proteins which were predicted to be highly insoluble (73%) turned out to be highly soluble (~80%) when expressed in *E. coli* BL21 (DE3) and BL21 (SI).

TABLE 1
Solubility analysis of the target, carrier and fusion proteins in *E. coli*

(a) Predicted solubility of target protein, VP2(HVR)

Target protein	Probability of solubility or insolubility[a]
VP2(HVR)	57% insoluble

(b) Comparison of the predicted solubility with experimental value

Carrier protein	Probability of solubility or insolubility[a]	Soluble protein[b] in *E. coli* strain
NP	72% insoluble	99% [TOP 10]

Fusion protein	Probability of solubility or insolubility[a]	Soluble protein[b] in different *E. coli* strains
NP-VP2(HVR)-trc	64% insoluble	10% [TOP 10]
NP-VP2(HVR)-T7	73% insoluble	81% [BL21(DE3)]
NP-VP2(HVR)-T7	73% insoluble	80% [BL21 (SI)]
Nus-NP-VP2(HVR)-T7	63% soluble	97% [Origami B]

[a]The revised Wilkinson-Harrison solubility model (Davies *et al.*, 1999) was used to predict the probability of solubility or insolubility of the proteins produced in *E. coli* cells.
[b]The percentage of soluble protein was determined by the Quantity One Quantitation software (BioRad, USA).
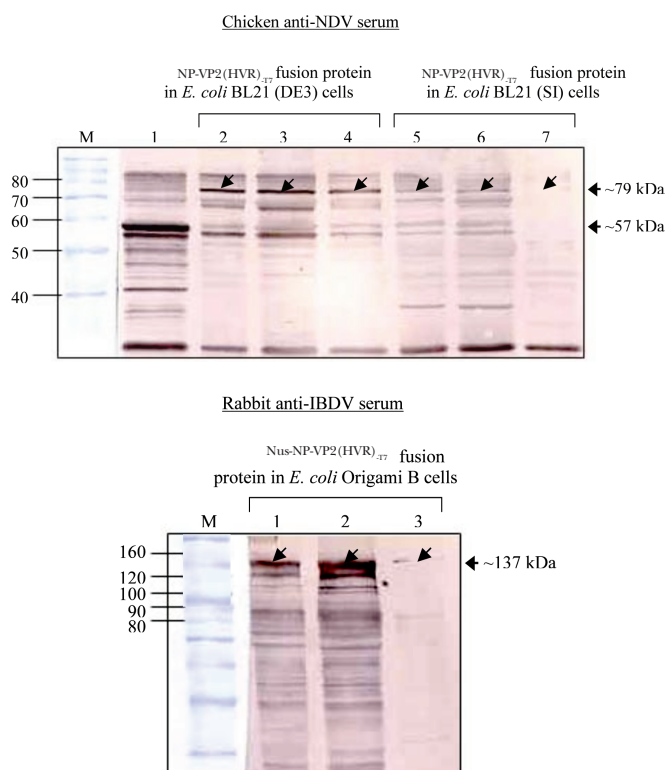
Fig. 5: *Solubility analysis of the NP-VP2(HVR)-T7 fusion protein in* E. coli *strains BL21 (DE3) and BL21 (SI) (a); and Nus-NP-VP2(HVR)-T7 fusion protein* E. coli *strain Origami B (b). The cells were grown to OD600 of 0.8 and induced with IPTG (1 mM) or NaCl (0.3 M). Cell lysates were analysed by SDS-12% PAGE, electrotransferred to a nitrocellulose membrane and probed with the respective sera. (a) Lanes: M, molecular weight markers in kDa; 1, NP protein; 2, total protein fraction; 3, soluble protein fraction; 4, insoluble protein fraction; 5, total protein fraction; 6, soluble protein fraction; 7, insoluble protein fraction. (b) Lanes: 1, total protein fraction; 2, soluble protein fraction; 3, insoluble protein fraction. Arrows indicate the expected protein bands*

DISCUSSION

A wide range of protein fusion partners has been developed in order to simplify the expression of recombinant proteins. Fusion proteins which include a partner or tag linked to the target protein can be purified easily by specific affinity purification strategies (Terpe, 2003). However, most protein designers have incorporated the fusion partners into their recombinant proteins to improve the solubility (Davis *et al.*, 1999; Makrides, 1996). Wilkinson and Harrison proposed a model for the theoretical calculation of solubility percentages of recombinant proteins expressed in *E. coli* cytoplasm (Wilkinson and Harrison, 1991). Although many proteins are highly soluble, they are not all effective as solubility enhancers. Therefore, this study is of importance for protein engineering as it explores the potential of NP protein of NDV in enhancing the solubility of VP2(HVR) protein of IBDV. The ability of the NP protein to confer solubility on the insoluble protein provides further insight into its application as solubility enhancer as well as a general carrier for viral antigen.

Each of the bacterial clones containing the respective recombinant plasmids was able to express the VP2(HVR) fusion protein. However, no band was observed on the Western blot when the fusion proteins probed with anti-VP2 Mab IBDV 3. This might be due to the specificity of the epitope on VP2 Mab used (Egbert M. pers. comm., 2003). In addition, Mab IBDV 3 is probably very specific to the virus isolate used in the development of the hybridoma and not to the hypervariable region of the VP2 protein (Becht *et al.*, 1988; Heine *et al.*, 1991).

Since polyclonal anti-sera, in general, can bind to many epitopes of a given antigen, it was no surprise that the VP2(HVR) fusion proteins expressed in *E. coli* TOP 10, BL21 (DE3), BL21 (SI) and Origami B interacted well with the rabbit anti-IBDV serum used in this study. Öppling *et al.* (1991) and Schnitzler *et al.* (1993) showed that all of the Mab-escaped IBDV mutants which were resistant to neutralization by the specific Mabs were still neutralized efficiently by vaccinated or convalescent chicken sera or mouse and rabbit hyperimmune sera.

Rabu *et al.* (2002) have shown that the level of the expressed NP fusion protein carrying the HN and F proteins of NDV was relatively high compared to the fusion proteins made in this study. The low expression level might be due to the size of the insert, which is about twice the size (the largest HN fusion protein has 96 amino acids) used by Rabu *et al.* (2002). For the proteins displayed on hepatitis B virus capsid, the insertion capacity of small peptides appeared to be limited to their inability to disrupt the folding of the core protein (Kratz *et al.*, 1999). Similarly, the nucleocapsid (N) of measles virus (MV) could not be assembled in *E. coli* when it was fused to either β-galactosidase or the maltose-binding protein (MBP) (Warnes *et al.*, 1995).

The experimental data of NP-VP2(HVR)$_{-T7}$ produced in *E. coli* BL21 (DE3) and BL21 (SI) revealed that the soluble proteins were much higher than the predicted solubility. The difference suggests that the cellular environment in which the proteins are synthesized is extremely complex compared to that of the predicted data. The approximate charge average should be slightly more electropositive than the actual charge due to different pH of the media used (7.5-7.9) (Wilkinson and Harrison, 1991).

About 90% of the VP2(HVR) fusion protein expressed in *E. coli* Top 10 were insoluble. This is probably due to the sub-optimal redox conditions, differences in the cell culture or an inadequate folding machinery of the host cell which resulted in the formation of inclusion bodies (Baneyx, 1999; Miroux, 1996). However, the amount of the soluble VP2(HVR) fusion proteins increased dramatically in *E. coli* BL21 (DE3), BL21 (SI) and Origami B. This result indicates that the protease-deficient host strains [which lack of the outer membrane protease (*OmpT*)] could improve the solubility of the fusion protein as they are less prone to form inclusion bodies within the cells.

The highest amount of the soluble Nus-NP-VP2(HVR)$_{-T7}$ fusion protein in *E. coli* Origami B (97%) might be due to the co-expression of Nus•A-tag and NDV NP proteins. The Nu•A protein (55 kDa) has been successfully exploited for its intrinsic solubility (Davis *et al.*, 1999; Wilkinson and Harrison, 1991). VP2(HVR) protein contains five cysteine residues and some of these cysteines may form disulfide bonds. The *E. coli* Origami B host strain that carries the *trxB/gor* mutations is able to facilitate disulfide bond formation in the cytoplasm and further improve the solubility (Sorensen and Mortensen, 2005).

CONCLUSIONS

The NP protein of NDV is able to increase the solubility of VP2(HVR) protein through the application of tightly regulated T7 promoter and introduction of the recombinant plasmid into protease-deficient host strains. Although the Nus•A protein has sufficiently improved the VP2(HVR) solubility in *E. coli* Origami B, the potential of the NP protein as a fusion partner to enhance the solubility cannot be ruled out as the increase in the solubility was still significant in the absence of Nus•A. Therefore, the ability of NP protein in improving the solubility of VP2(HVR) fusion protein could represent another means to produce soluble proteins in *E. coli*.

ACKNOWLEDGEMENTS

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Cloning of a Near Complete *Isochorismate synthase* (ICS) cDNA from *Morinda citrifolia* L.

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ABSTRACT

Isochorismate synthase (ICS) is a key enzyme that catalyses the conversion of chorismate to isochorismate which is then channelled to other secondary product such as the anthraquinones. A near complete cDNA was isolated through RT-PCR technique. Characterization of this gene is important in characterising its role in the production of anthraquinones in the Rubiaceae plant family. Anthraquinones are known for their medicinal properties and can be found in Rubiaceae especially in roots. In this study, total RNA was extracted from roots of 'mengkudu' using modified CTAB method. The total RNA was subjected to first strand synthesis using oligo-dT$_{18}$ primer and M-MuLV reverse transcriptase. Subsequently, PCR technique using primers designed from conserved ICS domains from other plants were used to isolate an internal conservative region of 426 bp. The cDNA was subsequently sequenced and verified using BLASTn program through the NCBI Genebank database, which showed a high sequence identity (72%) to the ICS from *Catharanthus roseus*. Based on this sequence, 3'RACE was performed to obtain the 3'-end of the gene and a 1036 bp 3'-fragment was generated. Apart from that, another PCR managed to generate a fragment of 491 bp upstream of the cDNA. Both fragments were sequenced and verified. Contig analyses and assembly of the partial cDNAs generated showed a near complete cDNA of 1872 bp. Sequence analysis of this partial cDNA showed a high degree of identity with ICS cDNA from other plants with the highest identity of 72% with ICS from *C. roseus*. Deduced amino acid showed a high similarity with *Rubia cardifolia* ICS of 85%.

Keywords: *Isochorismate synthase*, Rubiaceae, 'mengkudu', anthraquinones, PCR, 3'RACE

INTRODUCTION

'Mengkudu' or *Morinda citrifolia* is a plant belonging to the family of Rubiaceae. The plant has a height of between 3 and 12 m, the leaves are oval shaped and the fruit is fleshy, bumpy-structured, green in colour and changing to translucent upon ripening and produces odour. 'Mengkudu' is used in many cultures because of its medicinal properties for both traditional and modern medicinal purposes. Various parts of the plant are used such as the juice of the fruit and leaves (for arthritis, asthma, lumbago, regulate menstrual flow, heated leaves are used to relieve coughs, nausea, and colic); flowers, roots and bark (to treat eye problems, skin wounds, throat problems, respiratory ailments, constipation, stomach pains).

Modern research in 'mengkudu' is focussed in the application of the secondary products activity for various illnesses. Research on 'mengkudu' is mainly focussed on the therapeutic properties of its secondary products. 'Mengkudu' properties that have been reported include; analgesic and sedative properties (Younos *et al.*, 1990); anticancer properties (Hirazumi *et al.*, 1994); inhibitory properties against virus early-antigen activation (Hiramatsu *et al.*, 1993). Bioactive compounds from 'mengkudu' in the form of extracts have also been used to search for anti–malarial (Ancolio *et al.*, 2002; Tona *et al.*, 2001), anti-microbial (Jayasinghe *et al.*, 2002), anti-inflammatory (McKoy *et al.*, 2002) and anti-diabetic properties (Olajide *et al.*, 1999). In addition, 'mengkudu' has potential use in treating

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hyperkalemia (Mueller *et al.*, 2000) and cancer preventive treatment (Wang and Su, 2001) including promotion of immune responses in cancer treatments (Wong, 2004).

Metabolism and production of anthraquinones in 'mengkudu', which is normally stored in the root of the plants is of particular interest (Stalman *et al.*, 2003). In the plant family Rubiaceae that include species such as *Morinda, Rubia, Cinchona* and *Galium* species, anthraquinones are considered to be synthesised from chorismate (Han *et al.*, 2001). In this study, a near complete cDNA encoding *isochorismate synthase* was isolated and characterised. The enzyme is in the shikimate pathway that is involved in the biosynthesis of both primary and secondary metabolites. The ICS is involved in the conversion of chorismate to isochorismate and subsequently the conversion to 2,3-dihydroxybenzoic acid, phylloquinones and anthraquinones (Poulsen *et al.*, 1991).

Until now, there is no any information about gene encoding ICS from Morinda in gene bank but limited information about *ICS* in others plants is available. Recently some *ICS* cDNA have been isolated from plant species such as *Arabidopsis thaliana* (AF078080), *Catharanthus roseus* (AJ006065), *Capsicum annuum* (AY743431), and partial sequences from *Nicotiana tabacum* (AY740529) and *Rubia cardifolia* (EF090619). Isolation of *ICS* cDNA will enable a better understanding of the role(s) of the enzyme in production of anthraquinones in 'mengkudu'.

MATERIALS AND METHODS

Plant Materials

Root samples of 'mengkudu' were collected from the UNIMAS Plant House. All the samples were washed with 70% ethanol then rinsed with distilled water before RNA isolation.

RNA Isolation

Total RNA from 'mengkudu' root tissues was extracted using a modified CTAB method described by Zeng and Yang (2002). The quality and quantity of the extracted RNA were verified by agarose gel electrophoresis and spectrophotometry.

