DEVELOPMENTAL AND GENETIC STUDIES OF THE GENUS *MACROBRACHIUM* BATE

by

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Doctor of Philosophy

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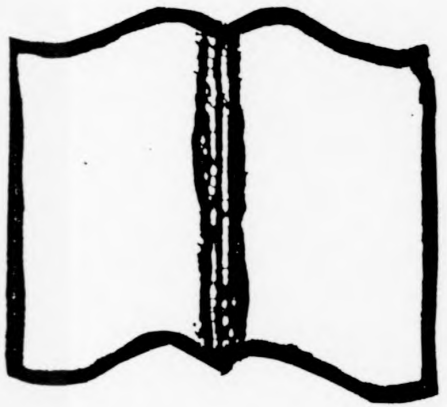
THIS VOLUME HAS A VERY TIGHT BINDING



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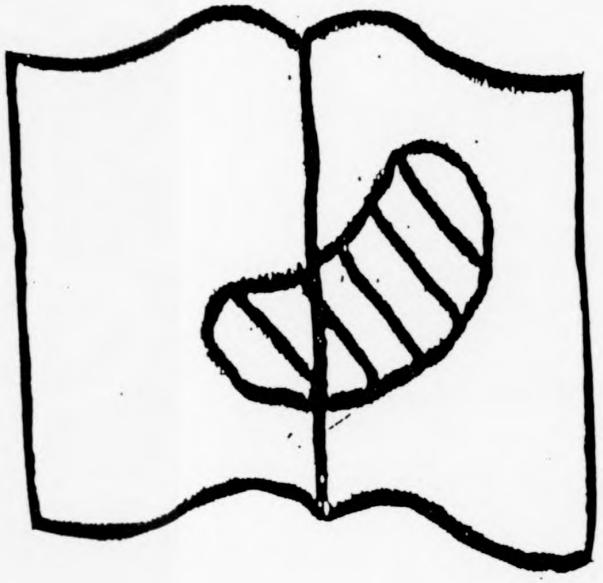

TO MY PARENTS

ABSTRACT

Macrobrachium, as a genus, has evolved comparatively recently from the marine into the freshwater environment. Biogeographic and morphological analysis suggested that the genus has evolved in the present-day Indo-West Pacific area and spread subsequently to West Africa, Eastern America and Western America. The present thesis investigated the larval development and genetics of the species and related the results to both evolution and aquaculture of the genus.

M. lanchesteri was found to have nine zoeal stages before metamorphosing to the postlarval stage. This is similar to the other species in the genus with an extended type of larval development. However, it is one of the few species of this group that can complete the larval phase in freshwater. *M. hainanense*, on the other hand, has the abbreviated type of larval development, all pereopods and pleopods (non-larval forms) are already present in the first post-embryonic stage. Phylogenetic trees constructed from allozyme data suggest that the 'hainanense' group (species with similar larval development to *M. hainanense*) is directly related to a primitive group within the genus, the 'rosenbergii' group, rather than to other species in the Indo-West Pacific. The comparatively fewer species with intermediate type of abbreviated larval development, coupled with inference from allozyme data, suggests that the genus has probably 'invaded' the freshwater environment in more than one wave. Phylogenetic trees drawn from larval developmental data and allozyme data also have a high degree of congruence. As the degree of abbreviated development relates to the history of

advancement in freshwater (except 'rosenbergii' group), the congruence between the larval developmental data and the allozyme data confirms the polyphyletic nature of the genus.

Larvae of a population of M.nipponense was found to metamorphose in a range of salintiy, including freshwater (reduced survival rate). A relatively high realised heritability was observed for larval freshwater tolerance of M.nipponense. This suggested that some species (some populations) still have a high potential for further adaptation to the freshwater environment.

Considerable genetic differentiation was observed between populations of M.nipponense and M.rosenbergii. Hydrographic conditions are suggested to have contributed to the population differentiation in M.rosenbergii. In M.nipponense, the two pond/lake populations have much lower values of genetic diversity than the two estuarine populations.

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Prof. L.B. Holthuis (Leiden), as an authority on the taxonomy of Macrobrachium, has been most helpful throughout the course of the present study. He also corrected two major mispelling of mine, Macrobachium (for Macrobrachium) and Holthius (for Holthuis), both being essential to the subject of concern in here. I am also indebted to Dr. D. I. Williamson (Liverpool), as an authority on crustacean larval morphology, for his advise and constructive criticisms throughout the study. Both Prof. L.B.Holthuis and Dr. D.I.Williamson, as well as Mr. J.C. Bostock, have also reviewed different parts of the present thesis.

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The present study involved a large number of samples (natural populations) from overseas. As I did some of the

collecting myself, I have come to realise that the task does require much effort (and probably resistence to mosquito). I am most thankful to the following persons who have helped either in the actual collection or subsequent transport of prawn samples : Mr. Mike Akester and his parents for samples from Mexico; Mr. Dave Sampson for samples from Bangladesh; Mr. John Bostock for samples from Tanzania; Mr. Matt Briggs for samples from Kenya; Mr. L. Balasursya, Dr. R. Subrasinga, and Dr. James Muir for samples from Sri Lanka and Thailand; Mr. Johannes Hutabarrat, Dr. Janet Brown and Mr. S. Budi for samples from Indonesia; Mr. and Mrs. T.P. Tang and Mr. S. Kobayashi for samples from Japan and Sabah; Mr. Richard Cheung and Mr. T.K. Mok for samples from Hong Kong; Prof. PAN Jin-pei, Prof. ZHU Zhoyin and Prof. WU Wei-sang for the samples from P.R.China; and Dr. I. Anderson and Dr. G. Nash for samples from Malaysia.

The present study in the taxonomy and evolution of the genus Macrobrachium has involved the hunting of many inaccessible references (even through the excellent system of inter-library loans). Miss Rowena White (U.C.N.W.), Prof. L.B. Holthuis, Dr. D.I. Williamson and Prof. T.J. Lam (Singapore) has generously provided many of such references. Dr. Joseph Felsenstein (University of Washington) generously provided his computor program PHYLIP freely for my use in phylogenetic analysis.

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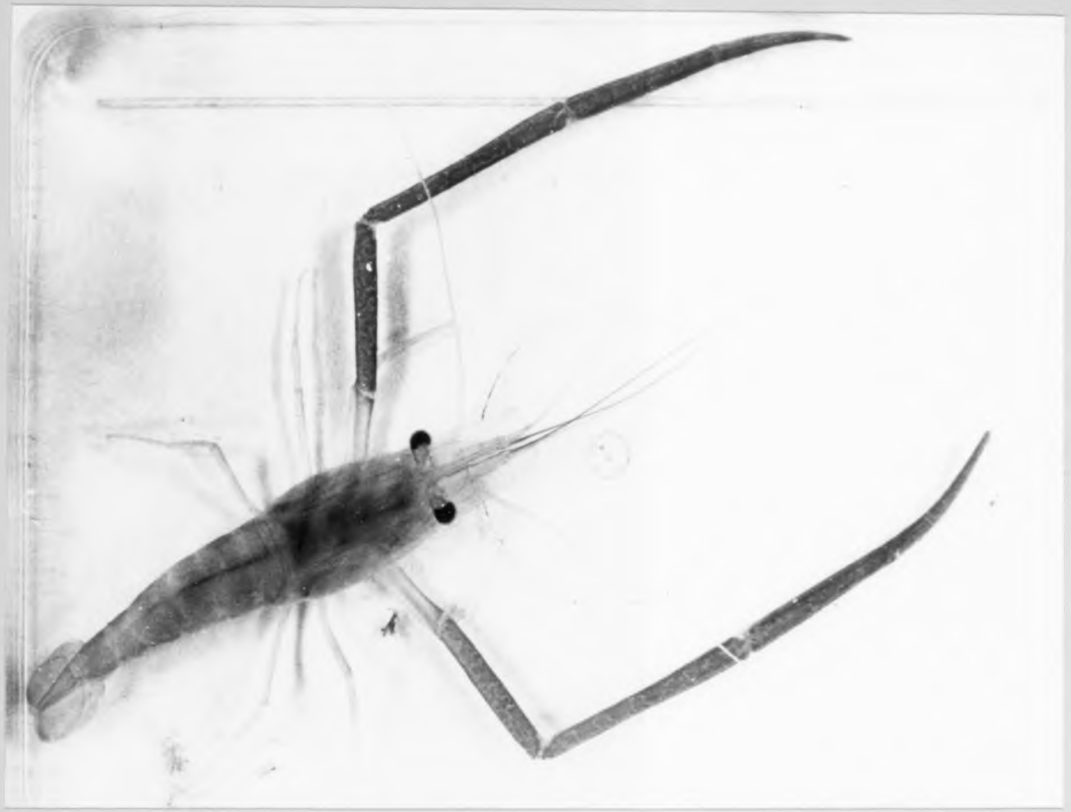
PLATE (I) :

Male and female *Macrobrachium nipponense*
(about actual size)



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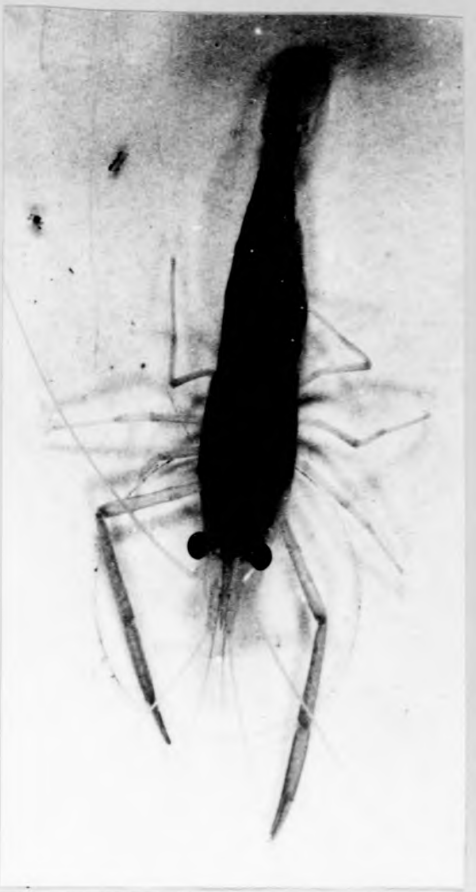

PLATE (II) :

A Chinese artist's impression of *Macrobrachium nipponense*.

Original painting has a dimensions of 1m x 0.5 m and was a work by the gifted painter Mr. CHU shun in 1984 (at the age of 15). Presented to the author at the beginning of this study.


蝦戟圖
天任吾兄笑正
甲子秋 弟 熏 遠 作

'In the shallow water, even the dragon would be toyed by the little prawn.'

- a Chinese folkore

CHAPTER ONE :

GENERAL INTRODUCTION

(1.1) Of Prawns and Man

In the history of Man, it is generally recognised that prawns had their well-defined niche wherein even the aforementioned dragon could not replace (the Chinese folklore). Thus, Macrobrachium nipponense (de Haan), the common freshwater prawn of China, has inspired Chinese painters for centuries (see Plate I & II). The catching and trapping of the same species in riceland, rivers and waterways, on the otherhand, provides an endless mean of entertainment for rural children. Furthermore, the well proclaimed delicacy of Chinese prawn paste and its production method, was recorded in the monumental work of "Chhi Min Yao Shu" (Essential Techniques for the Peasantry, by Chia Ssu-Hsieh, 535 A.D.) and has no doubt contributed significantly to the rural economy through the eras.

As human society transformed from a 'hunting' mode to a 'farming' mode, various livestock was domesticated at various times in history. It is generally recognised that prawn culture probably began in the form of trapping-holding-growing in coastal areas of South East Asia, some several hundred years ago (Ling,

1977). Considering the long history of M.nipponense as the 'holotype' of Chinese painting, the long history of fish ponds in China (first recorded in 'The Monograph of Fish Culture' by L. Fan, about 500 B.C.), and the proliferation of this species in southern China, the accidental trapping-holding-growing of freshwater prawns probably has a still longer history. However, the scientific culture of prawns began in more recent times. This is undoubtedly attributable to the complicated larval phase of these crustaceans. Thus, it was only after thirty years of research, with the introduction of algae and brine shrimp as larval food, that the first mass culture techniques of the kuruma shrimp, Penaeus japonicus Bate, was developed by Dr.M.Fujinaga in 1967 (Shigueno, 1974). The giant freshwater prawn, M.rosenbergii(de Man), while attracting much attention for its large size(up to 350mm), had failed all early attempts at culture. It was not until the well-publicised introduction of soya sauce into the culture medium by Dr.S.W.Ling, supplemented by ecological data on the species, that the first laboratory larval rearing became successful in 1967 (Ling, 1977). With these pioneer works, standardization of methodologies followed, and prawn culture has been adopted worldwide. The present world production of prawn from aquaculture approaches 300,000 tons (Ling, FAO). Given the high price and the still insatiable demand, prawns are now generally regarded as a major source of valuable foreign exchange in many countries.

Apart from the species M.rosenbergii, most attention has been given to the species of Penaeid prawns as candidates for aquaculture. However, as marine species, Penaeid prawns only

occur in the coastal areas and their culture is restricted for this reason. Species of the genus Macrobrachium, however, can be found in various freshwater habitats throughout the tropics. Many species, unlike M.rosenbergii, spend their entire life cycle in freshwater and have wide ranges of environmental tolerance. Most species are also caught traditionally and are accepted as food wherever they occur (Holthuis, 1980). It is probable that many species would be suitable candidates for culture. However, partly because of their worldwide distribution and their occurrence in many less accessible inland waters, little is known about the majority of the species.

(1.2) The Genus Macrobrachium Bate, 1868

Literally, 'macro-brachium' means big arm in Latin. Indeed, species of the genus, and especially the adult males, are generally characterised by the comparatively pronounced size of the second pereopod. According to the classification of recent crustaceans by Bowman and Abele(1982), the taxonomic status of the genus can be summarised as follows:

Superclass Crustacea Pannont, 1777
Class Malacostraca Latreille, 1806
Superorder Eucaridea Calman, 1904
Order Decapoda Latreille, 1803
Suborder Pleocyemata Burkenroad, 1963
Infraorder Caridea Dana, 1852
Superfamily Palaemonioidea Rafinesque, 1815
family Palaemonidae Rafinesque, 1815
Subfamily Palaemoninae Rafinesque, 1815
Genus Macrobrachium Bate, 1868

Four subfamilies, Euryrhynchinae Holthuis 1950, Typhlocaridae Annandale & Kemp 1913, Pontoniinae and Palaemoninae

are included in the family Palaemonidae by Holthuis(1950). The subfamily Palaemoninae in turn consists of fourteen genera (Holthuis, 1950): Desmocaris Sollaud,1911a; Creaseria Holthuis,1950; Leander E.Desmarest,1849; Leandrites Holthuis,1950; Palaemon Fabricius,1798; Palaemonetes Heller,1869; Troglocubanus Holthuis,1949; Leptocarpus Holthuis(1950); Cryphiops Dana,1852; Pseudopalaemon Sollaud,1911b; Brachycarpus Bate,1888 and Macrobrachium. A detailed scheme to separate the various subfamilies and genus can be found in Holthuis(1950). Some distinguishing features of the genus Macrobrachium are the absence of both supraorbital and branchiostegal spines, the presence of the hepatic spine (except M.hilderbrandti(Richters), and dactylus of last three legs simple. Before the work of Holthuis(1950), much confusion prevailed on the classification of the subfamily Palaemoninae. In his 1950's work, Prof.Holthuis pointed out the correct adoption of the name Palaemon for the genus with the type species Palaemon squilla(L.) and divide the otherwise defined genus Palaemon (by Kemp 1925) into Macrobrachium and Cryphiops. The latter two genera differ by the presence and absence of the hepatic spine respectively. While some authors still persist in adopting the name Palaemon(e.g. Tiwari 1955) for some species of Macrobrachium, the position has since been clarified.

Some specific areas on the taxonomy within this genus will be discussed in Chapter 3, Chapter 5 and Chapter 6. A list of the known species of Macrobrachium is included in Table(1.1). The authorities for each species would only be mentioned once in this table and omitted subsequently in the thesis. Additional species

to that included in Holthuis(1950,1952) were compiled with detailed search in Zoological Records, Biological Abstracts and Aquatic Science and Fisheries Abstracts. The list has also been confirmed by Prof. L.B.Holthuis (Pers. Comm., 1987). However, as the dates of some of these species would suggest, many new species may have been recorded since the preparation of the list. Also, some species would probably be synonyms with each other. As far as the alphabet is concerned, the genus is desperately lacking species beginning with the letter 'x'.

(1.3) Life Cycle and General Ecology

The life cycle of Macrobrachium, as in many other crustaceans, consists of embryonic(egg), larval, post-larval and the adult phases. Mating occurs between a post-moult mature female and a mature male. The female is guarded between the long chelipeds of the male throughout the pair formation for several hours. In an aquarium, alas, it is usually the biggest male who gets the post-moult female. Copulation occurs with the male turning the soft-shelled female to a ventral position(i.e. face to face) and then pressing down from above for 2-3 seconds. This behaviour, when observed in an aquarium, is usually repeated several times, resulting in the deposition of the spermatophores into the thoracic region of the female. Detail description of mating behaviour of M.nipponense can be found in Ogawa et al.(1981) and Chow et al.(1982).

Egg laying and fertilisation occur within a few hours after

Table (1.1) : Known species and subspecies of Macrobrachium

M.acanthochirus Villalobos,1966
M.acanthurus (Wiegmann,1836)
M.acherontium Holthuis, 1978
M.adscitum (Riek, 1951)
M.aemulum (Nobili, 1906)
M.altifrons altifrons (Henderson, 1893)
M.altifrons ranjhai Tiwari, 1964
M.amazonicum (Heller, 1862)
M.americanum Bate, 1868
M.andamicum (Tiwari, 1952)
M.aracamuni Rodriguez, 1982
M.asperulum (Van Martens, 1868)
M.assamense assamense (Tiwari, 1955)
M.assamense peninsulare (Tiwari, 1955)
M.atabapense Pereira, 1986
M.atactum atactum Riek, 1951
M.atactum ischnomorphum (Tiwari, 1955)
M.atactum sobrinum Riek, 1951
M.australe (Guerin, 1836)
M.australiense australiense Holthuis, 1950
M.austrliense eupharum Riek, 1951
M.austrliense crassum Riek, 1955
M.austrliense cristatum Riek, 1955
M.banjaree (Tiwari, 1955)
M.bariense (De Man, 1892)
M.birai Lobao, Melo & Fernandez, 1986
M.birmanicum (Schenkel, 1902)
M.borellii (Nobili, 1896)
M.brasiliense (Heller, 1862)
M.caledonicum (Roux, 1926)
M.callirhoe (De Man, 1898)
M.canarae (Tiwari, 1955)
M.carcinus (Linnaeus, 1758)
M.carvenicola (Kemp, 1924)
M.chevalieri (J.Roux, 1935)
M.choprai (Tiwari, 1949)
M.clymene (De Man, 1892)
M.cocoense Abele et Kim, 1984
M.cortezi Rodriguez, 1982
M.cowlesi Holthuis, 1950
M.crenulatum Holthuis, 1950
M.crybelum Chace, 1975
M.dayanum (Henderson, 1893)
M.dierythrum Pereira, 1986
M.digueti (Bouvier, 1895)
M.dux (Lenz, 1910)
M.edentatum Laing et Yan, 1986

(to be continued)

Table (1.1): (continue)

M.equidens (Dana, 1852)
M.eriocheirum Dai, 1984
M.esculentum (Thallwitz, 1914)
M.faustinum (De Saussure, 1857)
M.felicinum Holthuis,1949
M.ferreirai Kensley et Walker, 1982
M.fluviale (Streets, 1871)
M.foai (Coutiere, 1902)
M.formosense Bate, 1868
M.fukienense Liang et Yan, 1980
M.gallus Holthuis, 1952
M.glypticum Riek, 1951
M.gracilirostre (Miers, 1875)
M.grandimanus (Randall, 1849)
M.guangxiense Liang et Yan, 1981
M.hainanense (Parisi, 1919)
M.hancocki Holthuis, 1950
M.hendersodayanum (Tiwari, 1952)
M.hendersoni hendersoni (Tiwari, 1952)
M.hendersoni cacharense (Tiwari, 1952)
M.hendersoni platyrostre (Tiwari, 1952)
M.heterochirus (Wiegmann, 1836)
M.hildebrandti (Hilgendorf, 1893)
M.hirtimanus (Olivier, 1811)
M.hirtimanus (Tiwari, 1952)
M.holthuisi Genofre et Lobao, 1978
M.horstii (De Man, 1892)
M.idae (Heller,1862)
M.idella idella (Hildgendorf, 1898)
M.idella georgii Jayachandran & Joseph, 1985
M.iheringi (Ortmann, 1897)
M.inca Holthuis, 1950
M.indicum Jayachandran & Joseph, 1986
M.inflatum Liang et Yan, 1985
M.inpa Kensley et Walker, 1982
M.insulare (Parisi, 1919)
M.intermedium (Stimpson, 1860)
M.japonicum (De Haan, 1849)
M.jaroense (Cowles, 1914)
M.javanicum (Heller, 1862)
M.jelskii (Miers, 1877)
M.jiangxiense Laing et Yan, 1985
M.johnsoni Chong and Khoo, 1987
M.joppae Holthuis, 1950
M.kempi (Tiwari, 1949)

(to be continued)

Table (1.1): continued

M.kistnenssis (Tiwari, 1952)
M.kiukianense (Yu, 1931)
M.lamarrei lamarrei (H.Milne Edwards, 1844)
M.lamarrei lamarroides (Tiwari, 1952)
M.lanceifrons (Dans, 1852)
M.lanceifrons montalbanense (Cowles, 1914)
M.lanchesteri (De Man, 1911)
M.lar (Fabricus, 1798)
M.latidactylus
M.latimanus (Von Martens, 1868)
M.lepidactyloides (De Man, 1892)
M.lepidactylus (Hilgendorf, 1897)
M.longidigitum Dai, 1984
M.lorentzi (J.Roux, 1921)
M.lucifugum Holthuis, 1974
M.lujae (De Man, 1912)
M.macrobrachion (Herklots, 1851)
M.maculatum Laing et Yan, 1990
M.malcolmsonii malcolmsonii (H.Milne Edwards, 1844)
M.malcolmsonii choprai (Tiwari, 1949)
M.malcolmsonii kotreeanum Johnson, 1973
M.mammillodactylus (Thallwitz, 1892)
M.manipurense (Tiwari, 1952)
M.meridionalis Laing et Yan, 1983
M.microps Holthuis, 1978
M.mieni Dang, 1975
M.minutum (J.Roux, 1917)
M.mirabile (Kemp, 1917)
M.moorei (Calman, 1899)
M.naso (Kemp, 1918)
M.nattereri (Heller, 1862)
M.natulorum Holthuis, 1984
M.nepalense Kamita, 1974
M.niloticum (P.Roux, 1833)
M.nipponense (De Haan, 1849)
M.nobilii (Henderson& Matthai,1910)
M.novaehollandae (De Man, 1908)
M.obtusifrons Dai, 1984
M.occidentale Holthuis, 1950
M.oenone (De Man, 1902)
M.ohione (Smith, 1874)
M.olfersii (Wiegmann, 1836)
M.palaemonoides Holthuis, 1950
M.palawanense Johnson, 1962
M.panamanense Rathbun, 1912
M.patsa (Coutiere, 1899)

(to be continued)

Table (1.1): continue

M.pectinatum Pereira, 1986
M.peguense (Tiwari, 1952)
M.petersii (Hilgendorf, 1879)
M.petiti (J.Roux, 1934)
M.petronioi Melo, Labao & Fernandez, 1986
M.pilimanus (De Man, 1879)
M.pinguis Dai, 1984
M.placidulum (De Man, 1892)
M.placidum (De Man, 1892)
M.poeti Holthuis, 1984
M.potiuna (Muller, 1880)
M.praecox (J.Roux, 1928)
M.pumilum Pereira, 1986
M.quelchi (De Man, 1900)
M.raridens (Hilgebdorf, 1893)
M.rathbunae Holthuis, 1950
M.reyesi Pereira, 1986
M.rodriguezi Pereira, 1986
M.rogersi (Tiwari, 1952)
M.rosenbergii (De Man, 1879)
M.rude (Heller, 1862)
M.scabriculum (Heller, 1862)
M.shokitai Fujino et Baba, 1973
M.sintangense (De Man, 1898)
M.siwalikense (Tiwari, 1952)
M.sollaudii (De Man, 1942)
M.sophronicum Holthuis, 1950
M.srilankense Costa, 1979
M.sulcicarpale Holthuis, 1950
M.superbum (Heller, 1862)
M.surinamicum Holthuis, 1948
M.tenellum (Smith, 1871)
M.therezieni Holthuis, 1965
M.thysi Powell, 1980
M.tolmerum (Riek, 1951)
M.transandicum Holthuis, 1950
M.trompii (De Man, 1898)
M.veliense Jayachandran et Joseph, 1986
M.venustrum (Parisi, 1919)
M.villalobosi Hobbs, 1973
M.villosimanus (Tiwari, 1949)
M.vollenhovenii (Herklots, 1857)
M.weberi (De Man, 1892)
M.yeti Dang, 1975
M.yui Holthuis, 1950
M.zariquieyi Holthuuis, 1949

copulation, ranging from 1-6 hours for M.nipponense (Uno, 1971; Ogawa et al., 1981) and 6-20 hours for M.rosenbergii (Ling, 1969). Ova were extruded from the gonophores at the base of the third pereiopods and are fertilised on passing over the spermatophores attached between the third and the fifth coxas. During egg-laying, the abdomen is flexed and the pleopods extended so that the first and second pairs of pleopods overreach the sperm receptacle area, forming a "floor" for the egg passage to the brooding chamber (under the abdomen). Detailed description of spawning and post-spawning behaviour of M.nipponense can be found in Ogawa et al.(1981).

The egg mass is attached to the pleopods , vigorous beating of which helps oxygenation of eggs. Embryonic development takes 11-22 days for M.nipponense, depending on the water temparature (Uno 1971). In this laboratory, egg-incubation of M.nipponense generally takes 14 days at 28'C. Detail description of embryonic development for M.carcinus was reported in Rauh Muller(1984).

Hatching of the eggs result from both mechanical beating of the pleopods and osmotic inflow of water. Ogawa et al. (1981) and Katre and Pandian(1972) reported details of the process for M.nipponense and M.idae.

For many species with planktotrophic larvae requiring a brackishwater existence, the gravid females also perform a spawning migration. Some published examples are M.ohione (Reimer, et al., 1974), M.digueti and M.rosenbergii (Raman, 1964). For M.carcinus a species which requires brackishwater for larval development, Hughes and Richard (1975) proved that while males and non-gravid females have a positive rheotaxis upstream, gravid

females have a rheotaxis downstream. In the Mississippi, M.carcinus, a species which requires brackish water for its larval development, can be found 325 km upstream (Horne and Beisser, 1977).

Most Macrobrachium, as in many other species of shrimps, also have a diurnal change in activities, hiding understones, vegetation, etc. during daytime, and emerging at night. In a 24 hour trapping study in the Shimanto River, Japan, the density of M.nipponense, M.formosense and M.japonicus increased just as the light level become zero at 22:00 and decreased at dawn (5:00) (Ohno et al., 1977).

Apart from spawning and diurnal migration, many species of the taxa also have seasonal fluctuation in their abundance. In sub-tropical/temperate area, abundance of Macrobrachium tends to fluctuate with water temperature, with spawning and population peaks corresponding to high temperatures. In the Atchafalaya Rivar, Louisiana, M.ohione was most abundant between March and September, when water temperature was greater than 20'C, especially between April and June (Truesdale and Mermilliod, 1979). In the Lam Tsuen River, Hong Kong, abundance of M.hainanense was correlated with water temperature with a 1-2months lag effect (Dudgeon, 1985). In both Korea and Lake Kojima, Japan, M.nipponense spawned between May and September, and peaked in July (Chung, 1972; Ogawa and Kakuda, 1986). In Lake Kasimigawa, Japan, M.nipponense also underwent a seasonal migration, being most abundant in shallow water during the summer months when the water temperature was between 22'C-30.3'C but moved offshore in October as the water temperature became lower

(Kubo 1949). In the sub-tropical area of southern China, Qi(1977) observed two peaks in the number of berried females, one in April/May, and a lesser one in October.

In tropical areas, however, where variation in the seasonal water temperaure is less pronounced, most species can breed throughout the year. The main breeding season and population peak are affected, in this case, by the timing of the rainy/wet season. In the Lower Niger River, spawning of M.felicinum mostly took place during July to October, the rainy and flood period (Inyang, 1984). In the St.Paul River, Liberia, the main spawning season also occurred between August and December. In the Amazon Igapo, populations of both M.inpa and M.natteri peaked between the rise and fall of the water level between June and July (Walker and Ferreira, 1985).

While population abundance is affected by environmental factors as mentioned, species of Macrobrachium are generally one of the most prominant group of macroinvertebrates wherever they occur. These prawns are generally regarded as omnivorous and scavengers, but can actively pursue smaller animals (e.g. insects and fish by M.inpa; Walker and Ferreira, 1985). The natural diet of Macrobrachium generally consists of both vegetative and animal materials (e.g. M.digueti, M.tenellum; Abele and Blum, 1979). In the habitat of the Igapo of the Amazon, Macrobrachium are 'the largest invertebrate present in appreciable number' and they occupy an intermediate position in the chain of trophic levels (Walker and Ferreira, 1985). The same authors also inferred that "the relative high density of shrimp population must therefore be regarded as a reservoir which buffers the dangerous fluctuation

of a weak flow through an excessive unstable habitat". In Lake Biwa, Japan, M.nipponense, which again was the prominent macroinvertebrate, probably contributed significantly to the nitrogen-regeneration of a macrophyte community; especially in summer when both the production of macrophyte and prawn population were at their highest (Miura et al., 1978).

The ecological importance of the group was best summarised by Welcome(1985): "Ecologically they (refering to river decapods) may be considered together with fish on the basis of their size, position in the food chain, behaviour and economic importance as food otganisms. Species of Macrobrachium particularly form the basis of fisheries in many tropical rivers".

(1.4) Aquaculture and genetics

In the last decade, yields from aquaculture have doubled to about 8 million tons and a five to ten fold increase is projected by the year 2000 (WCED, 1987). Aquaculture can be carried out at all commercial levels - individual, family, co-operative or large business, and can be integrated to traditional agriculture. The intensity of capital investment also varies from intensive input to the more extensive type which involves high labour but relatively low investment. As aquatic products generally contribute more significantly to human protein diet in developing countries, aquaculture can produce a better livelihood in these countries through the provision of jobs and cash crops, as well as providing a better diet.

While most agricultural livestock has a long history of domestication, most cultured aquatic species (especially prawns)

are very recent in the 'domestic' scene and many species are still being 'hunted' in the wild. On the one hand, this situation necessitates basic biological data and husbandry techniques to be developed by empirical means. On the otherhand, there is the unique opportunity to apply to aquaculture many modern genetic selection/improvement techniques which have been responsible for the success of traditional agriculture. Apart from this fundamental difference with traditional livestock, many aquatic species also have several inherent characteristics which make them particularly suitable for genetic manipulation:

(1) Cultivatable aquatic animals, which are invertebrates or poikilothermic vertebrates, generally have higher genetic variability than the domesticated livestock, which are generally homeothermic vertebrates. Heterozygoscity and polymorphic loci of crustaceans and molluscs are generally at levels of 0.15 and 0.35 respectively, while that of vertebrates are 0.05-0.06 and 0.1-0.2 respectively (Nelson and Hedgecock,1980; Wilkins, 1981). This implies that the amount of variation available for manipulation is greater in aquatic species;

(2) Sex determination is also more flexible in aquatic invertebrates and fish. The production of a monsex population, which is desirable for many commercial traits in many species, can be readily attained (at least in fish) by hormonal treatment coupled with genetic manipulation;

(3) External fertilisation and larval development of many aquatic species implies the possibility to manipulate the gametes. Inter- and intra-specific crosses between species and sub-

species can be produced as potential candidates for aquaculture. Transgenic progenies can also be produced by microinjection of desired genes or nuclear transplantation. Both techniques would be easier in most aquatic species with external fertilisation and embryonic development, as compared to placental vertebrates;

(4) Also related to external fertilisation, second meiotic division of many aquatic species occurs only after fertilisation. This facilitate artificial manipulation of both meiotic and mitotic cell division via various chemical and physical agents (e.g. thermal shock). Also, the DNA content of the gametes can be denatured by irradiation without losing the mobility of the gametes. By the manipulation of maternal/paternal DNA, coupled with the manipulation of early cell divisions, triploid, tetraploid, gynogentic and androgenetic individuals can thus be produced. While triploidy generally implies sexual sterility to the individual and can be exploited for population control, tetraploid can be used to produce 'natural' triploids by mating with normal haploid gametes. Gynogenesis and androgenesis, can also be used to produce monosex population , and are generally regarded as shortcuts to produce highly inbred lines for selection programmes;

(5) Fecundity in fish and aquatic invertebrates are generally higher than domestic livestocks. Large numbers of progenies can be produced from the aforementioned genetic manipulations. This will enable the applications of classical selection, as well as other modern genetics.

Domestication is in effect the imposed evolution of cultivable species in artificial environments. Genetic manipulation then has its role in guiding this evolution to further benefit mankind and has its role in all the phases of aquacultural development, from choosing the species, assessment of stocks, prevention of inbreeding, further improvement of existing stocks, to the 'creation' of new stocks. More aspects of the possible application of modern genetics in aquaculture will be given in chapter 4 and chapter 5.

(1.5) Larval development, genetics, evolution and aquaculture

Larval development in many benthic invertebrates is planktotrophic, and is the dispersal phase of the life cycle (Cameron, 1986). The actual distance of dispersal is affected by the larval duration, physiological requirements/tolerance to environmental parameters (e.g. salinity, temperature) and the prevailing environmental conditions (e.g. current). In general, dispersal and gene flow are greater in species with planktotrophic larvae than in species with non-planktotrophic larvae (Hedgecock, 1986; see also Chapter 5). The level of gene flow among geographically separated co-specific populations, in turn, may have important implications for the evolutionary responses of a species to differences in selective forces among local environments (Crisp, 1978). Thus, the mode of larval development has strong implications for the dispersal, gene flow between populations, evolution and speciation of a species/taxa. This influence can be expressed in the inter- and intra- specific

variation of the taxa concerned which, in turn, affect the responses to selection in a cultured species. The genus Macrobrachium is generally believed to have invaded the freshwater environment from the marine environment. The mode of larval development and larval freshwater tolerance would represent special evolutionary traits. The larval phase is also the most capital and labour intensive part of the whole crustacean aquaculture operation. The supply of a sufficient number of juveniles is a prerequisite to a successful venture; an understanding of larval development is important to aquaculture on its own.

(1.6) The theory of speciation and evolution

The study of phylogeny aims to purport on the historic path of evolution and speciation. It is thus imperative to give a brief introduction to the contemporary theory of evolution and speciation.

(1.6.1) The theory of speciation

The biological species concept (Mayr, 1942) defined species as groups of actually or potentially interbreeding populations, which are reproductively isolated from each other. 'Sibling' species are morphologically indistinguishable, yet differ in other biological properties, and do not interbreed. The evolution of new species is, in essence, the evolution of genetic barriers.

The generally recognised modes of speciation are related to

the presence or absence of geographical separation in speciation. Two major schemes of classification have been proposed. the Mayr(1942) classification (table 1.2) and the population genetic classification of Templeton(1981). The various natural forces (natural selection, random genetic drift, mutation, gene flow) that are interpreted in population genetics will be discussed in more details in Chapter 5.

The chromosomal mutation, as pointed out by Futuyama(1986), even under the most intense selection pressure, probably relates more to a gradual speciation event. The 'Transilience' events of Templeton(1981), apart from the chromosomal and hybridisation events, also includes the 'founder effect' (see Chapter 6) as postulated by population genetics. The 'Divergence' events of Templeton(1981) relates to the gradual speciation processes of

Table (1.2) : Modes of speciation according to classification of Mayr (adapted from Mayr,1942; 1963)

1. Hybridization : speciation by hybrids between two existing species;

2. Instantaneous speciation (through individuals)
   a. Genetically by mutation
   b. Cytologically - (i) Chromosomal mutation
      (ii) Polyploidy

3. Gradual speciation (through populations):
   a. Sympatric speciation
   b. Parapatric speciation
   c. Allopatric speciation - (i) By isolation of a colony
      (ii)Division of range by extrinsic barrier or extinction of intermediate populations.

Mayr. Sympatric speciation is thus habitat divergent without isolation by distance. Adaptive divergent and clinal divergent with isolation by geographical distance is, in essence, allopatric speciation. However, divergence by non-adaptive processes (e.g. random genetic drift) was not included by Templeton(1981).

The relative importance of the different modes of speciation is a highly debated subject. Mayr(1963) suggested that most speciation was caused by geographical separation. White(1973) favoured chromosomal changes (including polyploidy) as the most important mechanism.

(1.6.2) The theory of evolution

Darwinisn implies that evolution of adaption takes place by natural selection. Neo-Darwinism, on the otherhand, added the mechanism of heredity, the central dogma of which is DNA makes RNA make proteins (Dawkins,1982).

The general mathematical models of evolution (e.g. Maynard Smith (1978,1982) relate the average fitness of a population under a certain biological strategy to the contemporary environment. Wright(1932,1982), however, modelled evolution in an adaptive landscape of allele frequencies in which peaks and troughs represent possible genetic compositions of a population for which the average fitness (w) is high and low respectively. As the average fitness increases, the population moves uphill to a peak. However, as selection can only increase average fitness, the population cannot move from peak to peak. A change in

environment changes the whole adaptive landscape, a previous peak may become a trough and vice-versa. Random genetic drift, however, can move a population downhill; and together with natural selection, can move a population from peak to peak in the adaptive landscape until the highest peak is attained. This essentially constitute the shifting-balance theory of evolution (Wright,1977,1982).