First Strand cDNA Synthesis

Total RNA was treated with DNaseI (Fermentas) prior to cDNA synthesis. Five microlitres of DNaseI-treated total RNA was mixed with 1 µl oligo-dT$_{18}$ primer (100mM), distilled water was added to bring to a final volume of 13 µl and heated at 70°C for 5 min. The mixture was cooled on ice for 5 min, followed by addition of 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTPs mix and 1 µl of M-MuLV Reverse Transcriptase (Fermentas) (200 U/ µl). The reaction was conducted in a final volume of 20 µl. Reverse transcription was performed for 60 min at 42°C and the reaction was stopped by heating at 70°C for 10 min. The single stranded cDNA was stored at -20°C until further use.

Internal Conservative Fragment Cloning

PCR was carried out in a total volume of 25 µl containing 2.5 µl of 10X buffer, 2 µl of 25 mM MgCl$_2$ 2.5 µl of 2mM dNTP mix, 10 pmol forward primer, 10 pmol reverse primer, 1 U of *Taq* polymerase (Fermentas) and 0.5 µl of first strand cDNA product. The PCR amplification was performed using a Mastercycler Personal unit (Eppendorf) with an initial denaturation at 94°C for 3 min, 35 cycles of 30 s denaturation at 94°C, 45 s annealing 55°C and 1 min 45 s elongation at 72°C and a final elongation at 72°C of 7 min. PCR of cDNA internal sequence was conducted using primers ICSha-2F (5'-TGGTTCCTCAG GTTGAGTTTGAT-3') and ICSha-3R (5'-TCTGGAGTGTTTCCAATGAATGC-3'). All primers that were used in this work was designed from sequence identity between *A. thaliana* [AF078080] and *C. roseus* [AJ006065].

3'RACE-PCR

Based on the internal ICS sequence obtained, 3'RACE was performed according to Ambion RLM-RACE Kit. The gene specific primers used in the RACE were designed based on the internal sequence of ICS obtained from the Internal Conservative Fragment. A combination of primers; ICSha-1F:ICSha-2R, ICSha-1F:ICSha-3R and ICSha-5F:ICSha-5R, were used for the 3'RACE and the PCR was performed according to the manufacturer's instructions with some modifications. The list of primer sequences are presented in Table 1. First strand synthesis was carried out according to the protocol mentioned above but using 3'Adapter primer instead of oligo-dT.

TABLE 1
Listing of primer name and sequences used in the
PCR and 3'RACE

Primer name	Sequence 5' – 3'
ICSha1-F	GCATTGGCCATGGAACGTCT
ICSha2-R	ATCAAACTCAACCTGAGGAACCA
ICSha5-F	ACAGAACGACGTTGTCAGTGTT
ICSha5-R	AGGCTTCGTCATGTTCTCTTGT

Cloning PCR Product
PCR products were cloned into the pGEM-T
Vector (Promega). The positive clones were
screened via PCR using gene specific forward and
reverse primers. Clones corresponding to the
expected size were selected for DNA sequencing.
The clones were grown overnight and plasmid
isolation was performed according to the modified
methods from Birnboim and Doly (1979).

DNA Sequencing and Bioinformatics Analysis
DNA Sequencing service was obtained from First
BASE Laboratories Sdn Bhd. All sequencing
reactions were performed on double-stranded
plasmid DNA by using BigDye Ò Terminator v3.1
Sequencing Kit and analyzed on ABI PRISM Ò
377 Genetic Analyzer. BLASTn, ClustalW ver1.82
and ChromasPro programmes were used for
sequence analysis, editing and alignment.

RESULTS AND DISCUSSION
For cloning of ICS cDNA, a 2-step RT-PCR
strategy was used. The initial step taken was to
isolate the internal sequence by PCR
amplification using specific primers designed
based on the conserved region of complete
coding sequences of ICS gene from the gene
bank (*A. thaliana* [AF078080], *C. roseus*
[AJ006065]). Primers ICSha-2F and ICSha-3R
were used to isolate the internal sequence (*Fig.
1*) and 426 bp cDNA fragments (fragment B)
was obtained and sequenced. This sequence was
verified against published sequences in NCBI
GeneBank database using the BLASTn program.
This cDNA fragment showed a 72% sequence
identity to the *ICS* cDNA derived from *C. roseus*
(accession no. AJ006065). Apart from that,
another cDNA sequence of 490 bp (fragment A)
was isolated using primers ICSha-1F and ICSha-
2R. An 891 bp cDNA (fragment C) was amplified
using primer ICSha-1F and primer ICSha-3R.
The fragment C sequence confirmed the
sequence generated by fragment A and B, whilst
fragment D was PCR to confirm the sequences
between the junction of fragment A and B.

From this initial internal conservative
sequence (fragment B), ICS1 primer was
designed and used as forward primer to isolate
the 3' end. Subsequent 3'RACE-PCR managed
to produce a fragment with 1036 bp that includes
the poly(A) tail (Plate1, fragment E). Sequence
analysis of this fragment showed high identity
with *ICS* from *C. roseus* (72%), *C. annuum* (65%)
and *A. thaliana* (65%).

Contig assembly analyses of fragments A, B
and E, showed a near complete ICS cDNA of
1872 bp size. Sequence analysis of this partial
cDNA showed a high degree of identity with *ICS*
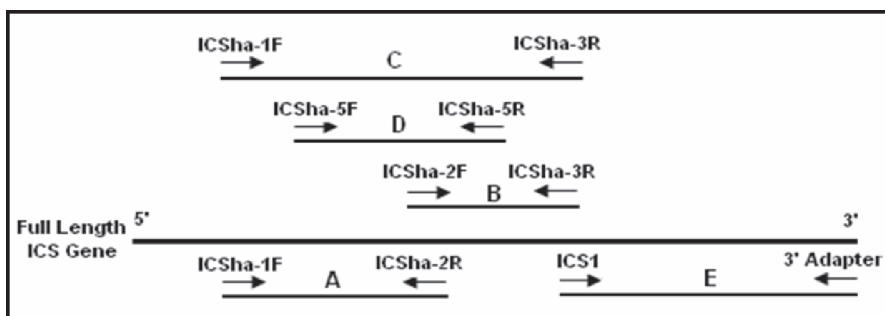open reading frame from other plant species.

*Fig. 1: Schematic representation of the cloning strategy of partial ICS cDNA by RT-PCR and 3'RACE. PCR was
undertaken using different combinations of primer sets (Fragment A to D) and
3'RACE-PCR were performed to obtain the partial cDNA (Fragment E)*

The highest identity was found to be 72% with ICS from *C. roseus*. Also ICS from *C. annuum* and *A. thaliana* were highly similar (67% and 62% respectively). A deduced amino acid sequence generated from the cDNA is also presented in *Fig. 2* and the percentage of amino acid similarity with other plant species is listed in Table 2 with the highest found to be with *R. cardifolia* (85%).
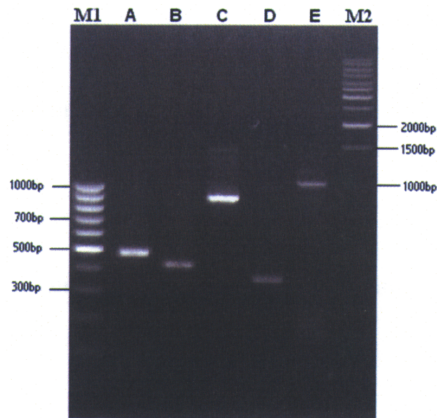

Plate 1: Amplification product of partial ICS cDNA via RT-PCR and 3'RACE. Lanes A to E represent the fragments labelled A to E in Fig. 1. M1 is the 100bp DNA ladder and M2 is 1kb DNA ladder (Fermentas)

Fig. 2: Predicted amino acid sequence from partial ICS cDNA of M. citrifolia. *Asterix indicates possible termination codon for the deduced amino acid sequence*

TABLE 2
Sequence similarity of predicted amino acid from partial *ICS* cDNA of
M. citrifolia with *ICS* from other plant species

Species	Length of deduced amino acid	% similarity	Accession no.
Catharanthus roseus	580	72	CAA06837
Capsicum annuum	576	66	AAW66457
Arabidopsis thaliana	622	59	NP_974143
Rubia cardifolia	252	85	ABK79678
Nicotiana tabacum	302	66	AAW67000

CONCLUSIONS

A near complete cDNA encoding *isochorismate synthase* have been isolated from *Morinda citrifolia.* The sequence shows a high sequence identity to the *ICS* cDNA from other higher plants (75% of nucleotide identity and 85% amino acid similarity to *C. roseus* and *R. cardifolia* respectively). A 5'RACE-PCR is currently in progress in order to get the full-length sequence of the gene.

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Isolation of Transcripts Related to Floral Scent Biosynthesis from Cempaka Putih (*Michelia alba*) Flower Using Subtractive Hybridization Approach

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ABSTRACT

Floral scent plays an important role in the reproductive processes of many plants and contributes a considerable economic value in guaranteeing yield and quality of many ornamental plants and cut flowers by enhancing their aesthetic properties. It is determined by a specific complex mixture of volatile low-molecular-mass molecules which fall into the terpenoid or phenylpropanoid/benzenoid classes of compounds. Although volatile compounds have been identified in several flower species, little is known about the enzymes and genes controlling the biochemical synthesis of floral scent production and the molecular mechanisms involved, which may differ from species to species. In this preliminary study, we have identified four genes associated directly with the monoterpene scent biosynthesis pathway in the local flower, Cempaka putih (*Michelia alba*) including geranyl diphosphate synthase, deoxyxylulose-5-phosphate synthase, cytochrome P450 and (+)-pulegone reductase, and two more, including benzoyl coenyme A: benzyl alcohol benzoyl transferase and salicyclic acid methyltransferase (SAMT) that might be involved with the phenylpropanoid/benzenoid scent pathway using subtractive hybridization. We have also identified four other genes that might be indirectly related to scent metabolism in this flower including lipoxygenase, peroxidase, heat shock protein and myb transcription factor.

Keywords: *Michelia alba*, monoterpene scent biosynthesis pathway, linalool biosynthesis, menthol biosynthesis, geranyl diphosphate synthase, deoxyxylulose-5-phosphate synthase, cytochrome P450, (+)-pulegone reductase, benzoyl coenzyme A: benzyl alcohol benzoyl transferase, salicyclic acid methyltransferase (SAMT), subtractive hybridization, bioinformatics

INTRODUCTION

Many plants emit floral scents which attract a variety of animal pollinators, mostly insects. They may also play a critical role in plant defenses against herbivores and pathogens (Wink, 1999). Floral fragrances vary widely among species in terms of number, identity and relative amounts of constituent volatile compounds. Humans find an aesthetic value in certain types of floral scents, and while there is certainly a wide variation in human taste, most people prefer the scents described as 'sweet smelling' (Knudsen and Tollsten, 1993). Unfortunately, very few plants are currently cultivated primarily for their scent. Moreover, a large number of commercial flower

varieties have lost their scent due to focus on maximizing post-harvest shelf-life, shipping, characteristics and visual aesthetic values (color, shape) and a lack of selection for the scent trait.

The chemical composition of floral scents has been extensively investigated for hundreds of years because of the commercial value of floral volatiles in perfumery. However, research on plant scents has been hampered mainly by the invisibility of this character, its dynamic nature, and the complex mixtures of components that are present in very small quantities. Gas chromatography and mass spectrometry (GC-MS) findings have shown that the floral scents are almost always a complex mixture of small

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(approximately 100-200D) volatile molecules and are dominated by terpenoids, phenylpropanoids/benzenoids compounds (Pichersky and Dudareva, 2007). These metabolites may be produced at specific stages of flower development and the volatile compounds escape directly into the atmosphere.

Terpenes are synthesized from isopentenyl diphosphate by different mono and sesquiterpene synthases (Vainstein *et al.*, 2001) via two alternative pathways: the mevalonate pathway from acetyl-CoA, and the methylerythritol phosphate pathway from pyruvate and glyceraldehyde-3-phosphate (G3P) (Rodriguez-Concepcion and Boronat, 2002). One of the best studied examples are the monoterpenes, the C_{10} members of the terpenoid (isoprenoid) family of natural products. Monoterpenes such as linalool, limonene, myrcene, and trans-β-ocimene, and also some sesquiterpenes such as farnesene, nerolidol, and caryophyllene are common constituents of floral scent. They are colorless, lipophilic, volatile substances responsible for many of the characteristic odors of plants (Hay and Waterman, 1993) and are also frequent constituents of oils and resins (Fahn, 1979). The phenylpropanoids constitute another large class of secondary metabolites in plants, several of which have been found to be volatile. Work on *Clarkia breweri* flowers (Pichersky and Dudareva, 2000) have resulted in the identification and characterization of three volatile enzymes that catalyze the formation of the floral volatiles, (iso)methyleugenol, benzylacetate and methylsalicyclate, from this group.

Although the chemical structures of many floral scent compounds have been determined (Guterman *et al.,* 2002), there have been few studies concerning the biochemical synthesis of floral scent compounds and the genes that control these processes. In the last few years, genes encoding the enzymes responsible for the synthesis of many monoterpenes and sesquiterpenes have been identified and characterized (Bohlman *et al.*, 1998). All of the enzymes responsible for the first dedicated steps of monoterpene (isoprenoid) biosynthesis from the deoxyxylulose-5-phosphate (DXP) pathway (Lange *et al.*, 1998, 1999) in peppermint, *Mentha × Piperita,* have been well established by *in-vitro* and cell-free studies (Colby *et al.*, 1993) including linalool synthase (LIS), (4S)-limonene synthase (LMS), myrcene synthase (MYS), 1,8-cineole synthase (CIS) and (-pinene synthase (PIS).

Earlier investigations on the floral scent production in *C. breweri* (an annual plant native to California) and *Antirrhinum majus* (cultivated snapdragon) have reported the isolation of several genes involved in the de novo synthesis of scent compounds in these flowers (Pichersky and Dudareva, 2007). Some of the genes encoding enzymes such as LIS, benzylalcohol acetyltransferase and 2-methyltransferases, involved in the biosynthesis of C. breweri scent volatiles (Dudareva and Pichersky, 2000) and methyltransferase that catalyzes methyl benzoate formation in the petals of the snapdragon, *A. majus* (Dudareva *et al.*, 2000) have been isolated and characterized. Other findings reported include enzymes involved in phenylpropene metabolism in sweet basil, *Ocimum basilicum* (Gang *et al.*, 2001), diterpene synthesis in Stevia rivaudiana of Asteraceae (Brandle *et al.*, 2002), terpene synthase in *Arabidopsis* (Aubourg *et al.*, 2002; Chen *et al.*, 2003) and sesquiterpene synthase from rose petals (Brandle *et al.*, 2002; Guterman *et al.*, 2002).