The 'Red Queen' hypothesis (e.g. Stenseth,1985), however, proposed that most organisms are unlikely to be at their adaptive peaks (but close), since the Wright's adaptive landscape changes as a result of evolution in co-existing species, as well as a result of altered physical conditions. The rate of evolution then depends upon the evolutionary load - the difference between the contemporary fitness of the species to that of the theoretical optimum.

A new paradigm, evolution by 'punctuated equilibrium' (also stasis-plus-punctuation), as suggested by some paleotologists (Eldredge and Gould,1972; Williamson,1981a,b) has gained momentum in the last decade. The theory suggested that evolutionary changes, as inferred from fossil records, are not gradual (as suggested by population geneticists) and continuous. Instead, long periods in which little or no changes occur (stasis) and are interrupted by rather sudden changes (punctuation). The later is purported to occur more likely in peripheral populations, at a time of lineage splitting (speciation).

The Darwinian view favoured gradual changes for two reasons. A finely graded variation is required to produce a detailed

adaptive fit to current conditions. A large change is unlikely to improve adaptation. Also, existing organisms are mostly presumed to be close to their adaptive peaks (Maynard Smith,1983). One major argument of the punctualist is the possible contribution of developmental constraint to stasis. Undoubtedly, developmental constraints do exist (e.g. the widely quoted example of the gastropod shell). However, such constraints merely limit the kind of possible changes, but do not rule out all changes nor variation, and has no inference as to the possible 'rate' of change.

Recently, Sheldon(1987) reported the phyletic gradualism of Ordovician trilobites which varied in the number of pygidial ribs. Sheldon also criticised that the detection of gradualism in other fossils might have been hindered by the binomial taxonomy of fossil. Maynard Smith(1987), suggested the study of Sheldon to be a major evidence for gradualistic theory (population genetic) of evolution and that " Darwinism stays unpunctuated". However, Sheldon(1988) commented that how much of each (gradual versus punctuated evolution) remained to be investigated.

(1.7) The aim of this study

The present thesis aims to investigate the phylogeny and evolution of the genus Macrobrachium, with special considerations on the implications to its aquaculture. In considerations of the phylogeny of a taxa, it is desirable that evidence is drawn from a variety of data which may include, among others, larval-morphological characters, adult morphological characters, physiological data, biochemical data and karyological data. The main bodies of evidence in the present thesis will be drawn from analysis of larval development and biochemical genetics of the genus.

Biogeographical analysis, which may give insights to the phylogeny of the genus as well as possible candidate for aquaculture, will be reported in Chapter 2.

Larval developmental characters, which is an important consideration to both evolution and aquaculture (section 1.5), will be investigated in Chapter 3. Experiments on larval salinity tolerance and larval selection for freshwater tolerance will be carried out in Chapter 4.

Variation is the basis for selection, both naturally and artificially. Population genetics of two species will be surveyed in Chapter 5, using allozyme data. Considerations of population differentiation would be made in relation to geographic distribution of the species, hoping to generate information on speciation processes.

In Chapter 6, phylogenetic relationships of a number of species would be constructed, using allozyme data as well as data from previous chapters. Comparisions between the possible

phylogeny contructed from the various types of data would be made with concluding comments. The overall implications of the findings to aquaculture would also be discussed in Chapter 7.

"Even from the Shrimps to the Leviathan
Enquiry ran."

-Thomas Heyward
in 'Searching for God'

"In considering the distribution of organic beings over the face of the globe, the first great fact which strikes us is that neither the similarity nor the dissimilarity of the inhabitants of various regions can be accounted for by their climatal and other physical conditions."

-Charles Darwin
in 'The Origin of Species'

CHAPTER TWO :

BIOGEOGRAPHY OF *MACROBRACHIUM*

(2.1) Introduction

Biogeography is the study of geographical distribution of organisms in space and time (Cox *et al.*, 1973). Not only does the biological properties of a species imply its present day adaptation, they also reflect historical characteristics of its ancesters. Similarly, the geographical distribution of a species also incorporates the products of both historical events and physiological limitations asserted by the environment of the species. "Evolutionary changes and geographic range changes befall a species simultaneously; each process affects and is affected by the other" (Pielou, 1979).

Abele(1982), in his recent review of crustacean biogeography, inferred that 'migrations and dispersal of crustaceans appears to be related more to the size of the species pool and to the ecological conditions than to competition.' *Macrobrachium* is a sizable taxa with a geographical spread round the tropics and from south-east Siberia to southern Australia. A knowledge of the biogeography of the taxa can probably reflect on the physiological characteristics of a species, which may in turn

reflect on their aquacultural suitability.

It is generally accepted that the genus has evolved from the marine to the freshwater environment. General evidence of this includes:

(1) Many freshwater species of the genus still process larvae that require saline water for sucessful development to postlarva while larvae of marine species can only metamorphose in seawater (see Chapter 4);

(2) Species with more 'advanced' and 'abbreviated' larval development tend to occur in 'more' freshwater environments (see Chapter 3);

(3) Many species that complete their larval development totally in freshwater can survive in saline water while larvae of brackishwater species (e.g. M. rosenbergii) will die in freshwater within a day or two;

(4) Most species have a coastal distribution (see present Chapter).

The present chapter aim to review and summarise our present knowledge of the biogeography of Macrobrachium, at various geographic scales, with particular consideration of the evolutionary biology and aquaculture of the group. The biogeogeographic aspects in relation to larval salinity tolerance, intra-specific variation and phylogeny of the genus would be discussed further in chapter 4, chapter 5 and chapter 6 respectively.

(2.2) Habitat preferences and regional distribution in Macrobrachium

Adults of Macrobrachium species can be found in a variety of

aquatic habitats. Most species are freshwater inhabitants, generally found in rivers, streams and lakes. However, numerous examples exist in which other habitats are also explored by the genus. M.crybelum leads a troglodytic existence in the subterranean caves of the Dominican Republic (Chace, 1975). M.inpa lives inside accumulated leaves of inundated Amazonian forests (inpago) (Kensley and Walker, 1982). M.intermedium, on the otherhand, is one of the exceptions of the genus which leads a marine existence in shallow waters (Holthuis, 1980).

While most species of the genus inhabit rivers, streams and lakes, each species tends to have a specific preference of a different microhabitat. When co-existing in the same river, different species tend to vary in their distribution up the river. In the Shimanto River of Japan, M.nipponense was only found near to the river mouth while M.formosense and M.japonicus were found up to 50 and 100 km respectively from the sea (Ohno et al., 1977). In Hong Kong, M.nipponense was found mainly in the lower course of the Lam Tsuen River while M.hainanense was found in the upper part (Dudgeon, 1985).

Apart from inter-specific differences, habitat differentiation also exists between adult and juveniles of the same species. In those species which require saline water for their larval phases, the newly metamorphosed post-larva probably have to pass through the coastal or estuarine habitats before migrating to the adult grounds (e.g. the upper reach of a river). Even in species which can metamorphose totally in freshwater, their juveniles often inhabit slightly different areas to that of the adults. In the Lam Tsuen River of Hong Kong, adults of

M.hainanense took up guarded territories in mid-stream areas while juveniles were mainly found among the submerged portion of marginal vegetation (Dudgeon, 1985). Juveniles of M.natteri were found in leaf litter of leaves in the inpago of the Amazon while adults inhabited the main stream or river (Walker and Ferreira, 1985).

Most of the regional keys to the identification of Macrobrachium would have notes on their distribution (e.g. Holthuis, 1950). However, only a few studies have been devoted totally to the regional distribution of the genus.

Shokita(1979) investigated the distribution and speciation of inland water shrimps from the Ryukyu Islands. He related their distribution to their early life history and catagorised them into three types:

a. Small sized-egg type - which were amphidromous or land-locked, occurred in marine, river or lake habitats;
b. Medium sized-egg type - which were amphidromous or landlocked, occurred in rivers, lakes and subterranean waters;
c. Large sized-egg type - lead a land-locked life cycle, and mainly inhabitants in rivers.

In addition, Shokita(1979) also identified four catagories of prawns in relation to their distribution in the longitudinal section of a river (i.e. the lower,middle,upper and ubiquitous).

Similarly, Tiwari(1955) also grouped the Palaemon (=Macrobrachium) in the Indo-Burmese region into two groups:

a. those with small eggs inhabiting the coastal regions;
b. those with eggs greater than 1mm inhabiting inland river systems.

Hunte(1978), in his investigation of the distribution of Jamaican freshwater prawns, identified three catagories:

a. those inhabiting high-gradient streams, mainly in eastern and north-eastern Jamaica;
b. those inhabiting low-gradient stream, mainly in the western and south-western Jamaica;
c. those which are found throughout Jamaica.

As suggested by their names, high and low gradient species tend to be associated with environments with either high or low current speed respectively. With further evidence on salinity tolerance and estuarine hydrographic conditions, Hunte(1978) also inferred that "preferences for physical characteristics of particular streams as well as salinity requirements during larval development could have evolved together".

It is evident that the distribution of Macrobrachium, at least on a regional scale, is much related to the larval salinity (including freshwater) tolerance and probably to the types of larval development (in relation to egg size). These aspects will be investigated later in the thesis.

Johnson(1967) conducted a detailed investigation on the physical and chemical factors (cations, pH, oxygen, temperature, salinity and waterflow) affecting the distribution of Malaysian Macrobrachium. Again, the author inferred a clear connection between high salinity tolerance of the species(at some stage) and their distribution. Two groups were identified:

a. species with fully freshwater adults but a brackishwater larval phase whose distribution resembled that of fully marine species;
b. other species having limited salt tolerance.

While there were no clear-cut correlations between distribution and oxygen tension, altitude & temperature, Johnson(1967) distinguished between torrential and non-torrential species. It was also inferred that while fast water-flow prevented some forms becoming established in torrents, there was no evidence that

those forms which are limited to fast waters demand considerable water movement as such. There was also a good correlation between the ability of a species to survive in acid or soft waters and its general distribution in Malaya. Harder water species such as M. lanchesteri and M. sintangense were rare in southern Malaya while species like M. tompii and M. geron (=M. malayanum), which could surive in waters with no detectable calcium, were abundant.

It is generally accepted that strong water current is one of the factors which may have prevented many species from establishing in the torrent zone. However, no comparative studies are known which dealt with the adaptive mechanism concerned with torrenticolous species of Macrobrachium. In freshwater crayfish, a probable ecological analog of Macrobrachium , those species of fast-flowing habitats were found to have special behavorial, morphological and physiological adaptation (Maude and Williams, 1983; Caine, 1978). It is probable that the same degree of multivariate adaptation are ivolved in torrential species of Macrobrachium, as in the freshwater crayfish.

(2.3) Global distribution of Macrobrachium

From the forgoing discussion, it is evident that, while there exists microhabitat preference between species in relation to some physical and chemical parameters, the overall present-day conditions of the tropic should bear no theoretical restriction to the distribution of the genus. Any restricted pattern of worldwide distribution, as can be seen in the next section, would thus be related to historical events. The rest of the chapter would aim to relate present-day distribution to such processes.

(2.3.1) Analysis of global distribution by classical Wallace's regions

Wallace(1876) was among the first investigators to have analysed the biogeography of organisms and constructed major 'biogeographic' regions based on their broad faunal similarities. Subsequent workers, with further information on the distribution of both plants and animals, have constructed slightly different schemes. Schmidt(1954) demarcated five major regions while Pielou(1979) delimited eight biogeographic regions based on both plant and animal distribution. However, the six faunal regions of Wallace(1876), as shown in Fig.(2.1), still remain a major framework for biogeographical analysis (Watts, 1984). Also based on this scheme, a species or taxa can be classified as Endemic, Characteristic, Semi-cosmopolitan and Cosmopolitan upon whether its range extend to 1,2,3-4 or 5-6 of the Wallace's regions respectively (Rapoport et al., 1976).

The present analysis of the distribution of the genus Macrobrachium will also be based on the faunal regions of Wallace(1876). To take into account the coastal distribution of the genus, four of the regions are sub-divided to represent the left or right margin of a continent. the regions are Neartic (1a&b), Neotropical(2a&b), Ethiopian(3a&b), Oriental(4a &b), Australasia(5) and Palaeartic(6). The oriental region is also sub-divided to account for the historical identity of the Indian subcontinent. The distributional data was based on Holthuis(1950, 1952 and 1980), as well as other original records of species. The fact that many new species are still being reported and the absence of complete data from many parts of the world hinder an unequivocal analysis. Individual distributions of each species

are briefly listed in Table(2.1). A summary of the analysis of the whole genus is presented in Table(2.2). The list only included 139 species of which detailed geographical distribution were accessible to the present author. While not all the species of table(1.1) were included, the present list would probably consist of 90-95% of all the known species, and would thus give a fair representation. The distribution of the subspecies listed in table(1.1) was treated as a single species. In the thesis, the present analysis would be referred to as 'analysis of the genus', rather than acknowledging the 90-95% in each of the statement involved.

The majority of Macrobrachium species have tropical distribution. Species richness decreases with increasing latitudes, as in the overall distribution of marine decapods (Abele, 1982). The Neartic and Palaeartic regions together, account for only 10% of the whole genus.

Among the biogeographic regions, the Oriental region has the highest score. Each of the other tropical regions, Neotropical, Ethiopian and Australasia, has roughly half of its number of species. No species are found in the Western Palaeartic region. M.niloticum is found in Lake Chad, Lake Rudolf, Lake Turkana and River Nile, but it is uncertain whether the species can be found in the Mediterranean. The fact that great intra-specific variation in adult morphologies exists between population at the various localities, coupled with the ability to metamorphose in freshwater and the advanced larval morphologies (Williamson, 1972; see also Chapter 3, esp. on epigastric spines)), M.niloticum has probably evolved from African species in recent

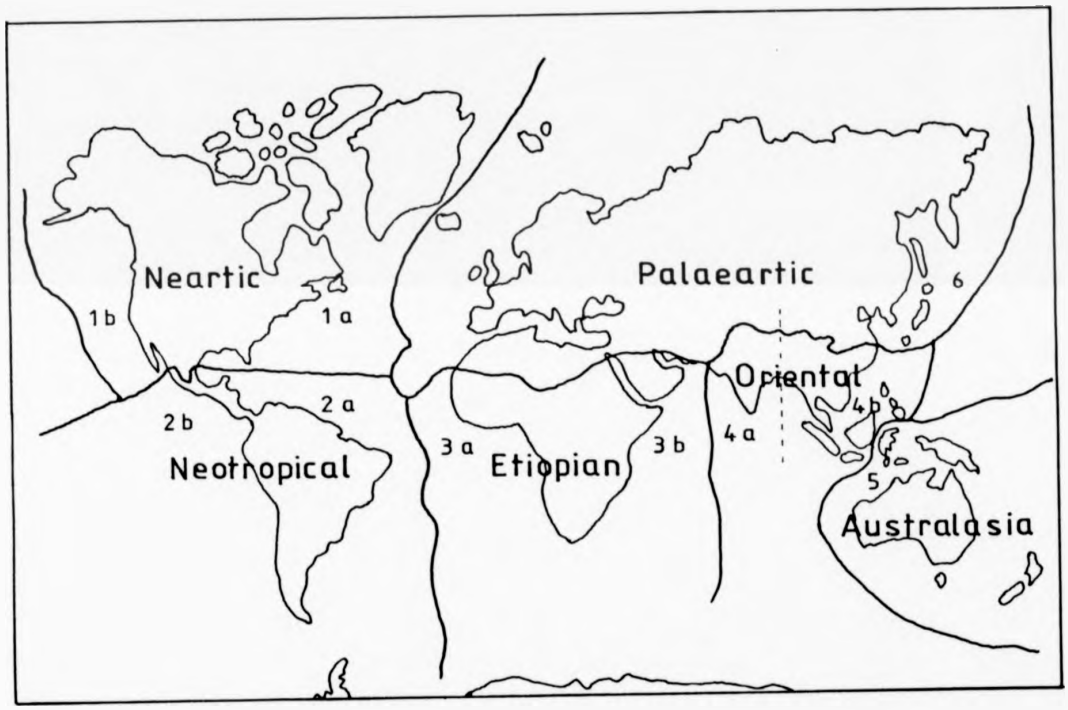

Fig. (2.1): Wallace's biogeographic regions (after Watts, 1984) Numberings refer to analysis by oceanic regions (Table 2.3)

Table(2.1) : **Distribution data of the genus Macrobrachium**

SPECIES	DISTN1	DISTN2	DISTN3
M.acanthochirus	1b	W.Am.	South-West Mexico
M.acanthurus	1a,2a	E.Am.	N.Carolina to Brazil,Caribbea
M.acherontium	1a	N.E.Am.	Mexico
M.adscitum	5	IWP	Queensland,Australia
M.aemulum	5	IWP	New Caledonia
M.altifrons	4a	IWP	North India only
M.amazonicum	2a	E.S.Am	Venezuela to Paraquay
M.americanum	1b,2b	W.Am.	Baja California to N.Peru
M.asperulum	6,4b	IWP	China,S.E.Siberia,Taiwan
M.atabapense	2a	E.S.Am.	Venezuela
M.atactum	5	IWP	Queensland,Australia
M.australe	3a,4ab,5	IWP	Madagascar to Polynesia
M.austrliense	5	IWP	Queensland,N.S.W.,Australia
M.bariense	4b	IWP	Malay Arch.
M.birmanicum	4a,b	IWP	E.India,Bangladesh to Burma
M.borellii	2a	S.E.Am.	Paraquay,Argentina,Uruquay
M.brasiliense	2a	C.&S.Am.	Surinam,S.W.Brazil,E.Ecuador
M.caledonian	5	IWP	New Caledonia
M.callirhoe	5	IWP	N.Borneo
M.carcinus	1a,2a	E.Am.	Florida to S.E.Brazil
M.carvenicola	4a	IWP	Siju Cave, Assam
M.chevalieri	3a	W.Afr.	Cape Verde Is. & Angola
M.choprai	4a	IWP	Ganges,Brahmaputra R.,India
M.clymene	4b	IWP	Sarawak
M.cocoense	2b	W.S.Am.	Coco Is.
M.cowiesi	4b	IWP	Manila
M.crenulatum	2a	C.&S.E.Am.	Caribbean,Panama,Venzuela
M.crybelum	2a	S.E.Am.	Dominican Republic
M.dayanum	4a	IWP	Pakistan,India,Bangladesh
M.dierythrum	2a	E.S.Am.	Venezuela
M.digueti	1b,2b	C.W.Am	Mexico to Ecuador
M.dux	1b,2b	W.Afr.	Rio Muni to Zaire
M.edentatum	4b	IWP	Siuchuan Province,China
M.equidens	3b4ab5,6	IWP	Madgascar,S.China to Caledoni
M.esculentum	4b,5	IWP	Celebes,Indonesia,Phillipines
M.faustinum	2a	C.E.Am.	Caribbean
M.felicinum	3a	W.Afr.	Gold Coast, Angola
M.ferreirai	2a	S.E.Am.	Amazon Basin,Brazil
M.foai	3a	W.Afr.	Upper Congo basin
M.formosense	4b,6	IWP	Japan,Taiwan,China
M.fukienense	4B	IWP	Fukien Province
M.gallus	2b	W.S.Am.	Ecuador
M.glyticum	5	IWP	North Queensland,Australia
M.gracilirostre	5	IWP	Fiji
M.grandimanus	4b,5	IWP	Ryukyu,Hawaiian Arch.
M.guangxiense	4b	IWP	Guangxi Province,China

(to be continued)

Table (2.1) : (continue)

M.hannocki	2b	C.&W.S.Am.	Costa Rica to Columbia
M.hendersodayanum	4a	IWP	India
M.hendersoni	4a	IWP	E.Himalayas to Yunan
M.heterochirus	1a,2a	E.Am.	Mexico to S.Brazil,Caribbean
M.hildebrandti	3b	IWP	Madagascar
M.hirtmanus	3b,4ab,5	IWP	Oceania to Mauritius
M.holthuisi	2a	E.S.Am.	Brazil
M.horstii	4b	IWP	E. Malay Archipelago
M.idae	3b,4ab	IWP	Madagascar to Indonesia
M.idella	3b,4a	IWP	E.Afr. to India
M.iheringi	2a	E.S.Am.	Brazil
M.inca	2b	W.S.Amer.	Chile,N.Peru
M.inflatum	4b	IWP	Jiangsu Province,China
M.inpa	2a	S.E.Am.	Brazil
M.insulare	4b	IWP	Taiwan
M.intermedium	5b	IWP	W.&S.Australia to Tasmania
M.jacobsonii	4b	IWP	Java,Sumatra
M.japonicum	4a,6	IWP	Japan, Taiwan, Rykukus
M.javanicum	4a,b	IWP	India,Thailand to Indonesia
M.jazoense	4b	IWP	Indonesia,Philippines
M.jelskii	2a	S.E.Am.	Venezuela to French Guiana
M.jiangxiense	4b	IWP	Jiangxi Province,China
M.johnsoni	4a	IWP	E.India
M.joppae	4a	IWP	Nias
M.kiukianense	4b	IWP	China
M.lamarrei	4a	IWP	India,Bangladesh
M.lanceifrons	4b	IWP	Philippines
M.lanchesteri	4a,b	IWP	Thailand,India,Malay
M.lar	3b,4ab	IWP	Madagascar to Ryukyu
M.latidactylus	4b	IWP	Malay Peninsula & Arch.
M.latimanus	3b,4ab	IWP	India to Malay to Ryukyu
M.lepidactyloides	4b,5	IWP	Malay, Fiji
M.lepidactylus	3b	IWP	East.South-east Africa
M.lorentzi	5	IWP	New Guinea
M.lujae	3b	W.Afr.	Zaire
M.macolmsonii	4a	IWP	Pakistan, India & Bangladesh
M.macrobrachion	3a	W.Afr.	French Guinea to Angola
M.maculatum	4b	IWP	Fujian Province,China
M.mammillodactylus	4b,5	IWP	Philippines,Indonesia,Guinea
M.meridoionalis	4b	IWP	Hainan,China
M.microps	5	IWP	Danmin Cave,New Ireland
M.mieni	4b	IWP	Vietnam
M.mirabile	4ab	IWP	East India to Borneo
M.moorei	3b	IWP	Lake Tanganyika, E.Africa
M.nattereri	2a	S.E.Am	Brazil,Guiana
M.nepalense	4a	IWP	Nepal
M.niloticum	3a	N.E.Afr.	Nile, Lake Chad,Lake Rudolf
M.nipponense	4b,6	IWP	S.China,Japan,Taiwan,Korea
M.nobilii	4a	IWP	India
M.occidentale	2ab	C.Am.	Guatemala,San Slavador,Panama

(to be coninued)

Table (2.1) : (continue)

M.oenone	5	IWP	
M.ohione	1a	N.E.Am.	U.S.A.
M.olfersii	1a,2a	E.Am.	U.S.A.,Mexico to Brazil
M.palaemonoides	4ab	IWP	Nepal,Sumatra
M.panamanense	2b	W.S.Am.	Honduras to Ecuador
M.patsa	3b	IWP	E.Africa, Madagascar
M.pectinatum	2a	S.E.Am.	Venezuela
M.petersii	3b	S.E.Afr.	S.E.Africa
M.petiti	3b	IWP	Madagascar
M.pillimanus	4b	IWP	Malay to Java
M.placidulum	4b	IWP	Sumatra
M.potuina	2a	S.E.Am.	S.E.Brasil
M.praecox	2a	S.E.Am.	Colombia,Venezuela
M.pumilum	2a	S.E.Am.	Venezuela
M.quelchi	2a	S.E.Am.	Mazaruni River,British Guiana
M.raridens	3a	W.Afr.	Guinea to Nigeria
M.rathbunae	2a	C.&S.E.Am.	Panama,Columbia
M.reyesi	2a	S.E.Am.	Venezuela
M.rodriguezi	2a	S.E.Am.	Venezuela
M.rosenbergii	3b,4ab,5	IWP	N.W.India to N.Australia
M.rude	3b,4a	IWP	E.Africa to Bangladesh
M.scabriculum	3b,4ab	IWP	E.Africa to Bangladesh,Sumatr
M.shokitai	4b	IWP	Ryukyu Is.
M.sintangense	4ab	IWP	Nepal,Thailand to Indonesia
M.sollaudii	3a	W.Afr.	Cameroons,Zaire
M.sophonicum	4b	IWP	Malay to Rykuku
M.srilankense	4a	IWP	Sri Lanka
M.sulcicarple	4b	IWP	Celebes
M.surinamicum	2a	S.E.Am.	Columbia,Venezuela,Guiana
M.tenellum	1b,2b	W.Am.	S.California to N.Peru
M.therezieni	3b	IWP	Madagascar
M.tolmerum	5	IWP	Queensland, Australia
M.transandicum	2a	S.E.Am.	Columbia
M.trompii	4b,5	IWP	Malay,Sumatra,Borneo
M.veliense	4a	IWP	south-west India
M.villalobosi	1a	N.E.Am.	Mexico
M.vollenhovenii	3a	W.Afr.	Senegal to S.Angola
M.yeti	4b	IWP	Vietnam
M.yui	4b	IWP	Yunan, China
M.zariquieyi	3a	W.Afr.	Gulf of Guinea

DISTN1 : distribution data in relation to Wallace's faunal regions (see Fig. 2.1)

DISTN2 : distribution data in relation to continental margins. (Am.=America ; Afr.=Africa ; IWP=Indo-West-Pacific ; N.,E.,S.,W. are directions; C.=Central)

DISTN3 : Brief discription of distribution by countries

Table (2.2) : **Analysis of the distribution of Macrobrachium by faunal regions of Wallace(1876)**

	Endemic species	Sub-Pandemic species	Pandemic species	total number
(1) NEARTIC				
East Neartic (1a) (N.W.Atlantic)	2	0	4	6
West Neartic (1b) (N.E.Pacific)	1	0	3	4
regional total*	3	0	7	10
(2) NEOTROPICAL				
East Neotropical(2a) (S.W.Atlantic)	23	0	4	27
West Neotropical(2b) (S.E.Pacific)	5	1	3	9
regional total*	28	1	7	36
(3) Ethiopian				
West Ethiopian (3a) (East Atlantic)	10	0	0	10
East Ethiopian (3b) (IWP-1)*	9	3	0	12
regional total *	19	3	0	21
(4) Oriental				
West Oriental (4a) (IWP-2)	15	7	10	32
East Oriental (4b) (IWP-3)	24	7	19	50
regional total *	39	7	21	67
(5) Australasia (IWP-4)	13	0	10	23
(6) Paleoartic (IWP-5)	0	0	5	5
OVERALL TOTAL *	102	8	29	(139)

* IWP = Indo-West-Pacific region
* All totals have taken into account of pandemic nature of the various species (and not arithmetic sum)

in recent time, bearing in mind that Macrobrachium species are found in equal latitudes in eastern Palaeartic region, their absence in the western side is probably related to palaegeographic or other ecological limitations.

The distribution of Macrobrachium was further analysed by the scheme of Rapoport et al. (1976) (see Table 2.3). Over 75% of the species, which have distribution within only one biogeographic region, were classified as Endemics. Also, Endemic and Characteristic species, when considered together, would account for about 97% of the whole genus. Not a single species of the genus has a circumtropical or even Cosmopolitan distribution. In contrast, 10% of marine decapods are circumtropical in distribution (Abele, 1982). On the whole, the very low value for the degree of Cosmopolitanism calculated (0.05) reflects the fact that many species are true freshwater inhabitants, with little long range dispersal via the marine environment. However, as discussed in the last section, it is generally observed that the genus Macrobrachium can be grouped into those that are and those that are not tolerant to salt in their larval phase. The global distribution of the genus also reflect this distinction. Prawns that are intolerant of salt-water tend to have restricted ranges. M.shokitai, a land-locked freshwater species, is restricted only to the Ryukyu Islands (Shokita, 1979). On the other hand, prawns that are salt-tolerant, like M.rosenbergii, M.lar, and M.equidens, tend to show a marine type of distributional pattern, extending to much of the Indo-Pacific area.

(2.3.2) Analysis of distribution by oceanic regions

The fact that the genus consists of both inland-freshwater and coastal species implies that its biogeographic analysis requires considerations by both the classical system, which is more by land distribution, as well as by the oceanic regions. The division of the classical faunal regions into left and right sections facilitates an easy 'oceanic' analysis, which is summarised in table(2.4). Of the four margins of the the two main oceans, the Indo-West Pacific(IWP) region alone accounts for over 60% of all the species of Macrobrachium while the West Atlantic accounts for another 23%. Assuming that the place of the highest species richness is the centre of origin, the genus may be inferred to have evolved in this region (Shokita, 1985).

On the other hand, species richness is also strongly affected by the available area concerned. According to Abele(1982) larger area would increase the species richness by increasing the habitat heterogeneity, increasing the population size (hence reducing the probability of becoming extinct), and also by increasing the sample size concerned. For crustaceans on a whole, there is a strong correlation between species richness and the shelf area of the four ocean margins (Abele, 1982). When the number of species is divided by the shelf area in the region, it is surprising to note that Western Atlantic, Eastern Atlantic and Eastern Pacific are almost identical in the 'Species Density' obtained while the Indo-West Pacific scores only half of the value (see Table 2.4). However, the Indo-West Pacific region consists of several dissimilar sub-regions, IWP-1 to IWP-4, in accordance with the Wallace's biogeographic regions

(see table 2.2). However, the analysis of the Indo-West Pacific included data from the Australasia (IWP-4) and Paleoartic (IWP-5) regions, both probably restricted by climatical and other ecological factors in relation to the distribution of Macrobrachium. Assuming that these various sub-regions are roughly equal in their shelf areas (i.e. 6,570,000 km/4), the calculated density of each sub-region then confirm the highest score for IWP-3, the Oriental region (table 2.4). Also, the analysis of species diversity in relation to the shelf areas may underestimate the relative value for IWP region, as the distribution of Macrobrachium may be related more to the area of river beds and estuarine areas.

The above analysis of distribution (both classical and oceanic analysis) were based on reports up to the presnt date. Due to the inaccessibility of many tropical localities, more Macrobrachium are likely to be discovered. From the recentness of the reports of species from various sub-tropical regions of China (by Prof. Laing of the Shanghai Fisheries College), it is likely that more species will be reported from this region. A similar situation probably applies to South American species. There also seems to be a paucity of species being reported from West Africa. The distribution of Macrobrachium can be divided into various sub-regions in accordance with Wallace(1876), including Madagascar, Malaysian Peninsula, Hainan Island, etc. In particular, the species of the Australasia Region can be divided into the Oceanic, the Australia and the Austro-Malaya subregion. However, considering the paucity of distributional data in many parts of the world, no attempts

Table (2.3) : Computation of Rapoport et al.(1976)'s degree of cosmopolitanism

No. of regions occupied by species (r)	No. of Species (Y)	Percentage of the Genus (%)
1 (Endemic) *	108	77
2 (Characteristic)	27	19.3
3 (Semi-cosmopolitan)	3	2.9
4	1	0.7

Average r = 167/139 = 1.21

$$C = \frac{\left(\sum_{r=1}^{r=4} r\,Y/T\right) - 1}{5*} = 0.05$$

* = TOTAL NUMBER OF REGIONS - 1 = 5
endemic species include sub-pandemic species of table (2.2)

Table(2.4) : **Distribution of species by oceanic margin and the corresponding species density**

Oceanic margin (shelf area : X 1000 km2)	Number of species (% of Genus)	Species density (X 10^{-5})
West Atlantic (1280) = 1a+2a	29 (20.9%)	2.3
East Atlantic (400) = 3a	10 (7.2%)	2.5
East Pacific (380) = 1b+2b	10 (7.2%)	2.6
Indo-West Pacific (6570) =3b+4a+4b+5+6	90 (64.7%)	1.4

Sub-regions of Indo-West Pacific

	No. of species		Species Density
IWP-1	19		1.2
IWP-2	29	> 65	4.0
IWP-3	51		
IWP-4	23		1.4
IWP-5	5		3

* Faunal regions of Wallace (see Table 2.1)

will be made to map the distribution of the genus further into sub-regions of the classical scheme. It is hope that with further studies of the genus, a fuller picture can be revealed in the near future.

(2.4) Phylogenetic inference from biogeography

From the foregoing discussion, the Oriental region of the classical biogeographic area has the highest species diversity of Macrobrachium. Within the Oriental region, the eastern sub-region accounts for over 60% of the regional share of species, as opposed to the western region, which is the Indian sub-continent. From an analysis of evolutionary morphologies (rostrum and second pereopod) and biogeography of the 'hendersoni' group of Palaemon (=Macrobrachium) in northern India, Tiwari(1952,1955) also concluded that the the Eastern Himalayan zone was the centre from where dispersal to other parts of India began. The species of the Palaeartic region, which are only present on the eastern side of the region, are mostly pandemic species that can be found in the Oriental region. Furthermore, a sizable proportion of the species in adjacent region, Eastern Ethiopian and Australasia, are pandemic species shared by the Oriental region. From the viewpoint of species density, species richness and species affinity, it may be justified to suggest that the genus Macrobrachium evolved in the area represented by the present eastern Oriental region. Intra-specific variation and geographic distribution of a 'primitive' species, Macrobrachium, also supports this hypothesis (Lindenfelser,1984; also see Chapter 5).

As to the other oceanic margins, while there is not a single

species common to any two margins, there are definitely some "very related" species present in at least three of them (Holthuis 1951,1952). The species cocerned are listed in table(2.6).

Most of the Eastern Pacific species are closely related to the western Atlantic species. There are only two known Macrobrachium species that are not directly related to the eastern forms (Holthuis, 1952). This lead to the speculation that at least some species in the region might have evolved from some common stock of the genus, and probably at some time in history when dispersal to all the three regions was still an ecological possibility. This implies that the genus probably evolved before the closure of the sea passage between north and south Americas in the Pliocene, some 5-7 million years ago (Pielou,1979) and after the continental drift between Africa and South America (about 100 million years ago). However, as the later event commenced earlier in the southern parts of both continents, it is possible that exchange between South America and Africa (especially at the latitude of Brazil) was still possible at some time after the initial separation event.

Scheltema(1986a), using data from continental drift and palaeotological ocean currents (direction and velocity), estimated the time required for passively transported larve (e.g. gastropod larvae) to cross the Atlantic (table 2.7). The larval duration of contemporary species of Macrobrachium can be as long as 50-65 days (see Chapter 3 and 4), suggesting that larval exchange across the tropical Atlantic may still have been possible in early Miocene (and possibly later). This is possibly

a conservative estimate as larval Macrobrachium can swim actively and that Scheltema(1986a) assumed a big reduction in current veleocity in early Miocene, based upon tank model studies (and hence a big dicontinuity in the estimate of transverse time between Miocene and Oligocene). From the assertions suggested so far, the genus has evolved before transverse transport in the Atlantic has become impossible, some 15-20 millon years ago. Considering the fact that many crustacean larvae can delay their larval duration under adverse conditions (see Chapter 4), some occasional exchange of larvae may still be possible in the late Miocene, some 10-15 million years ago.

The absence of Macrobrachium and their fossils in the Mediterranean may also suggest that the genus has evolved after Europe and Africa rejoined in the Miocene, some 17 million years ago (Pielou, 1979). This has assumed that the conditions of the Mediterranean, past or present, have not precluded the distribution of the genus. This, coupled with the other inferences, would suggest that the genus has evolved some 15-17 million years ago. Broken pieces of unidentified appendages in Oligocene bed of Panama have been assigned to be fossil Macrobrachium by Rathbun(1918). Without more and unequivocal evidents, such findings should not be included in the estimation of evolutionary time.

Tiwari(1952,1955) considered the phylogeny of species Palaemon (=Macrobrachium) in in the Indian subcontinent, based upon their morphological differentiation, distribution and historical events in the region. He gave particular attention to

Table (2.5) : Related species of Macrobrachium on both sides of tropical Americas and the Atlantic ocean (adapted from Holthuis, 1952)

East Pacific		West Atlantic		East Atlantic
M.tenellum	-	M.acanthurus	-	M.macrobrachion
M.occidentale	-	M.heterochirus	-	M.chevalieri
M.digueti	-	M.olfersii	-	M.felicilum
M.hancocki	-	M.crenulatum	-	M.zariquieyi
M.americanum	-	M.carcinus	-	M.vollenhoven
M.panamense	-	M.amazonicum		
M.transcendicum	-	M.surinamicum		

Table (2.6): Time required for passively transported larvae to cross the tropical Atlantic Ocean at different geological epoch (adapted from Scheltema, 1986a)

Geological epoch or period	Average width of ocean (km)	Time required to tranverse Atlantic (weeks)
Upper Cretaceous to Tertiary	2,248	1.8-3.6
Paleocene to Eocene	2,507	2.1-4.2
Eocene to Oligocene	3,215	2.5-4.2
Early Miocene	3,852	6.4-19.1 *
Late Miocene	4,194	8.3-25.0
Present	4,752	9.2-28.9

* paeleological current veleocity was based upon tank model studies; a significant drop in current veleocity was inferred at early Miocene due to the closure of Tethys Sea

the 'hendersoni' group of species, which inhabit hilly locations in northern parts of the subcontinent. The Western Ghats contain species identical to the eastern Himalayan region, suggesting that dispersal occurred from the later area to the former, before the existence of the present geographical barriers (mountains). Based upon the inter-specific and intra-specific differences in Macrobrachium observed in the region, and the larval developmental types (inferred from egg size) of the species concerned, Tiwari strongly suggested that the genus has dispersed to freshwater in more than one wave (similar to freshwater fishes in the region). Those inland species with larger egg size have evolved into the freshwater environment much earlier than those coastal species with a smaller egg size. Considering the glacial and inter-glacial periods which affect this part of India in much of the Pleistocene, Tiwari also suggested that the genus has evolved in the subcontinent in early Pleistocene. "It seems fairly certain that the genus, as understood at present, is polyphyletic in origin and the two main groups appeared at different time."