In general, expression of the genes involved in the synthesis of scent compounds have been found to be highest in petals and is restricted to the epidermal cell layers of floral tissues (Dudareva *et al.*, 1996; 1999; Dudareva and Pichersky, 2000). In *C. breweri* flowers, the expression of these genes were also found to be temporally and spatially regulated during flower development. The emission of the bulk of the volatiles was shown to occur from the petals of both the *C. breweri* and snapdragon flowers within few days of anthesis and thereafter declining gradually. LIS enzyme was found to increase in maturing buds and young flowers, then declined in old (5d) flowers, but activity remained relatively high even though emissions of linalool ceases. Accordingly the mRNA levels, encoding LIS enzyme, first detected in petal cells just before flower opening, increases until they peak at or around anthesis, then begin to decline (Dudareva *et al.*, 1996, 1998a; Wang *et al.*, 1997). Peak levels of mRNAs for this gene occur 1-2 days ahead of enzyme activity peaks and emission of corresponding compounds. Overall, a good positive correlation has been found between amount of mRNA, amount of protein and enzyme activity for each of these enzymes, and emission of corresponding components up to

2nd or 3rd day post anthesis. After that scent enzymes remain relatively high despite declining levels of corresponding mRNAs and also without concomittant emission of volatiles (Dudareva *et al.*, 1996, 1999). Thus, the level of enzyme activity involved in scent production (indirectly scent emission) is regulated mainly at mRNA levels at the site of emission in this flower.

In addition to the chemical composition, the physiological factors that regulate the production of these natural products have to be examined as well. In most cases that have been analyzed, the scent of flowers has been shown to be markedly reduced soon after pollination. The cessation of scent emission is often due to the senescense and wilting of petals (which usually constitute the bulk of the flower and the main source of scent emission), stigma and style. The effect of temperature on fragrance emission has also been shown to have a strong effect on the quantity of fragrance emitted. However, it is not clear if the increase in emission is due solely to the greater volatility of these compounds at the higher temperature, or if it is also due to biological processes, including increased synthesis.

In this report, we describe the combined use of subtractive hybridization and bioinformatics to partially deduce the scent biosynthesis pathway/s in the local flower Cempaka putih *(Michelia alba)* by subtracting RsaI-digested cDNAs of stage 1 flower buds from the full bloom flower at stage 10. This is a first attempt to identify the scent-related genes in this flower species and we hope that the results will provide useful biochemical insights into the scent biosynthesis pathway/s that exists in this flower.

MATERIALS AND METHODS

All plant materials were collected from the outskirts of Serdang town, Selangor. The development of *M. alba* flowers was divided into 12 stages (*Fig. 1*) according to size and morphology. At stage 1, the flower bud is very small and closed. The petals are green and no fragrance emitted. At stage 10, *M. alba* flowers are in full bloom and have a strong fragrance. Stage 1 was chosen as the driver so as to remove as many ribosomal proteins and housekeeping genes that might mask the low copy number of fragrance genes. Stage 10 was chosen as the tester as previous studies on GC-MS (*Fig. 2*)

(Suri Roowi, personal communication) had shown that the major fragrance compound, linalool, increased sharply from stage 8 and peaked at stage 10 before declining towards stage 11 when the flower begins to senesce.

Fig. 1: Michelia alba *at different stages of flower development*

Subtraction Hybridization

Total RNA was extracted from flower stages 1 and 10 using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Chang *et al.*, 1993). Poly(A) mRNA was isolated using the µMax Poly(A) RNA Isolation kit (Miltenyi Biotech, Germany). The subtraction hybridization was carried out using the BD PCR-Select cDNA Substraction Kit (BD BioSciences Clontech, USA). RsaI-digested cDNAs of stage 1 flower buds were subtracted from those at stage 10 and the cDNA clones synthesized were non-directionally mass cloned into the pGEM-T vector system (Promega, USA).

DNA Sequencing and Analysis

Plasmid DNAs were purified from overnight cultures using the Qiagen miniprep kit (Germany). The sequencing of the plasmid clones was outsourced (First Base Sdn. Bhd.) and sequencing was performed from the 5' end of the sense strand using the T7 universal primer. Raw sequence data was analyzed using our in-house iDNAs customized sequencing bioinformatics software (KooPrime Pte Ltd., Singapore). The PHRED and LUCY programs (Ewing *et al.*, 1998; Chou and Holmes, 2001)
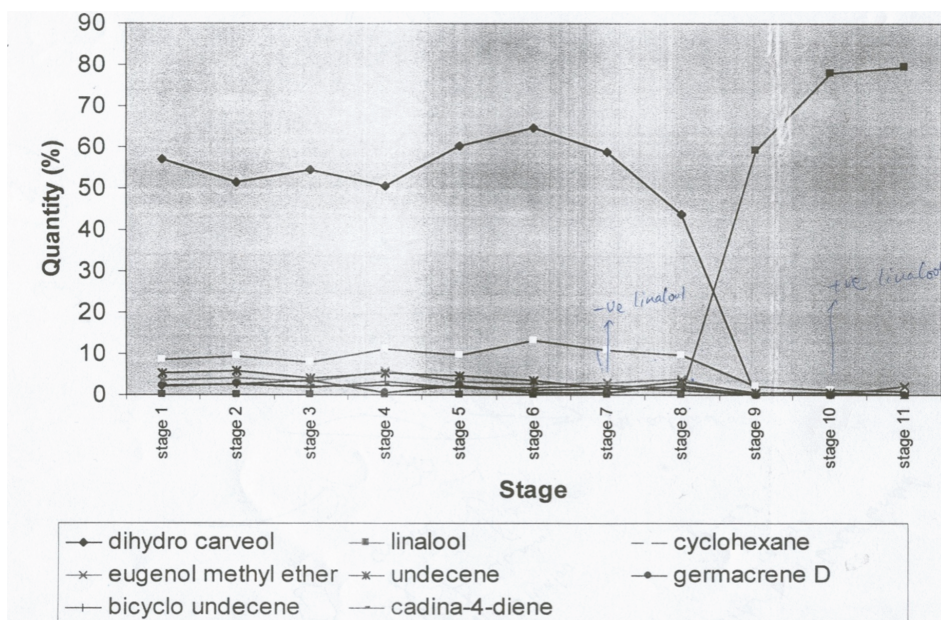
Fig. 2: Percentage of scent-related compounds obtained at different stages of M. alba *flower development using GC-MS*

were employed for base calling and sequence quality assessment (remove vector sequences, poly(A), adaptors and ambiguous sequences), respectively. CAP3 fragment assembly program was used to organize the redundant complementary DNA (cDNA) sequences into unigenes of overlapping contigs (Huang and Madan, 1999). The individually trimmed sequences were then submitted to BLASTX (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) analysis against our in-house nucleotide non-redundant protein database updated on 23rd May 2007 from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to search for similarities. The sequences were functionally characterized using Gene Ontology (GO) (http://www.geneontology.org) (Ashburner *et al.*, 2000). The putative floral biosynthesis enzymes deduced for *Phalaenopsis bellina* (Hsiao *et al.*, 2006) were used as a guide for the identification of the scent-related genes.

RESULTS AND DISCUSSION

A total of 420 cDNA clones were retained after subtraction. Vector trimming and homology search revealed 413 sequences varying in length between 50 and 773 bp. Fragment assembly of the clones generated 154 unigenes: 81 contigs and 73 singletons. 80.9% (334) of the clones showed similarities to known sequences in the non-redundant GenBank database, (E-value $\leq$ 1.0×10^{-4}), while the remaining 19.1% (79) had very low or no significant match (E-value $> 1.0 \times 10^{-4}$) based on the highest scoring results.

Putative functions were assigned to the above sequences based on the classification proposed by GO. Details of the gene species included in each group are given in Table 1. GO allowed 36.1% of the total sequences to be placed in the molecular function category, 22.8% in the biological process category and 0.5% in the cellular component category. The remaining 46.7% either showed insufficient similarities to any proteins (no hits, 19.1%) or hit proteins without a GO identifier (unclassified, 27.6%). Among the molecular functions, the categories most highly represented were the other enzyme activity excluding transferases, synthases, hydroxylases, oxidases and oxygenases (15.0 %), transferases (8.0%) and synthases (4.6%). Among the biological processes, the largest proportion (13.3%) of functionally assigned sequences fell into the other metabolic processes (excluding protein metabolism, DNA metabolism, electron transport, energy pathways and transcription);

TABLE 1
Functional classification of *M. alba* flower genes from stage 10 using GO
Classification and based on first hit blast results (E-value $\leq 1 \times 10^{-4}$)

Categories	Putative functions	Frequency	%
Molecular process	Protein binding	3	0.7
	DNA binding	4	1.0
	Other binding	2	0.5
	Transporter activity	16	3.6
	Synthase activity	19	4.6
	Transferase activity	33	8.0
	Other enzyme activity	62	15.0
	Structural molecule activity	10	2.4
Biological process	Protein metabolism	16	3.6
	DNA metabolism	2	0.5
	Electron transport	3	0.7
	Energy pathway	5	1.2
	Transcription	6	1.5
	Other metabolic processes	55	13.3
	Other physiological processes	5	1.2
	Other cellular processes	2	0.5
Cellular process	Other cellular components	2	0.5
Unclassified	Unknown, unnamed, hypothetical and others	114	27.6
No hits		79	19.1
TOTAL		413	

other cellular processes excluding signal transduction, cell organization and biogenesis and transport accounted for only 0.5% while the physiological processes accounted for 1.2%. Together, these two categories of molecular and biological processes accounted for 58.9% of the assigned sequences (Table 1).

Identification of Scent Biosynthesis Pathway/s in M. alba *Using the iDNAs Customized Bioinformatics Package*

Monoterpene synthase genes have been identified in both floral and vegetative organs of several angiosperms and gymnosperms (Aubourg *et al.*, 2002; Iijima *et al.*, 2004). The terpene synthases are of special interest, which are a large class of enzymes that appear to be responsible for most of the structural variation among terpenes (Wise and Croteau, 1999). In comparison to the floral scent biosynthesis pathway deduced in *P. bellina,* (Hsiao *et al.*, 2006), we identified four transcripts that were directly involved in monoterpene scent biosynthesis including deoxylylulose-5-phosphate synthase (DXPS), geranyl diphosphate synthase

(GDPS), cytochrome P450 and (+)-pulegone reductase (PR), and two others including benzoyl coenyme A: benzyl alcohol benzoyl transferase and salicyclic acid methyltransferase (SAMT) that might be involved with the phenylpropanoid/benzenoid scent pathway. Four other genes including lipoxygenase (LOX), peroxidase, heat shock protein and myb transcription factor were also identified that might be indirectly involved in scent biosynthesis (Table 2). GDPS was significantly expressed (4.36%) followed by DXPS (3.39%), cytochrome P450 (2.18%), LOX (1.21%), peroxidase (0.48%), heat shock protein 90 (0.24%), (+)-PR (0.24%), benzoyl coenzyme A: benzyl alcohol benzoyl transferase (0.24%) and SAMT (0.24%) (Table 2).

Analysis of volatiles by GC-MS data obtained earlier (Suri Roowi, personal communication), have shown that linalool (>80.0%) is a major fragrance compound of *M. alba* flower scent at stage 10 flower development as shown in *Fig. 2*. The high expression level of GDPS in *M .alba* flowers also suggests that scent biosynthesis in this species is predominantly due to production of linalool from GDP. GDPS has been shown to

pyruvate + glyceraldehyde 3-phospate

↓ *DXPS*

deoxyxylulose-5-phosphate

⇣

dimethylally diphosphate

↓ *GDPS*

Geranyl diphosphate

↙   ↘

(-)- limonene   linalool

↘ *CYTOCHROME P450*

⇣   linalool oxide
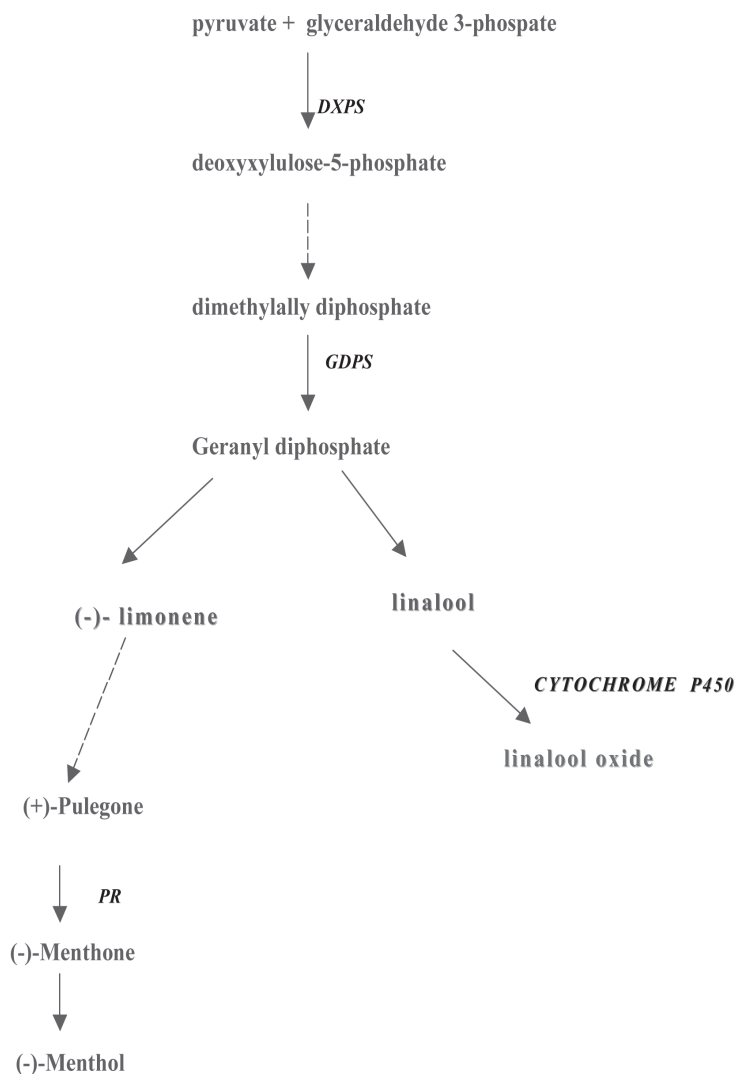
(+)-Pulegone

↓ *PR*

(-)-Menthone

↓

(-)-Menthol

Fig. 3: Putative metabolic pathway from pyruvate and glyceraldehyde-3-phosphate to monoterpene scent biosynthesis, and its related enzymes, in M. alba. *DXPS: deoxylylulose-5-phosphate synthase; GDPS: geranyl diphosphate synthase; cytochrome P450; PR: Pulegone reductase*

participate in the biosynthesis of monoterpenes in plastids (Sommer *et al.*, 1995) primarily by supplying the essential precursor. Based on the above findings and the fact that all monoterpenes are formed from geranyl diphosphate (GDP), which is synthesized from dimethylallyl diphosphate and isopentenyl diphosphate (Tholl *et al.*, 2004), it is therefore reasonable to speculate that monoterpenoids are biosynthesized in these flowers.

However, we were not able to detect the transcripts for linalool synthase or limonene synthase, the enzymes responsible for the formation of linalool and limonene en route to pulegone, respectively. These enzymes were also not detected in *P. bellina* (Hsiao *et al.*, 2006). They have been reported to belong to families with high diversity in non-conserved regions (Lange *et al.*, 1998) and therefore, the low sequence-relatedness among them (Iijima *et al.*, 2004) might have added to the difficulty in identifying them. Alternatively, the pool of subtracted clones obtained for *M. alba* may be insufficient, so that not all genes in the scent

TABLE 2

Major classes of scent and scent-related transcripts in *M. alba* flowers during full bloom (stage 10) based on first hit blast results (E-value $\leq 1 \times 10^{-4}$)

Scent and scent-related genes identified	Frequency	% (out of 413 sequences)
Deoxyxylulose-5-phosphate synthase (DXPS)	14	3.39
Geranyl diphosphate synthase (GDPS)	18	4.36
Cytochrome P450	9	2.18
(+)-pulegone reductase	1	0.24
Benzoyl Coenyme A: benzyl alcohol benzoyl transferase	1	0.24
Salicyclic acid methyltransferase (SAMT)	1	0.24
Lipoxygenase (LOX)	5	1.21
Heat shock protein 90	1	0.24
Myb transcription factor	1	0.24
Peroxidase	2	0.48

biosynthesis pathway were represented. It could also be due to regulation of the scent biosynthesis at the precursor level, and the enzymes responsible for synthesis are not transcriptionally regulated. Previous studies have shown that LIS levels and activities in *C. breweri* remain high while linalool emission decreases, suggesting that regulation of terpenoid precursors occurs in this species (Dudareva and Pichersky 2000; van Schie *et al.*, 2006).

Although we did not detect the transcripts for linalool synthase at this stage of flower development, the presence of cytochrome P450 indicated that linalool synthase could have been expressed much earlier. Our results showed that cytochrome P450 is involved with the formation of linalool oxide from linalool in *M. alba* as shown by Hsiao *et al.* (2006) (*Fig. 3*).This enzyme has also been reported to act as a hydroperoxide lyase and catalyze the cleavage of lipoxygenase products (fatty acid hydroperoxides), forming omega-oxoacids and volatile C6- and C9-aldehydes and alcohols (Noordermeer *et al.*, 2001).

Interestingly, we also detected (+)-pulegone reductase, a central intermediate in the biosynthesis of (-)-menthol, the most significant component of peppermint essential oil (Soheil and Rodney, 2003). Depending on environmental conditions, the cyclization of GDP, the universal monoterpene precursor will lead to the production of (-)-limonene and after a sequence of several steps, to produce the branch point metabolite, pulegone, which may be reduced to (-)-menthone en route to menthol, by PR.

Two different kinds of LOX transcripts (LOX1 and LOXC) accounted for the relatively high percentage (1.21). LOX genes may be involved in converting storage lipids into substrates for further oxidation to provide energy for scent emission as shown by the presence of many lipid bodies found in the petal epidermis of *P. bellina* by transmission electron microscopy (Hsiao *et al.,* 2006). LOX genes have also been reported to be involved in plant growth and development; biosynthesis of regulatory molecules such as jasmonic acid and traumatin; and biosynthesis of volatile compounds such as hexanal, hexenal and hexenol, which are involved in flavor, insect attraction and defense (Chen *et al.*, 2004; Feussner and Wasternack, 2002). The role of the LOX pathway in plant-pathogen interactions and their product, jasmonate, in resistance against insects and pathogens have been analyzed in numerous pathosystems (Howe and Schilmiller, 2002). Although the biological function of the relatively high levels of LOX expression in *M. alba* flowers is not clear, their expression may indirectly control the synthesis of some signal for flower scent formation or emission.

Our results also showed that peroxidase and heat shock protein 90 might be related indirectly to scent metabolism although they were found in very low abundance (0.48 and 0.24% respectively). Interestingly, anthocyanin colour biosynthesis genes were also identified although the flower is white. Transcripts encoding signal transduction factors (Table 1, cellular processes) such as membrane proteins were also identified,

suggesting that scent emission may be related to stimuli that causes a series of signal transduction processes leading to gene expression and scent production. We also detected Myb transcription factor (0.24%) which could be related to the Myb family protein (Table 2) shown to regulate the biosynthesis of petunia flower fragrance (Verdonk *et al.*, 2005).

From the chemical profiling, data mining and bioinformatic analyses, we partially deduced a monoterpene biosynthesis pathway of 4 steps in the *M. alba* flower, leading from pyruvate and G3P to GDP, linalool, limonene and their derivatives (*Fig. 3*). We also managed to show a weak existence of the phenylpropanoid/benzenoid scent pathway in this flower at this stage, although the 2 enzymes were present in relatively low abundance (0.24%). The sequences of these transcripts will be deposited in the public database after publications of these findings.

CONCLUSIONS

In this study, we have shown how a combination of genomics and EST database mining can be applied for the construction of a putative scent metabolism pathway in *M. alba* and the identification of the genes encoding the enzymes involved in this pathway. We used the customized in-house integrated DNA sequencing (iDNAs) bioinformatics package to identify the scent genes in *M. alba* including those for DXPS, GDPS, cytochrome P450 and (+)-pulegone reductase, all involved in the DXP-linalool-limonene pathway. For a non-model plant with no genomic information at all, EST analysis of its transcriptome profile becomes a very efficient and informative tool and may be applicable for comparative genomics.

It is clear from the above, that a major priority of scent research should be, to continue to understand the biochemical pathways leading to scent biosynthesis and the identification and characterization of genes controlling these pathways. In addition to this, the sub-cellular location of the synthesis of most of the scent compounds still needs to be determined, as well as the mechanisms controlling developmental changes of the pathways. It would also be useful to examine the molecular processes that bring about the variability in floral scent characteristics among different species, whether they are on the level of gene regulation, post transcriptional regulation, or protein evolution. Finally, the availability of scent genes should allow us to create transgenic lines with optimum fragrance production.

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Aquaculture in the Asia-Pacific Region: Applications of Molecular Population Genetics

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ABSTRACT

The utility of applied molecular population genetics in aquaculture has been questioned by some quantitative geneticists. They argue that there have been few examples where research in this area has directly resulted in development of improved strains used in culture. Here I suggest that this could be a narrow view of the diversity of issues that need to be addressed when new stock improvement programs for aquatic species are initiated. My belief is that this view has arisen due to fundamental differences in the relative starting points faced when new productive strains of terrestrial vs aquatic farmed species are developed. Aquatic species in general, possess very high fecundities and as a consequence of husbandry practices are therefore prone to lose genetic diversity much more rapidly in culture than comparable terrestrial farmed species. Additionally, natural populations of most aquatic farmed species are still common in the wild, unlike their terrestrial counterparts, and so can provide important genetic resources that can be exploited in sustainable ways to improve culture lines. Taken together, this implies that genetic diversity is a much more significant issue broadly speaking, for farmed aquatic species compared with terrestrial farmed species. Thus farming of aquatic species can benefit from application of well-conducted molecular population genetic research. Thus, the narrow view that some quantitative geneticists have taken on the utility of molecular population genetics in aquaculture, in my opinion may miss the broader applications of the technologies that can assist a move to development and farming of improved breeds of aquatic species while conserving natural gene pools in the Asia-Pacific region.

Keywords: Aquaculture, Asia-Pacific Region, aquatic species, genetic diversity

INTRODUCTION

Aquaculture is one of the fastest expanding industries worldwide and nowhere in the world is this more apparent than in Asia, a region that currently accounts for around 80% of total world production. The industry will continue to expand and aquaculture will be of growing importance for regional food security and national development in the Asia-Pacific region. While most farmed aquatic species in the region have yet to be improved, the trend in America and Europe is to move from farming essentially wild animals to production of genetically-improved, high performing aquatic breeds. In many parts of the world, the industry now focuses more and more on quantitative genetic approaches to improve the productivity of cultured species.

This development mirrors advances that have occurred in terrestrial farmed species over the last 60 years where significant advances have been made to the relative productivity of the major terrestrial species we produce in agriculture. For example, genetic improvement programs have; improved meat production in chickens by up to 200%, increased milk production in dairy cows by over 150% and meat production in pigs by around 100%, since 1940 (FAO, 2006). This rapid development follows approximately 10,000 years of domestication and genetic improvement of these species by humans, to a point where modern farmed terrestrial animals are physically unlike their now extinct wild relatives. In contrast, with a few notable exceptions, most farmed aquatic

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species are essentially wild organisms brought into husbandry. The future however, will be a move by industry to farming improved lines with traits often very different to phenotypes that are favoured in the wild.

When we examine the genetic characteristics of most farmed aquatic species, it becomes obvious very quickly, that most stocks have been developed with little regard for genetic attributes that would increase their value as farmed stocks. On closer inspection, levels of inbreeding are often high and as a consequence, genetic variation is generally low. These factors are often compromised by the husbandry practices we employ in culture that further erode the remaining genetic variation across generations in culture. As a consequence, many culture lines may have limited ability to respond to breeding programs when the industry moves to stock improvement (Frankham *et al.*, 2002 ; Allendorf and Luikart, 2007). Unintentional selection by animal breeders and farmers that results from husbandry practices can also compound the problem leading to fixation of unfavourable phenotypes. A widely acknowledged example is rapid declines in size at sexual maturation in female giant freshwater prawn (*Macrobrachium rosenbergii* - GFP). A characteristic of most aquatic farmed species, that has not been well understood, is the fact that many are very prone to rapid loss of genetic diversity, much more so, than are equivalent farmed terrestrial animal species.

Most aquatic farmed species are highly fecund with females capable of producing thousands if not hundreds of thousands of offspring from a single mating. This is not generally a problem in the wild where larval and juvenile survival is generally very low and populations and breeding numbers are generally high. In culture however, relative survival of offspring per mating can be very high and number of broodstock used to produce larvae and fry are often very limited. So the genetic relationship between surviving offspring can be very high, and over generations in the hatchery, is likely to increase significantly. This issue is often compounded by the fact that most broodstock used to produce the offspring for growout are unlikely to have been collected in a systematic way and the cost of collecting new broodstock and their maintenance over time, can result in their reuse and cross generational mating,

leading to compounding levels of inbreeding across generations. This problem is often not considered, or is not obvious to hatchery managers, because breeding registers are seldom employed. Across generations inbreeding levels are likely to increase and genetic diversity will decline potentially compromising future response to stock improvement programs.

High levels of inbreeding and low genetic diversity in many aquatic cultured stocks in comparison with their wild relatives does not necessarily mean that artificial selection programs are likely to fail. In fact, where aquatic species have been subjected to stock improvement programs, response to selection can be quite dramatic and it is not unusual to see very rapid genetic gains in excess of 10 or 20% per generation achieved. This is much faster that can be achieved for most farmed terrestrial species (TFAS) where genetic gains of 3 to 5% per generation are considered very good. This is because even given low genetic diversity and high levels of inbreeding relative to their wild cousins, genetic diversity levels in most cultured aquatic stocks are still orders of magnitude higher than equivalent TFASs, simply because they have spent many fewer generations in culture. The problem remains however, as most AFASs have greater potential to lose any remaining genetic diversity very rapidly, much faster than TFASs, if it is not managed carefully.

As interest has grown in many parts of the world to advance aquaculture from essentially farming wild animals to farming improved, domesticated breeds, both quantitative geneticists/animal breeders and population geneticists have become involved in this development. In particular, research by population geneticists has focussed on developing and applying molecular genetic marker analyses to assay genetic diversity in cultured aquatic species for a variety of applications. In recent times however, this type of study has been questioned by some quantitative geneticists working in developing countries and who gained their experience in stock improvement programs on terrestrial animal species, as being wasteful of limited resources for research. They argue that the majority of productivity gains in agriculture (and now, aquaculture) have come directly from animal breeding programs not molecular diversity studies. Thus, they have questioned the

relevance and value of molecular diversity research in aquaculture. While it is true that, in some instances, significant resources have been largely wasted in poorly-designed and conducted molecular diversity projects, particularly in the developing world in recent years, it is my view, that criticisms of the approach ignore some fundamental differences between the relative starting points for genetic improvement programs in agriculture and aquaculture. The focus of the current paper is to highlight these issues and to identify a more productive approach to future programs.