Shokita(1979) investigated the distribution and speciation processes in the Ryukyu Islands. Considering the morphological and ontological similarity between M.asperulum and M.shokitai and the data on palaegeography, he concluded that the latter evolved from the former in the Ryukyu Islands during early Pleistocene. The large egg size and the highly abbreviated larval development (see chapter 4) of the two species conform to the inland water group of Tiwari(1955). Shokita's estimation thus also supported the suggestion of Tiwari(1955) that this group of species (within

the genus Macrobrachium) have differentiated in early Pleistocene.

(2.5) Implications to Aquaculture

From the foregoing discussion, it would be evident that many species of Macrobrachium have very different patterns of distribution, both from the microhabitat scale and the global scale. Many patterns of distribution imply very different physiochemical requirements, which in turn has very strong implication for their suitability for aquaculture.

However, as pointed out by Malecha(1983), most useful intraspecific variation, especially in relation to temperature tolerances, would be found in species with latitudinal rather than longitudinal distribution. It is reasonable to assume that crustacean species with a north-south distribution would have strong physiological variation among different races. Species in this catagory would propbably include M.acanthurus of eastern Americas, M.equidens, M.nipponense and M.formosense from the Indo-West Pacific region. M.nipponense, a species already with considerable interests in many countries in both aquaculture and captive fisheries (e.g. Lake Biwa fishery, Yamane and Iitaka, 1987) will be given special attention in Chapter 3.

Useful intra-specific variation may also exist in species with large geographically separated populations (e.g. M.rosenbergii). This aspect would be discussed in both chapter 6 and chapter 7.

(2.6) Man-made Effect on the Biogeography of Macrobrachium

The activities of Man can severely alter the natural

biogeography of other living organisms. Man-made pollution and exploitation have already led to the destruction of many natural habitats. Other activities, like the construction of canals and dams can easily lead to the extinction or spread of exotic species through a geographical barrier.

Aquaculture itself may have implications for the pattern of distribution of Macrobrachium. Lideu de Paniza of the National Directorate of the Republic of Panama observed that "M.rosenbergii that had escaped from ponds to the nature had showed a different behaviour and growth rate" (Paniza pers.comm., 1986). M.rosenbergii, being one of the largest species of the genus, can possibly replace many local species in many tropical countries where it is being introduced.

The western Palaeartic region is devoid of any Macrobrachium species, probably for a non-ecological reason. Some species of the eastern Palaeartic sub-region would probably be able to establish in this region. One species, M.nipponense, has been found as far north as south-east Siberia and can be envisaged to thrive in many locations outside its natural range. As the species is also highly favoured by the aquarium trade, it will not be too long before isolated populations of the species are found in Europe and North America.

The Mediterranean is probably prone to both the introduction of exotic species by aquaculture (both Penaeus and Macrobrachium) as well as from the Lessepian migration from the Indo-West Pacific region through the Suez Canal. Fifteen species of Indo-Pacific brachyurans can now be found in the Mediterranean (Almaca, 1985). Macrobrachium, being mostly brackishwater

species, might be prohibited from establishing in the Red Sea and Mediterranean by their high salinities. However, fully marine species are known in the genus (e.g. M. intermedium) and the possibility cannot be precluded.

Populations of M. equidens, an Indo-Pacific species, have already become established in West Africa (Powell, 1986). While the exact mode of spread cannot be traced, Powell suggusted the ballast water of oil tanker to be a possible agent. If such is true, exotic species of Macrobrachium may also be found in other areas of high tanker traffic (e.g. Mediterranean).

"The man reaches the peak of his development, and displays perfect grace through the true expression of his character without pretensions. He understand the patterns of human frailties."

-from the 'I Ching', about 1200 B.C.
(translation by R.G.H.Siu 1968)
MIT Press

CHAPTER THREE:

LARVAL PHASE : DEVELOPMENTAL MORPHOLOGY AND MODES OF DEVELOPMENT

(3.1)Introduction

(3.1.1) Evolution and the modes of decapod development

Crustacean larval development, for those species that undergo such processes, generally involves the addition of posterior segments and the formation of particular appendages, until the final metamorphosis in which the larva is transformed into a juvenile (Knowlton,1974; Rabalais and Gore,1985). Regular development is defined as the predominant type of larval development in a particular group. Different groups of Decapoda can have very different type of larval development. Palinurid and scyllarid lobster regularly undergo an 'extended ' type of development in which the species pass through a large number of discrete instars (larval stages), coupled with a long larval duration. However, there is a general tendency in most groups towards the reduction of larval stages (Gurney,1942) and that very few Eucaridea pass through a total of more than 9 stages. A total of 9-11 zoeal stages seems likely to represent the ancestral condition in Eucaridea (Williamson, 1982).

'Accelerated' development involves species with a shorter type of development, probably by the elimination of a complete stage or more. In the 'advanced' type of development, the young are still hatched as a zoea, but in a morphologically advanced stage., so that the larval development is often shortened both durationally and ecdysially. In 'direct ' development, no free swimming larval stages are produced, and the hatchling resemble an adult. Both the direct and the advanced type of development are classified as 'abbreviated' types of development. However, the demarcation between the different types of development seems to be too vague to classify many cases of decapod larval development (e.g. M.hainanense in the present study), which is the general problem of imposing an artificial system on to a natural way of life.

There is now considerable neotological and paleontological evidence that the mode of larval development is an instrinsic biological trait that strongly influences evolutionary rates in at least some marine invertabates (Jablonski, 1985). The planktonic and non-planktonic types of development denote very different ways of life, the former may result in a greater geographic range, greater species longitivity, higher gene flow and lower speciation rates than the later. Work on other marine invertebrates has led to large amounts of data, from which many theories have been put forward to account for the different types of larval development. Strathmann(1986) reviewed and summarised most of the conceptual issues. Many assertions, which would be applicable to the present consideration of Macrobrachium, are summarised in the followings: (adapted from Strathmann, 1986)

1. The planktonic existence is generally safer than the epibenthos for unprotected newly hatched young, and offers

more food and oxygen than the interstitial benthos;

2. A major function of a feeding larva is to convert a small young (just hatched from an egg) into a juvenile with a larger size. A reduction in larval size may permit higher fecundity, but may impose higher risks from a longer period of larval feeding. The balance between fecundity, growth and survival is affected by historical characteristic of the taxa concerned;

3. Feeding larval forms originated long ago and have been very conservative. A feeding larval stage is often lost in a lineage but rarely regained;

4. The form of relationships between egg size, development time, growth, and mortality rates also creates barriers to transitions in the type of development. Models have predicted that a transition state would result in adaptive troughs between peaks (see Chapter 6) under a wide range of conditions;

5. Reproductive effort is not a constraint on the type of larval development;

6. Selection among individuals does not favour large scale dispersal, which would involve loss from favourable grounds.

For a particular taxa, the mode of larval development may also be related to an optimal time for spawning and for settlement ("Settlement-Timing" hypothesis; Todd and Doyle, 1981). Similarly, larval behaviour can also drastically affect their dispersal and settlement. Larvae of M.novaehollandiae were photopositive during the first two stages and strongly photonegative after stage ten, "a mechanism that can prevent large scale seawards movement down an estuary" (Thorne, et al., 1979). Larval behaviour as well as hydrographic conditions (Hunt, 1980) may in effect retain planktonic larvae near to the parental grounds in an estuary.

The evolution events leading to abbreviated (advanced or direct) development would involve modifications under the influence of developmental constraints, physical constraints as

well as historic factors (Raff, 1987). Physical constraints are exogenous factors which limit parameters (e.g. body size, cell numbers, biochemical functions) through the properties of materials used for the construction of body parts. Developmental constraints result from internal factors of the development program, such as the interaction of genetic and epigenetic processes. The magnitude of developmental constraints generally depends upon the interaction between processes (e.g. the differentiation of one tissue being induced by the formation of another) but can be dissociated from each other in evolutionary shifts (e.g. heterochronies). If such nonconstrainted modifications occur, the same overall evolution any change would arise by different pathways (e.g. the evolution of direct development in different crustacean taxas). Constraints may also be more rigid in some stage of development than others, giving rise to highly conserved characters and less conserved characters in other stages (see discussion). In addition to all these factors, the historical characteristics of development of a taxa then form the basis for development. Many characters may be related to historical factors, rather than subsequent constraints or change per se. Comparisons between direct and extended development in relation to tissue and molecular differentiation can be found in Raff(1987).

(3.1.2) Larval development and exogenous factors

The duration of larval development, as well as the number of morphologically recognisable stages, can also be affected greatly exogenous factors like salinity, temperatures and food.

Macrobrachium species have evolved from the marine environment to the freshwater environment, larval freshwater tolerance will thus constitute an evolutionary trait and would be investigated further in the next chapter. Within the physio-chemical range of physical parameters, the availability of food is generally regarded as the most important factor in determining the process of development (Knowlton, 1974).

Studies on the effect of starvation have led to the recognition of various critical points in decapod larval development, which can be recognised by distinct morphological and hormonal conditions in relation to the moulting cycle (Angers, 1987). The point of no return (PNR) refers to a point in the larval development between two stages, which if exceeded by starvation, would not be restored to normal development (probably death) by subsequent feeding. PNR generally occurs early in the larval development long before the energy reserves were ultimately depleted and is much affected by other environmental parameters (Anger et al., 1981). In relation to the general moulting cycle of crustaceans (A, Postmoult; B, Early intermoult; C, Late intermoult; D, premoult; E, Ecdysis), the Point of Reserve Saturation (PRS) occurs generally at the transition between substage C and beginning of substage D (Do) (Anger, 1987). PRS denotes the point in larval development at which prior sufficient food availability would automatically result in the development of the remaining substages. The substage Do is usually characterised by the onset of apolysis, but before the invagination processes near the base of setae has actually begun (D1).

Under laboratory rearing conditions, usually with an ample supply of Artemia nauplii, most larvae would probably proceed at a relatively fast rate. The data on larval development (especially larval duration) obtained under laboratory conditions should thus be interpreted with caution in relation to extrapolation to ecological conditions.

In species with direct development, all the critical points are passed in the embryological development.

(3.1.3) Larval morphology and phylogeny

Appearances of the same crustacean species in the larval and adult phase can be drastically, if not totally different. Many of the crustacean species recognised today were classfied previously as another species. Thus, the generic name Nauplius has been given to the newly hatched form (i.e.nauplius stage) of the copepod Cyclops by Muller(1876) (in Williamson,1982). In Macrobrachium, the hepatic spine, which differentiate the genus from closely related ones, is absent in all the larvae !

In some cases, classification of species, or even taxas, have been based on larval characters. Rhizocephala, the group of parasitic barnacles, was classified as Cirripedia(or even Crustacea) on the form of their nauplius and cypris larvae (Schmitt, 1965; Williamson, 1982). As adult and larval forms of crustacea would be separately subjected to the selection pressure of Nature, a comprehensive phylogeny of species or taxas should logically take into account the larval morphology.

The present chapter aims to report the larval development of two species of Macrobrachium, M.hainanense and M.lanchesteri,

and to review the published data in relation to evolution and aquaculture of the genus. The data compiled in this chapter would be used again in phylogenetic analysis of the genus in chapter 6.

(3.1.4) Definition of terminology

The definition of larval stages by Williamson(1969) would be adopted in the present thesis:

Nauplius : larva with first 3 pairs of cephalic appendages setose and functional , other appendages absent or rudimentary.

Zoea : larva with natatory exopods on some or all of the thoracic appendages, pleopods absent or rudimentary.

Megalopa : larva with setose natatory pleopods on some or all of the abdominal somites; in the present thesis, the term 'Post-larva' would be adopted which has been used as a synonym by most workers for megalopa (Williamson pers.comm., 1987a). In Macrobrachium, it is unlikely to find a clearly defined megalopal stage (Williamson pers.comm., 1987b).

Juvenile : young form, usually small and sexually immature, showing general resemblance to adult.

Thus, in general terms, crustacean larvae are categorised into nauplius, zoea and megalopa which swim with their cephalic, thoracic and abdominal appendages respectively. The adoption of this definition for M.hainanense would prove to be difficult.

Different authors have used different terminology for the various larval appendages. The present thesis would adopt those used by Williamson(1972,1982) and Shokita(1973). The term 'pereopod' would be used for thoracic legs, as suggested in the "Ecdysiast"(Volume 6, No..1).

Terminology of the modes of larval development are mainly based on Gore(1985) and are used in a comparative context within the genus Macrobrachium. Thus, 'direct' development occupy one end of the spectrum while 'extended' development occupies the

other end. 'Advanced' and 'accelerated' development then occupy the region in between with the former more advance than the latter. As in classification of stages, classification of larval developmental type would also prove to be difficult. The term 'metamorphosis' will be used to denote the final ecdysis in the larval development in which the larva is transformed into a juvenile.

(3.1.5) General Information on M.hainanense

The freshwater prawn M.hainanense has mostly been reported from southern China (Yu,1931) but Holthuis(1950) also recorded its presence in Java. Until recently, little was known about the environment in which the species lives. Dudgeon(1985) reported the population dynamics of carideans in the upper reaches of the Lam Tsuen River in Hong Kong and included M.hainanense. Larger adults were found to take up guarded territories in mid-stream areas while the juveniles were mainly found among the submerged portion of vegetation along the banks. However, larval development of the species,which may compliment ecological findings, has not been investigated.

(3.1.6) General Information on M.lanchesteri

The Palaemonid prawn M.lanchesteri is found in freshwater and brackish water habitats of the Indo-West Pacific (Holthuis, 1980). It is especially common in riceland and other open-country areas and is probably the most common freshwater prawn in many parts of Malaysia (Johnson, 1967). While the total maximum length of the species is only about 55mm, many workers have commented on

the potential of this species for aquaculture (e.g. Johnson, 1968). This is probably attributed to the fact that this species can tolerate a high temperature and very low oxygen content (Johnson, 1967). Johnson (1965) suggested that this small species of Macrobrachium probably occupied the same ecological niche as freshwater species of Palaemonetes, in the absence of the latter in many parts of the Oriental Region. Field trials and laboratory studies in the Philippines (Guerrero et al., 1982 and India (Ponnuchamy et al., 1983) also seems to suggest that the larvae of this species may be able to develop and metamorphose entirely in freshwater.

(3.2) Materials and Methods

Mature broodstocks of M.hainanense were collected from the upper Lam Tsuen River, Hong Kong and mated under laboratory conditions. Ovigerous females were kept individually in small tanks (20cm x 10cm) supplied with recirculated freshwater kept at 26-28°C. The hatched larvae were also kept in the same system with the females removed. Samples of the larvae were collected every 2-3 hours and preserved in 10% buffered formalin (pH 7.0).

Mature broodstock of M.lanchesteri were collected from fish ponds of Universiti Pertanian Malaysia, Selangor, Malaysia. Ovigerous females were kept individually in one litre round-bottom containers, supplied with recirculated freshwater kept at 27-28°C. The hatched larvae were kept in the same system with the females removed. Freshly hatched Artemia nauplii were fed to the larvae twice a day (9.00 a.m. and 6.00 p.m.) with a supplement

of micronised trout diet at noon. Samples of larvae were examined and staged three times per day. Individuals of each stage were preserved in 10% buffered formalin(pH 7.0).

All drawings were made with the aid of a projector-microscope. Measurements were taken by a binocular microscope with an occular micrometer. Body length was measured from the post-orbital margin to the posterior end of the telson(excluding setae). Each drawing was based on at least three specimens. Definition of stages was based on Williamson(1969).

(3.3) RESULTS

(3.3.1) Larval Morphology of M.hainanense

Among three ovigerous females of M.hainanense, broods of 30, 56 and 74 larvae were hatched. Mean incubation period was 37 days at a temperature of 26-28°C. Two larval stages were observed before metamorphosis to post-larva. Major characteristics of the stages were as follows:

Stage I (Fig. 3.1)

Body length: 4.06mm Carapace length: 1.21mm

Duration: 0.5-1.0 day

Behaviour: Settled to the bottom , generally remained stationary but responded to stimuli mainly by movement of telson.

Eye sessile . Rostrum completely curved downwards , reaching between the antennular peduncle ; both upper and lower margin without teeth. Carapace with branchiostegal and supra-orbital spines. Telson narrow and fan shaped with 26 plumose setae.

Antennular peduncle(Fig.3.1d) unsegmented ; main flagellum with a plumose setae ; outer flagellum with 3 plumose setae and 1 aesthetasc.

Antenna(Fig.3.1e) biramous , similar to adult shape; protopod unsegmented ; flagellum(endopod) with 35 segments and shorter than body length; antennal scale (exopod) with 10 plumose setae along the anterior-distal margin.

Mandible(Fig.3.1f). Incisor process with two rudimentary teeth; molar process simple.

Maxillule(Fig.3.1g) rudimentary; endopod simple; upper(distal) endite bigger than lower(proximal) endite, both simple.

Maxilla(Fig.3.1h). Endite represented by 3-lobes, lower lobe with one spine at the tip , other two lobes simple; endopod with a small setae on its outer margin , no terminal setae. Exopod(scaphognathite) with 36 plumose setae on entire margin.

First maxilliped(Fig.3.1i) simple; protopod bilobed, lower lobe smaller than upper ; endopod unsegmented with three terminal setae; epipod simple; exopod with 4 apical setae.

Second maxilliped(Fig.3.1j) biramous with a 5-segmented endopod; exopod unsegmented with 4 apical and 2 subapical pulmose setae.

Third maxilliped(Fig.3.1k) biramous; endopod 5 segmented and spines at the last three junctions; exopod with 4 apical and 2 sub-apical plumose setae.

Pereopods(Fig.3.1l-1p) uniramous; first and second pereopods with rudimentary chelae, second larger then the first , cutting edges of both chelae without teeth. Third pereopod with 2 rudimentary setae at the ultimate junction.

Pleopods(Fig.3.1l-1u). Second to fifth pleopods with appendices internae; endopods and exopod with small setae buds; endopod of first pleopod smooth and simple.

Stage II (Fig. 3.2)

Body length: 4.5mm Carapace length: 1.67mm

Duration 1-1.5 days

Behaviour: stayed mostly stationary, responded to stimuli mainly by movement of telson.

Eye stalked. Rostrum with 7-8 dorsal teeth and 1 ventral tooth. Carapace with both branchiostegal and antennal spines. Telson remaining mostly unchanged with 26 plumose setae. Outlines of rudimentary uropods can be seen within the telson.

Antennular peduncle(Fig. 3.2d) 3-segmented ; first segment longest , bearing a stylocerite with 4 setae and an outer distal marginal spine ; third segment with a dorsal distal knob with two setae ; inner flagellum 5-segmented ; outer flagellum (exopod) biramous , inner part 2-segmented with 4 apical aesthetascs , outer part 5-segmented.

Antennal flagellum(Fig. 3.2e) still shorter than body ; Antennal scale with 44 plumose setae and an outer distal marginal spine.

Mandible(Fig. 3.2f) still rudimentary.

Maxillule(Fig. 3.2g). Endopod cleft in inner proximal margin ; Upper and lower lacinia with 4 and 3 teeth, respectively.

Maxilla(Fig. 3.2h). Endite 2-lobed , each with 2 spines at tips, endopod bare. Exopod with 41 plumose setae.

Maxillipeds(Fig. 3.2i-k). Mostly similar in shape to the zoeal stage ; Exopodal lobe of the first maxilliped with 7 setae , endopod simple.

Pereopods(Fig. 3.2l-p). Third , fourth and fifth pereopods fully developed with sensory hairs ; First and second pereopods more developed than last stage but still rudimentary.

Pleopods(Fig. 3.2q-u). Endopod of first pleopod still bare ; Setation on second to fifth pleopods as 7,6,6,5 respectively ; that of exopods on first to fifth pleopods as 9,11,12,12,11 respectively.

Stage III (Fig. 3.3)

Body length: 4.76mm Carapace length: 1.52mm

Duration: 1 day

Rostrum bearing 8 dorsal and 1 ventral teeth. Carapace with both

branchiostegal and antennal spines. Telson broad, with 18 post-marginal plumose setae and 2 pairs of lateral marginal spines; uropods newly formed and biramous; exopod with a small terminal spine and 26-28 plumose setae; endopod slightly smaller than exopod, with 14 plumose setae. Movement mainly by propulsion with pleopods.

Antennular peduncle(Fig. 3.3d) 3-segmented , mostly as preceeding stage; inner flagellum with 8 segments; outer flagellum biramous, inner part 3-segmented with 3 apical aesthetacs, outer part having 9-segmented. Antennal flagellum(Fig. 3.3e) as long as body; antennal scale still with 44 pulmose setae as in preceeding stage.

Mandible(Fig. 3.3f) more developed; incisor process with 2 teeth, molar process with 3 teeth. Maxillule(Fig. 3.3g) mostly as preceeding stage, upper and lower lacinia with 8 and 3 teeth, respectively.

Maxilla(Fig. 3.3h). Exopod with 44 plumose setae; endopod still bare; upper lobe of endite with 5 setae: lower lobe with 6 setae.

Maxillipeds(Fig. 3.3i-k) with setation more developed; upper protopodal lobe of first maxilliped with 29 short spines; lower lobe with 2 plumose setae; exopodal lobe with 8 plumose setae; endopod with 2 lateral setae; exopod with 4 apical plumose setae. Second maxilliped with a 5 segmented endopod; width of ultimate and penultimate segments longer than length and with numerous short spines; exopod with 4 apical and 2 sub-apical plumose setae. Third maxilliped with 4-segmented endopod and with numerous setation; exopod with 4 apical setae and 2 sub-apical setae.

Pereopods(Fig. 3.3l-p) fully developed, each with numerous setae; fingers of first and second pereopods becoming more cylindrical. Pleopods(Fig. 3.3q-u) fully functional. Endopod of first pleopod with 2 small setae; setation on second to fifth pleopods as 7,8,7,6 respectively; that of exopods on first to fifth pleopods as 9,13,12,12,11 respectively.

Stage IV (Fig. 3.4)

Body length: 5.10mm Carapace length: 1.60mm

Duration: about 1 day

Rostrum bearing 8 dorsal and 2 ventral teeth; carapace with both branchiostegal and antennal spine.

Telson(Fig. 3.4c) with 14 plumose setae and 3 pairs of lateral spines; the most distal pair especially strong; Uropodal exopod and endopod with 33-35 and 25 pulmose setae, respectively.

Antennule and antenna(Fig. 3.4d-e) mostly as preceeding stage; inner antennular flagellum with 13 segments; inner and outer part of outer flagellum with 4 and 13 segments respectively.

Mandible(Fig. 3.4f) more developed.

Maxillule(Fig. 3.4g). Lower and upper lacinia with various spines; lower lobe of exopod with two setae. Maxilla (Fig. 3.4h) mostly similar to preceeding stage; exopod with 50 pulmose setae. Maxillipeds(Fig. 3.4i-k) and pereopods mostly similar to preceeding stage with higher degree of setation. Minute setae on cutting edge of fingers on both first and second pereopods.

Pleopods(Fig. 3.4q-u). Setation on endopod of first to fifth pleopods as 2,7,8,6,5 respectively; that of exopod as 9,11,14,11,11.

Fig. (3.1) : Post-embryonic Stage I of *M. hainanense*

(a) Lateral view; (b) Dorsal view; (c) Telson; (d) Antennule; (e) Antennae; (f) Mandible; (g) Maxillule; (h) Maxilla; (i) First Maxilliped; (j) Second Maxilliped; (k) Third Maxilliped; (l) First Pereopod; (m) Second Pereopod; (n) Third Pereopod; (o) Fourth Pereopod; (p) Fifth Pereopod; (q) First Pleopod; (r) Second Pleopod; (s) Third Pleopod; (t) Fourth Pleopod; (u) Fifth Pleopod.

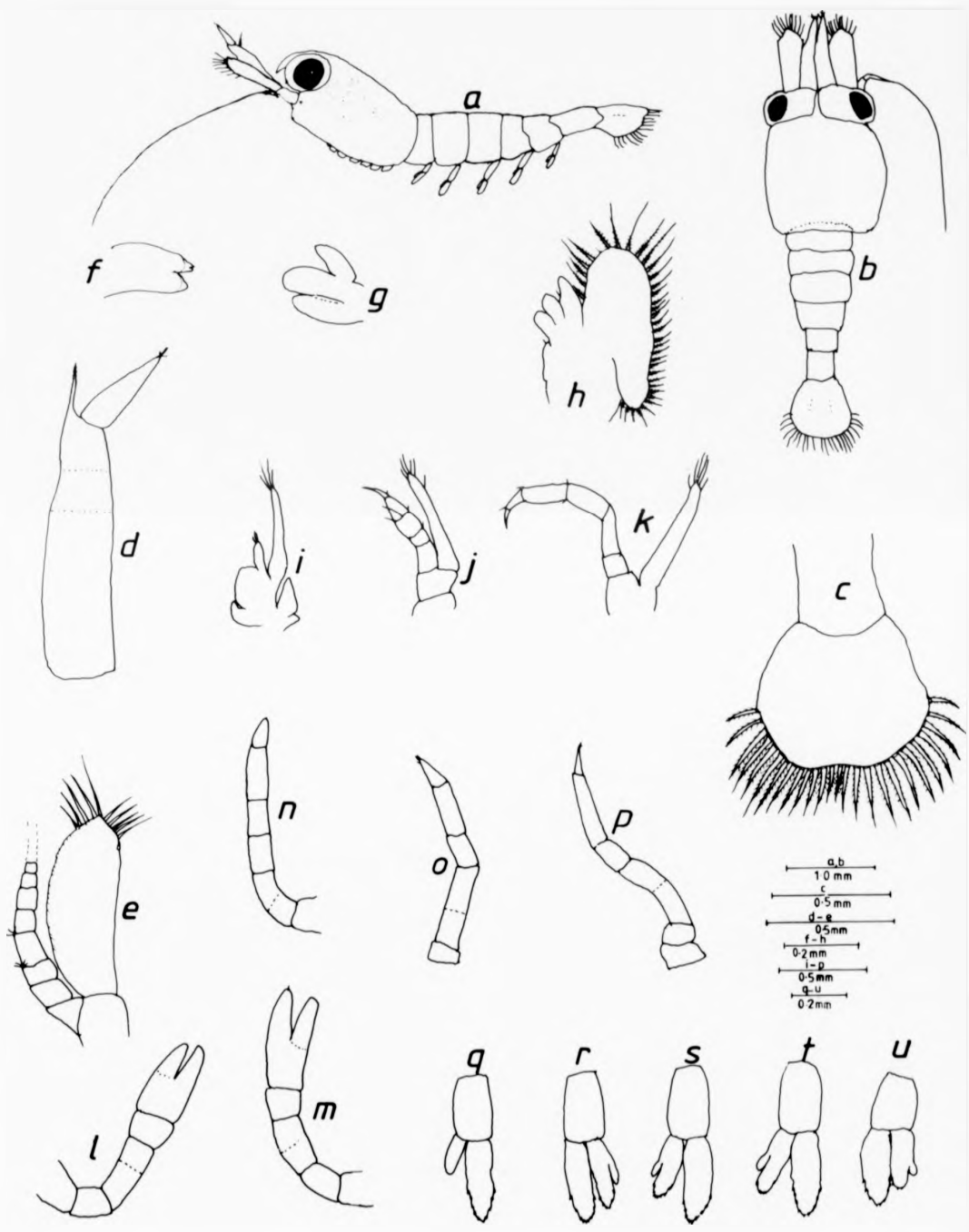
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Fig. (3.2) : Post-embryonic Stage II of *M.hainanense*

(a) Lateral view; (b) Dorsal view; (c) Telson; (d) Antennule; (e) Antennae; (f) Mandible; (g) Maxillule; (h) Maxilla; (i) First Maxilliped; (j) Second Maxilliped; (k) Third Maxilliped; (l) First Pereopod; (m) Second Pereopod; (n) Third Pereopod; (o) Fourth Pereopod; (p) Fifth Pereopod; (q) First Pleopod; (r) Second Pleopod; (s) Third Pleopod; (t) Fourth Pleopod; (u) Fifth Pleopod.

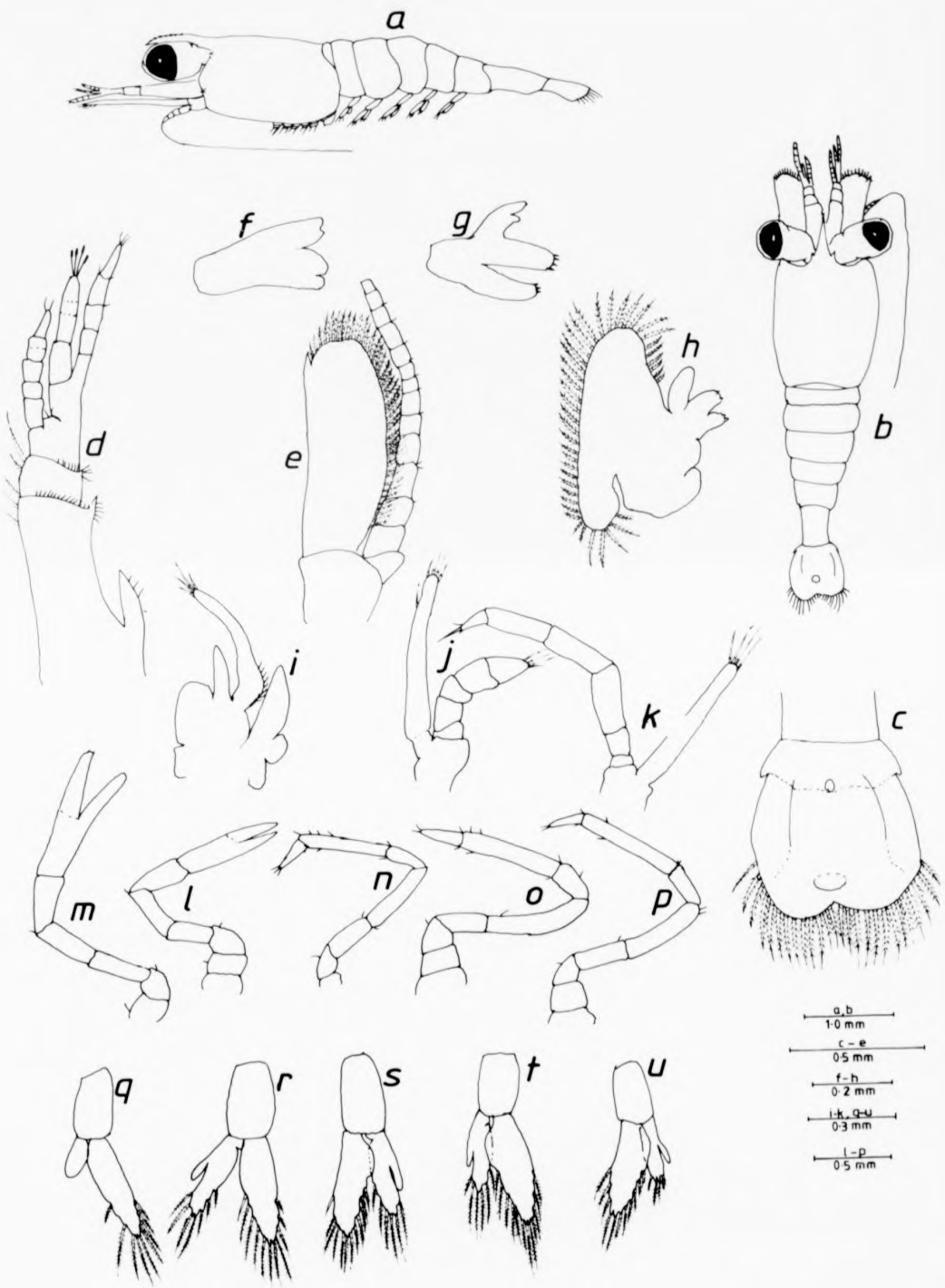
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Fig. (3.3): Post-embryonic Stage III of M.hainanense

(a) Lateral view; (b) Dorsal view; (c) Telson; (d) Antennule; (e) Antennae; (f) Mandible; (g) Maxillule; (h) Maxilla; (i) First Maxilliped; (j) Second Maxilliped; (k) Third Maxilliped; (l) First Pereopod; (m) Second Pereopod; (n) Third Pereopod; (o) Fourth Pereopod; (p) Fifth Pereopod; (q) First Pleopod; (r) Second Pleopod; (s) Third Pleopod; (t) Fourth Pleopod; (u) Fifth Pleopod.

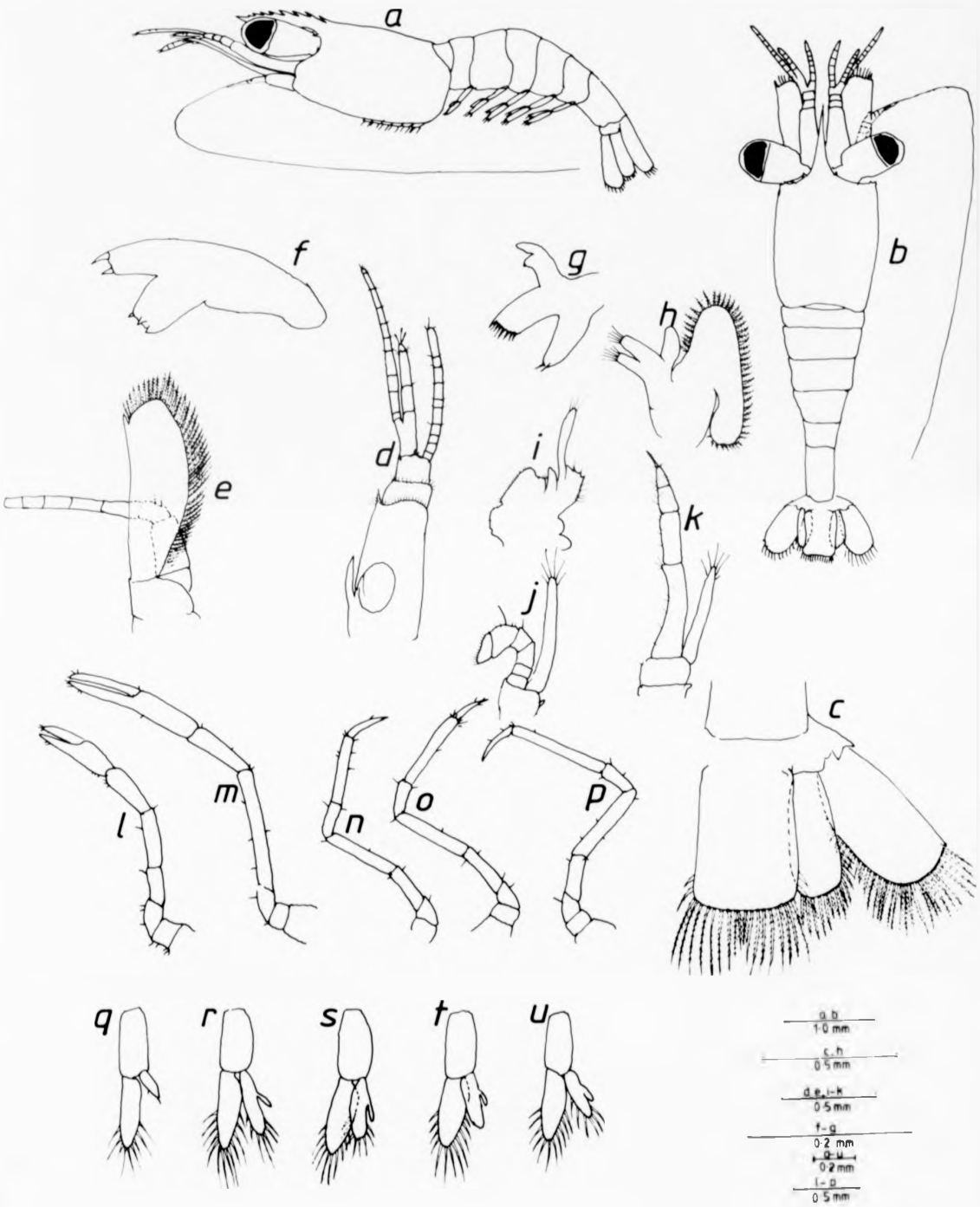
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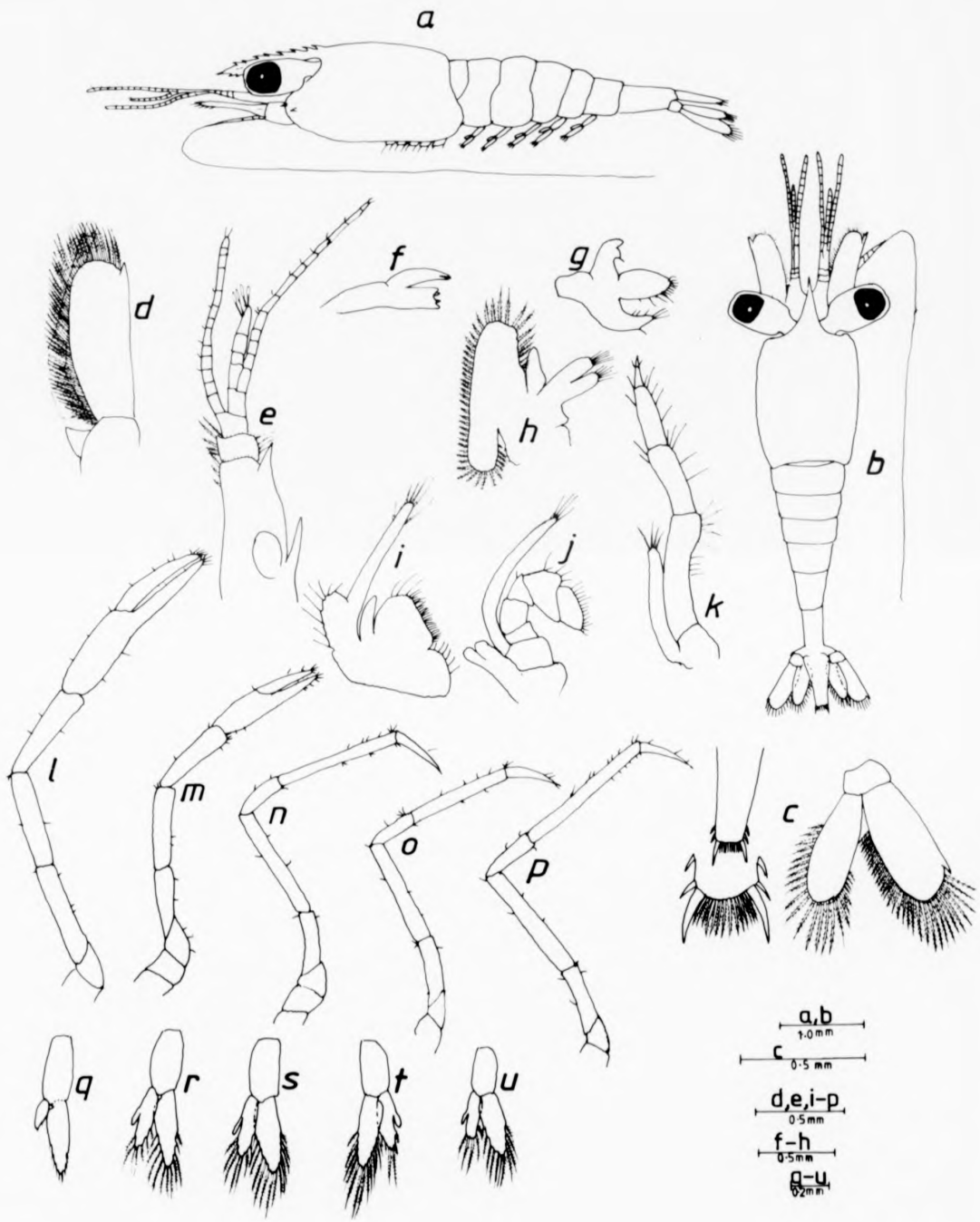
Fig. (3.4) : Post-embryonic Stage IV of *M.hainanense*

(a) Lateral view; (b) Dorsal view; (c) Telson; (d) Antennule; (e) Antennae; (f) Mandible; (g) Maxillule; (h) Maxilla; (i) First Maxilliped; (j) Second Maxilliped; (k) Third Maxilliped; (l) First Pereopod; (m) Second Pereopod; (n) Third Pereopod; (o) Fourth Pereopod; (p) Fifth Pereopod; (q) First Pleopod; (r) Second Pleopod; (s) Third Pleopod; (t) Fourth Pleopod; (u) Fifth Pleopod.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(3.3.2) Larval Morphology of M.lanchesteri

Several batches of larvae were successfully reared to metamorphosis in complete freshwater. The total duration of larval development in M.lanchesteri took about 30 days. Nine zoeal stages were observed before the first post-larval stage. The duration of each stage is summarised in Fig(3.5). In the diagrams, the hairs on plumose setae are not represented quantitatively.