When we compare the general situation that faces aquaculture with that in terrestrial agriculture as they relate to issues relevant to a move to farming improved breeds, immediately it is obvious there are some stark contrasts. While humans have been changing the attributes of farmed terrestrial animal species for thousands of years, the number of farmed terrestrial animal species is quite small (~6 to 10 species worldwide) compared with more than 100 AFASs that are either farmed or are being trialed in aquaculture today. Secondly, the wild relatives of most TFASs have been extinct for 1000s of years while healthy populations of most AFASs still exist in the wild. Thus, available wild genetic resources for most AFASs are extensive and largely unexploited, but ongoing genetic improvement of TFASs must depend on the limited genetic resources that remain in cultured stocks after many generations of inbreeding. While both TFASs and AFASs could benefit from transgenic options in the future, consumer acceptance issues are likely to remain a problem with this technology. Apart from the issues identified above, a major contrast will always be the fact that major life history traits in AFASs are usually quite different to those present in TFASs. Of particular relevance here, are differences in relative individual fecundity, a factor that seems to have been largely ignored by quantitative geneticists except when they benefit from the large number of offspring that can be produced from a very small number of breeders in stock improvement programs. For TFASs this has never been a problem, but for aquatic species it can be a genetic 'time-bomb', that can rapidly erode away any remaining genetic diversity including the important exploitable component. This is where

I believe, applied molecular population genetics, when used appropriately, has a major role to play in the development of modern aquaculture. Of specific relevance here, the science of molecular population genetics can provide important data that will assist with (1) better broodstock choice, (2) monitoring genetic diversity across generations to reduce inbreeding levels, (3) reducing effects of unintentional selection and help to maintain the selection response in a breeding program. In addition, molecular diversity studies can monitor and help to maintain healthy effective population size in the hatchery and assist biologists to better understand the reproductive biology and social traits of target species. Finally, modern genomic approaches can identify critical genes and even individual mutations that influence important quantitative traits and hence focus the attention of animal breeders on critical areas of the genome. Below I provide some examples where I believe molecular population genetic research has contributed to the productivity of cultured aquatic species.

Applications of Molecular Population Genetics in Broodstock Selection

Wild genetic resources of most cultured aquatic species are still extensive but are often poorly known. Most farmers source their broodstock from other breeders potentially compounding genetic diversity problems at the start of any stock improvement program. Knowing the genetic resources that are available in the wild and their relative levels of genetic diversity can allow breeders to make informed choices to optimise the diversity available to future breeding programs. These data can also assist in assessing the potential for obtaining hybrid vigour vs outbreeding depression outcomes in crosses made between genetically discrete stocks and for developing 'synthetic culture lines' that are genetically compatible but which possess high genetic diversity due to their divergent origins (an example is the 'GIFT' strain of Tilapia). Equally, genetic diversity studies of wild and cultured lines provide a reference point for assessing impacts that husbandry practices may have had on genetic diversity levels in cultured stocks, before inbreeding becomes a significant issue for farmers.

Case Study: Giant Freshwater Prawn (M. rosenbergii) *Culture in Asia*

GFP culture is the 6th largest culture industry in Asia and is worth close to USD 1 billion currently per year to the region. While most farmers in Asia have sourced their broodstock from local wild stocks in the past, the industry was initiated originally from 12 individuals from Malaysia in the 1970's and taken to Hawaii where the life cycle was closed and hatchery technologies developed. This stock was later translocated widely outside Asia to develop new culture industries in regions where the species is exotic. GFP are now cultured in over 43 countries worldwide on five continents. Genetic diversity levels in all stocks outside Asia are likely to be low however, due to the extreme bottleneck that the original introduced population was exposed to when the original Hawaiian culture line was developed. Impact of repeated translocations of this stock to new culture locations has most probably compounded this problem. In contrast in Asia, while local wild stocks were sourced for broodstock during development of local culture industries, this was done basically without knowledge of genetic diversity levels or a clear understanding of why or how to maximise diversity in culture. More recently, most new farms source their culture stock from existing farms and it is also common for only small numbers of breeders to be used in the hatchery. Thus genetic diversity levels in the Asian culture industry are also likely to have been impacted by the process of culture industry development and inbreeding levels may be high. This could explain the observation reported in many locations, that female size at sexual maturation has declined over years in culture, a sure sign that unintentional selection and inbreeding have resulted in early maturing, small-size females being sourced repeatedly as broodstock. Much of this has remained unstudied with the exception of a very recent paper by Chareontawee *et al.* (2007) on Thai GFP stocks.

Currently, there is growing interest in many countries for stock improvement programs for GFP, but if inbreeding levels are high, any response to selection could be compromised by low exploitable levels of genetic variation. Thus, developing a better understanding of genetic diversity in culture lines and their wild antecedents, will allow informed choices to be made about initiating new culture lines, high in genetic diversity, prior to stock improvement programs being initiated that may be compromised by low exploitable levels of diversity.

From a theoretical population genetic perspective, when life history characteristics of GFP (primarily freshwater with a short estuarine phase, life cycle confined to stream systems) are considered in the light of the extensive natural distribution of the species, levels of genetic diversity in wild stocks are likely to be high and may be structured, geographically. The natural distribution of GFP includes from Pakistan in the west across southern and SE Asia to central Vietnam, includes some Philippine islands and northern Australia, PNG and extends to some larger Pacific islands. Until recently nothing was known however, about genetic diversity in either wild or cultured populations of GFP. Recent studies by de Bruyn *et al.* (2004a ; 2004b ; 2005), resolved the patterns of genetic diversity in wild stocks of the species and this baseline data form a foundation for future comparative analyses of culture lines.

Taxonomists currently consider wild GFP populations to be monophyletic across the species' extensive natural range. Molecular analysis of broad-scale variation in wild stocks using the highly conserved 16S RNA mtDNA gene however, identified two major divergent lineages (*Fig. 1a*) referred to as 'eastern' and 'western' forms with distributions divided by Wallace's Line (extends to Huxley's line to the north – *Fig.1b*) (de Bruyn *et al.*, 2004a). A calibration of the theoretical time of separation (MRCA) of the ancestors of the two forms was 5 to 12 million years bp, indicating that the two lineages probably diverged in the Miocene, yet modern populations of the two different forms cannot be, or are difficult to, distinguish morphologically.

Analysis of diversity within the two major wild GFP lineages using a more rapidly evolving mtDNA gene (CO-I) identified additional, fine-scale geographic population structure. Within the 'western' or Asian lineage, a major genetic break was evident between wild populations north and south of a biogeographical zone, referred to as the 'Isthmus of Kra' in southern Thailand, an area of low topography that was inundated a number of times during sea level changes (eustasy) over the last 100,00 years (*Fig. 2* - de Bruyn *et al.*, 2005). Fine-scale population
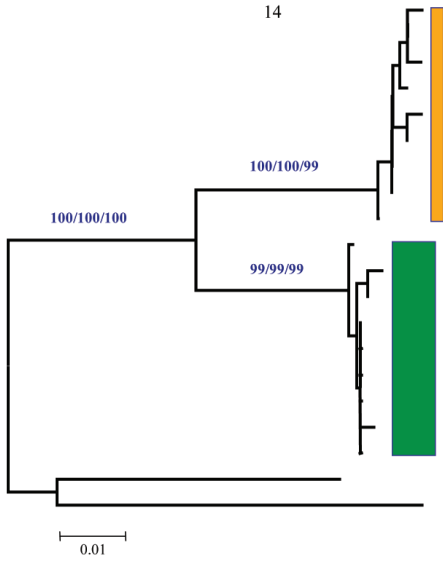
(a)

Fig. 1a, b: Neighbour-joining tree of molecular diversity in the mtDNA 16SRNA gene in wild stocks of M. rosenbergii *showing deep divergence between 'eastern' and western' populations*
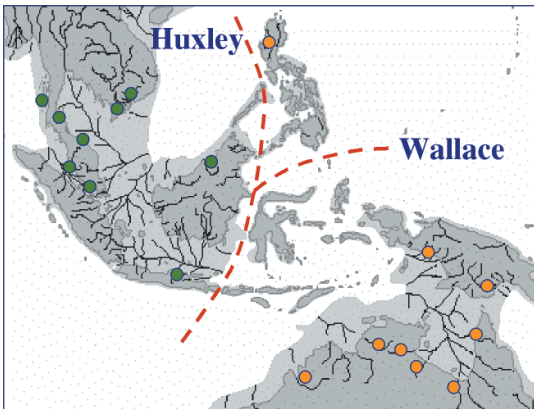
(b)

Fig. 1b: The geographic pattern of 16SRNA gene diversity in wild stocks of M. rosenbergii *from across the species natural range related to two major biogeographic regions*
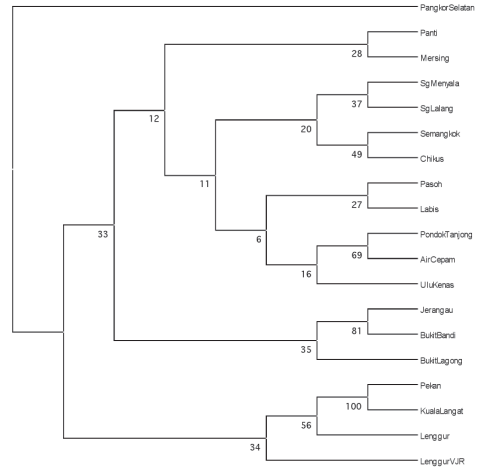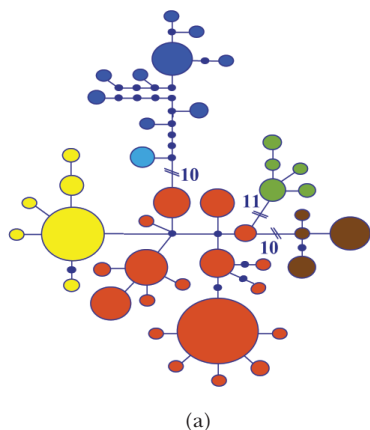

Fig. 2: Patterns of diversity in the 'western' form of M. rosenbergii *at the mtDNA CO-1 gene showing a break concordant with the 'Isthmus of Kra' region in southern Thailand*

structure within the 'eastern' form was greater with five divergent lineages identified, four of which occur in northern Australia and that have distributions correlated with known biogeographical regions (*Figs. 3a* and *3b* - de Bryun *et al.*, 2004b). When the analysis was extended to include nuclear markers (microsatellites) for the 'eastern' form, populations were structured at a similar regional scale to that identified with the fast-evolving mtDNA gene marker indicating that sets of specific nuclear alleles were restricted to the different geographical clades *Figs. 4a* and *4b*). If this is true for neutral, non-coding alleles at microsatellite loci, there is a strong chance that local selection and genetic drift will also have resulted in unique alleles at coding loci, perhaps even loci that may influence quantitative traits affecting productivity.

Data on patterns of wild stock diversity have relevance for the GFP culture industry because the patterns imply that to date, very little of the natural genetic diversity present in GFP wild stocks has been captured in culture (de Bruyn *et al.*, 2004a; 2004b; 2005). Of the diversity that has been captured, it is likely that a significant proportion may have been lost or eroded by high levels of inbreeding due to culture management practices. Given the extent of diversity revealed, potential for all possibilities from heterosis to outbreeding depression may be possible in crosses between discrete wild gene pools. Outbreeding depression can result from the mixing of incompatible genomes and can lead to poor outcomes in the progeny. For example, if outbreeding produces genetic incompatibilities, offspring may not survive, may be infertile or have low fertility relative to inbred progeny or may have adaptive incompatibilities
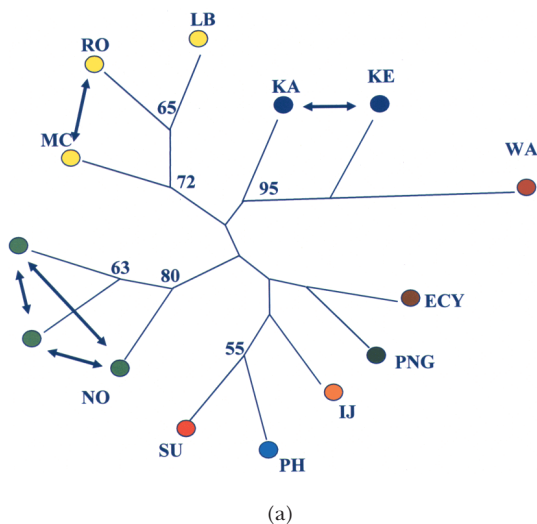
(a)

Fig. 3a: Minimum spanning network of CO-1 haplotypic diversity in the 'eastern' form of M. rosenbergii *indicating the distributions of four divergent clades across northern Australia*
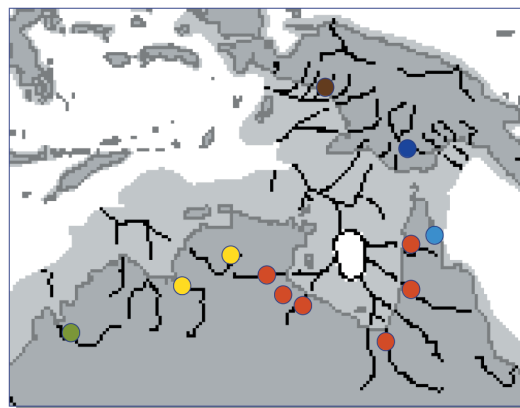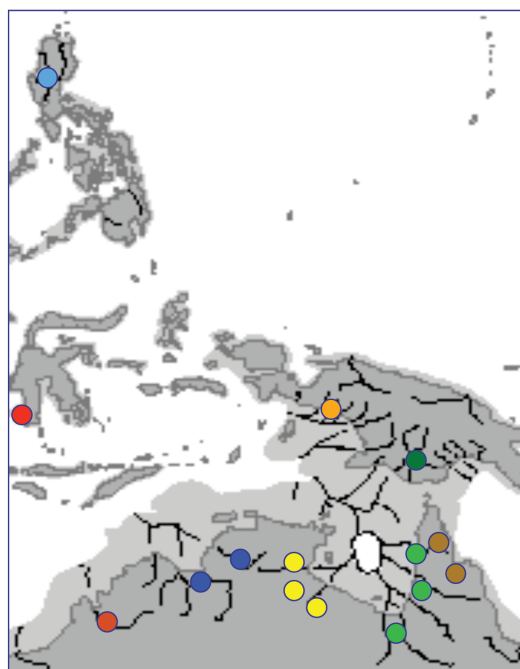

(b)

Fig. 3b: Patterns of diversity in the 'eastern' form of M. rosenbergii *across northern Australia and PNG at the mtDNA CO-1 gene*

(a)

Fig. 4a: Neighbour joining tree of microsatellite variation in the eastern form of M. rosenbergii

(b)

Fig. 4b: The pattern of geographic variation in microsatellite diversity in 'eastern' populations of M. rosenbergii *are concordant with mtDNA clades*

leading to low relative fitness. Unfortunately, potential for outbreeding depression is impossible to predict but in general terms, the more genetically divergent parents are, the higher is the probability of producing poor outcomes in the progeny. Since GFP have been translocated widely for culture and molecular analysis has indicated that the 'eastern' and 'western' forms probably last shared a common ancestor greater than 5 million years b.p., care should be taken in mixing GFP populations either for culture or when culture stocks escape to the wild. Even within the two major GFP

lineages, a conservative view should be adopted when crosses are considered for culture or stocks are translocated to new locations where wild stocks are still healthy.

Recent molecular diversity studies of GFP wild stocks provide data that allow preliminary predictions about potential for genetic compatibility between different wild GFP stocks and hence provide a geographical scale at which 'synthetic' culture lines might be developed. These data could form the basis for a new systematic approach for broodstock collection and a breeding program, particularly for the Asian region designed to produce a highly genetically diverse base population for a future culture stock improvement. Further work will be needed at fine spatial scales however, to better understand the significance of population divergence and to assess whether some local variation may be clinal or reflect isolation and independent evolution in the recent past.

A number of studies have been initiated recently with collaborators in the region to explore some of the practical applications of the GFP molecular diversity data. In Vietnam, we are trialing a diallelic cross among two genetically-discrete wild Vietnamese GFP strains and a third culture strain from Thailand, to relate genotype to strain performance and potential for crosses to show hybrid vigour. A new project will estimate relative levels of inbreeding in specific culture lines and the wild populations from which they were derived to quantify the impact that past management practices have had on genetic diversity in culture lines in the region. In late 2007 we plan to introduce new culture strains of GFP from Asia to the Pacific region under quarantine to assess their relative performance against the 'Hawaiian' strain that has been cultured there, and more widely around the world, since the 1980's, but that has showed some decline in productivity.

Applications of Population Genetics Studies on Genetic Diversity in the Hatchery

A number of recent molecular studies have reported significant declines in genetic diversity in hatcheries. The fact that this has been reported in diverse species from marine fish to mollusc species suggests that the phenomenon may not be uncommon. Factors identified as potentially contributing to this problem include; non-systematic choice of broodstock, use of small numbers of parents, unequal sex ratios in the parents, social factors in communal broodstock tanks (e.g. dominance hierarchies) leading to unequal contributions to fry and variation in family survival. Any of these factors in isolation or in combination, can significantly erode genetic diversity in the fry and rapidly increase inbreeding rates. Understanding the causal factors will be very important for sustaining culture productivity and can help with the design of better breeding strategies. Molecular diversity studies of specific species can provide these data and have been used to identify the likely causal agents.

A study by Hara and Sekino (2003) on Japanese flounder (*Paralicthys olivaceus*) using parentage assignment based on microsatellite genetic markers demonstrated that only 57% of hatchery broodstock spawning in communal tanks contributed to offspring. This resulted in a 29% loss in allelic diversity in the offspring compared with their parents and average heterozygosity was also significantly reduced. They observed that this was happening every hatchery cycle and so, even if genetic diversity had been relatively high in the parents at the start, it can be eroded very rapidly across generations. Since loss of genetic diversity is essentially random, even quantitative alleles that confer high fitness can be lost by this process. Another recent study of Sea Bass (*Lates calcarifer*) by Frost *et al.* (2007), reported that 55% of progeny were sired by a single male when 7 males were used in a communal breeding tank and whole families did not survive to fingerling stage. Family loss was apparently random and unrelated to relative growout performance, implying that even high performing families could be lost by chance. In addition, the effective population size (EPS) of the offspring was only ~ 50% that of their parents, implying that only a limited number of potential parents contributed their genetic diversity to the fry. Size-grading of the fry prior to stocking, can apparently further erode genetic diversity levels. Thus, hatchery practices can have major impacts on genetic diversity in offspring and significantly increase inbreeding rates over very few generations. The same outcome is unlikely to occur in terrestrial animal species due to major differences in life history traits. This problem has been largely ignored in most fish, mollusc and crustacean hatcheries until very recently, yet farmers often comment on losses in productivity in their culture stocks over time.

Molecular population genetic studies can also have an important role to play in assessing the impacts that hatchery genes may have on

wild gene pools in aquatic species. This is not a significant issue for farmed terrestrial animal species because wild gene pools for most species are long extinct. It is obvious that for aquatic species produced in hatcheries however, that accidental escapes to the wild or deliberate releases for stock enhancement or ranching purposes have the potential to lead to contamination of wild gene pools with hatchery genes. Since most hatchery-produced stock are less genetically diverse than their wild counterparts, when interbreeding occurs, levels of inbreeding are likely to increase and genetic diversity to fall consequently, in the mixed population. Even traits that are favoured in the wild may be lost by chance, as has been reported recently in salmon. Vasemagi *et al.* (2005) used molecular markers to identify interference by hatchery-reared fish that had been stocked in wild rivers in Europe on natural spawning of wild Atlantic salmon (*Salmo salar*) strains. As we produce more aquatic species in culture and stock enhancement is practiced more widely to replenish declining wild populations, it will be important not to compromise the fitness of wild adapted populations. Molecular population genetic analyses provide a powerful set of techniques for monitoring stocking impacts and to assess the extent of introgression of hatchery genes into wild gene pools.

Thus integrating research on molecular population genetics, quantitative genetics, animal breeding and nutrition provide the best option for new stock improvement programs on aquatic species. While to date, an integrated approach has been employed on only a relatively few species, where it has been practiced, outcomes have been very encouraging (e.g. Tilapia, Atlantic salmon, Pacific salmon and Channel catfish). Nile tilapia (*Oreochromis niloticus*) is probably one of the few species cultured widely in Asia that has benefited from such an approach to stock improvement. While Nile tilapia has been cultured in Asia for decades, declines in stock productivity due to high inbreeding levels and hybrid introgression led to the recognition that new germ-plasm was required to reinvigorate the industry. As wild stocks of Nile Tilapia were still plentiful in northern Africa a stock improvement project adopted an integrated approach to developing a new strain with high productivity for the industry in Asia. The new

strain referred to as the 'Genetically Improved Farmed Tilapia (GIFT strain) was developed in the Philippines as a result of integrated applied research in the fields of: molecular population genetics, animal breeding and quantitative genetics to deliver a culture strain that grew >60% faster than other tilapia culture lines available there (Eknath *et al.*, 1993). Molecular population geneticists collaborating on this project characterised the genetic diversity in wild african populations and these data were used to undertake strategic collections of compatible, yet highly diverse, wild river strains that, after evaluation in culture as inbred lines, were later combined into a synthetic culture strain that was taken through multiple generations of family selection to improve growth rate. This strain was later disseminated widely in the Asia-Pacific region to reinvigorate the culture industry. Some recent reports have suggested however that the productivity of some GIFT culture stocks have already declined and the suggestion is that this may result from poor stock management leading to high inbreeding rates and hybrid introgression with local strains. Ongoing monitoring of genetic diversity levels in GIFT culture stocks is rare, but had this been practiced, could have alerted the industry to the problem before it became a major issue. Atlantic salmon and Channel catfish stock improvement programs have not made the same mistake and routinely monitor genetic diversity in both cultured and wild stocks.

CONCLUSIONS

Thus, questions about the utility of population genetics in aquaculture has, in my opinion, focussed on a very narrow aspect of the application of the science, i.e. direct production of improved strains. As outlined above, there are other important related issues with farmed aquatic species where applied molecular population genetics research has a significant role to play. In many cases, these are not issues that are highly relevant for improvement programs in terrestrial farmed animal species. Maintaining healthy levels of genetic diversity in cultured aquatic species over the long term constitutes a much greater problem than for equivalent terrestrial animal species and molecular population genetics when applied appropriately, provides powerful tools to address

this issue. Specifically, the approach can (1) allow effective documentation of wild genetic resources, (2) assist in better broodstock selection, (3) be used to monitor the impact of hatchery practices on genetic diversity levels in the hatchery and growout cohorts, (4) be used to develop a better understanding of the social and reproductive behaviour of target species and (5) to design better breeding systems and (6) can be used to monitor impacts of hatchery genes on wild populations. Hence, I am confident that applied molecular population genetics has an important role to play in the future development of aquaculture in the Asia-Pacific region and should be more closely integrated into breeding programs and quantitative studies of aquatic species targeted for stock improvement programs in the future.

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Ti: Genetic Diversity Assessment of *Koompassia malaccensis*

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ABSTRACT

A genetic diversity study of *Koompassia malaccensis* based on 19 populations from 18 forest reserves in Peninsular Malaysia is reported. The genetic diversity assessment was based on six polymorphic microsatellites. Overall, all the populations showed high levels of genetic diversity. The allelic richness ranged from 6.0 (Pekan) to 9.3 (Lenggor) whereas the gene diversity ranged from 0.683 (Pekan) to 0.859 (Lenggor). The estimated coefficient of population differentiation (R_{st}) was 0.07, implying that 93% of the genetic diversity was partitioned within populations, with only 7% distributed among populations. From the cluster analysis among the populations, the two peat swamp populations (Pekan and Kuala Langat Selatan) formed a tight cluster even though they are not adjacent to one another. Further analysis including more populations and applying more microsatellites will generate more comprehensive genetic information.

Keywords: Kempas, genetic diversity, population genetics, simple sequence repeats

INTRODUCTION

Koompassia malaccensis Maingay ex Benth. (Leguminosae) is an important tropical timber species distributed in Sumatra, Peninsular Malaysia, Singapore and Borneo (Hou, 2000). It is locally known as kempas and grouped under medium hardwood. It is a very tall tree, easily reaching 55 m in height and has a diameter of 200 cm. It is found in lowland, hill, peat and freshwater swamp forests up to 800 m, but often favouring an altitude not exceeding 150 m. It flowers and fruits regularly and the main flower visitors are bees, *Apis* sp. (Appanah and Weinland, 1993). It produces flowers and fruits all year round with fruits surrounded by a papery wing that spins down. Under the IUCN (1994) version 2.3 criteria, kempas was assigned as lower risk/ conservation dependent (LR/cd) (IUCN, 2006). However, as the demand of its timber is high due to shortage of hardwood, proper conservation measures are crucial to ensure sustainable harvesting.

Knowledge of the distribution of genetic diversity within and among populations of tropical trees is essential in the development of conservation strategies (Hamrick, 1983). Microsatellite markers have been used in population genetic studies for a wide array of timber species (Al-Rabab'ah and Williams, 2002; Novick *et al.,* 2003; Wyman *et al.,* 2003; Lee *et al.,* 2006) due to their ability to detect and describe genetic differences between populations.

This study was carried out using a set of microsatellite loci newly developed for *K. malaccensis* (Lee *et al.,* 2006). The aims were, i) to estimate the genetic diversity levels of *K. malaccensis* in Peninsular Malaysia and, ii) to survey the distribution of genetic diversity within and among populations, and iii) to investigate whether *K. malaccensis* from the peat swamp forests are genetically distinguishable from those of the non peat swamp forests.

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MATERIALS AND METHODS

Leaves or inner bark tissues were collected from *K. malaccensis* trees from 19 natural forest reserves throughout Peninsular Malaysia with an average sample size of 24 (Table 1). Of all the populations surveyed, two were from the peat swamp forests (Pekan and Kuala Langat Selatan) while the rest were from the non peat swamp forests. *Fig. 1* shows the location of the sampling sites. Genomic DNA was extracted using modified Murray and Thompson (1980) method.

A total of six polymorphic microsatellite loci (*Kma089, Kma125, Kma127, Kma141, Kma156, Kma157*) were applied on all the 460 samples. Previous characterization showed no linkage among these six loci with a Bonferroni correction ($\alpha = 0.05/276 = 0.0002$) (Lee *et al.,* 2006). Polymerase chain reaction (PCR) amplifications were carried out according to the protocols described in Lee *et al.* (2006). The PCR products were subjected to fragment analysis using ABI PRISM 377 DNA sequencer. Allele sizes were assigned against GeneScan Rox 400 (Applied Biosystems) internal size standard using GENESCAN v3.7.1 and genotyped using GENOTYPER v3.7 software (Applied Biosystems).

Genotypic data generated were analysed using FSTAT v2.9.3 (Goudet 2001). Genetic diversity parameters measured include the average number of alleles per locus (A_a), allelic richness R_s, (Petit *et al.,* 1998) and gene diversity (H_e, Nei, 1987). Population differentiation coefficient was quantified using R-statistics (R_{st}; Slatkin, 1995; Goodman, 1997), an analogue of Nei's genetic diversity statistics (G_{st}; Nei, 1987) developed for microsatellite loci under the assumption of a stepwise mutation model, which is likely at many microsatellite loci (Jarne and Lagoda, 1996).

Cluster analysis using D_A genetic distances (Nei *et al.,* 1983) and neighbour-joining (NJ) method (Saitou and Nei 1987) was performed using the program PowerMarker (Liu and Muse, 2005). Relative strength of the nodes was determined using bootstrap analysis of 1000 replicates.