First Zoea (Fig.3.6)

T.L.: 2.88mm Duration: 1 day

C.L.: 0.74mm

Eye sessile. Rostrum smooth. Carapace with pterygostomian spine on the antero-ventral border.

Telson triangular(Fig.3.6c) and not separated from 6th somite; posterior margin with 7 pairs of plumose setae.

Antennular peduncle (Fig.3.6d) unsegmented; endopod with 4 apical seta; exopod in the form of a long plumose setae.

Antennal scale(exopod)(Fig.3.6e) with 5 distal segments (faint 6th) with 9 plumose setae along the antero-inner margin and long distal spine ; a small plumose seta from outer margin of fourth segment; endopod with 1 apical plumose setae and three aesthetacs.

Mandible(Fig.3.6f) rudimentary ; 2 teeth on incisor process; molar process bare.

Maxillule(Fig.3.6g) endopod unsegmented, terminating in 1 spine; the two endites each with two spines.

Maxilla(Fig.3.6h) exopod with 4 plumose setae and a strong

proximal seta at apex; coxal and basal endites each with two setae at apex; apex of endopod with one seta at apex, basal lobe also with one seta.

Maxillipeds(Fig.3.6i-k) with exopods longer than endopods; exopod of first maxilliped with 4 natatory plumose setae at apex and two sub-apical setae;endopod un-segmented ; exopods of second and third maxillipeds each with 4 natatory setae at apex ;endopods of second and third maxilliped 4-segmented(faint 5th) with stout terminal spine and 2 short subterminal setae.

Biramous buds of first, second and third pereopods (Fig.3.6l,m,o) present; Fourth pereopod not represented; Fifth pereopod(Fig.3.6n) uniramous and unsegmented;

All pleopods absent.

Second Zoea (Fig.3.7)

C.L.: 0.79mm Duration: 2-3days

T.L.: 2.89mm

Eyes stalked; carapace with a pronounced supraorbital and pterygostominal spines and a dorsal tubercle at the dorso-median border; telson with an additional(8th) pair of small setae along the posterior margin.

Antennular peduncle 4-segmented; endopod with 1 apical plumose seta and three aesthetasc; setation of each segment as in Fig.(3.7d).

Antennal scale(Fig.3.7e) 4-segmented with 11 plumose setae and an outer distal spine.

Mandible(Fig.3.7f) with 3-teeth on incisor process; molar process with pointed upper edge.

Maxillule(Fig.3.7g). Upper and lower lacinia with 4 and 2 teeth, respectively.

Maxilla(Fig.3.7h) mostly as Zoeal I.

Maxillipeds(Fig.3.7i-k) similar to preceding stage with a higher degree of setation; endopod of third maxilleped 5-segmented.

Pereopods(Fig.3.7l-p). First and second pereopods biramous, exopods with 6 natatory setae each, endopods 5- and 4- segmented respectively; third pereopod a biramous bud; fourth pereopod a rudimentary bud; fifth pereopod longer than third and very much longer than fourth.

Third Zoea (Fig 3.8)

C.L.: 0.9mm Duration: 3-6 days

T.L.: 3.53mm

Carapace with a second spine at the antero-lateral border, occupying the branchiostegal position, in addition to a supraorbital spine, a pterygostomian spine and a small dorsal tubercle.

Telson(Fig.3.8c) separated from the sixth abdominal somite; posterior margin with 8 pairs of seta; median pair naked, the rest plumose; uropods present, exopods with 10 plumose setae, endopod still rudimentary.

Antennular peduncle(Fig.3.8d) 4-segmented; stylocerite with developing spine; exopod with 1 apical plumose seta and 3 aesthetascs; endopod a small knob.

Antennal endopod(Fig.3.8e) 3-segmented with two apical spines; antennal scale unsegmented, longer than endopod and with 17

plumose setae.

Mandible(Fig.3.8f). Incisor process with three teeth; molar process with 5 minute teeth.

Maxillule(Fig.3.8g). Endopod with one spine; upper and lower lacinia with 5 and 2 teeth respectively.

Maxilla(Fig.3.8h). Endopod with 9 plumose setae; exopod with 3 plumose setae at apex, basal lobe with two.

Maxillipeds(Fig.3.8i-k) similar to preceeding stage.

Pereopods(Fig.3.8l-p). Endopods of first, second and third pereopods 5-segmented; fourth pereopod a biramous bud; fifth pereopod reaching anterior of carapace.

Fourth Zoea (Fig.3.9)

C.L.: 1.16mm Duration: 3-6 days

T.L.: 3.67mm

Rostrum with two epigastric teeth, separated from dorsal median tubercle. Carapace with spines similar to preceeding stage. Telson (Fig.3.9c) almost parallel-sided, concave posteriorly, with 5 pairs of plumose setae on posterior margin and 2 pairs of lateral spines; exopod and endopod of uropods with 16 and 9 plumose setae respectively.

Antennular peduncle(Fig.3.9e) 4-segmented, stylocerite well developed; endopod more developed, with an aesthetasc and a plumose seta at apex.

Antennal(Fig.3.9d) endopod 4-segmented; antennal scale with 18 plumose setae.

Mandibles(Fig. 3.9f1&2). Incisor processes of both mandibles with

4 teeth each; molar process of left mandible with 7 minute setae.

Maxillule(Fig. 3.9g) similar to preceeding stage.

Maxilla(Fig.3.9h). Exopod with 16 plumose setae.

Maxillipeds(Fig.3.9i-k)similar to as preceeding stage.

Pereopods(Fig.3.9l-p). Fourth pereopod fully developed, biramous; endopod 5-segmented; exopod with 4 apical plumose setae. Fifth pereopod reaching top of antennal scale.

Fifth Zoea (Fig.3.10)

C.L.: 1.17mm Duration: 3-7days

T.l.: 4.03mm

Rostrum with two teeth. Spines on carapace similar to preceding stage. Telson (Fig.3.10c) narrowed at posterior end; 4 pairs of plumose setae and 2 pairs of lateral spines on its posterior margin; uropodal exopod and endopod with 19 and 13 plumose setae respectively.

Setation of antennular peduncle as in Fig.(3.10d); a inner-ventral marginal spine present at proximal segment; endopod more elongated.

Antennal scale(Fig.3.10e) with 21 plumose setae and as long as endopod.

Mandible(Fig.3.10f). Incisor processes of left and right mandibles with 3 and 6 teeth respectively; molar processes bare with a tooth-like edge.

Maxillule(Fig.3.10g). Exopod with 1 seta; upper and lower lacinia with 5 and 3 teeth respectively.

Maxillal exopod(Fig.3.10h) with 25 plumose setae.

Maxillepeds(Fig.3.10i-k). First maxilliped well developed; exopod

with 4 plumose setae; exopodal(caridean) lobe with 1 seta; endopod with 3 apical and 2 sub-apical setae. Second and third maxillipeds similar to preceeding stage.

Pereopods(Fig.3.10l-p). Fourth pereopod fully developed; exopod with 4 plumose setae; endopod 5-segmented; other pereopods mostly as preceeding stage.

Sixth Zoea (Fig.3.11)

C.L.: 1.26mm Duration: 3-7 days

T.L.: 4.23mm

Rostrum and carapace similar to preceeding stage.

Posterior margin of telson(Fig.3.11c) with 5 pairs of plumose setae and 2 pairs of lateral spines. Uropodal exopod and endopod with 21 and 16 plumose setae respectively.

Antennule(Fig.3.11d) and antennae(Fig.3.11e) mostly as preceeding stage.

Mandibles(Fig.3.11f). Incisor processes with very strong teeth; molar processes of both mandibles with two small teeth and pointed lower edge.

Maxillule(Fig.3.11g) mostly similar to preceeding stage.

Maxilla(Fig.3.11h) with 31 plumose setae on the entire margin of exopod.

First maxilliped(Fig.3.11i) with 2 setae on exopodal lobe(caridean lobe).

Pereopods(Fig.3.11l-p) mostly unchanged, except in setation.

Pleopods present for first time in form of rudimentary buds.

Seventh Zoea (Fig.3.12)

C.L.: 1.50mm Duration: 3-8 days

T.L.: 4.43mm

Telson(Fig.3.12c) narrower, with 4 pairs of plumose setae on posterior margin and three pairs of lateral spines; uropodal exopod and endopod each with 20 plumose setae.

Antennular peduncle(Fig.3.12d) with biramous exopod; endopod more elongated.

Antennal scale (Fig.3.12e) with 25 plumose setae; endopod 6-segmented and longer than exopod.

Mandibles(Fig.3.12f 1&2). Incisor processes of both mandibles with 4 teeth; molar processes of left and right mandibles with 2 and 1 teeth respectively.

Maxillule(Fig.3.12g) and maxilla(Fig.8h) similar to preceeding stage.

First maxilliped(Fig.3.12i) with 4 setae on the exopodal lobe.

First and second pereopods(Fig.3.12l,m) with rudimentary chelae.

Pleopod buds(Fig.3.12q-u) biramous but unsegmented.

Eighth Zoea (Fig.3.13)

C.L.:1.52mm Duration: 4-12 days

T.L.:4.83mm

Telson(Fig.3.13c) with 5 pairs of plumose setae on posterior margin; uropodal endopod and exopod with 25 and 28 plumose setae respectively.

Antennule(Fig.3.13d). Inner rami of exopod 2 segmented; outer rami elongated but unsegmented; endopod 3-segmented.

Antennal scale(Fig.3.13e) with 26 plumose setae.

Mandibles(Fig.3.13f,1&2). Upper edges of incisor processes pointed to form a strong tooth; incisor process of left mandible also has a blunt stout tooth.

Maxillule(Fig.3.13g) with 5 teeth and 1 seta on upper lacinia; lower lacinia with 4 teeth.

Maxilla(Fig.3.13h) with 33 plumose setae on exopod; endopod with 3 setae; protopod 4-lobed with 3 setae each.

Pereopods(Fig.3.13l-p). Chela of first and second pereopods more developed.

Maxillipeds(Fig.3.13i-k) similar to preceeding stage.

Pleopods(Fig.3.13q-u) with rudimentary setae, mostly on exopods.

Nineth Zoea (Fig.3.14)

C.L.:1.63mm Duration: 7-13 days

T.L.:5.00mm

Telson(Fig.3.14c) similar to preceeding stage with eight plumose setae along posterior margin; uropodal endopod and exopod with 27 and 30 plumose setae respectively.

Antennular peduncle(Fig.3.14d) with 3-segmented endopod; outer ramus of the biramous exopod 4-segmented; inner ramus 2-segmented.

Antennal scale (Fig.3.14e) with 32 plumose setae ;endopod 10-segmented.

Mandibles(Fig.3.14f,1&2). Upper edge of left incisor process ending in blunt tooth; that of right mandible ending in pointed tooth.

Maxillule(Fig.3.14g) mostly similar to stage 8.

Maxillal exopod(Fig.3.14h) with 35 plumose setae.

Maxillipeds(Fig.3.14i-k) generally similar to stage eight, except

in size and setation; exopodal lobe of first maxilleped with 5 setae.

First and second pereopods(Fig.3.14l,m) with fully developed chela.

Pleopods(Fig.3.14q-u) with appendix interna (except first); all exopods and endopods with minute setae.

Post-larva (Fig.3.15)

C.L.: 1.65mm

T.L.: 4.93mm

Behaviour: settled to bottom and swam periodically motion by propulsion of pleopods.

Carapace with antennal and branchiostegal spines, the prominent supraorbital spine of the larval phase absent.

Rostrum formula 5/1.

Telson(Fig.3.15c) with only 2 pairs of posterior spines, outer much stronger than inner; 3 pairs of lateral spines; uropodal exopod and endopod each with 32 plumose setae.

Antennular endopod(Fig.3.15d) 5-segmented; exopod biramous, outer ramus 7-segmented, inner ramus 3-segmented.

Antennal scale(Fig.3.15e) with 28 plumose setae and an outer distal spine; exopod longer than body, with 23-25 segments.

Mandibles cleft between incisor and molar processes. Left mandible(Fig.3.15f/1); incisor and molar processes with 3 and 5 teeth respectively. Right mandible(Fig.3.15f/2); incisor and molar processes with 5 and 5 teeth respectively.

Maxillule(Fig.3.15g). Exopod bilobed with one seta on lower lobe; upper and lower lacinia with 8 and 5 teeth respectively; lower lacinia also has 3 lateral spines.

Maxilla(Fig.11h). Exopod with 45 plumose setae; endopod unsegmented and bare; endites elongated and deeply cleft in between.

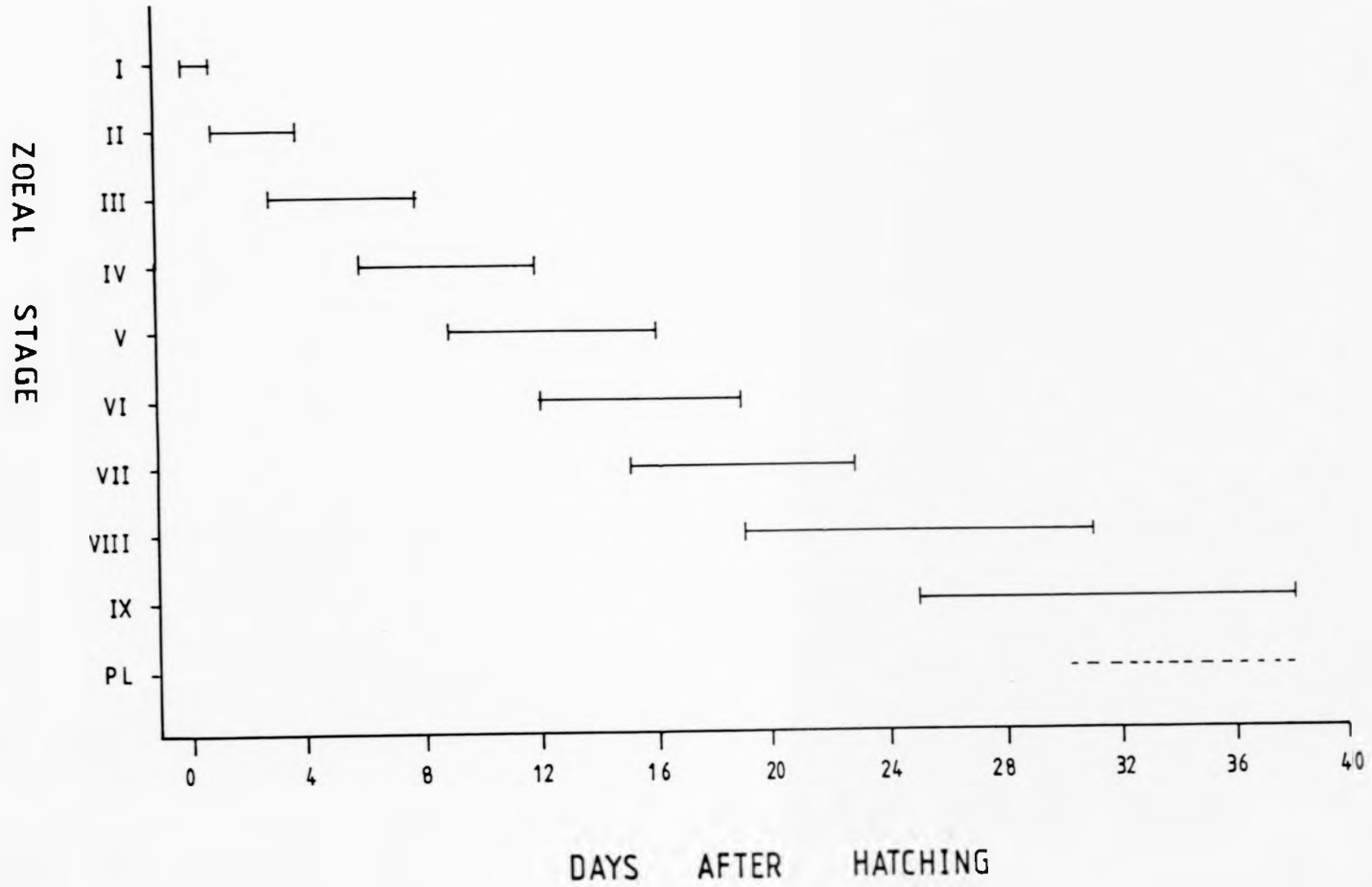
First maxilliped(Fig.3.15i) with 10 plumose setae on exopodal lobe; endopod with 3 lateral setae; endopods of second and third maxillipeds 5-segmented; ultimate and penultimate segments broadened with numerous short setae; exopods of all maxillipeds with 6 plumose setae.

First and second pereopods(Fig.3.15l&m) with fully developed chelae; exopods of first to fourth pereopods still present but small and with few setae..

Pleopods(Fig.3.15q-u) fully developed; setation on endopod of first to fifth pleopods 3,8,11,9 and 8,respectively; setation on exopod of first to fifth pleopods 15,18,19,18 and16 respectively.

Fig. (3.5):

Duration of larval stages of *M. lanchesteri*.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DAYS AFTER HATCHING

Fig. (3.6) : Zoeal I of *M. lanchesteri*

(a)Side view; (b)Dorsal view; (c)Telson; (d)Antennule; (e)Antenna; (f)Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Fifth Pereopod; (o)Third Pereopod.

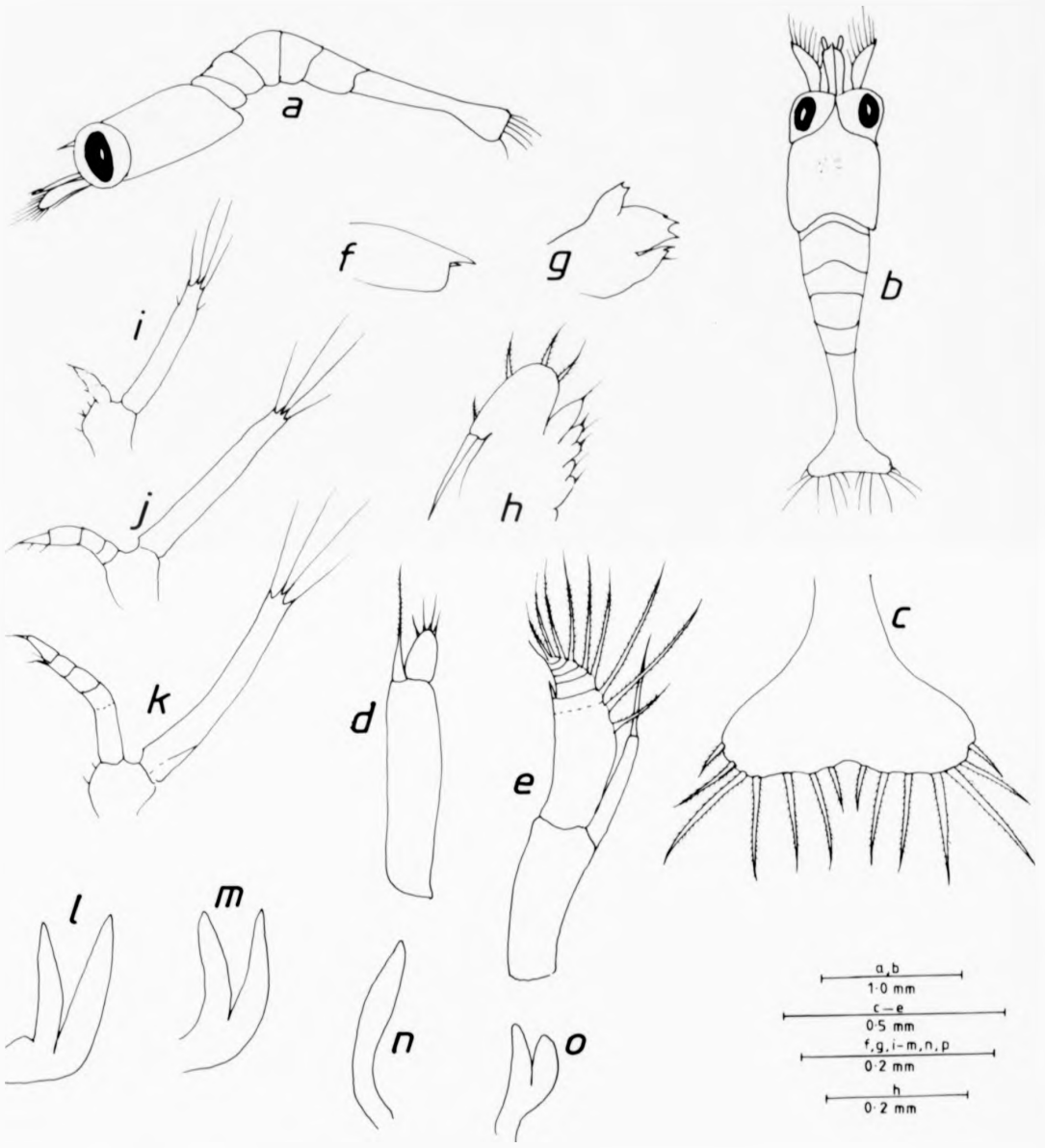
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Fig. (3.7) : Zoeal II of M. lanchesteri

(a)Side view; (b)Dorsal view; (c)Telson; (d)Antennule; (e)Antenna; (f)Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod; (p)Fifth Pereopod.

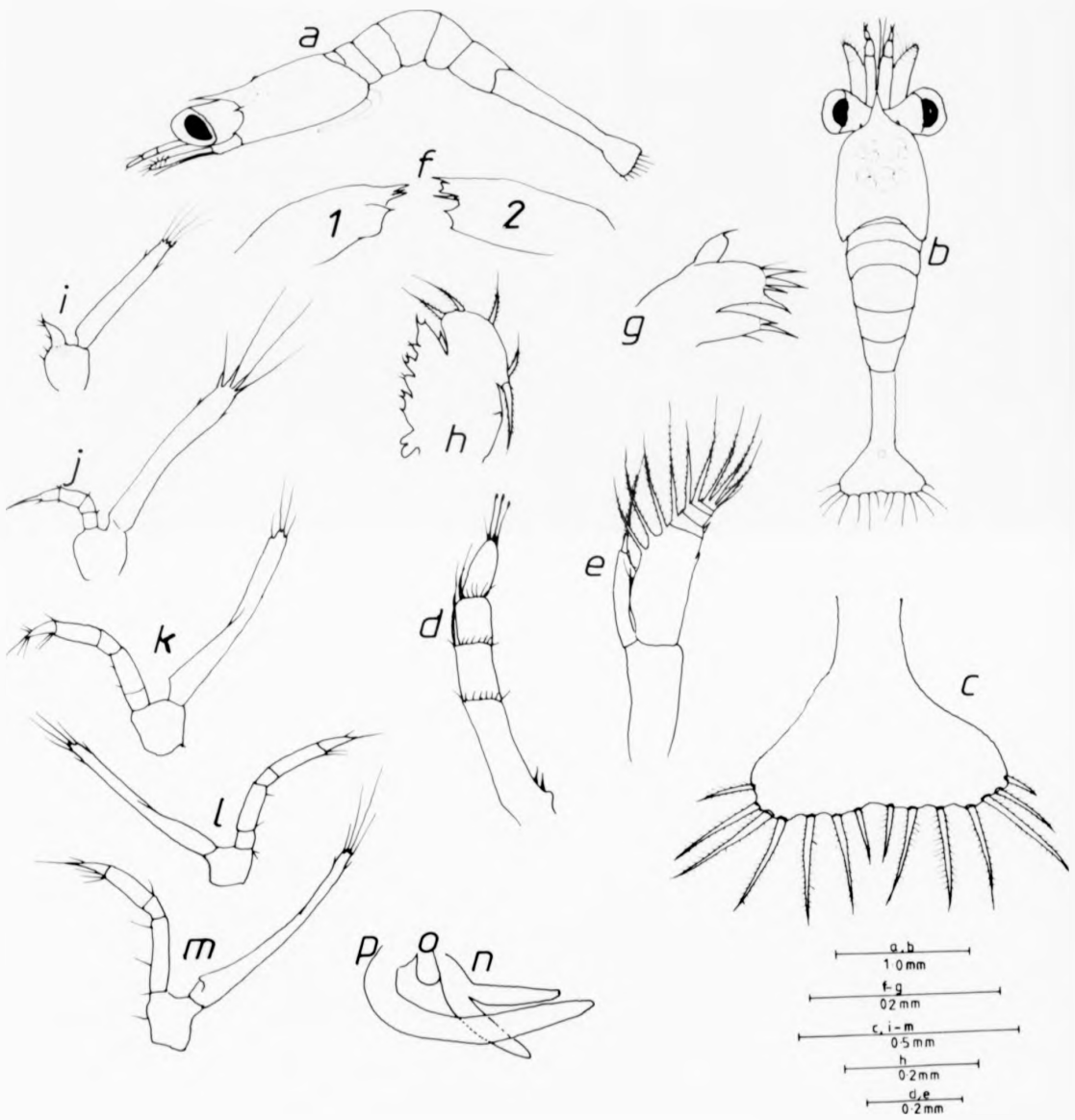
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Fig. (3.8) : Zoeal III of M. lanchesteri

(a)Side view; (c)Telson; (d)Antennule; (e)Antenna; (f)Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod; (p)Fifth Pereopod.

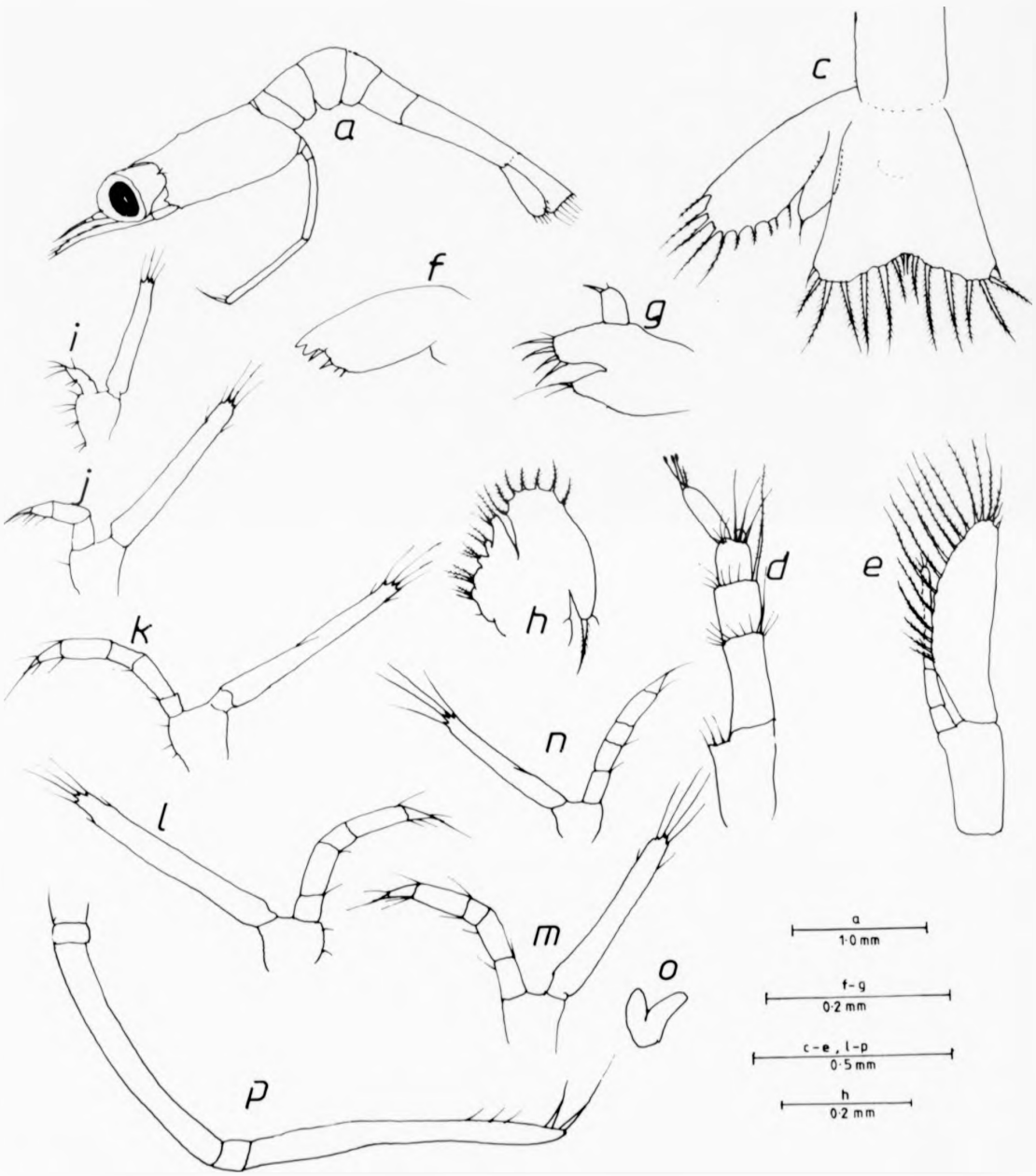
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Fig. (3.9) : Zoeal IV of M. lanchesteri

(a)Side view; (c)Telson; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod.

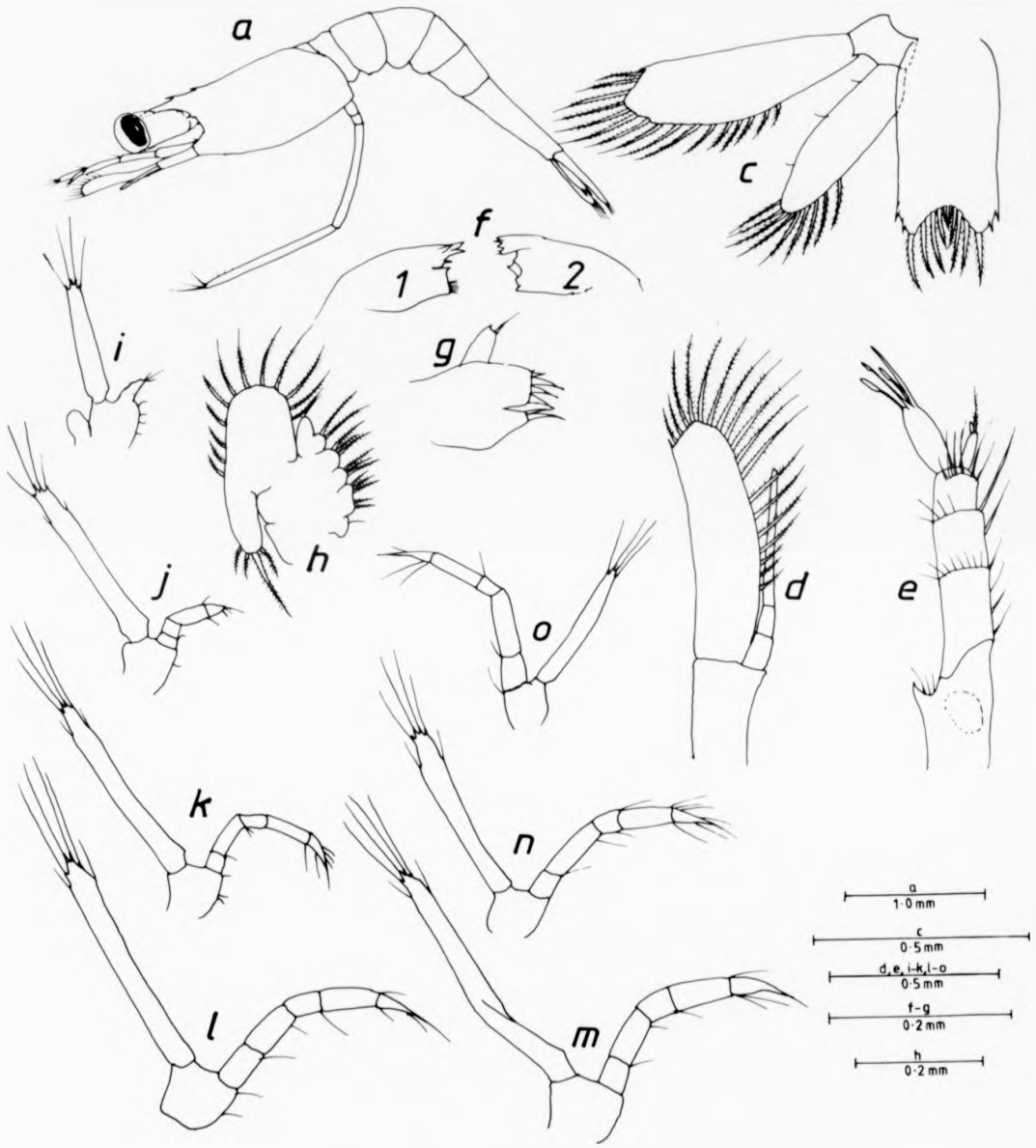
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Fig. (3.10) : Zoeal V of *M. lanchesteri*

(a)Side view; (c)Telson; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod.