RESULTS AND DISCUSSION

High levels of genetic diversity were observed in most of the populations surveyed (Table 2). The mean number of alleles per locus per population (A_a) was 10.2. Gene diversity (H_e) of the six loci

TABLE 1
Details of the *Koompassia malaccensis* populations investigated in this study

| Forest Reserve	State	Compartment No./ status
Labis	Johor	C9
Lenggor (VJR)	Johor	C231, VJR (unlogged)
Lenggor	Johor	C238, C241 (tagged for logging)
Mersing	Johor	C71 (unlogged, but tagged for logging)
Panti	Johor	C39, C41 (logged over)
Pasoh	Negeri Sembilan	50 ha ecological plot (unlogged)
Sungai Menyala	Negeri Sembilan	C9, C10, VJR (unlogged)
Pekan	Pahang	VJR (unlogged)
Air Cepam	Perak	C5 (tagged for logging)
Chikus	Perak	C44
Pangkor Selatan	Perak	Permanent Forest Reserve, Gazette No. 119
Pondok Tanjong	Perak	C10, C11
Ulu Kenas	Perak	Recreational Forest
Bukit Lagong	Selangor	C15, VJR (unlogged)
Kuala Langat Selatan	Selangor	C26, VJR (unlogged)
Semangkok	Selangor	C6 (unlogged)
Sungai Lalang	Selangor	C24, VJR (unlogged)
Bukit Bandi	Terengganu	Proposed as VJR (unlogged)
Jerangau	Terengganu	C10, VJR (unlogged)

VJR = virgin jungle reserve
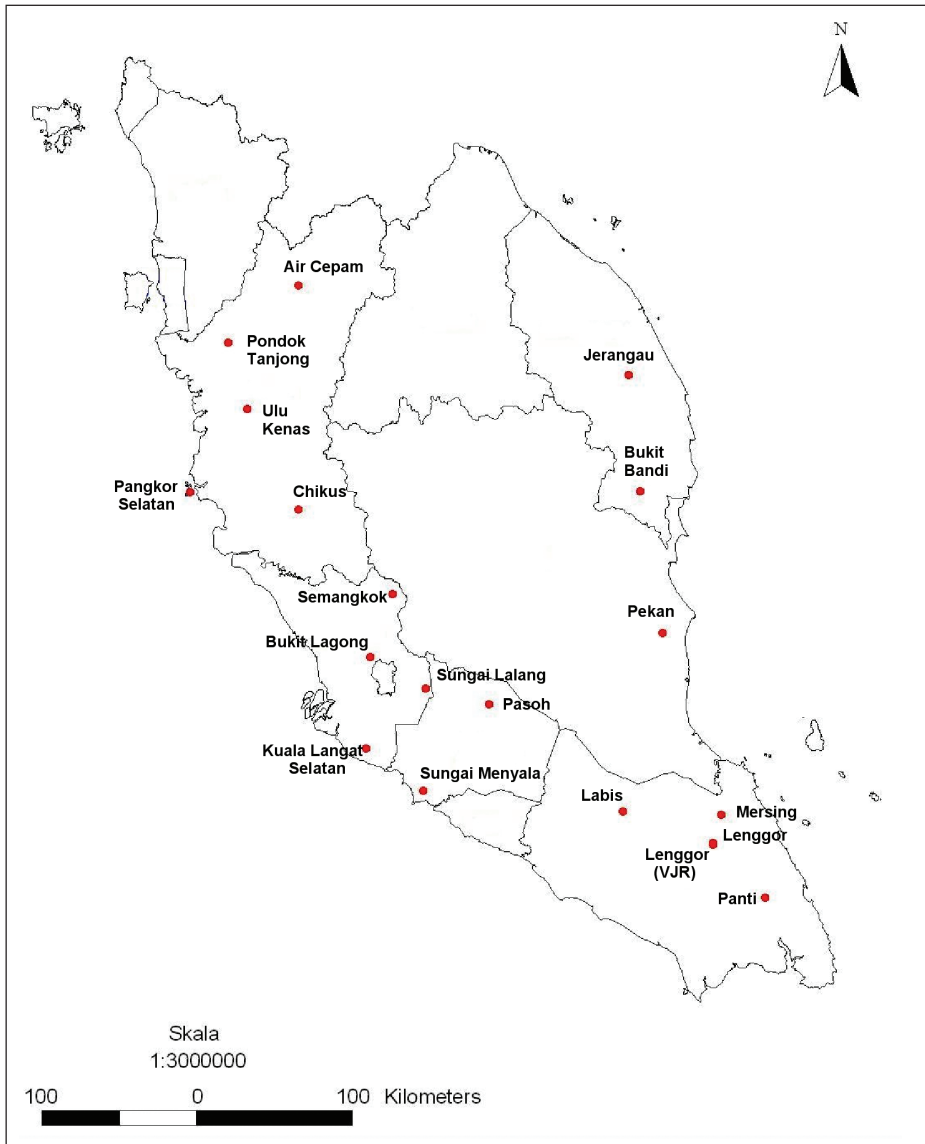
Fig. 1:   Map of Peninsular Malaysia showing the sampling sites of Koompassia malaccensis

ranged from 0.683 (Pekan) to 0.859 (Lenggor), with a mean of 0.798, whereas the mean allelic richness, R_s was 9.7, ranging from 6.0 (Pekan) to 9.3 (Lenggor). Gene diversity (H_e) obtained is comparable to *Swietenia macrophylla* (Lemes *et al.,* 2003) and *Jacaranda copaia* (Jones and Hubbell, 2003), but slightly higher than *Santalum austrocaledonicum,* an insular tree (Bottin *et al.,* 2005) and *Shorea lumutensis,* a rare dipterocarp (Lee *et al.,* 2006). Notably, the two peat swamp populations (Pekan and Kuala Langat Selatan) exhibited relatively low genetic diversity compared with the non peat swamp populations. In fact, Pekan showed the lowest values for all the three genetic diversity parameters estimated (A_a = 6.8, R_s = 6.0 and H_e = 0.683) and Kuala Langat Selatan is the second lowest in A_a (7.0) and R_s (6.3) (Table 2).

The population differentiation coefficient R_{st} was 0.07, i.e., 93% of the genetic diversity was partitioned within populations, with only 7% distributed among the populations. A previous study based on six populations using isozyme markers reported 4.5% of F_{st} (Lee *et al.,* 2007).

TABLE 2

Average number of alleles per locus (A_a), allelic richness (R_s) and gene diversity (H_e) of
Koompassia malaccensis from 19 populations surveyed based on 6 microsatellite loci

Population	No. of samples analysed	A_a	R_s	H_e
Air Cepam	19	10.1	8.8	0.847
Bukit Bandi	20	8.0	6.7	0.707
Bukit Lagong	20	9.8	8.7	0.839
Chikus	19	9.0	8.0	0.806
Jerangau	20	10.8	8.9	0.795
Kuala Langat Selatan	20	7.0	6.3	0.713
Labis	30	12.5	9.0	0.834
Lenggor (VJR)	20	10.3	8.9	0.815
Lenggor	25	12.0	9.3	0.859
Mersing	19	9.3	8.2	0.802
Pasoh	40	12.3	8.6	0.801
Panti	26	11.0	8.3	0.798
Pekan	20	6.8	6.0	0.683
Pangkor Selatan	37	10.0	7.4	0.785
Pondok Tanjong	34	10.3	7.9	0.806
Semangkok	16	10.3	9.2	0.829
Sungai Lalang	20	11.2	8.9	0.811
Sungai Menyala	43	14.3	9.0	0.813
Ulu Kenas	13	8.8	8.7	0.827
Mean	24	10.2	9.7	0.798

In comparison, Hamrick (1993) reported 13.5% of G_{st} for tropical woody species. According to Hamrick *et al.* (1992), woody species with large geographic range, outcrossing breeding systems, and wind or animal-ingested seed dispersal have more genetic diversity within species and populations but less variation among populations than those with other combination of traits. *Koompassia malaccensis* is wind dispersed, with fruits of twisted papery pods. To date, there is no reported study on the mating system of *K. malaccensis*, however Appanah and Weinland (1993) reported that bees, *Apis* sp. are the main flower visitors, which are generally long distance pollinators. Recent studies revealed that most tropical tree species are predominantly outcrossing (Doligez and Joly, 1997; Lee *et al.*, 2000; Loveless, 2002; Ward *et al.*, 2005).

Fig. 2 shows the neighbour-joining tree (Saitou and Nei, 1987) based on the D_A genetic distances (Nei *et al.*, 1983). Three clusters were observed with Pangkor Selatan, the only island population in this study as the outlier. The biggest cluster comprised of the western and southern populations. The other two clusters (Jerangau – Bukit Bandi – Bukit Lagong and Pekan – Kuala Langat – Lenggor – Lenggor VJR) did not correspond to the geographical locations. More stable dendrograms could be obtained by increasing the number of loci utilized (Koskinen *et al.*, 2004).

The cluster analysis also revealed close relationship between the two peat swamp populations, which formed a tight cluster with 100% bootstrap support value (*Fig. 2*). As they are not adjacent to one another (*Fig. 1*), the underlying factor for the high genetic similarity could be due to selection that might have had taken place in the process of adaptation to the habitat. In fact, morphologically, the boles of *K. malaccensis* from the peat swamp forests are generally cylindrical with steep plank-like buttresses. However, this speculation is not conclusive as microsatellite markers are generally selectively neutral. The application of more loci and/ or other molecular markers would further elucidate whether *K. malaccensis* of the two ecotypes (peat swamp and non peat swamp) are genetically distinguishable. Morgan-Richards and Wolff (1999) studied the genetic structure and differentiation of *Plantago major* and found a pair of sympatric sister species of two different ecotypes.
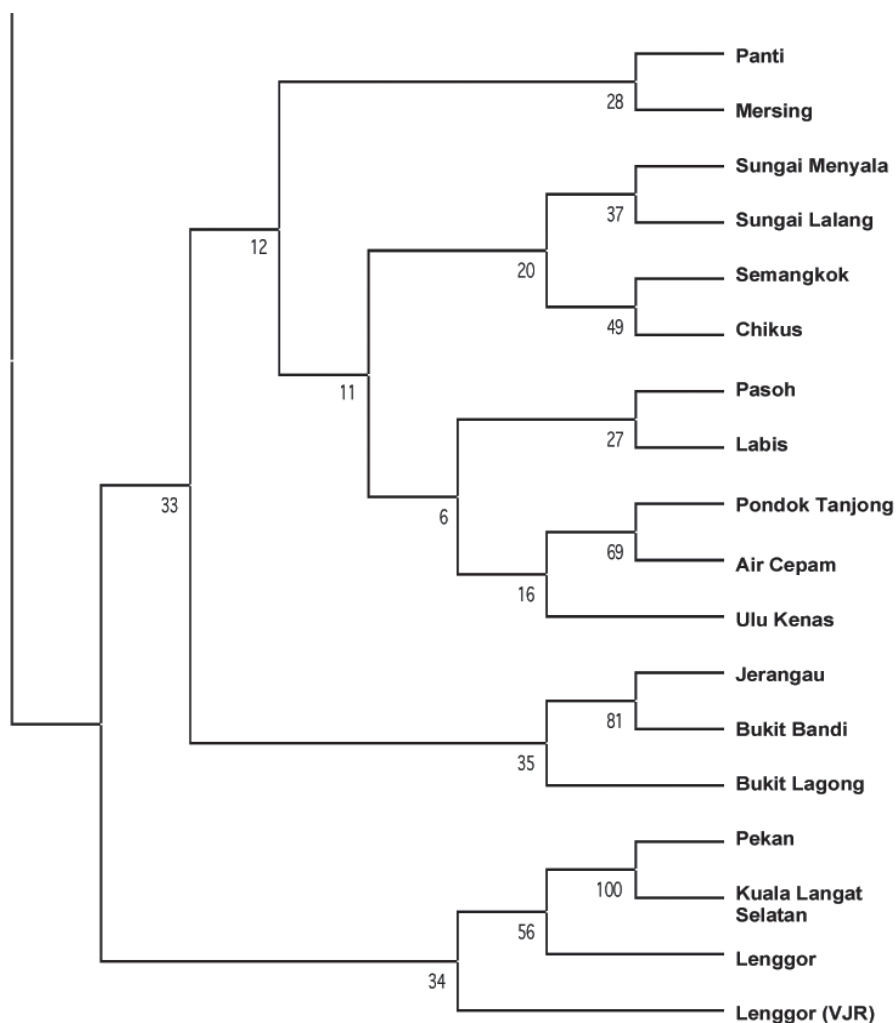
Fig. 2: Cluster analysis based on the genetic distances among the populations of Koompassia malaccensis *surveyed (bootstrap values were estimated based on 1000 replications)*

CONCLUSIONS

The application of more microsatellite markers and expansion of the study sites will generate more comprehensive genetic information for the planning of effective conservation and management programs for *K. malaccensis.*

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Y-chromosomal STR Variation in Malays of Kelantan and Minang

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ABSTRACT

Malays in Malaysia are a mixture of different races, caused by the history of migration centuries ago and may consist of 14 sub-ethnic groups. We used the Y-chromosomal STR (Y-STRs) to genotype two of the sub-ethnic groups namely, Kelantan and Minang Malays. In this ongoing study we investigated the polymorphisms of six Y-STR loci namely, DYS19, DYS388, DYS390, DYS391, DYS392 and DYS393 in the two populations mentioned. Twenty males (10 Kelantanese and 10 Minang) were analyzed by PCR amplifications followed by 8% non-denatured polyacrylamide gel electrophoresis. Randomly selected samples were sequenced for validation. Results revealed a total of 32 alleles, ranging from three (DYS19) to nine (DYS390). Allele frequency distributions ranged from 0.05 (DYS388, DYS 391, and DYS393) to 0.65 (DYS388). Although the level of polymorphisms of the two sub-groups were similar (average number of alleles, 4; average heterozygosity, 0.6), allele frequency distribution appeared to be imbalanced. Significant differences of allele frequency distributions were observed in loci DYS390, DYS391, and DYS393. None of the individuals shared the same haplotypes. However, errors of scoring and factors like small sample size should be considered. Preliminary results revealed polymorphisms in the six loci among the two Malay sub-ethnic groups. Significant differences of the allele frequency distributions were observed, but a further investigation with a larger sample size is warranted to confirm these findings.