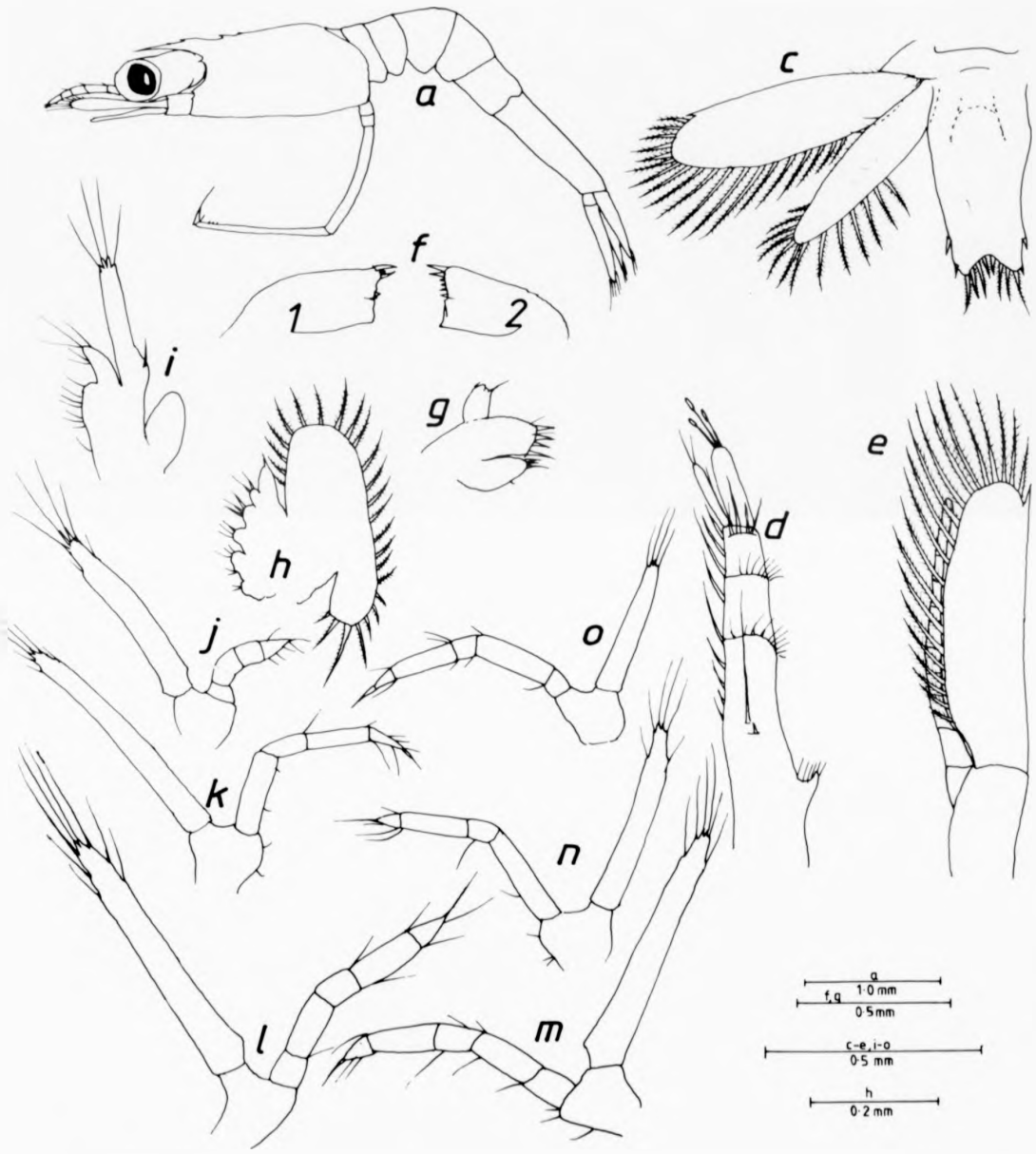
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Fig. (3.11) : Zoeal VI of M. lanchesteri

(a)Side view; (c)Telson; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod.

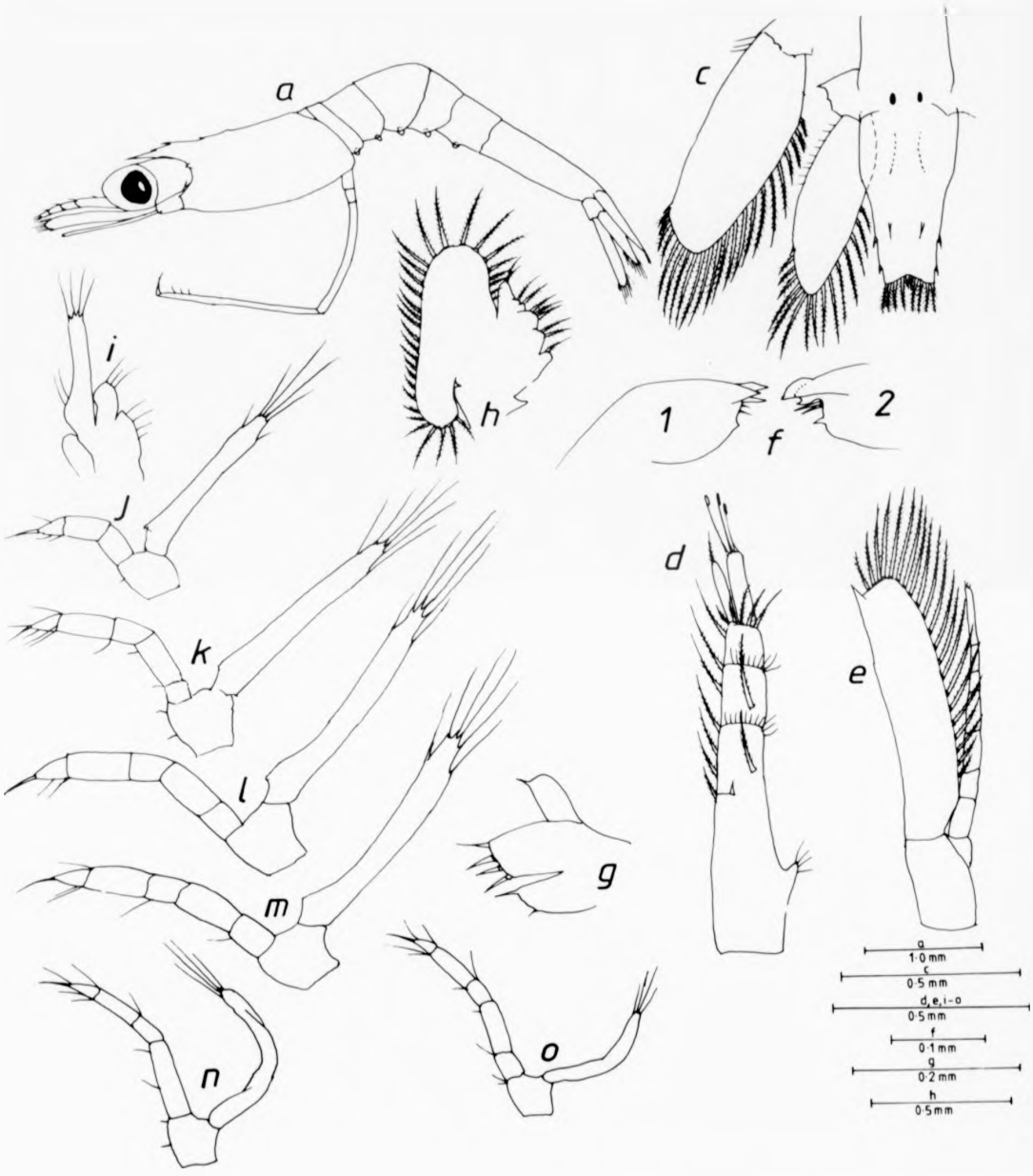
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Fig. (3.12) : Zoeal VII of *M. lanchesteri*

(a)Side view; (b)Telson; (c)Uropods; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod; (q)First Pleopod; (r)Second Pleopod; (s)Third Pleopod; (t)Fourth Pleopod; (u)Fifth Pleopod.

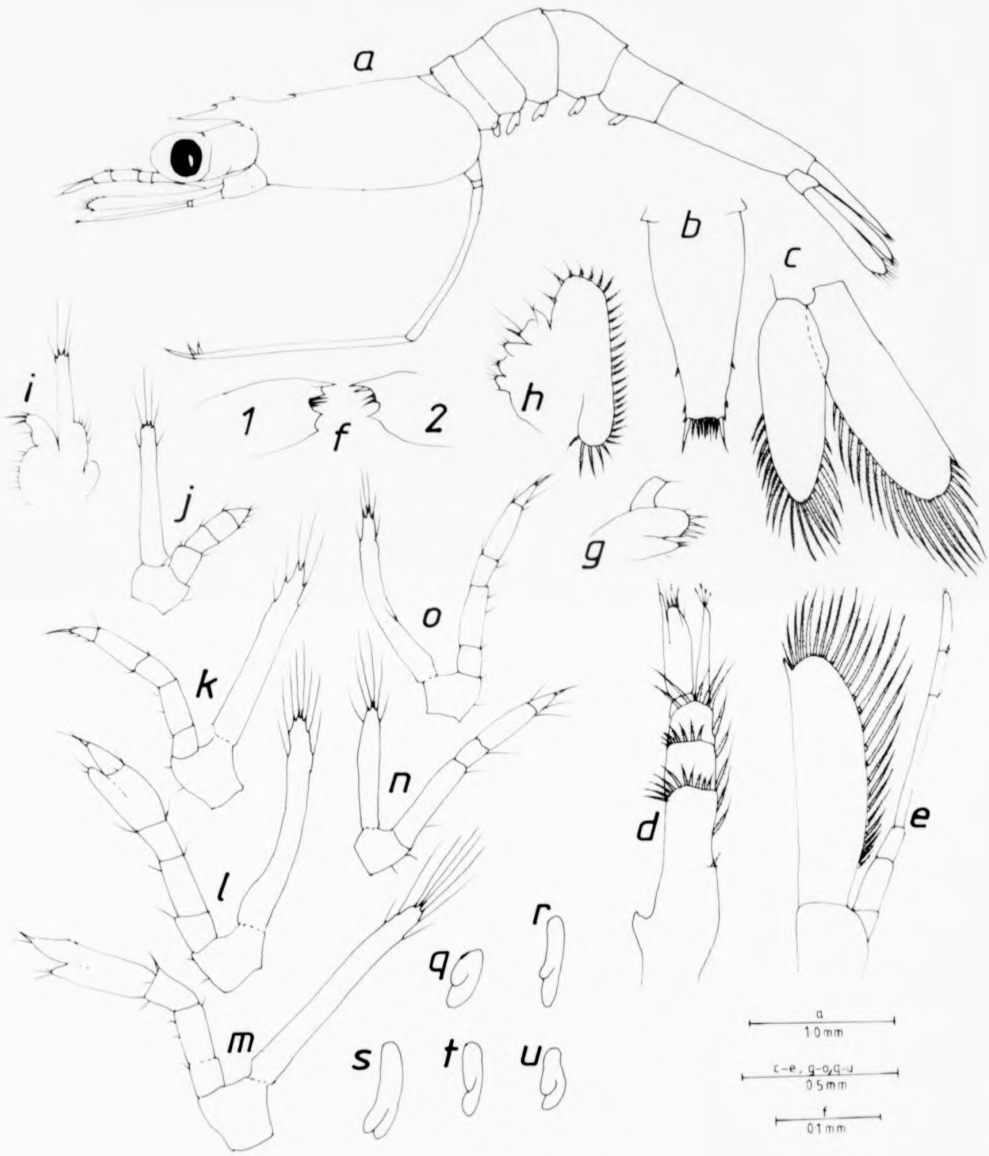
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Fig. (3.13) : Zoeal VIII of M. lanchesteri

(a)Side view; (b)Telson; (c)Uropods; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod (o)Fourth Pereopod; (q)First Pleopod; (r)Second Pleopod; (s)Third Pleopod; (t)Fourth Pleopod; (u)Fifth Pleopod.

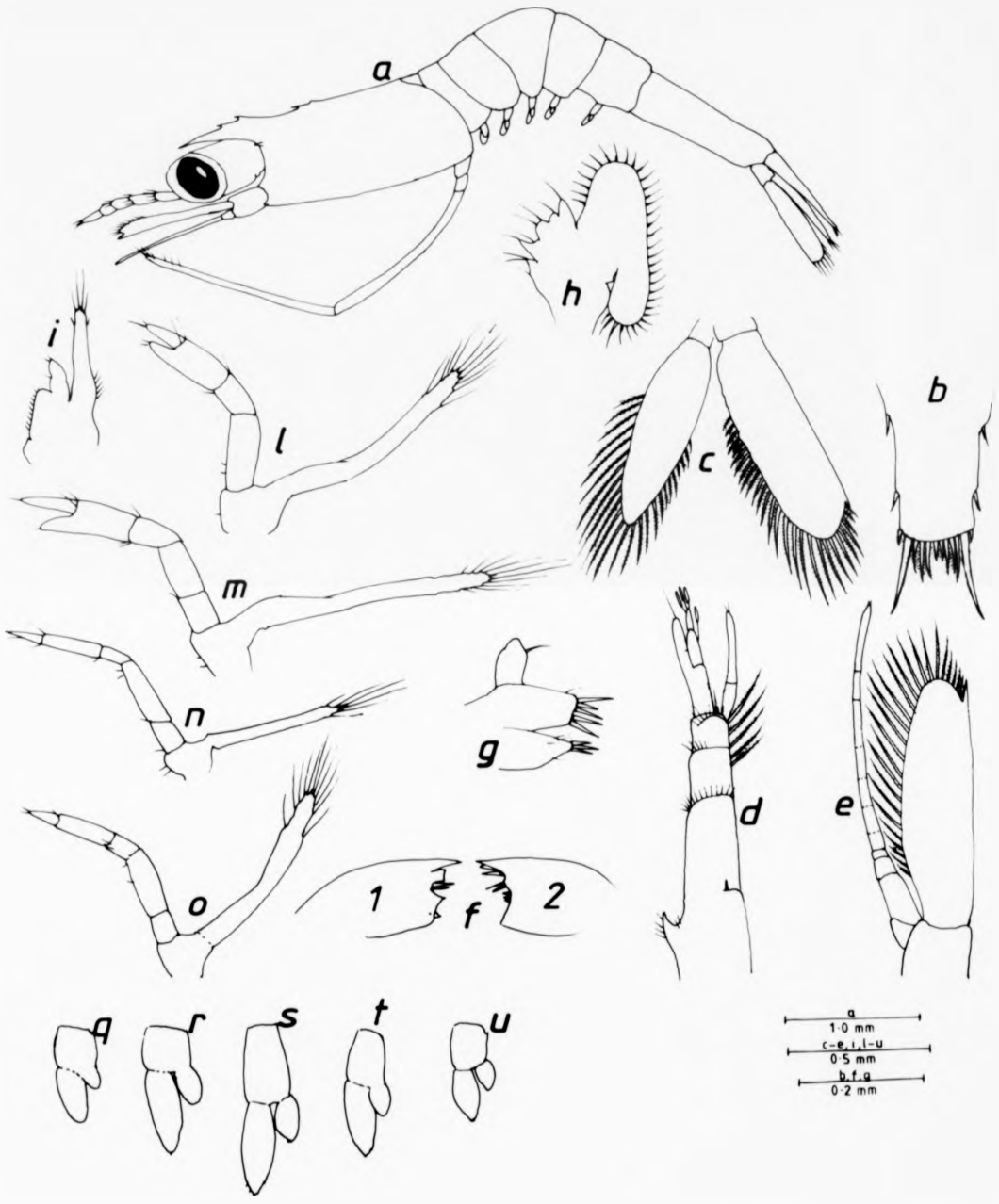
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Fig. (3.14) : Zoeal IX of *M. lanchesteri*

(a)Side view; (b)Telson; (c)Uropods; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod; (q)First Pleopod; (r)Second Pleopod; (s)Third Pleopod; (t)Fourth Pleopod; (u)Fifth Pleopod.

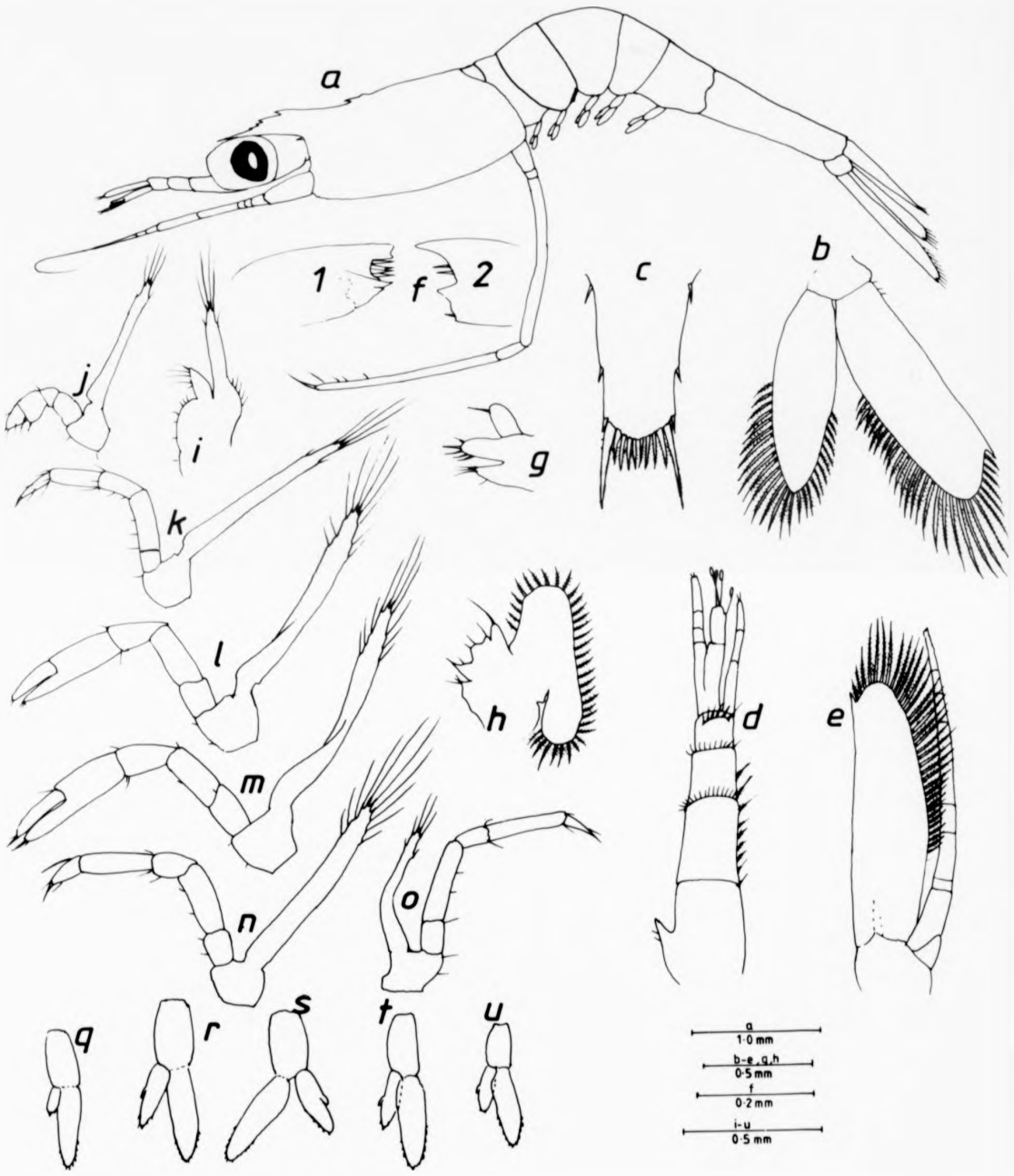
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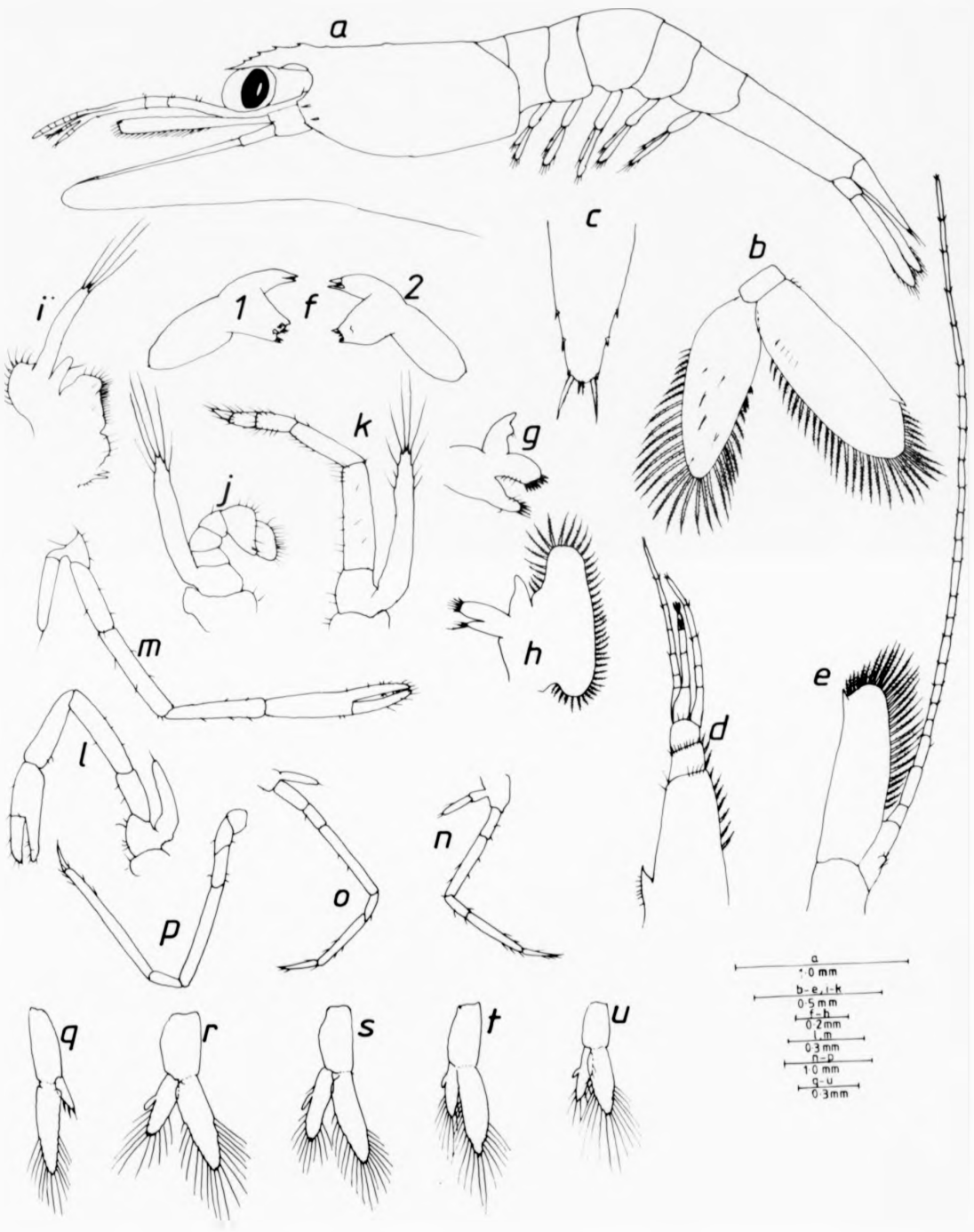
Fig. (3.15) : Post-Larva of M. lanchesteri

(a)Side view; (b)Telson; (c)Uropods; (d)Antennule; (e)Antenna; (f,1)Left Mandible; (f,2)Right Mandible; (g)Maxillule; (h)Maxilla; (i)First Maxilliped; (j)Second Maxilliped; (k)Third Maxilliped; (l)First Pereopod; (m)Second Pereopod; (n)Third Pereopod; (o)Fourth Pereopod; (q)First Pleopod; (r)Second Pleopod; (s)Third Pleopod; (t)Fourth Pleopod; (u)Fifth Pleopod.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(3.4) DISCUSSION

(3.4.1) Discussion on the larval development of M.hainanense

Amongst the species of Macrobrachium investigated, five others, M.asperulum, M.shokitai, M.pilimanus and M.malayanum (Shokita, 1973,1977; Chong and Khoo, 1987b,c; Zhang and Sun,1979). were found to have a similar pattern of larval development to M.hainanense. In general, the species have three similar stages after hatching and their major characteristics are summarised as follows: (1) Compared to other species of the genus, the first stage of these species were born with more advanced morphology including uniramous pereopods and biramous pleopods. On the other hand, some features of early larval characteristics were present, like sessile eyes, biramous antennules, and absence of uropods . (2) At the second stage, setation of pleopods is completed , antennules become tri-ramous, eyes become stalked but uropods are still absent. (3) At the third stage, uropods are formed, mouthparts and pleopods become functional. While the three species are similar in general developmental pattern, they do differ in details. Some of the major differences between the six species are tabulated in Table(3.1). The number of posterior-marginal plumose setae of telson varies and the rostral formula varies greatly between species. The number of plumose setae on the caridean lobe of stage III is also characteristic of each species. Also, there is an increase in size in the order of M.asperulum, M.pilimanus, M.malayanum, M.hainanense, M.asperulum(?) and M.shokitai. The major difference, however, is the absence or presence of exopod in the pereopods (except fifth pereopod). In M.malayanum, a

species in forest streams (slow and fast flowing), the exopods are reduced. In M.pilimanus, a species of torrential streams, the exopods are much reduced to rudimentary buds. In M.shokitai, M.hainanense, and M.asperulum (from Yangtze and from Taiwan), all exopods are absent. Also, M.pilimanus tended to to arch their bodies and cling to a roughened wall while M.malayanum tended to stay "stretched-out" at the bottom of containers (Chong and Khoo, 1987b,c). Thus, it is likely that the presence/absence of exopod is related to the environment of the prawns, as pointed out by Chong and Khoo (1987 b&c). The development of M.asperulum as reported by Shokita(1973) differed to that reported by Zhang and Sun(1979) in most characteristics listed in table(3.1). As M.asperulum is generally reported in China, and considering the isolated nature of the Island of Taiwan, it is likely that the species reported by Shokita(1973) belongs to a separate species or subspecies. The species reported by Shokita(1973) will subsequently be referred to as M.asperulum(?) in this thesis. In relation to the spines on the carapace, it is likely that all the 6 species developed branchiostegal spine in the first stage, and both antennal and branchiostegal spines at the second stage. It is generally accepted that the branchiostegal spines would migrate subsequently to form the hepatic spines in the juvenile stage (Williamson, 1972; also next section). Nomenclature of these spines should thus represent their actual position in the larval stage (to prevent further confusion) rather than in their subsequent stage as adopted by Chong and Khoo(1987b & c).

The definitions of larval names by Williamson(1969) are mainly based on the mean of movement, and especially the

development of pleopods. In addition to many typical larval characteristics, the pleopods of the first stage of M.hainanense are not fully setose, qualifying it to be a zoeal stage. At its second stage of development, as in the other 5 species, juvenile characters like the 3-branched antennule appears and the fact that pleopods are fully setose. On the otherhand, the telson is a zoeal character. Also, as observed by Shokita(1973) for M.shokitai, both first and second stage larva responded to stimuli by jumping and mostly by movement of the telson. While slight movement of the pleopods might be occasionally observed, it would be debatable whether they are fully functional. While zoeal II was employed by Shokita(1973, 1977), Shokita(1985) recently acknowledged the existence of only one zoeal stage for both M.asperulum and M.shokitai. At the third stage of development, as in M.asperulum and M.shokitai, M.hainanense swim by the movement of pleopods. Other juvenile characters like uropods and antennule statocyst are also present. As also pointed out by Kobalais and Gore(1985) while refering to Shokita(1977), the third stage 'megalopa', with functional pleopods and the presence of uropods, posesses many more 'postlarval' than larval characteristics. Thus, M.hainanense hatches as a zoeal stage and develops into a juvenile at stage III, with a stage II which processes both zoeal (e.g. telson) and juvenile characters. Stage IV is generally very similar to stage III, only of a different size and with a narrow telson. Based on the fact that pleopods at stage II are already fully setosed, the stage is considered as post-larval, but only with hesitation. In all, the hatchlings of M.hainanense, with both pereopods and pleopods, resemble an adult

rather than a larval form (except sessile eyes). The post-embryonic phase of M.hainanense conceptually approaches more to a direct mode of development than an abbreviated mode, but technically and marginally fails to be classified as such. In relation to this, Zhang and Sun(1979) reported on four "young stages" (in Chinese) for M.asperulum while Chong and Khoo (1987b,c) adopted the Shokita's system for M.malayanum and M.pilimanus. The arguments given above could also applied be to these three species. M.malayanum, having reduced exopod on its pereopods (but present), could technically satisfy the definition of Zoeal stage than the other 5 species, but this is still conceptually unsatisfactory as the exopods were obviously not functional (natatory).

Abbreviated larval development was also described for M.iheringi with three stages described before the juvenile stage (de Siqueina Bueno, 1980). Pleopods were at least partially setose and uropods were present at stage three. Also, biramous pleopods were present at the first stage. It is possible that larval development of M.iheringi and M.potiuna (Muller, 1892; in de Siqueina Bueno, 1980) is very similar to the abbreviated type described in this paper. However, as no diagram was presented in de Siqueina Bueno's paper, no conclusive comparision can be made. Other species of Macrobrachium are also known to have some form of advanced or abbreviated larval development. In M.lamarrei and M.australiense, after three larval stages, pleopods become fully developed with setation at the fourth stage (postlarva) (Fielder, 1970; Jalhal et al., 1980). In M.kistnensis, while having a similar development to M.australiense, the 'fourth' zoeal

described by Ngabhushanum and Kulkarni(1979) has "pleopods well developed" and Shokita(1985) only ascribed three zoeal stages to this species. M.jelski was also reported to have two zoeal and one post-zoel stage before reaching the first juvenile stage and "pleopod buds" were present at stage one (Gamba, 1984). The extremity of abbreviation is represented by M.hendersondayanum which hatch with setose pleopods already developed. With the exception of the absence of uropods, this stage mostly resembles a small adult. It is likely that more species of Macrobrachium will be discovered to have newly hatched forms which "resemble adults to a considerable degree in behaviour and morphology" as in M.inpa reported by Walker and Ferreira(1985).

Of this latter group of species, only the larval morphologies of M.australiense, M.kistnensis and M.lamarrei are reported sufficiently for comparision. The three species have a certain degree of similarity in their pattern of development. While only having 3 or 4 zoeal stages, the first stage still hatch with biramous(unsegmented) pereopods which become formed(natatory) by stage two. The pleopods also develop slightly later than in the M.hainanense group. The number of setae on the telson, caridean lobe, scaphocerite and scaphognathite also differ between species, as in the case of the species with abbreviated larval development.

Table (3.1) : Comparision of major developmental characteristics between 6 species (or subspecies) of *Macrobrachium* with abbreviated larval development similar to *M.hainanense*

	ASP	ASP?	SHO	HAI	MAL	PIL *
First Stage						
body length	3.50mm	4.70mm	4.92mm	4.06mm	4.30mm	4.25mm
telson	26p.s.	33p.s.*	33-35p.s.	26p.s.	20p.s.	18p.s.
exopod of pereopod(1)	a	a	a	a	r	rud
Second Stage						
body length	4.00mm	4.70mm	5.13mm	4.50mm	4.50mm	4.30mm
rostrum formula	3-4/0	7-8/1	9/0	7-8/1	3-5/0	1/0
telson	?26p.s.	33p.s.	33-35p.s.	26p.s.	20p.s.	18p.s.
Third Stage						
body length	?	4.90mm	5.50mm	4.76mm	4.75mm	4.40mm
rostrum formula	7/0	7-8/1	8-9/0	7-8/1	4-6/0	6-7/0
Caridean lobe	0p.s.	1p.s.	6p.s.	8p.s.	0?	1p.s.
telson	22-24p.s.	33p.s.	33-35p.s.	26p.s.	18p.s.	16p.s.
Fourth Stage						
body length	5.00mm	5.17mm	5.95mm	5.10mm	4.80mm	4.70mm
rostrum formula	7/3	8-9/1	7-9/0	7-8/2	6-7/0	8-9/1
telson	18-20p.s.	28p.s.	29-30p.s.	14p.s.	12p.s.	10p.s.
Reference	Zhang& Sun (1979)	Shokita (1977)	(1973)	present thesis	Chong& Khoo (1987b)	(1987c)

* p.s.= plumose setae; for telson implies posterior marginal plumose setae.

abbreviations : ASP=*M.asperulum*; PIL=*M.pilimanus*
HAI=*M.hainanense*; SHO=*M.shokitai*
MAY=*M.malayanum*

(1) a = absent; r = much reduced; rud = rudimentary

(3.4.2) Discussion on the larval morphology of M. lanchesteri

Among the species of Macrobrachium investigated for larval development, a number have been reported to have 8-10 zoeal stages before metamorphosis to post-larva (see Table 3.2), including M. acanthurus, M. amazonicum, M. equidens, M. formosense, M. grandimanus, M. idella, M. intermedium, M. japonicum, M. nipponense, M. novaehollandae, M. niloticum and M. lanchesteri. These species share a similar overall pattern of development but differ in the timing of the appearances of some appendages and their setation. Eyes become stalked at stage II while uropods appear in stage III, as in all Eucaridea. All the maxillipeds are functional at stage I and become more setated through the larval phase. First and second pereopods appear in stage I as rudimentary buds, becoming biramous and segmented in stage II. Other pereopods and abdominal pleopods, differ in the stages of first appearance and formation. The chelipeds on the first and second pereopods develop at stage VI onwards becoming fully developed just before metamorphosis into post-larva. The third pereopod first appeared as rudimentary buds between stage I-III and become funtional at stage III-V. In M. lanchesteri, the third pereopod first appeared at stage I and became functional at stage III. The fourth pereopod first appeared as rudimentary bud between stage II and V and they become functional between stage V and VII. Again, the fourth pereopod developed comparatively earlier in M. lanchesteri and M. niloticum, first appearing at stage II in both species. The fifth pereopod first appears between stage I and stage IV but mostly at stage II and becomes functional between stage II and stage V (mostly at stage IV). In

three species, M.acanthurus, M.intermedium and M.japonicumn, the fifth pereopod only appeared at stage III and stage IV. In M.niloticum and M.lanchesteri, the fifth pereopod become functional by stage II and stage III respectively. Pleopod buds first appear between stage V and stage VII, becoming segmented in one or two stages afterwards. In M.lanchesteri, these appendages tend to appear and to develop comparatively earlier than the other species in this group of Macrobrachium. It is the only species of the group with a rudimentary bud of the fifth pereopod appearing in the first stage and fully formed by the third stage. The fifth pereopod also seems to be comparatively longer than that of other species at corresponding stages. Whether this is a special adaptation to planktonic food can only be supported by further ecological investigations. It is one of the only two species (M.niloticum)) which can complete the larval phase totally in freshwater.

The presence (or absence) of various spines on the carapace also differ between species. The two spines on the antero-ventral border of the carapace, as pointed out by both Williamson(1972) and Ngoc-Ho(1976), vary in their order of appearance. In some species(e.g. M.niloticum, M.rosenbergii, M.equiden and M.lanchesteri), the more ventral of the two spines developed first, occupying the pterygostomian position throughout the larval phase, and it moved towards the hepatic position in the post-larval phase. The more dorsal one appears in a later stage and becomes the antennal spine in the post-larval stage. In other subfamilies of Palaemonidae, this pattern of development is only known in the Pontoniinae. In other species(M.intermedium),

however, the more dorsal spine developed first, also in the pterygostomian position at first, moving towards the branchiostegal position during the larval phase. The more ventral one developd later, displacing the former one in the pterygostomian position. For those species reported (Table 3.2), apart from the order of development, the first appearance of each spine also differs between species.

The appearance of the epigastric(dorsal-median) spine(s) and processes also differ between species in this group of Macrobrachium. For some species(e.g. M.niloticum), the epigastric spines and tubercle are absent throughout the larval phase. For others, there may be one or two epigastric spines making the first appearance at different stages of the larval phase. In many cases with two epigastric spines(e.g. M.nipponense), however, the more posterior spine tends to appear earlier than the anterior one. In others, both appear at the same stage (e.g. stage IV of M.idella). In addition to the epigastric spines, a dorsal tubercle is also present in many species, which generally develops earlier or at the same than the former in the larval phase (e.g. M.amazonicum). In some species (e.g. M.equidens), no epigastric tubercle occur throughout the larval development while in M.novaehollandae, two tubercles("humps") occur at stage III. It is evident that both the epigastric spines and the antero-ventral spines vary greatly in their form and pattern of development and this is probably of phylogenetic importance within the genus.

(3.4.3) Overall Discussion on Larval Morphology of Macrobrachium

Within the genus Macrobrachium, larval morphologies of 30 species have been reported. Of these, 15 species have 8-11 zoeal stages while 10 species have under eight. There is a continuum of larval developmental types, from direct development in M.handersodayanum (Jalihal and Sankoli, 1975) to extended development in M.carcinus (Choudhury, 1971a). Thus, as in other Eucaridea, there is a general reduction in developmental stages and that the 9-11 stages probably represent the ancestral conditions (Williamson, 1982).

The published works on the larval development of Macrobrachium are summarised in Table (3.2). Apart from the meristic data (e.g. the number of setae), the developmental data are represented by the post-embryonic stage at which the corresponding larval appendages appeared and became functional. For the abbreviated development of M.asperulum, their pereopod would thus classified as O(O) as they hatched with post-functional (larval) pereopods. Comparision of different species within the 'extended' group and the 'abbreviated' group of species have already been presented in the last two sections. The setation on the telson, scaphocerite and scaphognathite of zoeal I are identical among species with extended larval development (respectively 14, 9 and 5) but different among species with abbreviated and advanced development. The 6+6 formula of the post-larval telson processes are also universal among species with extended larval development. M.lamarrei, a species with only 3 zoeal stages, also has 12 plumose setae on the posterior margin of the post-larval telson. However, the two cases are different

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as the 6+6 formula includes also the dorso-lateral spines and all the posterior processes while in M. lammarei, the twelve processes are of comparable sizes along the posterior margin only. The lateral spines at the 5th abdominal somites of stage II are also absent in species with the non-extended type of larval development. Both the epigastric spines and the branchiostegal spines, which vary greatly in their development in species with extended development, tend to be absent in species with abbreviated development. The supra-orbital spine, however, persists in most species. Rostral teeth generally tend to appear in the postlarval stage but in some species with extended development (e.g. M. rosenbergii), they make their first appearances in late zoeal stages. The caridean lobe first appears at stage V or later in species with extended development, IV or V in species with advanced development and at stage III even in species with abbreviated development. Pereopods rarely become fully formed(natatory) before stage V(except M. lanchesteri) in species with extended development, at stage II in species with advanced development and born as uniramous appendages in species with abbreviated development. Similarly, pleopod buds first appear much later (later than stage IV) in species with advanced development (generally at stage I). Thus, as in other Eucaridea, the stalked eyes, uropods and segmentation of antennule peduncle seem to be the characters reluctant 'to be abbreviated', and this is probably related to developmental constraint of these appendages.

While the variation of larval sizes or even the number of moults may be attributed to variation in environmental factors (

e.g.Broad, 1957; see also section 3.1), morphological variation is probably related to intrinsic events. For M.acanthurus, Choudury(1970) reported 10 larval stages in a duration of 32-45 days while Wong et al.(1979) reported 12 stages in a duration of 33-65 days. However, no description of morphology was given in the latter paper and that different feeding regimes were used in the two investigations. Also, both Chouduhry(1970) and Dobkin(1971) acknowledged that molting in the larvae of M.acanthurus was frequent and that little morphological changes were evident, except in the first few molts and the last larval moult. The development of the third pereopod, the fourth pereopod, the fifth perpeopod, the pleopods, the caridean lobe and rostral formula of M.nipponense also differ among the reports of Kwon and Uno(1969), Zhang and Sun(1979) and Ge(1980). The total absence of epigastric processes has also been observed in some individuals of the same species(pers.obs.). Rajyalakshmi(1961) and Jalihal et al.(1982) also reported different development of the caridean lobe and the rostal formula in M.lamarrei. Thus, within the genus Macrobrachium, intra-specific variation do exist in many species. authors commented on the lack of variability in the larval stages of palaemonids, in comparision to 'considerable' variation in the number of stages, size and morphology in species with more extended development (Dobkin, 1971; Fielder, 1970). It may be related to the fact that most studies were made in the laboratory with sufficient supply of food and hence the moulting cycle in relation to PRS (section 3.1) is not upset. On the other hand, non-obligatory stages of development is generally associated with the more extended types

of development (Rabalais and Gore, 1985). Larval development of M.carcinus differs drastically between the reports of Lewis and Ward(1965) and Choudhury(1971). However, Choudhury(1971) already pointed out that the former report had obviously missed out certain stages (e.g. hatched with stalked eyes !) and should not be treated as intra-specific variation as purported by Rabalais and Gore(1985, p.87).

Most of the Macrobrachium species reported can be accounted for by two patterns of development, the abbreviated development as in M.hainanense and the extended development(8-11 stages) as in M.lanchesteri. Bearing in mind that there is a gradual acceleration of developmental patterns within the groups with 8-11 stages (as in M.lanchesteri and M.niloticum), as well as in the 'asperulum' group (in relation to the reduction of exopods on pereopods), the apparent continuum of larval developmental stages seem to be very weak (if not broken) in species having 3-7 stages (and especially 5-7). Tiwari(1955), by comparing the egg size and adult morphology of the Indian species of Palaemon(=Macrobrachium) with consideration of paeleogeography, concluded that the present-day Macrobrachium probably migrated to the freshwater in more than one wave. The slight 'discontinuity' of larval developmental stages between the two popular groups may merely reflect the different length of evolutionary history in freshwater environments. The latter group only advanced to freshwater very recent in its evolutionary history and many still perform a seaward spawning migration. An alternative explanation (which can incoporate the first explanation) would be a sta-blizing selection favouring the two ends of the developmental

developmental spectrum (Strathmann, 1986; see Chapter 6.4).

Crustaceans with abbreviated larval development have been recorded from a variety of habitat types (boreal, terrestial, montane, semi-terrestial, freshwater, estuarine, rocky intertidal shores, marine, deep sea, hypersaline etc) (Rabalais and Gore, 1985). There is no general rule, among all crustaceans where one would find abbreviated larval development. It is thus, in general terms, a convergent developmental pattern and has no phylogenetic significance (for higher taxas) in nature (Rabalais and Gore, 1985). However, within a certain taxa or even a sub-taxa, certain trends can be recognised. "The fact that larger eggs may signify abbreviated development in a species is most evident among closely related species where some exhibit abbreviated development and others do not"(Rabalais and Gore, 1985). Speciation after the acquisition of abbreviated development would result in phylogenetic relationships between species with such development. Among those species with abbreviated development in Macrobrachium (including the advanced type of development), the 'asperulum' group of species has a much higher similarity in their developmental mode than the rest of the species, which is unlikely to be the result of convergent evolution by physical and developmental constraints. The adult morphologies also showed a high degree of similarity, the 'pilimanus' complex (consisting of several species in addition to M.pilimanus and M.malayanum) has long been recognised for its taxonomic difficulty (Holthuis,1950; Johnson,1960; Chong and Khoo,1987a). In addition to a high degree of similarity, M.shokitai and M.asperulum, two land-locked species from Ryukyu Islands and Taiwan respectively, have been

observed to cross-breed with each other (one of the few instances when different species of the genus are reported to cross-breed). Thus, within the genus Macrobrachium, abbreviated larval development do bear phylogenetic significance. Another phylogenetic group would be the 'rosenbergii' group with the similar extended type of development (over 10 stages) (see chapter 6).

The foregoing discussion has presumed the reduction of larval developmental pattern in the process of evolution from the marine to the freshwater regime. Many previous workers have speculated on the possible biological significance of abbreviated larval development. Most of the relevant inference from marine invertebrate data are summarised in section (3.1). Dudgeon(1985), commenting on the abbreviated larval development of Neocaridina serrata(Stimpson), which co-exist with M.hainanense in the upper course of the Lam Tsuen River, speculated that "such larval development may allow the hatchlings to grasp trailing vegetation and roots, thereby avoiding being swept away during spates". Macrobrachium species, with abbreviated larval development or large-size eggs, are indeed reported from the upper courses of rivers (e.g. Dudgeon, 1985; Shokita, 1973; Walker and Ferreira, 1985; Johnson, 1967). The environment in upper reach of a river (e.g. current, physiochemical parameters), while being 'freshwater' as in the lower course, is quite different to that of the latter (e.g. Dudgeon, 1985; Johnson, 1967 and Walker and Ferreira, 1985). Such habitats would exert on the adults a different selective pressure to that of the lower course of a river.

The fact that species with abbreviated larval development generally survive on yolk reserves until the post-larval stage, also points to the possibility that such adaptation may also be resource related. The observations that hatchlings of M.inpa "took refuge within leaves" and "catch live prey within 48 hours of hatching" led Walker and Ferreira(1985) to suggest such a mode of reproduction as an adaptation to benthic habitats in "plankton-poor" waters. To go further, Rabalais and Gore(1985), commenting on M.shokitai, even speculate that abbreviated larval development may have developed "simply because favorable prey was scarce at sometime in the evolutionary history of a species".

The possible biological significances of abbreviated larval development can obviously be diverse and it is unlikely that any single one would solely be responsible for the selective pressure on random-drifted genotypes. The number of larval stages in a present-day species of Macrobrachium is probably the result of selective pressure which keeps in balance the pros and cons of the various factors like intra-specific resource partitioning, larval surival, etc (see section 3.1). As a species of marine ancestry invades freshwater, it is probably faced with a regime(e.g. a coastal pond) with more limited space, and perhaps a more limited choice and quantity of food per individual. Thus, the selective advantages of a large number of progeny with a long larval duration would probably be lost. An increase in larval survival in freshwater, a shortening of larval stages and a decrease in larval duration were observed for the more 'freshwater', as compared to brackish water, populations of M.nipponense (Mashiko, 1983a, Ogasawara et al.,1979). The fact

that some freshwater species have far fewer larval stages than others probably implies different freshwater regimes and a different evolutionary history in freshwater. Tiwari(1955) inferred that the present-day freshwater prawns probably migrated to the freshwater in more than one wave. Thus, the use of M.nipponense as an example to disprove the association of abbreviated development and freshwater by Rabalais and Gore(1985, p107) is not justified without consideration of the evolutionary history of the genus, the existence of different 'freshwater' habitats and published data on inter-population differences (e.g.Mashiko,1983a,b,c). On the otherhand, it is possible that some species of high-gradient streams can rely to on coastal hydrographic conditions to return to the same river of origin, without larval adaptation to freshwater (Hunte, 1978).

Attributed to the long larval duration of M.rosenbergii, commercial hatcheries of the species have to bear the expense of seawater, planktonic diet(green water system or Artemia) and intensive labour. Many authors have thus suggested the use of species with abbreviated larval development as candidates for aquaculture (e.g.Dobkin, 1969; Williamson, 1969). While the suggestion is logical, practical difficulties can be envisaged. Species with abbreviated larval development generally have an increase in egg size and a parallel decrease in egg numbers. Species like M.asperulum, M.shokita and M.hainanense generally have brood size of about 100(if not less) while species with extended development generally have a fecundity several magnitudes higher. Thus, in order to match the juvenile production of one M.rosenbergii, say 50,000 to 200,000, about

1000 pairs of broodstock would be required(even with higher survival rate). Holding such a number of broodstock individually would be impossible. Thus, unless a 'fingerling' production system similar to that of the tilapia arena system, the use of species with abbreviated development is limited. However, such prawns are ideal for the aquarium and M.hainanense has been known in the ornamental fish trade in Hong Kong.

A possible solution then is to introduce the character of abbreviated development into species with aquaculture potential and higher fecundity. Attempts of hybridization between species would be reported and discussed in later chapters.

The advance of molecular biology in recent years has led to the understanding, at least in part, of the molecular mechanism that controls development in other arthropods (i.e.Drosophila). The control of segmentation by the Homeobox gene complex have been much researched for Drosophila and some vertebrates (see review by Davidson, 1986). Similar cell lineages, using monoclonal antibodies, in embryos of species of sea urchin with extended and direct development have also been identified (Raff,1987). Similar investigation in crustaceans may lead to an understanding and possible control of larval development. Meanwhile, much more basic work is still needed just to identified the pattern of larval development of other species of Macrobrachium.

Table (3.2): Summary of published data on larval development (a)

Species	stage	LDur	Temp	Salinity	Eggsize
M.acanthurus	10	32-45		60%sw	
M.amazonicum	11	33-86	26-28	D 1.005	
M.americanum	11				
M.asperulum	1(2)	2-3	28.3	0	1.9*1.4
M.asperulum	1(2)	2-3	25-27	0	1.8*1.4
M.australiense	3	6-9	21-28	0	0.8-1.0
M.birmanicus	5?	20-25	30-32	10-20	
M.carcinus	12	56-66	24-28	14-16	
M.equidens	10	36-53	26-29	32.9	
M.formosense	9	20-22?	24-28	13-16	
M.grandimanus	9	25-27	27	17.5-35	0.85*0.55
M.hainanense	1	3-4	28	0	
M.hendersodayanum	0	0	25-28	0	
M.idella	10	42	25-28	12-18	0.42*0.64
M.iheringi	1(3)	4		0	2.2*1.6
M.intermedium	10?		18	sw	
M.japonicum	9	42-47	24.2-31.	6.0Cl @	
M.jelskii	1?	9-15			2.17*1.58
M.kistnensis	3(4)	4-5	24-26	0	0.9*0.5
M.lamarrei	3	5-6		0	1-1.28*1.3-1.8
M.lamarrei	3			0	1.36*0.91
M.lanchesteri	9		28	0	
M.lar	>11	>89	23-26.9	35.5	.85-.92*.58-.62
M.longipes	8	15-19	25	33%sw	
M.malayanum	1(2)	5	28-30	0	1.75x1.35
M.malcolmsonii	>16	>45	28-32	10-70%sw	
M.niloticum	8			0	.7*.8, 1*1.1
M.nipponense	9	15-20	27.8-28.	8.26-9.3	0.54*0.67
M.nipponense	9	16, 21	28.5,31	0	.54-.56*.67-.92
M.nipponense	9	25	23-30	0	0.65-0.8
M.novaehollandae	10	41-58	15-28	23	.72*.57-.9*.65
M.olfersii	>12	47-65	30	21	0.4-0.75
M.pilimanus	1(2)	5	22-26	0	1.85x1.20
M.rosenbergii	11	36	28	12-12.3	
M.shokita	1	20hrs	28-29.5	0	2*1.4-2.3*1.6
M.spp	12	>40	26-29.2	32.5	
M.vollenhovenii	>8	>45		5-6.6Cl	

Table (3.2): continue (b)

Species	first	chela	third	fourth	fifth	pleopods
M.acanthurus	I(II)	VII(VIII)	II(V)	IV(VI)	II(IV)	VII(VII)IX
M.amazonicum	I(II)	VII(VIII)	II(V)	IV(VI)	III(IV)	VI(VII)X
M.americanum						VI(VII)VIII
M.asperulum	O(O)	O(O)	O(O)	O(O)	O(O)	O(I)II
M.asperulum	O(O)	O(O)	O(O)	O(O)	O(O)	O(I)II
M.australiense	I(II)	I(II)	I(II)	I(II)	I(II)	I(II)III
M.birmanicus	I(II)	IV(V)	II(III)	II(V)	II(V)	IV(V)
M.carcinus	I(II)	IX(X)	II(IV)	IV(VI)	III(V)	VII(X)XII
M.equidens	I(II)	VI(VII)	II(IV)	III(VI)	II(IV)	V(VII)
M.formosense	I(II)	V(VII)	II(IV)	IV(V)	II(IV)	V(VI)VII
M.grandimanus	I(II)	VIII(IX)	II(IV)	III(V)	II(IV)	VI(VII)IX
M.hainanense	O(O)	O(O)	O(O)	O(O)	O(O)	O(I)II
M.hendersodayanum	O(O)	O(O)	O(O)	O(O)	O(O)	O(O)O
M.idella	I(II)	IX(IX)??	III(IV)	V(VI)	III(IV)	VI(VII)X
M.iheringi	O(I)?	O(I)?	O(I)?	O(I)?	O(I)?	O(I)I?
M.intermedium	I(II)	VI(VII)	II(V)	V(VII)	III(V)	V(VI)?
M.japonicum	I(II)	VIII(IX)	III(IV)	V(VI)	IV(V)	VIII(IX)X/P
M.jelskii	I(I)?	I(I)?	I(I)?	I(I)?	I(I)?	I(I)I?
M.kistnensis	I(II)	I(II)	I(II)	I(II)	I(II)	I(III)IV
M.lamarrei	I(II)	I(II)	I(II)	I(II)	I(II)	I(II)IV
M.lamarrei	I(II)	I(II)	I(II)	I(II)	I(II)	I(I)III
M.lanchesteri	I(II)	VII(VIII)	II(III)	III(IV)	I(III)	VI(VII)IX
M.lar	I(II)	IX(X)	II(IV)	III(VI)	II(V)	VI(VIII)X
M.longipes						
M.malayanum	O(O)	O(O)	O(O)	O(O)	O(O)	O(I)II
M.malcolmsonii	I(II)	XIV(XVI)	II(IV)	II(VI)	II(IV)	X(XI)XVI
M.niloticum	I(II)	VII(VIII)	I(III)	II(V)	I(II)	V(?)?
M.nipponense	I(II)	VI(VII)	II(III)	IV(VI)	II(IV)	V(VI)VII
M.nipponense	I(II)	VI(VII)	II(IV)	IV(VI)	II(IV)	V(VI)VII
M.nipponense	I(II)	VI?(VIII)	III(IV)	V(VI)	III(IV)	V(VI)?
M.novaehollandae	I(II)	VII(IX)	II(IV)	IV(VI)	II(IV)	VII(VIII)IX
M.olfersii	I(II)	VIII(IX)	II(V)	V(VII)	III(V)	IX(X)
M.pilimanus	O(O)	O(O)	O(O)	O(O)	O(O)	O(I)II
M.rosenbergii	I(II)	VIII(X)	II(IV)	III(VI)	II(IV)	VI(VII)IX
M.shokita	I(I)	I(I)	I(I)	I(I)	I(I)	I(I)I
M.spp	I(II)	VII(IX)	II(IV)	MV(VI)	II(IV)	VII(VIII)X
M.vollenhovenii	I(II)	VI?(VII)	II(III?	IV(V)	II(IV)	V(VI)VIII?

Table (3.2): continue (c)

Species	Antvensp	Episp	Carlobl & 2 & 3			telson		1&2	max
M.acanthurus	I(III)	O(III)IV	VII	5	7	14	6+6	5	9
M.amazonicum	II(III)	III(IV)VII	V	2	8	14	5+5?	5	9
M.americanum									
M.asperulum	O(I) ?	na	IV	1	0	33	33	35	34
M.asperulum	O(II)?	na				26	26		
M.australiense	II(?)	O?(O?)	IV	4	4	14	12	10	9
M.birmanicus	II?(?)	?	V	3	?	14	?	5	9
M.carcinus	I(III)?	O(III)IV	VII	1-2	?	14		5	9
M.equidens	I(III)	O?(III)IV	VI	0-1	8	14	6+6	5	9
M.formosense	I(II?)	O(II)IV	V	1	11	14	6+6	5	9
M.grandimanus	I(III?)	II(I)IV?	V	1	13	14	6+6	5	9
M.hainanense	I(II)	na	II	7	0	26	26	36	35
M.hendersodayanum	O(I)	O(O)O	I	7	7	11-12	11-12	41	35
M.idella	I(II)	II?(III)IV	VI	3	10	14	6+6	5	9
M.iheringi		na				18	18		9
M.intermedium	III(I)	II(II)IV				14		5	9
M.japonicum	?(I?)	O?(III)IV	VII	5	10	14	6+6	5	9
M.jelskii	na								
M.kistnensis	III(?)	O?	V	3-5	0	22	18	9-12	14
M.lamarrei	I(III)	O(II)O?	III	3	4?	14	12	20	11
M.lamarrei	I(I)?	III?	III?	8?	8	14	12	22-25	11
M.lanchesteri	I(III)	II(II)V	V	1	11	14	6+6	5	9
M.lar	II(II)?		V	2		14		5	9
M.longipes		III(IV)?				14	6+6		9
M.malayanum	II(I)	na	IV?	3-4?	0?	20	20	23-26	12
M.malcolmsonii	I(V?)	III(III)IV	VI	2		14		5	9
M.niloticum	I(III)	@(@)@				14	6+6	5	9
M.nipponense	I?(II)	O?(III?)IV	V	1	9	14	6+6	8?	5
M.nipponense	I(III)	III(III)IV	VII	4	9	14	6+6	8?	5
M.nipponense		@(III)IV				14	6+6		
M.novaehollandae	III(IV)	II(II)IV	VII	3	10	14	6+6	5	9
M.olfersii	II(III)	@(III)III	@!	@!		14		5	9
M.pilimanus	II(I)	na	III	1	0	18	18	15-20	11
M.rosenbergii	I(II)	@(III)IV	V	2	17	14	6+6	5	9
M.shokita	II?(I)	na	III	6	0	33-35	35	32	35
M.spp	I(III)	III(III)IV	V	1	11	14	6+6	5	9
M.vollenhovenii	II(II)	'?(III)V?				14	5+5?		9

Table (3.2) : continue (d)

Species	Rost1 & 2		Zlsize	Plsize	Reference
M.acanthurus	VII?	7-8/2-3	2.3	5.5-6.5	Choudhury(1970)
M.amazonicum	P?	5-6/0	1.7	5.9	Magalhaes(1985)
M.americanum	X				Monaco(1975)
M.asperulum	X	8/0	6.14	6.55	Shokita(1977)
M.asperulum	II	3-4/0	3.5	5	Zhang & Sun(1979)
M.australiense	IV?	6-8/0	3.2-3.33	3.75-4.1	Fielder(1970)
M.birmanicus	4/0?	IV			Khan et al.(1984)
M.carcinus	XI		2.0-2.1	6.5-8.0	Choudhury(1971)
M.equidens	XI,P	9/1-2	.58-.65	4.9-5.2	Ngoc-Ho(1976)
M.formosense	IX	9-10/1-2	1.80	6.32	Shokita(1970)
M.grandimanus	IX	11/1	2.24	7.59	Shokita(1985)
M.hainanense	II/P	7-8/1	4.06	4.50	present study
M.hendersodayanum	I	6/2	na	7.0	Jalihal&Sankoli (1975)
M.idella	XI/P	9/1	1.92-2.1	6.5	Pillai&Mohamed(1973)
M.iheringi	II				Siqueira Bueno (1980)
M.intermedium	2.2-2.3				Williamson(1969)
M.japonicum	X/P	9/3	1.7	6.6	Morizane&Minamizawa (1971)
M.jelskii					Gamba(1980)
M.kistnensis	IV	1/0	4.2-4.4	5.2	Nagabhushanam&Kulkani (1979)
M.lamarrei	II?	4-6/0	4.2-4.5	5.2-6.2	Jalihal et al.(1982)
M.lamarrei	III	4/1-2	4.077	5.11	Rajyalakahmi(1961)
M.lanchesteri	X/P	5/1	2.88	4.93	present study
M.lar	2.26-2.3				Atkinson(1977)
M.longipes	VIII?	9/1	1.87-2.0	6.7-7.0	Shen(1979)
M.malayanum	II	3-5/0	4.3	4.5	Chong & Khoo (1987b)
M.malcolmsonii					Kewalramani(1971)
M.niloticum	VIII	10/1	3.5t	9t	Williamson(1972)
M.nipponense	X/P?	9/1	2.06	5.31	Kwon and Uno(1969)
M.nipponense	VIII	9-10/1-2	2.1	5.4	Ge(1980)
M.nipponense	IX		2.3		Zhang and Sun (1979)
M.novaehollandae	X	7/3	1.76	5.31	Greenwood et al.(1976)
M.olfersii	1.75				Dugger & Dobkin(1975)
M.pilimanus	II	1/0	4.25	4.3	Chong & Khoo(1987c)
M.rosenbergii	X	11/5	1.92	7.69	Uno and Kwon (1969)
M.shokita	II	9/0	4.92	5.5	Shokitai(1973)
M.spp	XII	9/3	1.9-2t	5.5-7t	Ngoc-Ho(1976)
M.vollenhovenii	VI		1.7	6.6	Ville(1971)

Explanation to Table (3.2)

Stage - number of **zoeal** stages of larval development
LDur - duration of larval development observed in laboratory
Temp - temperature used for larval rearing
Salinity - salinity used for larval rearing
Eggsize - observed egg size

The following characters refer to the stage at which the corresponding **larval** appendage first appear and first became functional :

first - first pereopods
- chelipeds on first and second pereopods
third - third pereopods
fourth - fourth pereopods
fifth - fifth pereopods

The following characters refer to the first appearance of the corresponding body parts:

pleopods - pleopod buds, pleopod with setae buds, pleopods become setated
Antvensp - antennal spine, branchiostegal spine
Episp - epigastric spine
Carlob1 - caridean lobe
Rost1 - rostral teeth

The following characters are meristic characters of the correspondig appendages :

Carlob2 - the number of setae on caridean lobe, when first appeared
Carlob3 - the number of setae on caridean lobe in post-larvae
telson1 - the number of posterior plumose setae on first post-embryonic stage
telson2 - the number of posterior plumose setae on first post-larva
max - the number of plumose setae on maxilla in first post-embryonic stage
ant - the number of plumose setae on the antennal scale in the first post-embryonic stage
Rost2 - rostral formula in the first appearance of rostral teeth
Zlsize - total length of first post-embryonic stage
Plsize - total length of first post-larval stage

? - unclear from the original publication
na - not applied to this species
@ - probably none

"For since each virtue shines by its own proper light, The merit of tolerance is resplendent with very special glory."

John of Salisbury (1120-1180)
in "Polecraticus",
(translation by John Dickinson)

CHAPTER FOUR :

LARVAL PHASE : SALINITY AND FRESHWATER TOLERANCE

(4.1) Introduction

(4.1.1) Evolution and salinity tolerance in crustaceans

Salinity tolerance, or the ability to osmoregulate at a certain range of salinity, is a prerequsite of existence for aquatic crustaceans, as in other aquatic organisms. Being a recent inhabitant of the freshwater environment, salinity tolerance of Macrobrachium is an important life history trait related to the evolutionary path and history of the genus. Larval salinity tolerance can also have important implications on the dispersal, and ultimately population differentiation of the species.

Different species can obviously have very different tolerance/preference to salinity, generally reflecting the characteristic range of the environemnt concerned. Much work has been done on the salinty tolerance of various crustaceans and various schemes have been proposed to classify crustaceans in relation to their salinity tolerance. Dorgelo(1976), based upon the survival curve at a range of salinity, catagorised

crustaceans into five types of salt tolerance (Fig. 4.1):

(I) Polystenohaline, true oceanic species. The 'pointed' curve has rather steep slopes. The steeper the slope, the more truely oceanic the representatives are;

(II) More or less euryhaline species from hypersaline, marine, intertidal or estuarine environments. The optimum curve has a distinct plateau with a centre lying around sea-water concentration;

(III) Extremely euryhaline species that can tolerate the entire range from marine to limnetic conditions equally well. The left side drops suddenly and steeply towards very dilute media;

(IV) More or less euryhaline, genuine brackish water species. The top of the plateau of the curve lies in between seawater and freshwater concentrations and ust be separated from both extremes;

(V) Oligostenohaline, true freshwater species. The curve drops very adruptly at the left side.

Similarly, Gilles and Prequeux(1983) classify crustaceans into limnetic stenohaline(<0.5%0), limnic euryhaline(0-5/8%0), euryhaline(0-40%0), marine euryhaline(18-40%0) and marine stenohaline(30-40%0) species. Both schemes would be useful for comparative analysis of cogenic species, especially species with laboratory data but very little ecological data. Specific examples can be found in the two reviews.

In addition to variation of salinity tolerance between different species, intra-specific variation of the same magnitude can also exist between different life-history phases of the same species. The fact that many Macrobrachium species perform spawning migrations (see Chapter 2) to the estuarine environment suggests that, at least in some species, the larval phase would have very different salinty tolerance to that of the adult phase. For example, M.petersi in Keiskamma River perform a breeding

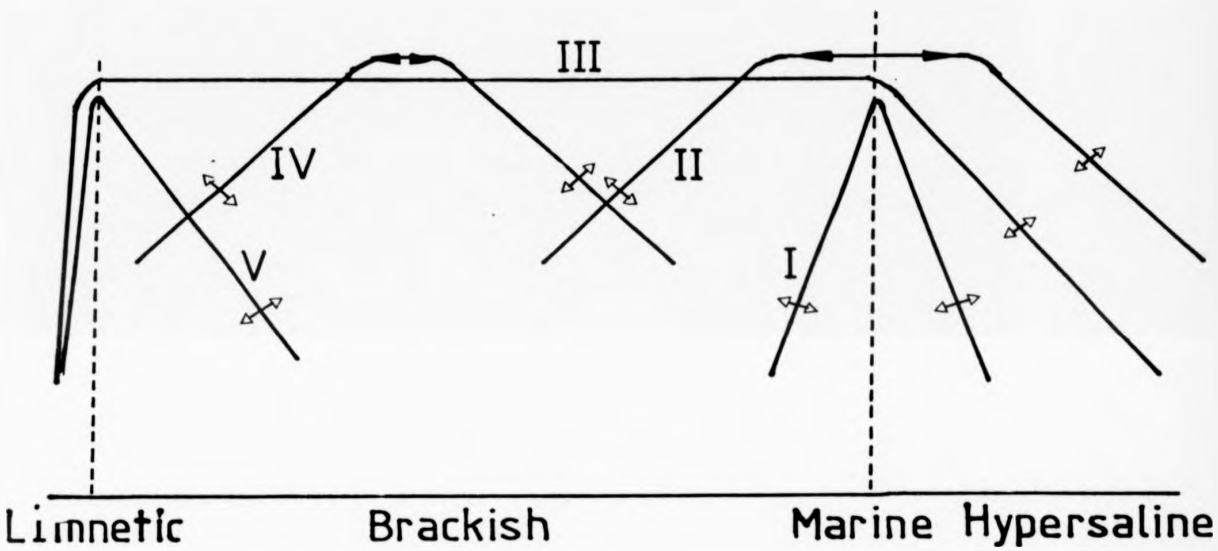


Fig. (4.1): Classification of salinity tolerance of crustaceans by Dorgelo (1976)

migration downstream and an upstream migration to avoid the elevated salinity during dry season (Read, 1985).

Among the various developmental modes of the genus, the more 'advanced' forms tend to be species from the 'more' freshwater environment (see last Chapter). An analysis of larval salinity toleranoe can readily verify the various degree of adaptation to freshwater of different species, and probably in relation to different developmental modes.

"Attempts to understand the evolution of life history strategies must ultimately examine the heritability of their component traits" (Hines, 1986). The migration of Macrobrachium from the marine to the freshwater environment has involved the change of life history traits in many species, from an ancestral marine species, to species which perform obligatory spawning migration, to complete freshwater species with direct development. The knowledge of the heritability of larval freshwater tolerance would give an indication to the possible rate of freshwater advancement. However, artificial selection can only be made on population with an existed degree of freshwater tolerance already. The present chapter aims to review the published data on salinity (including frshwater) tolerance of Macrobrachium, with considerations of evolution and aquaculture of the genus. The larval salinity tolerance and its artificial selection will be investigated experimentally. As discussed in the last chapter, larval development can be affected by many exogenous factors, apart from salinity. Temperature, apart from its own effect on larval development, can also affect osmoregulation (Dorgelo, 1976). Much of the data on the effect of

temperatures on salinity tolerance of crustaceans were reviewed in Dorgelo(1981). Most of the Macrobrachium species are tropical species and most the previous investigations have carried at tropical temperatures. The effect of temperatures(23-34.9'C) on larval salinity tolerance of M.nipponense was investigated by Yagi and Uno(1981). The experiment in the present section mainly aims to characterise the salinity tolerance of a population of M.nipponense in Hong Kong, with the intention to select for larval freshwater tolerance.

(4.1.2) Mechanism of Osmoregulation

On advancement to the freshwater environment, the ancestral marine Macrobrachium have to face both fluctuating salinity in the estuarine environment and the dilute media in freshwater. The ability to hyperosmoregulate in freshwater and to maintain cell volume in a media of fluctuating salinity are thus the prerequisite for the advancement.

The regulation of cell volume in crustacean tissue acclimatised to salinity change results from different processes acting both at the cell level and and at the blood(haemolymph) level. It is recognised that free amino acids generally play the most significant part as non-permanent ions in animal osmoregulation. The first stage of response to hyperosmotic stress is the regulation of cell volume by a rapid accumulation of intracellular free amino acids.

However, much controversy has arisen concerning the mechanism by which intracellular amino acid concentration are lowered in response to hypo-osmotic stress, and of the fate of

the amino acids. Many authors have suggested that, under hypoosmotic conditions, the leaked amino acids can be stored in the form of blood proteins (e.g. Gilles and Pequeux, 1981), coupled with an increase in oxidation and catabolism of amino acids. Other workers support a series of processes to account for hyper-osmoregulation (from Rankin and Davernport, 1981):

1. As a result of osmotic swelling, there is an increase in cell membrane permeability to at least some amino acids, which are transported by blood and coupled with an increase in deamination and oxidation of amino acids;
2. Amino acids not regulated by permeability changes may be broken down by the action of oxidizing enzymes within the cell itself. The breakdown products being able to diffuse out into the blood;
3. The amount of production of non-essential amino acids within the cell is reduced.

Control of amino acid metabolism within the cell appears to be directly controlled by the prevailing intracellular ionic concentration. In M.olfersii, the increase in metabolic rate in response to high and low salinity has already been proved to be controlled neuroendocrinally via two antagonistic factors synthesised in the supra-oesophageal ganglion (Souza and Moreira 1987).

It is also evident that different amino acids are selectively used as osmolytes by crustaceans (or different animal groups), notably alanine, glycine and proline. It is also purported that these solutes create a cellular microenvironment conducive to optimal enzyme function and structure (Bowles and Somero, 1979). The production of these amino acids are metabolically complex but at least partially dependent upon the catabolism of intracellular proteins and the transamination of the subsequent amino acids to those that are osmotically active.

The enzymes responsible for these steps then form the first line of control of osmoregulation. Lysosomal amino peptidase and Glutamate pyruvate transaminase are respectively responsible for the hydrolysis of a variety of amino acids and the final step of alanine synthesis and transamination. Polymorphism in Amino peptidase 1 in mussel Mytilus edulis and glutamate pyruvate transaminase in intertidal copepod Trigriopus californicus have been linked to physiological differences in salinity tolerance of different populations (Burton and Feldman, 1983 ; Koehn and Siebenaller, 1981).

(4.1.3) Osmoregulation in adult Macrobrachium

Measurement of haemolymph osmotic concentration (HOC) in relation to different salinities can readily give the ability of osmoregulation of the species concerned. Published studies on osmoregulation of adult Macrobrachium by monitoring HOC are summarised in table (4.1). Three general patterns can be recognised:

(I) Most of the catadromous species of Macrobrachium belong to this group. HOC maintained at hyperosmotic to medium at low salinities (hyper-osmoregulate), but hypo-conform in salinities above the isosmotic point. Members of this group include M.carcinus (Moreira et al., 1981), M.acanthurus, M.heterochirus and M.potuina (Moreira et al., 1983), and M.petersi (Read, 1984).

(II) The second group shows hyper-hypo-osmoregulatory capability, i.e. haemolymph maintained hyperosmotic to medium at low

salinities and hypoosmotic at high salinities within a certain limit. This pattern is typical of brackishwater species and include M.olfersii (Moreira et al., 1983) and M.equiden (Denne 1968).

(III) The third group includes M.australiense (Denne, 1968), M.kistnensis (Nagabhushanan and Kulkarni, 1970), M.rosenbergii (Singh, 1980) and M.ohione (Castile and Lawrence, 1981). The HOC remains hyperosmotic at low salinities, as in the first group, but increases sharply just before the isosmotic point, typified true freshwater decapod tolerance limit.

Read (1985) depicted a possible evolutionary course of osmoregulation of the genus Macrobrachium. The breakdown of osmoregulatory ability of M.equidens at low salinities suggests it is a recent invader of the estuarine environment, probably not too dissimilar to the ancestral form. M.olfersii, which can hyper-osmoregulate at low salinity probably represent the next step. This then would be followed by species like the osmoregulatory pattern of group (I), and finally the 'advanced' pattern of group (III) with the loss of hypo-osmoregulatory ability in some true freshwater species at intermediate salinities.

While M.olfersii can hyper-osmoregulate at freshwater, HOC of M.equiden decreased sharply below an external salinity of 10‰. This reflected that the population of M.equiden at St.Lucia was a true brackishwater population which was known to migrate to water of higher salinity at rainy season(Denne,1968). However, it is known that M.equiden, at least in some populations, do exist

Table (4.1) : Summary of osmo-regulatory data on adult *Macrobrachium*

Species	HOC1 (mOsm)	S1 (‰)	T (°C)	S2 (‰)	HOC(ISO) (mOsm)	Reference
M.acanthurus	440	0-35	20	0-7	640	Moreira *et al*(1983)
M.australiense	475	0-25	22?	0-17	520?	Denne(1968)
M.carcinus	461	0-35	28		492	Moreira *et al*.(1981)
M.equidens		0-40	22?	7-27	529	Denne(1968)
M.heterochirus	425	0-35	20	0-7	647	Moreira *et al*.(1983)
*M.kistnensis**	484	0-10	27	0-4	537	Nagabushanan&Kulkani(1970)
M.ohione	462	0-30	25	0-13	643	Castile&Lawrence(1981)
M.olfersii	520	0-35	20	0-21	620	Moreira *et al*.(1983)
*M.petersi**	495	0-35	24	0-30?	495	Read(1984)
(postlarva)	457	0-35	24	0-30?	515	Read(1984)
(juvenile)	457	0-35	24	0-30?	498	Read(1984)
M.potuina	493	0-35	20	0-14	552	Moreira *et al*.(1983)
M.rosenbergii						
(postlarva)	479	0-27	28	0-17?	?	Sandifer *et al*.(1975)
(juvenile)	473	0-27	28	0-17	515	Sandifer *et al*.(1975)
(juvenile)	450		27		693	Armstrong *et al*.(1981)
(adult)	360	0-17	?	0-17	485	Singh(1980)

Abbreviations:
HOC1 - Haemolymph osmotic concentration in freshwater
HOC(ISO) - Haemolymph osmotic concentration at isosmotic point
S1 - Experimental salinity range investigated
S2 - Lower and upper regulation limit
T - Experimental temperature
* - recaluculated from freezing point depression

freshwater (Holthuis, 1950). It probably indicated that intra-specific variation in osmoregulation or salt tolerance exist between different populations of the species. Similarly, Mashiko(1983) reported the reproductive incompatibility between a freshwater and a brackishwater population of M.nipponense.

Both M.kistnensis and M.australiense, two hololimnetic species with abbreviated larval development (see last chapter), share the same pattern of osmoregulation (group III). M.potuina, another hololimnetic species with abbreviated larval development, however, has an osmoregulatory pattern of group (I), hyper-osmoregulating at low salinity and hyperosmoregulate at medium salinty. In considering the evolutionary path of ancestral marine species into the freshwater, it can be assumed that the acquirement of hyper-osmoregulation probably occur before the disappearance of the ancestral ability to hypo-osmoregulate in high salinities. It may then be purported that M.potuina has a more recent history in the freshwater than M.australiense and M.kistnensis. It is possible that different species with similar mode of larval development, but dissimilar pattern of osmoregulation, can have different history in the freshwater biotopes. Adult species with extended and abbreviated type of development, e.g. M.rosenbergii and M.australiense, can complete adaptation to freshwater, implying that species with both types of larval development can have similar evolutionary history in the freshwater environment. Also, it probably implies that some species, like M.rosenbergii, has adapted specially for freshwater existence via spawning migration, adult osmoregulatory ability in freshwater, and probably larval behaviour (see last

chapter), without resort to abbreviation of larval development. However, such life history strategy probably still restrict the species from the upper course of river and as far as the 'rosenbergii' group is concerned, may be related to developmental and reproductive constraints of large size. M.rosenbergii, while having an extended type of development, is probably fairly 'ancient' in nature, as suggested by Johnson(1973) (see also Chapter 6). Based on these assumptions, and provided that these species are representatives of their respective areas, it can be further speculated that species in South America have perhaps evolved comparatively more recent to those of the Indo-Pacific area, as suggested by biogeographical analysis. However, more data are required to support this hypothesis.