Keywords: Y–STRs, polymorphisms, sub-ethnic groups, PCR amplifications

INTRODUCTION

The Malays in Malaysia are a mixture of different races, caused by history of migration centuries ago. Speculations were made on their pre-historic migration patterns to the Southeast Asia (SEA) region. Dental morphological traits suggested that two migrations into SEA originated from China about 30,000 years before present (Turner, 1987); while the linguistic groups proposed two major wave of migrations (Bellwood, 1985). Today, the Malays are heterogenous. We postulated that they may consist of 14 sub-ethnic groups namely, Melayu Kelantan, Minang, Bataq, Jambi, Kurinchi, Jawa, Riau, Melayu Yunnan, Mendeleng, Banjar, Bugis, Acheh, Champa, and Rawa.

The Y-chromosome, much like mtDNA, is inherited in a sex-specific manner. Its most attractive feature has been an apparent inability to undergo genetic recombination, making the task of assessing the extent of genetic variation in living human relatively straight forward as the accumulation of mutations is fairly by evolutionary forces over the period.

In this ongoing study, we investigated the polymorphisms of two of the unique Malay sub-ethnic groups namely, the Malays of Kelantan (Melayu Kelantan) and the Minangs (Melayu Minang) using six Y-STR markers.

MATERIALS AND METHODS

Sample Collection

Ethical approval was obtained from the Research and Ethics Committee of USM Health Campus. After obtaining the informed consent, 20 healthy and unrelated male subjects were recruited (10 for each sub-ethnic group). The samples of the Malays of Kelantan were collected from various districts of Kelantan including Machang, Kuala Krai, Rantau Panjang, Bachok and Kota Bharu);

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while the samples of Minangs were collected from Seri Menanti and Lenggeng, Negeri Sembilan. Subjects were interviewed to confirm their family history. They must be born at the specified location and their family at least three generations. Those with unknown family history, mixed marriage and consanguineous marriage and consanguity marriage were excluded from this study. Blood samples were collected from these subjects and DNA was extracted using commercial kit (QIAamp ® DNA Blood Mini Kit, Germany).

PCR Amplification

A total of six Y-STR markers were chosen based on the study done by Thomas *et al.* (1999). Details of the selected markers are outlined in Table 1. PCR was performed in a total volume of 10 ml consisting 20 ng DNA, 1 unit of *Taq* polymerase (Promega, Madison, MI, USA), 1x PCR reaction buffer (Promega, USA), 0.5 mM dNTPs (Promega, USA), appropriate concentration of Mg^{2+}, and forward and reverse primers (Table 1). The PCR protocol comprised of 5 min 95°C predenaturation; 38 cycles of 94°C denaturation (1 min); appropriate annealing temperature, and 72°C extension (30 sec; except DYS390, 90 sec). Amplification products were separated by vertical gel electrophoresis through 8% non-denaturing polyacrylamide gel along

with 20 bp DNA ladder. Some alleles, which were subsequently used as references, were later confirmed by DNA sequencing.

Statistical Analysis

Allele frequency of the six loci for each ethnic group were estimated and compared. Genetic diversity (h) in each ethnic group was estimated as $1 - \Sigma p_i^2$, where p_i represents the frequency of the ith allele at the locus.

RESULTS

The allele frequency distributions of the six Y-STR loci in the two Malay sub-ethnic groups are summarized in *Fig. 1*. Results revealed a total of 32 alleles, ranging from three (DYS19) to nine (DYS390) of the 20 individuals analyzed. Both groups seemed to have similar diversity both in terms of the number of alleles and their allele frequencies. The allelic variations at one of these loci are shown in *Fig. 2*.

Table 2 represents the diversity statistics for six Y-STR loci in the two Malay sub-ethnic groups studied. Table 3 indicates the haplotype data for both Minang and Kelantan Malays. Interestingly, none of the individuals shared the same six-locus haplotypes. However, sample sizes are too small to permit a complete analysis of the Y-chromosomal structure of these two sub-populations.

TABLE 1
Summary of loci selected, indicating the primer sequences, repeat motif, expected allele sizes, primers and $MgCl_2$ concentrations and annealing temperatures of PCR

Locus	Primer sequence (5' – 3')	Repeat motif	Exp allele sizes (bp)	[primers]+	$MgCl_2$ (mM)	T_a * (°C)
DYS19	F: CTACTGAGTTTCTCTGTTATAGT R: ATGGCATGTAGTGAGGACA	$(TAGA)_n$	186	50 ρmol	2.25	58
DYS388	F: GTGAGTTAGCCGTTTAGCGA R: CAGATCGCAACCACTGCG	$(ATT)_n$	127	50 ρmol	2.25	57
DYS390	F: TATATTTTACACATTTTTGGGCC R: TGACAGTAAAATGAACACATT	$(TCTA/TCTG)_n$	215	40 ρmol	1.5	54
DYS391	F: CTATTCATTCAATCATACACCCATAT R: ACATAGCCAAATATCTCCTGGG	$(TCTA)_n$	171	40 ρmol	1.5	57
DYS392	F: AAAAGCCAAGAAGGAAAACAAA R: CAGTCAAAGTGGAAAGTAGTCTGG	$(TAT)_n$	176	40 ρmol	1.5	57
DYS393	F: GTGGTCTTCTACTTGTGTCAATAC R: AACTCAAGTCCAAAAATGAGG	$(TAGA)_n$	128	50 ρmol	2.25	57

+[primers], concentration of primers; *T_a, annealing temperature
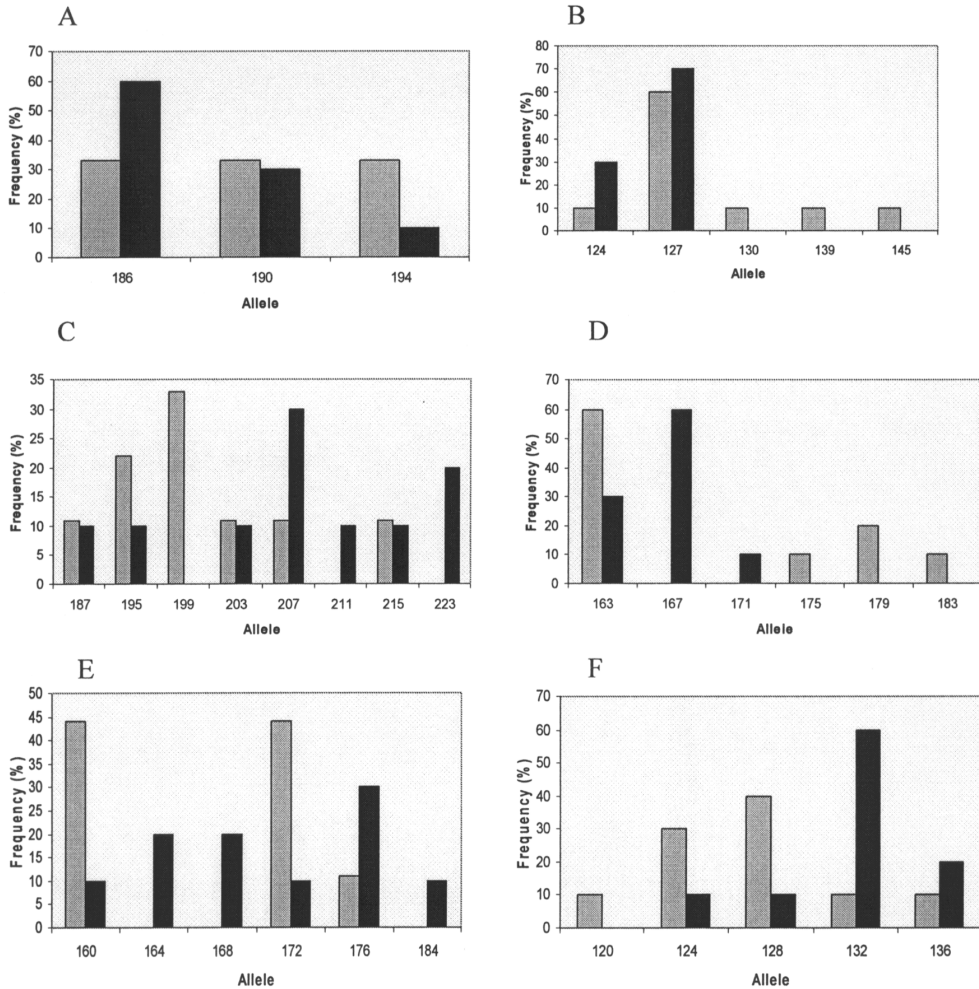
Fig. 1: Allele frequency distribution of locus (A) DYS19, (B) DYS388, (C) DYS390, (D) DYS391, (E) DYS392 and (F) DYS393, among Malays of Kelantan (bright colour) and Minang (dark colour)
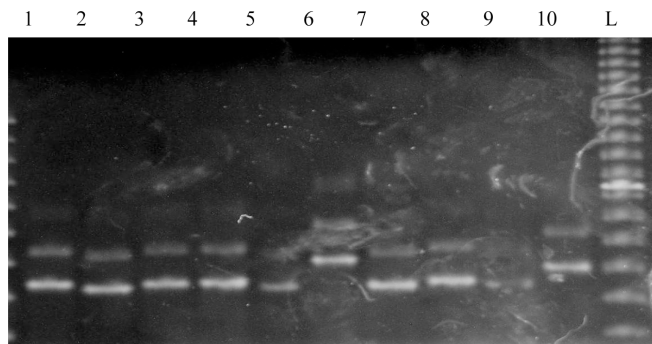

Fig. 2: Resolution of DYS393 on native polyacrylamide gel electrophoresis Lanes 1 to 5, Subjects of Malays of Kelantan; Lanes 6 to 10, subjects of Malays of Minang; Lane L, 25 bp ladder

TABLE 2
Diversity statistics for six Y-chromosome microsatellite loci in two Malay sub-ethnic groups

Population	Average heterozygosity	Total number of allele	Average number of alleles
Melayu Kelantan	0.658	26	4.333
Melayu Minang	0.617	25	4.167

TABLE 3
Y-STR haplotypes in the males subjects of the Malay sub-ethnic group

Sample		Locus*					
		DYS19	DYS388	DYS390	DYS391	DYS392	DYS393
	1	194	127	199	179	160	128
	2	194	124	207	175	160	132
Melayu	3		127		175		136
Kelantan	4	186	127	215	183	156	124
	5	186	127	199	171	160	124
	6	190	145	199	163	172	128
	7	194	127	203	163	172	124
	8	190	130	187	163	172	120
	9	190	127	195	163	184	128
	10	186	139	195	163	176	128
Average locus diversity						0.5926	
	11	186	127	207	165	164	136
	12	194	127	207	163	184	132
	13	190	127	187	163	164	132
Melayu	14	186	127	195	171	172	132
Minang	15	190	127	203	167	176	132
	16	186	127	223	167	176	132
	17	190	124	215	163	168	132
	18	186	124	207	167	160	132
	19	186	127	211	167	176	124
	20	186	124	223	167	168	136
Average locus diversity						0.8000	

* Locus haplotypes were indicated as the size of amplicon, bp

DNA sequencing performed on the selected samples confirmed the number of repeat motifs of the loci (*Fig. 3*).

DISCUSSION

The Minangs originated from West Sumatra, Indonesia. They are believed to be one of the largest martilineal groups in this modern time. Traditionally, the wife remained with her maternal relatives after marriage and inheritance are passed through the women. Islam was brought into the Minangs as a result of increased external trade with India, Acheh, and Melaka; and now they are among the most committed people to practice traditional Islam in the archepelago (Gall, 1998). Their ethnic traditions, was believed to be derived from animistic and Hindu-Buddhist beliefs. During the late 17th and early 18th centuries, migration of the Minangs from West Sumatra to the state of Negeri Sembilan, Peninsular Malaysia took place and their descendants now form the main sub-ethnic group in this state.

Fig. 3: DNA sequencing performed on a selected sample confirmed presence of the repeat motif
$(TAGA)_{11}$ *of the loci*

Meanwhile, long before the emergence of the Melaka Sultanate, Kelantan was a crucial centre of overland trans-peninsular trade route. Kelantanese also embraced Islam earlier than Melaka and was reputed as the centre of Islamic learning and scholarship. History resources showed that the ancient Hindu-Malay Empire of Langkasuka was centred in Pattani, which encompassed of Modern Malaysia states of Kelantan, Terengganu and Northern Kedah, as well as the provinces of Pattani, Yala, Narathiwat, Songkhla and Satun in Thailand. The Pattani Malays are very much similar in ethnicity, culture and language to the Malays of Kelantan.

Although the level of polymorphisms of the two sub-groups were similar (average number of alleles, 4; average heterozygosity, 0.6), allele frequency distribution appeared to be imbalanced. The differences of the history of settlement and their socio-cultural practices could probably explain this phenomenon.

Significant difference of locus diversity was observed in DYS392, where Kelantan appeared to have lower locus diversity (h = 0.5926) than Minang (h = 0.800). This could be an interesting locus to investigate its ability to differentiate the two sub-ethnic groups. Previous studies found that the locus diversity of this locus ranged from 0.7 – 0.75 (Yong *et al.*, 2006; Chang *et al.*, 2007). Meanwhile, Srikummool *et al.* (2000) reported a relatively lower frequency of allele 132 bp in DYS393 among the Thai population studied. Interestingly, this was found to be similar with the the Malays of Kelantan in the current study, with a contrasting result from the Minangs, which revealed a significantly higher frequency for allele 132 bp, proposing a closer affinity of the Malays

of Kelantan to the Thai population. In addition, allele 167 bp of locus DYS391 was also interesting since it was a common allele in Minang but was not found in Malays of Kelantan. Few of these differences may be significant even with the small number of samples and loci currently examined but the consistency of the results, however, is reassuring.

The 8% PAGE offered a rapid, sensitive, and reproducible method for genotyping these six Y- STRs locus. Due to overall large fragment sizes, the point mutations were ignored, as they do not change the migration behaviour in the native PAGE seperation. However, technical errors like miscoring and null alleles should be considered.

In summary, our preliminary results revealed polymorphisms in the six loci among the two Malay sub-ethnic groups. Significant differences of the allele frequency distributions were observed, but a further investigation with larger sample size is warranted to confirm these findings.

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