For M.rosenbergii, enough data has been reported to detail the ontogenetic development of osmoregulation after metamorphosis to post-larva. Juvenile and adult M.rosenbergii have osmoregulatory pattern as in group I, with isosmotic point at about 17-18‰ (Singh, 1980; Stephenson and Knight, 1980). Large post-larvae and juveniles M.rosenbergii increase their metabolic rate at low salinities (Nelson et al., 1977), probably indicative of osmoregulation. On the other hand, Harrison and Lutz(1980), by comparing 1-day old and 7-day old post-larvae, revealed that the hyper-osmoregulatory ability was not immediately developed upon metamorphosis to post-larva. Read(1984) also detailed the the ontogenetic changes of osmoregulatory ability of the catadromous M.petersi. The ability to osmoregulate over a wide range of salinities was strongest at the post-larval (and

first larval stage) stage, with group (II) pattern of osmoregulation, and developing into a group (III) pattern in the juvenile and the adult stage.

Read(1984) also purported that a low HOC in freshwater may be a further step in the colonisation of freshwater by Macrobrachium. While it is true that the average HOC (in freshwater) of M.acanthurus and M.heterichirus were lower than M.olfersi, as pointed out by Read(1984), HOC of both M.australiense and M.kistnensis were of a similar range. While a trend of decreasing HOC has been observed in other crustaceans, further data are required before the extension of the theory to Macrobrachium. As to the isosmotic point of the different species, there does not seem to be any definite trend in relation to the environmental salinity except that the hololimnetic species tend to have slightly lower values.

It is generally observed that metabolic rate of aquatic crustaceans can be much affected by osmotic stress (see also last section). If osmotic work were the sole cause of variation of metabolic rate in response to salinity, the lowest values should therefore be recorded at the isosmotic concentration. Indeed, many species have been reported to exhibit such a pattern (e.g. Penaeus aztecus, Bishop et al., 1980) while many others are not. Moreira et al.(1983) also reported on the effect of salinity on the metabolic rate of Macrobrachium species. M.potuina had the lowest value close to the isosmotic point. In contrast, M.acanthurus and M.olfersii had the highest value at the isosmotic point while the metabolic rates of M.heterochirus did not vary between 7-28‰ salinity (isosmotic point at 22.6‰).

While the metabolic peak at isosmotic concentration cannot be explained, it nevertheless explains the increase in moulting rate of M.olfersi at 21‰ (McNamara and Moreira, 1981). Several workers (e.g. Pannikar, 1968) have speculated on the use of isosmotic conditions for aquaculture. While not all species have their lowest metabolic rate at isosmotic concentration (as reported by Moreira et al., 1983), Singh(1980) also conclusively proved that increased salinity up to the isosmotic point actually retarded the growth rate of M.rosenbergii.

(4.2) Material and Methods for Salinity Tolerance Experiment

Adult broodstock were collected from fish ponds in the Au Tau area of the New Territorries , Hong Kong. This area and the nearby region of Yuen Long have been traditionally used for various fresh and brackishwater aquaculture . However , because of pollution in recent years , many fish ponds , including the ones sampled in this study , obtain an increasing amount of water from the public supply rather than from natural sources. These ponds , while being subjected to an increasing amount of freshwater inflow , still have an average salinity of about 2-10% (personal observation) . This is probably attributed to diffusion of salts through the soil banks .

The prawns were collected , flown to Stirling and kept in a recirculated system at 27-30°C and under a 12hr light/12hr dark regime. Ovigerous females were observed closely everyday for the development of the eggs. As the egg mass became more transparent and eyed , the female was transferred to a static-water tank with aeration.

Newly hatched larvae were then transferred , step by step , to the various experimental salinities. Six experimental salinities were investigated , 0‰, 5‰, 7.5‰, 10‰, 12.5‰ and 15‰. Stock solutions of the various salinities were made up with aquarium salts and rechecked by a refractometer with an accuracy of ±1‰ .

Three replicates were employed for each treatment and 50 larvae were used for each replicate. Each replicate was contained in a one litre beaker at the required salinity and with gentle aeration. The number of surviving and metamorphosed larvae was

counted every other day. The culture medium was also renewed at the same time . The number of post-larvae obtained was recorded until all the surviving larva had metamorphosed. The larvae were staged according to Kwon and Uno(1969). Two separate batches of larvae from different broodstocks used in two replicated-sets of experiments. This is to investigate the possible differences between different broodstocks.

The feeding regime consisted of feeding newly hatched Artemia nauplii at 9.00 a.m. and 6.00 p.m. as well as micronised trout pellet at miday every day. The density of Artemia nauplii was kept between 5-10/ml in all the treatments throughout the experiment.

(4.3) Results of salinity tolerance experiments

Experiment 1

The average survival rates in the different treatments at two day intervals are plotted in Fig. (4.2). Most of the mortality occurred within six days after hatching while most of the larvae were still at the fifth stage of development or below. The cumulative and bi-daily percentage of metamorphosed larvae (post-larva) are represented in Fig. (4.3). Post-larvae were first observed at 16 days after hatching. For most treatments (5‰ , 7.5‰ , 10‰ and 12.5‰) most of the post-larva were obtained by day 20. The development in treatment 0‰ and 15‰ was much delayed .

The best overall average rate of survival to post-larva, 54±S.E.3.5% was obtained in 10‰. T-values calculated infer significant differences ($p=0.05$) between the overall survival

rates of 10%o and that of 0%o, 5%o and 15%o. No significant differences (p=0.05) was inferred between the survival rates of 10%o, 7.5%o and 12.5%o. At 1% (p=0.01)level , there were also no significant different between 10%o and 5%o.

Experiment 2

The average survival rate of experiment 2 are plotted in Fig. (4.4). Again, most of the mortality occurred within 6 days after hatching. The cumulative and bi-daily percentage of metamormorphosed larvae(post-larvae) are represented in Fig. (4.5). Post-larvae were first observed at 16 days after hatching and most were accounted for by day 20. The rate of development in treatment 0%o and 15%o were also much delayed, the first post-larva appeared at day 22 and day 20 respectively.

T-tests between all the treatments infer significant differences (p=0.05) between treatment 0%o and all other treatments. No significant difference (p=0.05) was inferred among treatment 5%o, 7.5%o, 10%o, 12.5%o and 15%o.

The major difference between the results of experiment 1 and experiment 2 is the result for treatment 15%o; the results for the other treatments corresponded fairly well with each other.

Fig. (4.2): Plot of Larval Survival of *M. nipponense* with Time after Hatching (Experiment 1)

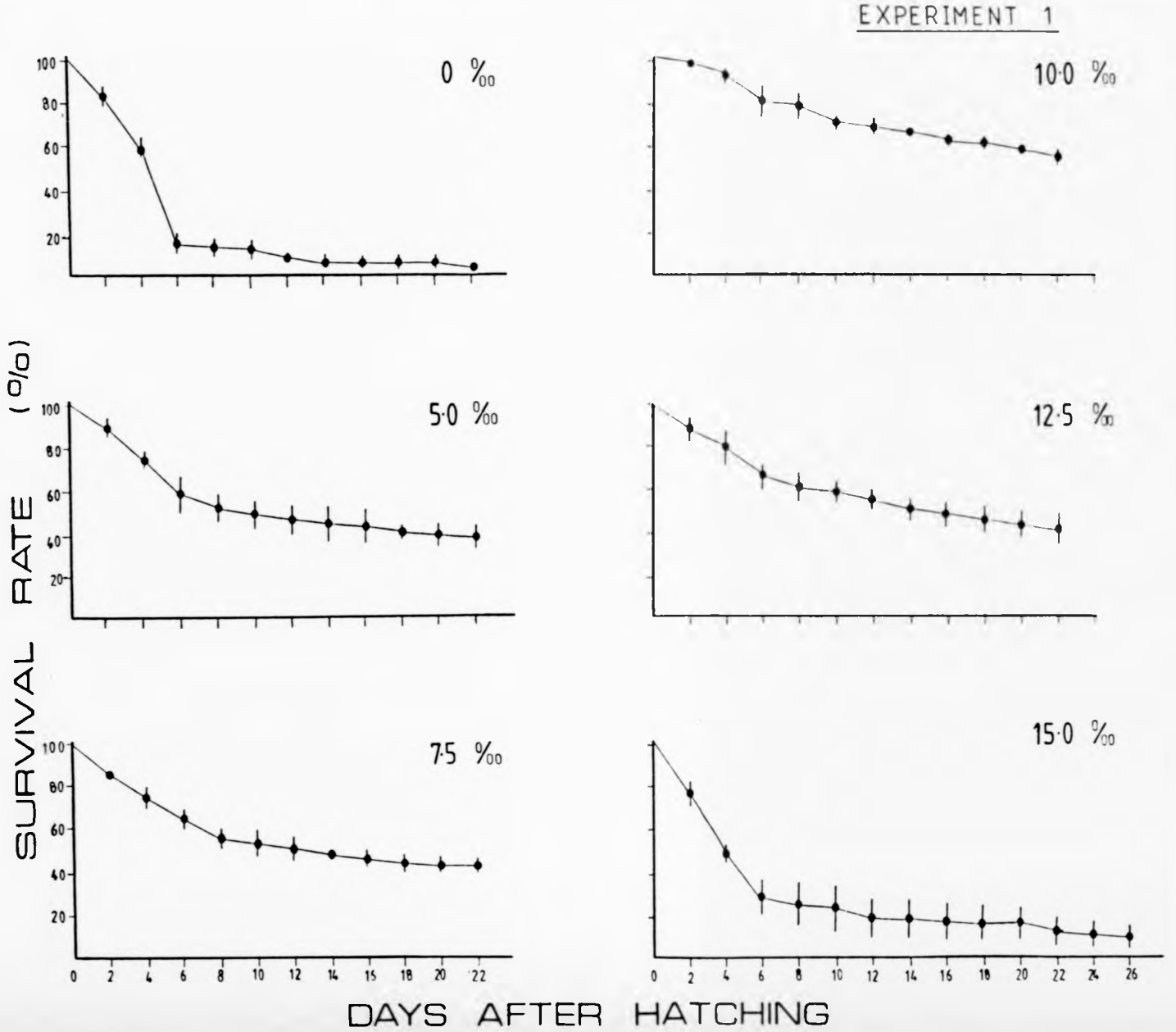

EXPERIMENT 1
0 ‰
10·0 ‰
5·0 ‰
12·5 ‰
7·5 ‰
15·0 ‰
SURVIVAL RATE (%)
DAYS AFTER HATCHING

Fig. (4.3): The Cumulative and Bi-daily percentage of Metamorphosed Larvae (Experiment 1)

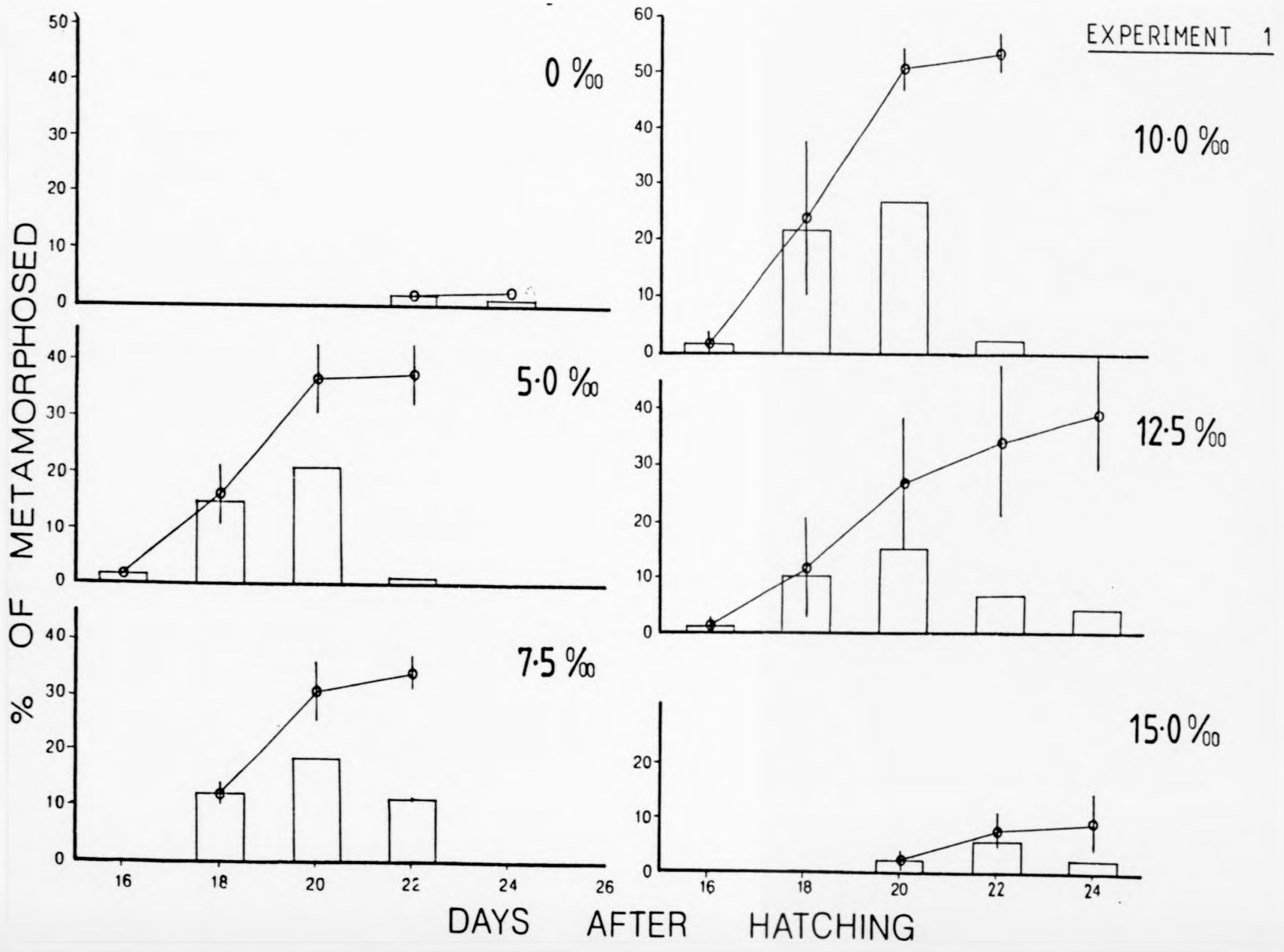

EXPERIMENT 1
0 ‰
5·0 ‰
7·5 ‰
10·0 ‰
12·5 ‰
15·0 ‰
% OF METAMORPHOSED
DAYS AFTER HATCHING

Fig. (4.4): Plot of Larval Survival of M.nipponense with Time after hatching (Experiment 2)

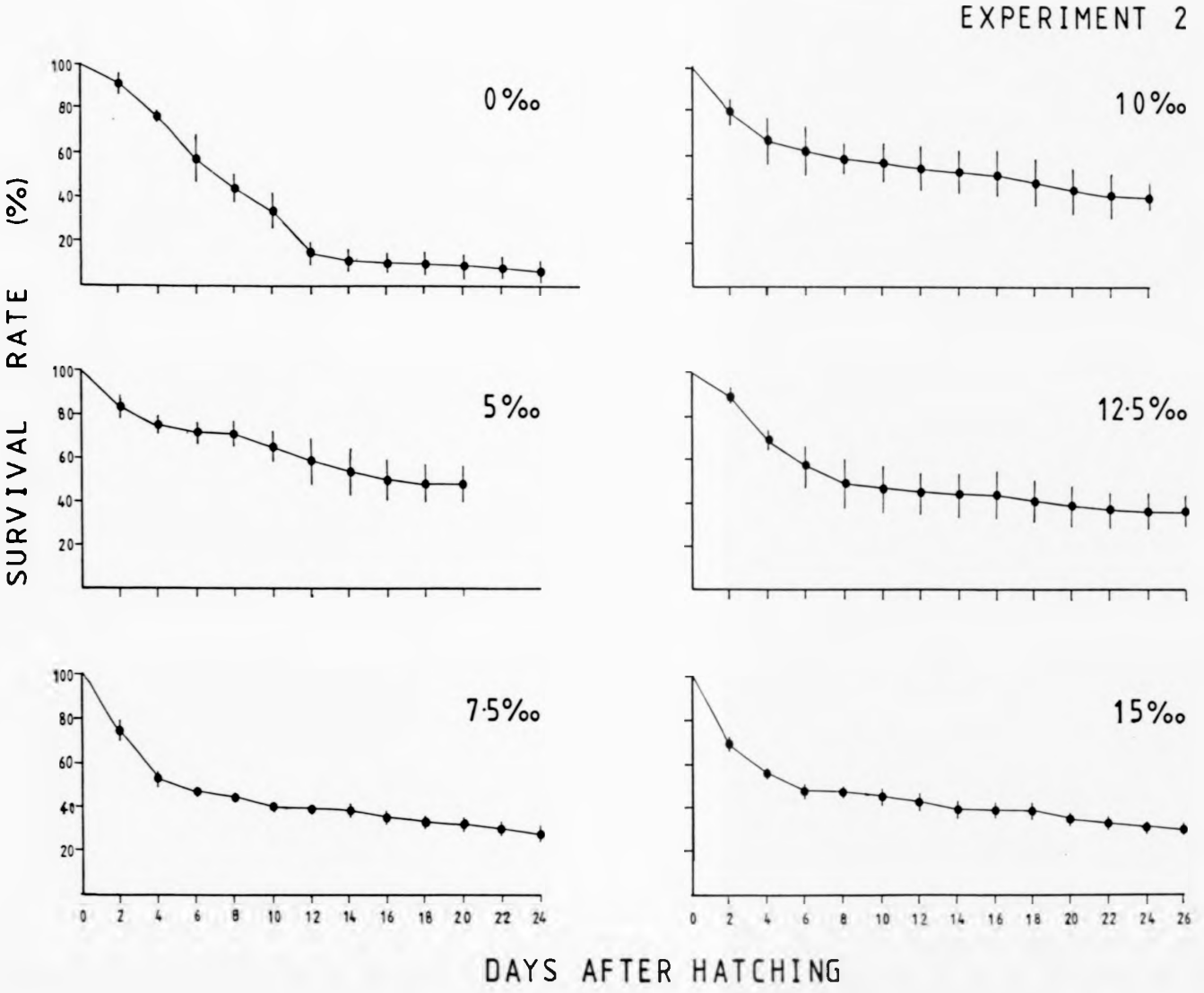
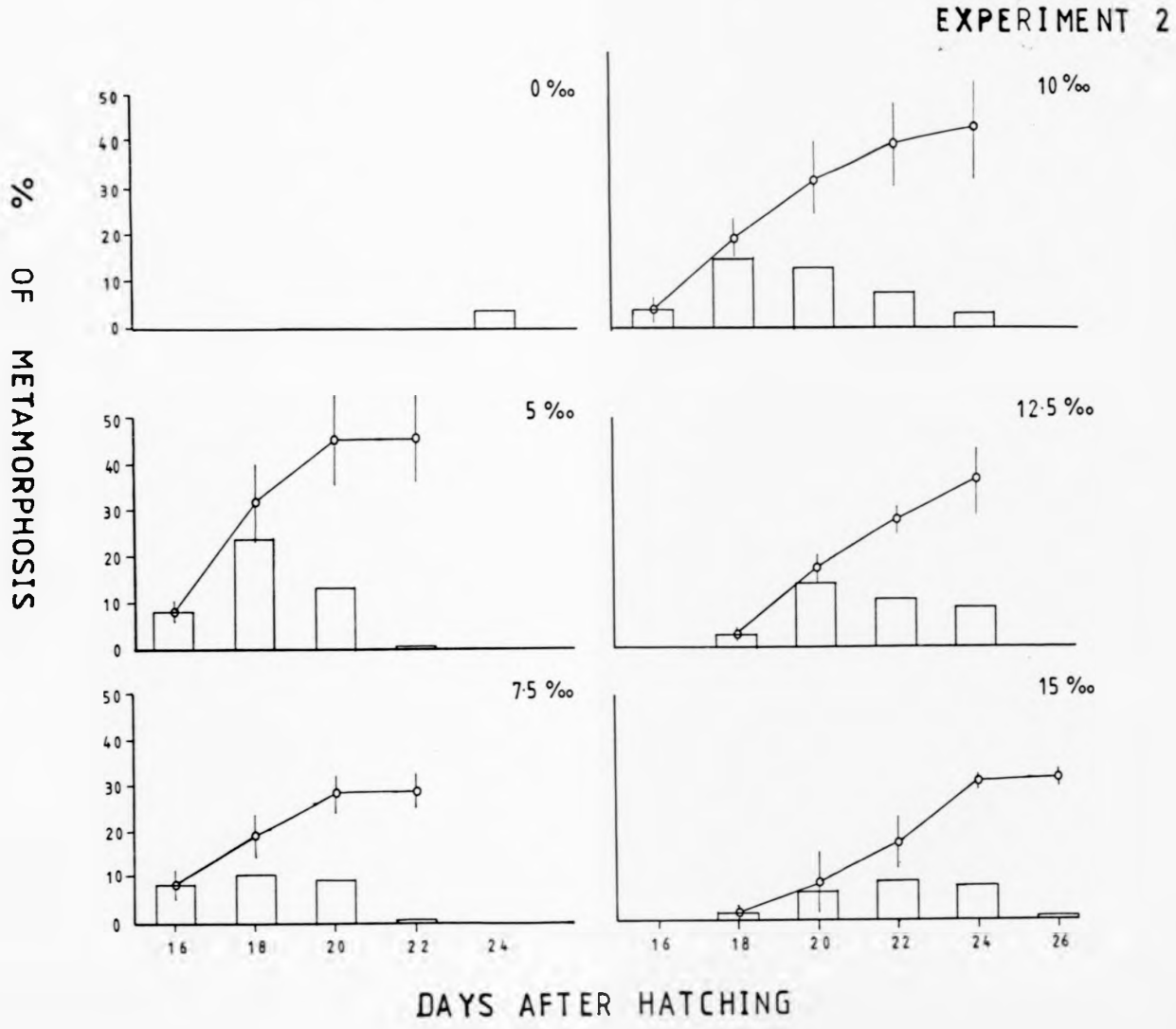

EXPERIMENT 2
SURVIVAL RATE (%)
0‰
10‰
5‰
12·5‰
7·5‰
15‰
100
80
60
40
20
0 2 4 6 8 10 12 14 16 18 20 22 24
0 2 4 6 8 10 12 14 16 18 20 22 24 26
DAYS AFTER HATCHING

Fig. (4.5): The Cumulative and Bi-daily percentage of metamorphosed Larvae (Experiment 2)


EXPERIMENT 2
0 ‰
10 ‰
5 ‰
12·5 ‰
7·5 ‰
15 ‰
% OF METAMORPHOSIS
DAYS AFTER HATCHING
0
10
20
30
40
50
16
18
20
22
24
26

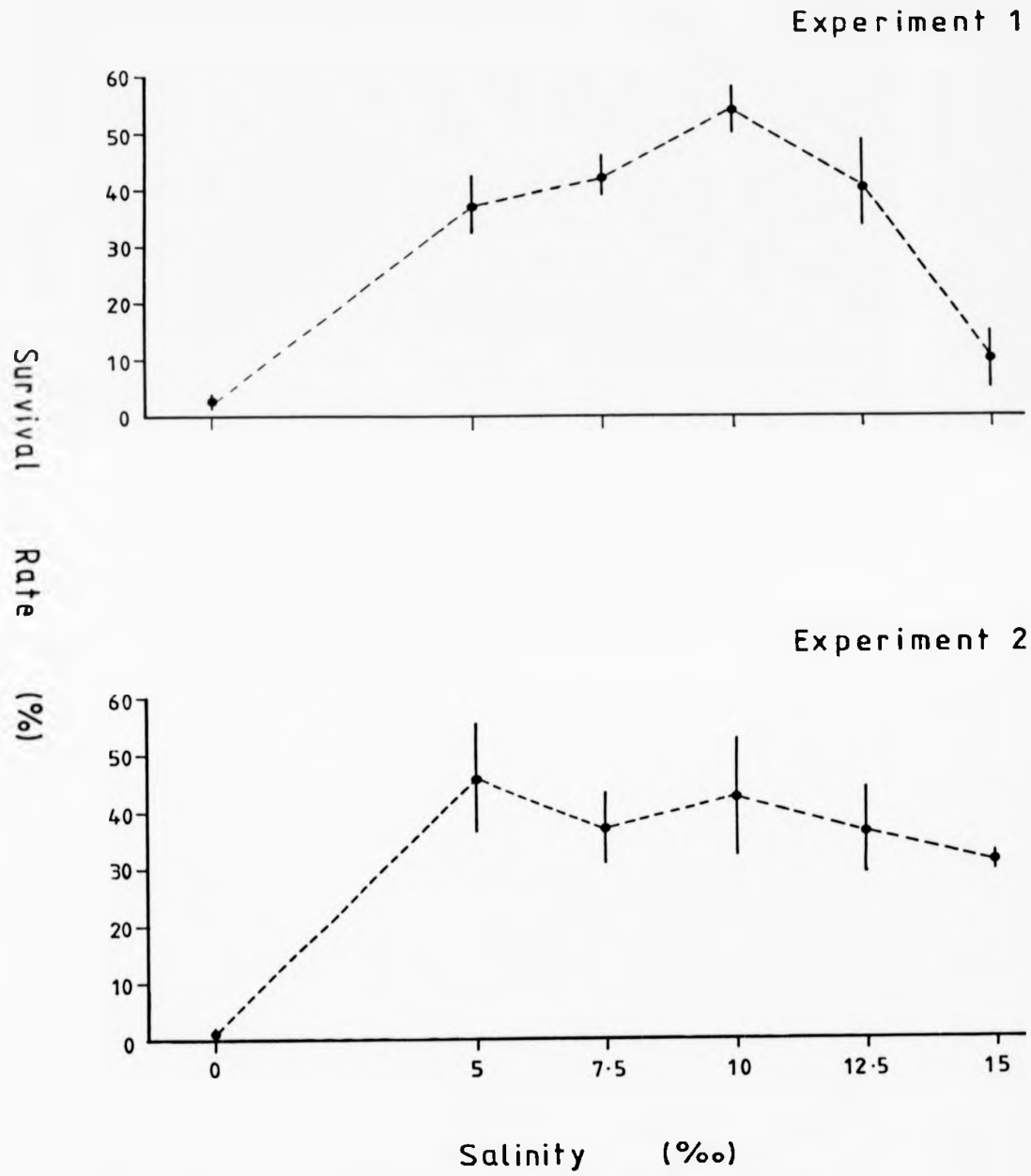


Fig. (4.6) : Salinity-Survival curves of M.nipponense

(4.2) Discussion for salinity tolerance experiment

Results of Experiment 1 and Experiment 2 generally agree well with each other. No significant results were inferred between results of the same salinity treatment, except for 15‰. The latter indicates that slight individual variation exist in the tolerance to more saline conditions. The survival-salinity curves (Fig. 4.6) corresponded well with the type IV curve of Dorgelo(1976), which typified brackishwater species (Fig. 4.1), just that the survival plateau is much wider in the present case.

The results of the present study also correspond well with the findings of some Japanese populations of M.nipponense. Larvae from lake Kasumigaura which tended to survive better between 8-10 ‰ (Uno, 1971) or 30-50% seawater (Ogasawara et al., 1979). However, it is known that larvae of M.nipponense from different populations can have very different responses to salinity. Rothbard(1977) reared larvae very successfully at 15‰. Ogasawara et al.(1979) observed a difference in salinity preference between the larvae of Lake Kasumigaura and Shimanto River (40-70% seawater). In China , both Ge(1980) and Zhang and Sun(1979) successfully reared larvae to metamorphosis in freshwater. Recently , Mashiko (1983a) revealed a different response to salinity between two populations in the Sagami River and inferred a genetic distance between them. It is evident that the salinity response of M.nipponense varies greatly from population to population. Most types of survival-salinity curves described by Dorgelo(1976) can be found in different populations of M.nipponense, (probably except stenchaline marine), with the

present population representing the euryhaline type towards the freshwater end.

Previous works on the survival responses of larval Macrobrachium to salinity are summarised in table (4.2). Most species reported have survival-salinity curves similar to genuine euryhaline species with best overall survival about the brackishwater salinity of 15-25‰ (e.g. M.amazonicum, Guest and Durocher, 1979 ; M.acanthurus, Dugan et al., 1975). Another major similarity of larval responses to salinities shared by these catadromous species is the inability to survive in freshwater for more than a few days. The only exception that has been studied in detail so far is the larvae of M.australiense, with the highest survival at freshwater and lower salinity but fail to metamorphose at salinity higher than 15‰. Therefore, the present population of M.nipponense is highly characteristic among populations of M.nipponense as well as other species of catadromous Macrobrachium in having survival in both freshwater and brackishwater. However, larvae of other species have been reported to be able to metamorphose in freshwater, including M.lanchesteri, M.niloticum, M.hainanense, M.asperulum, M.shokitai, M.pilimanus, M.kistnensis, M.hendersodayanum, M.malayanum, M.lammarei, M.jelskii.

As in the adults, larval responses to salinity can also be investigated by monitoring the changees in metabolic rates. Dr.G.S.Moreira and her co-workers had carried out a series of studies on the effect of salinity on the metabolic rates of first zoea of Brazilian Macrobrachium using the cartesian diver microrespirometers . The metabolic rate-salinity (M-S) response

Table (4.2) : Published data on larval salinity tolerance of *Macrobrachium* based on survival test

Species	Exptal S (‰), Temp ('C)	Optimal S (‰)	Reference
M.acanthurus	14-20,29	16	Dugan *et al.*(1975)
M.acanthurus	0-33,23-27	20	Choudhury(1971)
M.amazonicum	0-30,28	12-18	Moreira *et al.*(1986)
M.amazonicum	0-15	7.5-12.5	Guest & Durosher(1979)
M.americanum	0-40,28	20	Holtschmit&Pfeiler(1984)
M.americanum	15,29.5	NA	Monaco(1975)
M.americanum	10,28	NA	Arana(1974)
M.australiense	0-35,15-35	0-15	Lee and Fielder(1981)
M.carcinus	6-22,27.5	12	Dugan *et al.*(1975)
M.carcinus	7-33	14	Choudhury(1971b)
M.carcinus	21	NA	Lewis & Ward(1965)
M.faustinum	0-27	19-20	Hunte(1980)
M.japonicum	4-19,23-35	15.3	Yagi & Uno(1983)
M.nipponense	4.1-19.5Cl, 23-34.9	7.9Cl	Yagi & Uno(1981)
M.nipponense	0-100%sw Kasumigawa	30-50%sw	Ogasawara *et al.*(1979)
	0-100%sw Shimanto River	40-70%sw	
M.nipponense	1.65=11.9Cl ,20-36	4.64Cl	Uno(1971)
M.nipponense	16-17	NA	Rothbard(1977)
M.nipponense	0 & 30.5		Mashiko(1983a)
freshwater population		0	
brackishwater population		30.5	
M.nipponense	0-15	5-10	present study
M.ohione	0-20,29	15	Dugan *et al.*(1975)
M.petersii	2.9-5.3	5.3	Read(1985)
	general	8-35	
M.rosenbergii	5-35	15	Nair *et al.*(1977)
M.vollenhovenii	0-20	10	Vile(1971)
M.hainanense??	0-23	7-23	Ge and Yu(1987)

curves reported can be catagorised into two groups. The first group exhibited an increase in metabolic rates in the lowest and highest salinities, forming 'U-shaped' curves over a certain range of salinities, with the range of salinity differing between species. This group so far includes M.amazonicum, M.holthuisi, M.acanthurus and M.olfersii (McNamara et al., 1983 ; Moreira et al., 1980 and Moreira et al., 1982). In contrast, the second group, consisting of M.carcinus and M.heterochirus (Moreira et al., 1982), showed an apparent decrease in metabolic rates at both high and low salinities. However, analysis of the results of the two species showed no statistical differences existed between the metabolic rates within each salinity range (Moreira et al, 1982). This difference in M-S pattern probably reflect differences in osmoregulatory mechanisms and is, for the species studied so far, in general agreeement with the general distribution of adult shrimps (Moreira et al., 1982). M.heterochirus and M.carcinus can regulate their metabolic rates in a wider range than M.acanthurus and M.olfersii. Accordingly, adult M.carcinus and M.heterochirus are generally encountered in 'swiftly flowing' rivers at their mouth while M.acanthurus and M.olfersii are more abundant in slow moving rivers with long estuarine areas (Moreira et al., 1982).

Catadromous Macrobrachium generally release their larvae somewhere near to the river mouth. Zoeal I larvae then have to face a quickly changing saline regime, probably more so than in the later stages. However, only a few studies have compared the salinity-tolerance ability of different larval stages. Holtschmit and Pfeiler(1984) reported that early stages of M.americanum survive best between a salinity of 20-30‰ while latter stages do

better at 15-20‰. By monitoring the larval HOC, Read(1984) concluded that while M.petersi displayed strong regulatory power in all larval stages, zoeal I and post-larva were the strongest stages, both regulating at freshwater and 35‰. However, this regulatory power in freshwater was lost in stage II, confining the species in estuarine water. All the findings correspond well with the fact that M.petersi was mostly found in the upper reach of the estuary (Read, 1984). It can be envisaged that evolution of larval tolerance to salinity would be much affected by the hydrography of river mouth and estuarine ares. Hunte(1978) related larval salinity requirements of Jamaican Macrobrachium to the hydrography of the different streams and river mouths. The same author concluded that species which inhabit high-gradient streams have larvae which prefer high salinity for development, those which inhabit low-gradient streams have larvae which prefer low salinities, and species inhabiting both low and high-gradient streams are more flexible in their larval salinity requirement.

Considering all the different types of data, larval salt tolerance of Macrobrachium can possibly be categorised into groups which may also depict the possible evolutionary steps in the past:

(1) Euryhaline larvae that can survive in both low (>7‰) and high (35‰) salinity. This group includes M.petersi, M.olfersii, M.japonicum and probably M.acanthurus. The additional fact that adults of these species can tolerate seawater also suggest that they are only recent invaders to freshwater biotopes. The overall survival plateau of this group probably occur between 15-25‰. Other species which

may belong to this group are : M.equidens, which was reported to metamorphose in 32.9‰ (Ngoc-ho,1974); M.lar, which was reported to metamorphose at a salinity of 35‰ (Atkinson,1977) and M.grandimanus, which was reported to metamorphose between 17.5-35‰ (Shokita,1985).

(2) Stenohaline larvae that survive in low to medium salinities, but cannot tolerate high salinity greater than 30‰. This group includes M.rosenbergii, M.carcinus, M.holthuisi,M.amazonicum and probably brackishwater populations of M.nipponense. The overall survival plateau of this group occurs between 10-20‰. From the studies on larval morphologies, it can be inferred that a whole list of species probably belong to this group: M.birmanicus, which was reported to metamorphose at 10-20‰(Khan et al.,1984); M.formosense, which was reported to metamorphose at 13-16‰ (Shokita 1970); M.idella, which was reported to metamorphose at 12-18‰ (Pillai and Mohamed,1973); M.longipes, which metamorphosed at 33‰sw (Shen,1979); M.malcolmsonii, which metamorphosed at 10-70‰sw; M.novaehollandae, which metamorphosed at 23‰ (Greenwood et al.,1976) and M.vollenhovenii, which metamorphosed at 5-6.6‰Cl (Ville,1971).

(3) Stenohaline larvae that survive best in freshwater but cannot tolerate salinities greater than 15-20‰. M.australiense is the only species reported so far.

In relation to larval development, species with extended larval development belong to both group 1 and group 2, while M.australiense have advanced larval development. It is

possible that with further investigations of larval salinity tolerance, especially in relation to ontogenetic changes, more patterns of salinity reponses can be revealed. M.lanchesteri and M.niloticum, for instance, can metamorphose in total freshwater, but have an extended types of larval development. Other species like M.hainanense, M.shokitai, M.asperulum etc. have larval development with higher degree of abbreviation than M.australiense, it is possible that they have different pattern salinity reponses in both larval and adult phases.

Ge and Yu (1987) recently reported on the salinity response of M.hainanense. However, I have severe doubts on the correct identification of his prawns. M.hainanense, as reported in the present thesis, has abbreviated larval development, did not feed until metamorphosis to Stage IV, and completed larval development completely in freshwater. The larvae of Ge and Yu, however, did not have abbreviated larval development, feeding began at stage II and had total mortality in freshwater. The authors did not mention the source of the broodstocks. As the experiment was carried out 4-5 years ago and that the first author has since transferred to another institute (Ge, pers.comm. 1987), it may be impossible to confirm these points. From the geographical location of Wuhan (previous address of the authors), the larval development and response to salinity, the species concerned seems likely to be M.formosense.

(4.5) Introduction to the Artificial Selection of Larval Freshwater Tolerance

(4.5.1) Artificial selection and estimation of heritability

Artificial selection for commercial traits is one of the most ancient genetical practices of traditional agriculture. The potential gain and the rate of gain by selection is generally governed by the genetic contribution to the phenotypic variation of the interested trait. The phenotypic variance (Vp) of a population for a specific trait can be partitioned into the environmental (Ve) and the genetic components (Vg). The genetic component can, in turn, be further partitioned into additive (Va), dominance(Vd), and interactive(Vi) components, representing the consequences of additive genetic effects, dominance genetic effects and epistatic genetic interaction respectively.

i.e. $Vp = Ve + Va + Vd + Vi$ (equation 4.1)

The ratio Va/Vp or heritability (h^2) of the trait then predetermine the outcome of the selection, under identical environmental conditions.

$R = h^2 S$ (equation 4.2)

Where R is the response to selection and S (selection differential) is a measure of the magnitude of selection, as the mean value of parents expressed as deviation to the population mean.

Artificial selection can be performed by different methods, based on different units of animals being selected. The various methods can be categorised into three groups:

1. Mass selection or 'individual selection' is based on the the selection of individuals with the best phenotypes of the chosen trait.
2. Family selection is based on the selection of the best families among families compared for the mean value of the chosen trait.
3. Progeny testing generally involves comparision of the phenotypic performance of the offsprings from different pairing of parents. The best combinations, instead of individual parents, are selected. Different methods of progeny testing are employed , including the simple testing of different pairs, testing of parents of one sex, incomplete diallele cross and complete diallele cross.

The appropiate method of selection depends upon the expected value of h^2. Mass selection is favoured when $h^2 > 0.5$ while family selection is favoured when $h^2 < 0.5$. Progeny testing, while widely used in farmed animals, double the generation time required. The mathematical appraisal and prediction of the different methods can be found in Falconer(1981). For most traits, however, family selection is more efficient than mass selection (Falconer,1981 ; Gjedrem,1983). In practice, a combined method of selection is generally the popular choice:

1. Families with small numbers of progenies are established, along with the records of the trait(s);
2. This is followed by mass selection within each families;
3. Broodstocks for the next generation are then chosen by progeny testing of the selected individual from (2).

Furthermore, it is unlikely that, under commercial conditions, only one single desired trait is selected. It is normal practice that a selection index between several traits is chosen. This also favours the combined method of selection (Refstie, 1986).

All these methods of selection generally require a large number of matings between different sires and dams. A large number of culture facilities, with identical conditions, are demanded to hold the parents and to grow the offsprings. Therefore, it is logical that the h^2 of the trait has to be estimated prior to the design of an expensive selection programme. As in the methods of selection, there are also different methodologies for the estimation of heritability. All the methods estimate the additive genetic component of the phenotypic variance by computing the correlation or covariance between close relatives and relating this to their known relationship with h^2. The different approaches can be catagorised into four groups:

1. Response to Selection where heritability of a trait is estimated from a small scale selection with a known selection differential S (see equation 4.2). However, the estimates relate to 'realised heritability' as the response would only be the additive variance utilised in the study.
2. Parent-Offspring Regression based on the regression coefficient b of a linear regression line $y=a+bx$ of the mean offspring phenotypes to that of the parents, which is equal to the heritability.
3. Correlation between Close Relatives, generally involves full-

sib and half-sib analysis. The correlation coefficient then relates to the known relationship with heritability. Half-sib analysis is generally recommended because of the impact of common environmental factors and maternal effect on the full-sib data.

4. Variance Analysis determine the heritability by analysis of phenotypic variance of families resulting from hierarchial or diallele crosses. The actual value is estimated from the known relationships between the different mean sum of squares to the different components of the phenotypic variance.

Most of the mathematical definitions are given in Falconer(1981). The componen-t estimated by the different methods are summarised in table (4.3). Full-sib analysis generally overestimates heritability because it confounds Va with Vd. Realised heritability, as estimated from response to selection, theoretically underestimates the true value, as it refers only to the additive genetic variance utilised in the selection. Offspring-parent regression, on the otherhand, is much affected by both maternal and common environmental effect. However, the final choice of the methods i generally overshadowed by the availability of the facilities and the reproductive characteristics of the animals invoved. Experiments can also be designed to generate data for computation simultaneously using more than one method.

Table (4.3): Comparision of the Major Approaches in Heritability Estimation

Approach	Heritability measured	Components of Vp that can be quantified
Full-sib	$\frac{Va + Vd}{Vp}$	(Va + Vd), Ve
Half-sib	Va / Vp	Va , Vd , Ve
Diallele and Hierachial crosses	Va / Vp	Va , Vd , Ve
Offspring-parent Regression	Va / Vp	Va
Response to selection	R/S	realised heritability (presumably Va)

From Falconer(1981) and Tave(1986)
For symbols, see explanation in text (equation 4.1 and 4.2).

(4.5.2) Artificial selection and heritability estimates in aquaculture

Since the scientific study of aquaculture in the last two decades, quite a number of investigations have reported on the artificial selection of quantitative traits. Most of the reports are summarised in Gjerdrem(1983), Tave(1986), Refstie(1986) and Purdom(1986). While the number of reports are still comparatively rare compared to their agricultural counterparts, the following brief summary can be inferred:

1. Studies concentrate, and rightly so, on economically important traits like growth rate (weight and length), food conversion efficiency, survival, resistence to disease, meat quality, age at maturation and recapture frequency.

2. At the most, only a handful of estimates are available for each trait. Estimates of most traits are available for atlantic salmon, rainbow trout, channel catfish and common carp.

3. Coefficient of variation (CV) are very high for body weight and lower for body length. CV of body weight is also higher in younger animals.

4. Heritability for body weight is generally higher in adults than in juveniles.

5. Mortality/survival generally exhibits low heritability.

6. Resistence to specific disease mostly show medium to high heritability.

7. Heritability of the age of maturation tend to differ between different species.

Notwithstanding advantages like high fecundity and external fertilisation, problems in artificial selection of cultured crustaceans are posed by their modes of reproduction. Oviposition is immediately followed by fertilisation with spermatophores already implanted in the female's thorax. Spawning

can take a considerable period of time and, in many species, the eggs are incubated by the females (e.g. Macrobrachium, lobster). Even with the electrical ejaculation of spermatophores in M.rosenbergii (Sandifer and Lynn, 1980), no more than two, and possibly three females can be mated by a single male in one day. Conversely, it would be impossible to mate a female simultaneously with more than one male. Thereby, in order to perform large numbers of crosses for an accurate estimation of heritability, it is a prerequsite to devise methods of in vitro fertilisation and in vitro incubation of eggs. The in vitro fertilisation of Penaeus aztecus (Clark et al., 1973) demonstrated the real possibility of such development. In vitro incubation of Macrobrachium eggs, however, can only be performed so far with eggs more than 5-6 days old (personal observation ; Balasundaram and Pandian, 1981). Because of frequent moulting, most of the tagging systems used for fish have been rendered redundant in the case of crustacean broodstock. Clipping of appendages can be used to differentiate between limited numbers of selected groups, but is not totally satisfactory. The highly cannabalistic character of most crustaceans necessitate a large number separate holding facilities with uniform conditions. Furthermore, in order to apply any heritability values estimated, the experimental conditions have to bear resemblance to the natural or aquacultural conditions, which would generally involves huge expenses.

In spite of these problems, heritability estimates have been given for several cultured decapods and are summarised in table (4.8). High heritability values were observed for the lobster

Homarus americanus (Hedgecock et al, 1976, 1978; Fairful et al. 1981). However, the results were all based on full-sib analysis and represent an upper limit. For M.rosenbergii, Malecha(1986) reported a difference in heritability for 311 day weight between the two sexes, 0.35 for females and not significantly different from 0 for males. Lester(1988) reported on heritability values for larval size in Penaeus vannamei and P.stylirostris. While his data probably suffered from the full-sib approach and the small sample size, it did indicate a significant contribution of additive genetic variance to the phenotypic variance for both species, and more so for P.stylirostris. All in all, while there is an understandably paucity of data on decapod growth, the few reports so far do indicate the presence of a significant additive genetic component in this trait.

As far as crustacean aquaculture is concerned, size is obviously an important trait, as in other species. However, production of many species have been more affected by heterogenous individual growth (Malecha et al., 1980) and cannibalistic mortality resulted from high stocking density under culture conditions. The ability to withstand high stocking density, probably being related to the aggresiveness between individuals. Preliminary study on the heritability of aggressive behaviour of lobster inferred a small value (Finley and Haley, 1983). Fairful et al.(1981) also reported significant values of heritability of molting mortality but low values of overall mortality in juvenile lobster.

Table (4.4) : Estimates of heretability in decapod crustaceans

Species & Traits	Ref., No. of Families	Method of Analysis	Heretability ±Standard Errors
Homarus Americanus (Fairful et al., 1981) (juveniles, molt-7,8,9)			
Time to molt			
10-ND (a)	20	Full-sibs	0.81±0.18
15-ND	20	Full-sibs	0.25±0.09
20-ND	20	Full-sibs	0.11±0.08
Size at molt			
10-ND	20	Full-sibs	0.52±0.15
15-ND	20	Full-sibs	0.62±0.18
20-ND	20	Full-sibs	0.39±0.26
50-day-weight			
10-ND	20	Full-sibs	0.44±0.08
15-ND	20	Full-sibs	0.33±0.07
20-ND	20	Full-sibs	0.12±0.04
150-day-weight			
10-ND	20	Full-sibs	0.52±0.09
15-ND	20	Full-sibs	0.44±0.11
20-ND	20	Full-sibs	0.10±0.05
Mortality (total)			
10-ND	20	Full-sibs	0.11±0.05
15-ND	20	Full-sibs	0.10±0.07
20-ND	20	Full-sibs	0.11±0.06
Molting mortality			
10-ND	20	Full-sibs	0.19±0.07
15-ND	20	Full-sibs	0.32±0.14
20-ND	20	Full-sibs	0.48±0.17
Post molt mortality			
10-ND	20	Full-sibs	0.20±0.05
15-ND	20	Full-sibs	0.34±0.14
20-ND	20	Full-sibs	0.19±0.09
H. americanus (21°C)	(Hedgecock et al., 1976) Hedgecock and Nelson(1978)		
100-day-weight	12	Full-sibs	0.33
90-day-weight	9	Full-sibs	0.38
H. americanus Agressiveness	(Finley and Haley, 1983)		
Latency	30	Full-sibs	0.13±0.03
Frequency	30	Full-sibs	0.11±0.05
Duration	30	Full-sibs	0.08±0.05

Table 4.3 : (continued)

Species & Traits	Ref. & No. of families	Method of Analysis	Heretability ±standard errors
M. rosenbergii	(Malecha, 1986)		
311-day-weight	16s;16d	Full/Half sibs	0.16±0.14
311-day-weight	16s;16d	Full/Half sibs	0.14±0.25
311-day-weight	16s;16d	Full/Half sibs	0.35±0.28
Penaeus vannamei	(Lester, 1988)		
Size of			
Protozoea-I	9-10	Full-sibs	0-0.64
Mysis-I	9-10	Full-sibs	0-0.18
Post-larvae-I	9-10	Full-sibs	0.84-1.02
P. stylirostris	Lester(1988)		
Size of			
Protozoea-I	6-9	Full-sibs	1.27-1.31
Mysis-I	6-9	Full-sibs	0.64-1.09
Post larvae	6-9	Full-sibs	0.84-1.02

The main basis of artificial selection is the presence of phenotypic and genotypic variances. Data on decapod crustaceans will be reviewed in Chapter 5. In many species (e.g. many Penaeus species), however, the life cycle have yet to be closed under cultured conditions and husbandry improvements are no less valuable than are genetic ones (Cf. Purdom, 1986).

(4.5.3) Larval freshwater tolerance as a selection trait

Selection for survival rate in itself is obviously important in any aquaculture situation (see discussion). Larval freshwater tolerance (LFT) is an equally important aquaculture trait, which can give insight into the possibility of the production of purely freshwater strains of Macrobrachium species for use in aquaculture. LFT is also an important life-history trait in relation to the evolutionary study of Macrobrachium. While the survival rate of M.nipponense larvae in freshwater in the present study was very low, the fact that some dic survive in freshwater as well as in brackishwater has not been reported in other populations of the species. This probably reflects an adaptation to the salinity regime of the Au Tau fish ponds, which have been increasingly freshened with tapwater in the last decade or so. Attempts to understand the evolution of life history strategies ultimately examine the heritability of their component traits (Hines, 1986a). To investigate the evolutionary biology of the genus Macrobrachium, which has advanced from the marine to the freshwater environment, many species are believed to have done so in very recent times. The knowledge of the heritability of larval freshwater tolerance may not give the true rate of evolution in the distant past, but can at least reflect the possible rate of

freshwater advancement in recent history. The trait probably does not represent the first step of advancement, as the adult populations have to establish themselves first in the freshwater environment.

Larval survival is generally affected by a large number of environmental variables (see Chapter 3 and 4) and as a fitness trait, is generally expected to have a small value of h^2 (Gjerdrem, 1983). A combined selection would generally be recommended as the possible selection method. Also, mass selection and diallele progeny testing would require massive number of holding tanks for the broodstock, incubators for larvae, and subsequent rearing facilities for the juveniles. However, to conduct the selection in a proper way, synchronous hatching and incubation of larvae, as well as a sizable number of mating pairs would still be required.

It is thus pertinent to attempt an estimation of the possible value of heritability. However, methods like half-sib analysis, hierachial analysis and diallele cross analysis generally require large number of facilities, as required by mass selection and progeny testing. The present population of M.nipponense can mature within 45-60 days (personal observations) It is thus practically possible to conduct a simple selection exercise through 2-3 generations, which would also be within the scope of time and available facilities. Also, inbreeding depression generally occurs after 2-3 generations in highly inbred lines, as in the present ones.

There is no data available on the overall population mean of larval survival rate in freshwater (population phenotypic mean),

and the fact that a small number of mating pairs would be involved, it would be difficult to consider the present trait as a continuous quantitative trait. Larval survival in freshwater, however, can be considered as a **threshold** character with two phenotypic classes (survived and dead) with a single threshold separating them (Ch.18 of Falconer,1981). The proportion of the affected individuals is known as the incidence. The underlying continuous variable, which is generally known as the liability, is both genetic and environmental in origin and is generally assumed to be normally distributed. Many studies on the heritability of human disease have been considered as such (e.g. He et al.,1987). Because of the unsuitability of survival data in percentages for the analysis of variance, data of incidences must be converted into liability. One way is to assign arbitrary values, 0 and 1, to the two phenotypic classes of a threshold character followed by transformation (e.g. arcsine square-root) to the liability scale (Cf. Kanis et al.,1976). This would then allow normal computation of ANOVAR and correlation as in a continuous quantitative character. In the second method, the deviation(x) from the population mean in the scale of standard deviation is calculated from the data of incidence, based on the assumed normally distribution of liability. Response to selection(R) between two populations can then be calculated by the difference between the deviations of the two populations, and expressed as standard deviation from the population mean. Also, as the parents of a new generation are chosen **randomly** from the affected individuals (among the incidents), the selection differential then can calculated as the average of all the

affected individuals (group mean). The realised heritability(h^2) is then equal to R/S. The conversion values between percentage, reponse to selection and selection differential in deviation from the threshold, based on normally distributed liability, are all tabulated in Appendix A of Falconer(1981). This method of computation will be used in the analysis of data in the present study.

Mathematical justification of the use of offspring-parent regression can be found in both Falconer(1981) and Becker(1968). Standard errors of heritability computed from the offspring-parent regression are calculated from the following formula, as defined by Becker(1968) :

$$s2 = \frac{\left[y2 - \frac{([xy)^2}{[x2}\right]}{N - 2} \quad \text{; (equation 4.3)}$$

$$S.E. = \left(s^2 / [x \right)^{1/2} \quad \text{(equation 4.4)}$$

where s^2 and S.E. are the standard deviation and standard errors respectively. x and y referred to the parameters of the two axis while N is the sample size (the number of regression points).

(4.6) Material and methods for artificial selection experiment

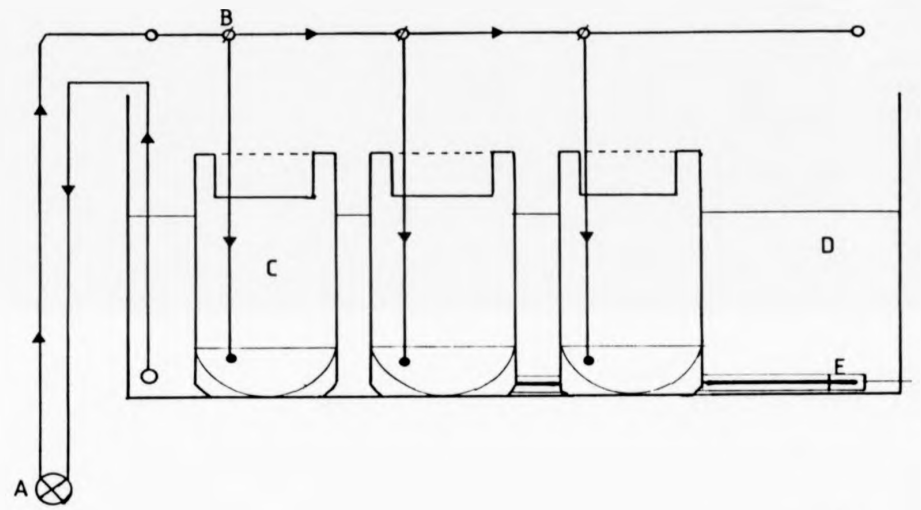
Three separate pairs of wild M.nipponense were used as broodstocks to produce three separate batches of larvae. 600 larvae from each batch were then counted to form the founding population of the first generation, 300 each (3 replicates of 100) in the freshwater group and the control group (at 10 %o). The postlarvae of the three replicates of each line were then

mixed and reared to sexual maturation. The broodstocks for the next generation were then chosen randomly (first three mating pairs) from each line. The process was then repeated for the third generation. The number of metamorphosed post-larvae was recorded everyday and transferred daily to the juvenile rearing system.

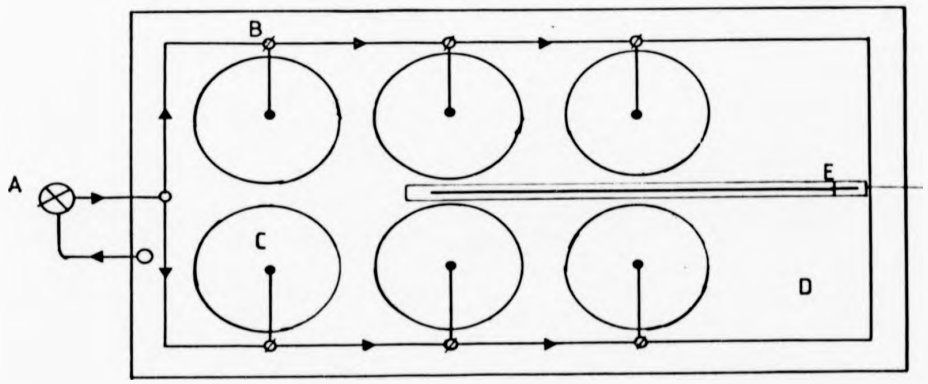
Two separate recirculation systems were used for larval rearing in both the freshwater and the control lines. Each system consisted of a reservoir tank and individual incubators (Fig. 4.7). Water in the system was continuously recirculated from the reservoir tank through an Eheim aquarium pump, through both physical and biological filters, and distributed via pvc pipes and plastic tubings into each individual incubators. Water in the reservoir tank was aerated and kept at 28°C by thermotatically controlled heaters. Water in each system was checked for salinity daily and evaporated water was replaced everyday with aged and aerated water to make up the required salinity. Similarly, 40% of the water of each system was also changed weekly. Saline water was made up using aquarium salt (Aquarientechnik Germany). Previous work on larval rearing and survival experiments have proved that identical systems, using the same water management regime and the same feeding regime were able to keep water quality parameters within favourable limits (Rahardjo, 1986 ; Maclean, 1986 and Shah, 1986). Incubators were made up with round-bottom 1-litre plastic beakers (Coke bottles) with 10cm diameter and fenced with 150 um mesh round the open top. Water was then supplied via the distributor from the pump into the bottom of each incubator, upwelled and overflowed

Fig.(4.7): Recirculated system used for the selection experiment for larval freshwater tolerance.
(a) side view;
(b) overhead view
(c) incubator
(d) reservoir tank
(e) submersible heater

a



b



through the mesh into the reservoir tank. The water-flow into each incubator was regulated by an aquarium valve at 180-200ml/min..

The feeding regime consisted of feeding newly hatched Artemia nauplii at 9.00 am and 6.00pm as well as commercial particulate diet (Zeiger 250 um) at every midday. The density of Artemia nauplii was kept at approximately 5-10/ml throughout the experiment. This feeding regime has been proved to be the most favourable among several that were tested by Maclean(1986) for larvae of M.nipponense.

The stocking density of larvae was kept between 50-100/l (i.e. 50-100 larvae per incubator) which was also proved to be the most favourable for larval rearing of M.nipponense using identical systems (Rahardjo,1986).

The rearing and feeding system of post-larvae to the next generation was carried out in a recirculated water system (constructed by the present author) equiped with both physical and biological filters, as well as temperature, aeration and flow control. Each batch was kept separately in identical tanks of the size of 30 x 50cm, at a maximum initial stocking density of 160/m^2. This stocking density was experimentally proved to be most favourable for the present system in separate experiments. The feeding regime, which was also proved experimentally to be viable in separate experiments, consisted of the followings:

Day 0 to Day 10	first hatched Artemia and micronised trout diet at 200% body weight,
Day 11 to Day 30	micronised trout diet at 100% body weight,

Day 30 onwards	trout pellets (Ewos Baker no.3) supplemented with fresh mussel once every week.

Feeding time was at 9:00 and 18:00 everyday for trout pellets and Artemia of the first 10 days was fed at 12:00 hours. Water temperatures was kept between 28°C to 30°C and the system was kept in a 12 hour light/12 hour dark illumination regime.

(4.7) Results of the Selection Experiment

(4.7.1) General comments on the results

The results of the larval survival rates for the selected and the control lines are both summarised in table (4.5). The overall average between the three control lines stayed between 59-61% for the control lines but increased from 24% to 50% in the selected lines. The average results of the control and selected lines are plotted in Fig. (4.8). Significant difference was inferred between the control and the selected lines at the first and the second generations but not at the third. The survival rates of line A was also significantly different to that of line B and line C in both the control and the selected groups. When challenged with freshwater, control line B gave survival rate of 30% and 36% respectively for the second and the third generation respectively.

Considering the fact that larval survival is a highly variable trait affected by many environmental factors, the variability between replicates were rather low.

Table (4.5) : Results of selection experiment on larval survival in freshwater

Control lines:	A	B	(fw)*	C
(10 %o medium)				
First Generation				
Batch size	100, 100, 100	100, 100, 100		100, 100, 100
% of survival	40, 23, 32	84, 84, 58		72, 72, 68
average±S.E.	31.7±4.91	75.3±8.66		70.7±1.33
Generation average : 59.2 ± 7.51				
Second Generation				
Batch size	60, 80	80, 100, 80	(80)	80, 80, 100
% of survival	41.7, 50	60, 76, 80	(30)	60, 70, 72
average±S.E.	45.9±5.87	72.0±6.11		68.0±3.05
Generation average : 61.0 ± 8.11				
Third Generation				
Batch size	80, 100	80, 100, 80	(100)	100, 80
% of survival	30, 46	82, 80, 72	(36)	62, 70
average±S.E.	38.0±8.00	78.0±5.29		66.0±4.00
Generation average : 60.7 ± 11.85				

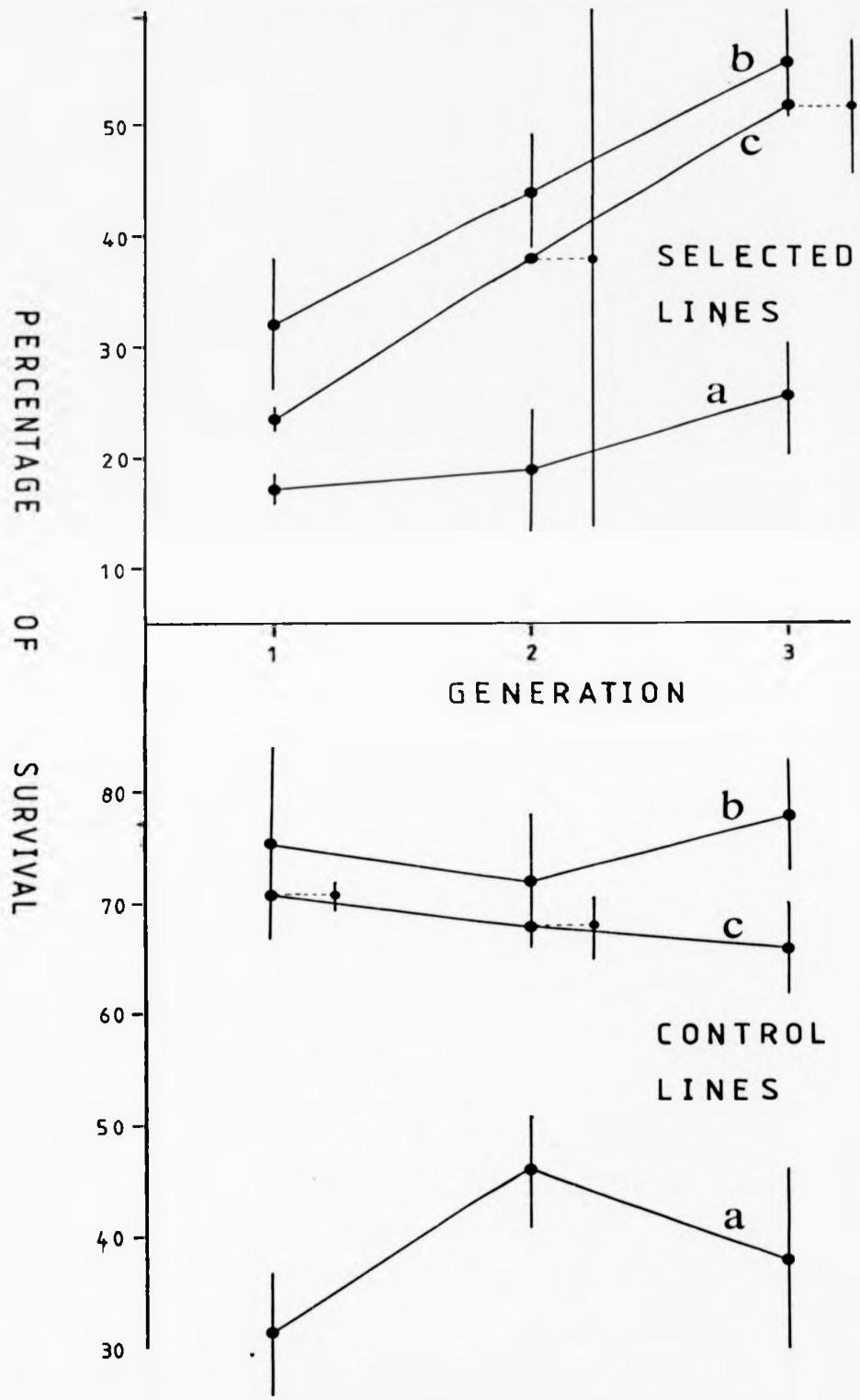
(fw)* - results of control line B challenged with freshwater

Selected Lines:	A	B	C
(freshwater)			
First Generation			
Batch size	100, 100, 100	100, 100, 100	100, 100
% of survival	20, 20, 12	22, 32, 42	22, 25
average±S.E.	17.3±2.67	32.0±5.77	23.5±1.22
Generation average : 24.4 ± 3.20			
Second Generation			
Batch size	70, 100, 50	80, 80, 100	100, 100, 100
% of survival	10, 18, 29	50, 40, 42	13, 61, 40
average±S.E.	19.0±5.51	44.0±5.29	38.0±24.00
Generation average : 33.7 ± 7.10			
Third Generation			
Batch size	100, 80, 100	100, 100, 100	80, 100, 80
% of survival	16, 30, 32	50, 53, 65	40, 56, 60
average±S.E.	26.0±5.03	56.0±4.58	52.0±6.11
Generation average : 44.7 ± 5.39			

Fig. (4.8):

Larval survival rates

in the selected and the control lines


PERCENTAGE OF SURVIVAL
50
40
30
20
10
b
c
a
SELECTED LINES
1
2
3
GENERATION
80
70
60
50
40
30
b
c
CONTROL LINES
a

(4.7.2) Computation of realised heritability from response to selection as a threshold character

In the present selection experiments, two phenotypic classes(survive and dead) were recognised and the parents of the next generation were selected randomly among the surviving individuals. The response to selection can thus be considered as a threshold character as described in Chapter 18 of Falconer(1980). The method of computation has already been described in section (4.5.3).

Realised Heritability was calculated between each generation (Table 4.5). The mean values for line B and line C were very similar to each other at 0.31 while that of line B was much lower at 0.108. The overall mean value of heritability was 0.244.

A similar calculation was carried out for the control lines, which is also summarised in table (4.5). Heritability values varied from -0.215 to 0.405. On average, realised heritability was negligable at 0.015.

Line B of the control line was also challenged with freshwater in the second and third generations. Similar calculation using the difference between selected and the control lines as the response, inferred a realised heritability of 0.34. This is similar to the results calculated from selection line B, using generation difference as the selection response.

(4.7.3) Computation of heritability by offspring-parents regression

As there are data from 3 lines and 3 generations, a regression analysis is possible, using the average data for each generation in relation to its offspring/parents.

The correlation coefficient for both the control and the selected lines are highly significant (p=0.05), at 0.923 and 0.967 respectively with d.f.=4.

The regression formula are:

Selected lines : $y = 1.51 + 1.30\ x$

Control lines : $y = 14 + 0.789\ x$

where x and y are survival rates of parental and offspring generation respectively.

The computed values for the selected and the control lines are almost identical at 0.055 and 0.054 respectively.

The heritability and its standard errors (from Becker, 1968) of larval survival in freshwater then, as estimated from offspring-parents regresssion are:

Selected lines : 1.30 ± 0.055

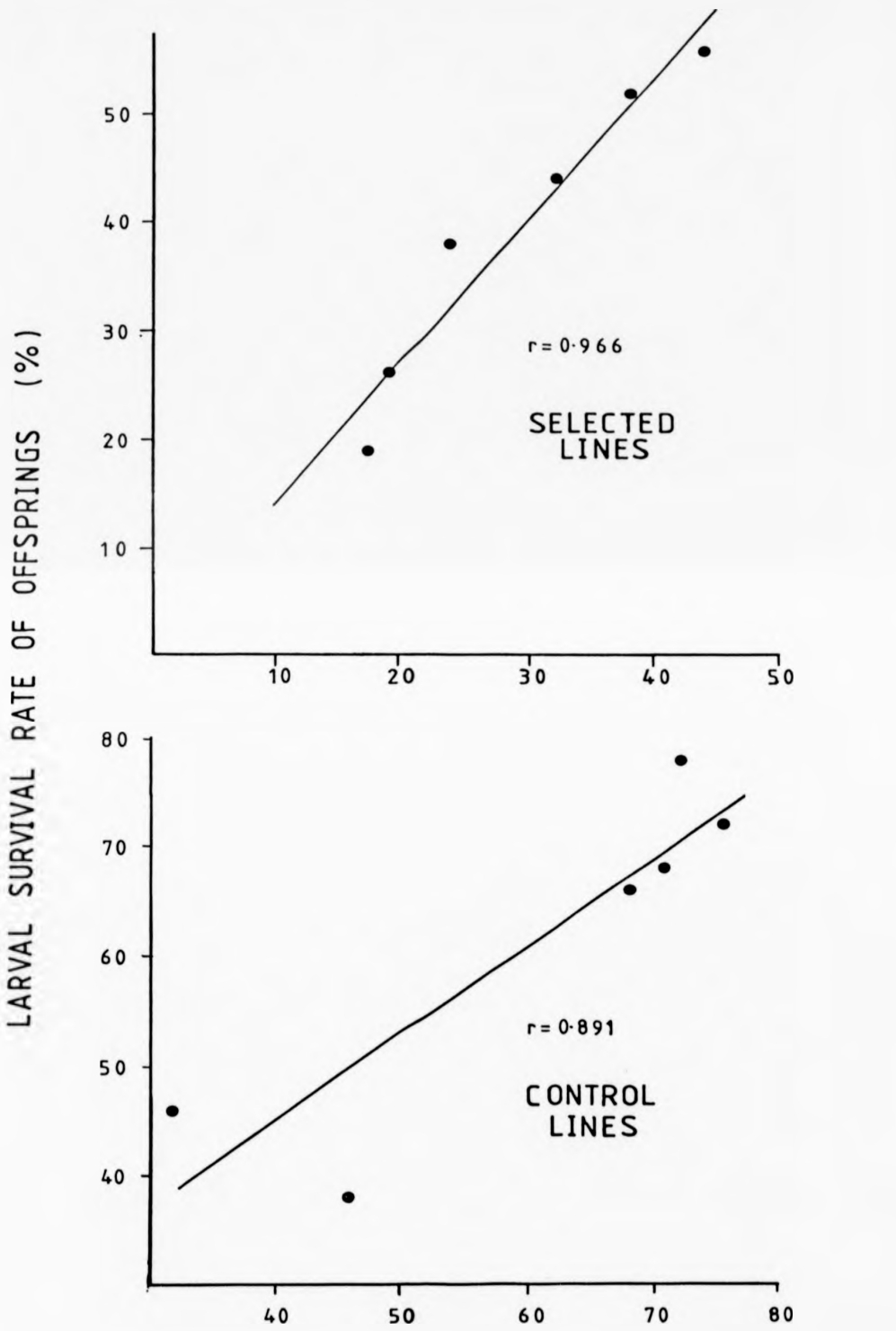
Control lines : 0.789 ± 0.054.

Table 4.6 : Computation of Heritability from Response to Selection of a Threshold Character

Average survival(%)	Deviation from Mean (σ)	Selection differential(σ)	Heritability
	Selected Lines (freshwa-ter)		
Line A			
G1 17.3	0.954	1.489	0.050
G2 19.0	0.880	1.430	0.166
G3 26.0	0.643		(average:0.11)
Line B			
G1 32.0	0.468	1.118	0.284
G2 44.0	0.151	0.896	0.337
G3 56.0	−0.151		(average:0.31)
Line C			
G1 23.5	0.723	1.320	0.316
G2 38.0	0.305	1.002	0.309
G3 52.0	−0.005		(average:0.31)
(Total average of selected lines = 0.24 ± SE 0.065)			
Line B (compared to freshwater)	control line B but challanged with		
G2 30.0 (C)*	0.524	1.159	0.32
G2 44.0 (S)	0.151		
G3 36.0 (C)	0.358	1.039	0.72/2 = 0.36 (2 generations)
G3 56.0 (S)	−0.151		(average:0.34)
	Control Lines (10%0 salinity)		
Line A			
G1 31.7	0.468	1.118	0.264
G2 41.7	0.228	0.948	−0.081
G3 38.0	0.305		(average:0.091)
Line B			
G1 75.3	−0.674	0.424	−0.215
G2 72.0	−0.583	0.467	0.405
G3 78.0	−0.772		(average:0.095)
Line C			
G1 70.7	−0.553	0.482	−0.176
G2 68.0	−0.468	0.526	−0.106
G3 66.0	−0.412		(average:-0.141)
(Total average of control lines = 0.015 ± SE 0.104)			

* (S)-selected ; (C)-Control ; G1-3 - Generation 1 to 3

Fig. 4.9: Regression Lines for Offspring-parent Regression


LARVAL SURVIVAL RATE OF OFFSPRINGS (%)
50
40
30
20
10
r = 0·966
SELECTED LINES
10
20
30
40
50
80
70
60
50
40
r = 0·891
CONTROL LINES
40
50
60
70
80
LARVAL SURVIVAL RATE OF PARENTS'S GENERATION (%)

(4.8) Discussion on the Selection Experiment

(4.8.1) Discussion on the computation of heritability

The fact that larval survival rate in freshwater has increased to over 50% (at least in line B) in three generations implies that additive genetic variance does contribute significantly to phenotypic variance. The apparent discrepency in the value of heritability computed by the two methods is inherently related to the nature of the trait as well as the small number of data available.

In the offspring-parent regression, data utilised were from family means, as survival rate was measured as the percentage of surviving post-larvae per batch, rather than individual survival. The heritability calculated was probably related more to the family, rather than to the individual.

Offspring-parents regression is likely to give rise to too high an estimate attributed to maternal effect and common environmental effect (Falconer, 1981), especially for fitness traits of post-embryonic stages. Thus, maternal effects on survival of young sprake hybrids were 0.78, 0.68 and 0.40 respectively for uneyed, eyed and alevin stage, all being high and decreasing with development (Ayles, 1974). Similarly, Mclaren(1976) also inferred a significant maternal effect on the heritability of pre-mature mortality in the copepod Eurytemora herdmanii. As larvae (zoeas, in the present case) is the first post-embryonic stage, and that most mortality (especially related to freshwater tolerance) generally tends to occur in the first few days (see last experiment) of development, a large maternal

effect can logically be expected.

Doyle and Hunte(1981) observed heritable changes of survivorship and fertility in a population of Gammarus lawrencianus as a result of 'domestication' under laboratory condition. Malecha(1986) also suggested that, as the larval survival rate of M.rosenbergii was markedly increased under cultured conditions, the gene pool had been 'flushed'. As the broodstock of the present study was obtained from the wild populations from the Au Tau fish ponds in Hong Kong, part of the apparent contribution to heritability would be attributed to common environmental effect of the general culture conditions (e.g. artificial diet and Artemia).

While the average survival rates of the control lines did not significantly increase with generation, offspring-parent regression gave significant correlation coefficient with estimated heritability of 0.789. It can thus be assumed that this value is attributed to maternal and common environment effects and thus be deducted from heritability estimate of the selected line. The value of heritability should thus be 0.511±0.1.

Offspring-parent regression generally requires a large number of data points (Falconer,1981). The present computation only utilised six data points. Furthermore, regression of offspring-parent would only be linear when data utilised were near to the mean (Kirpichnikov,1984). The fact that improvement occurred probably implies that families with high average values of survival rates were used. Therefore, the value of 0.511± probably still represent only the upper limit of the actual value.

In the computation of realised heritability from response to selection of a threshold character, the survival data was first transformed to deviation from the population mean while the selection intensity was also computed as the average of all deviations of the affected individuals. The response to selection was then calculated as the difference between the two deviation in the scale of standard deviation, thus any effect due to maternal or common environmental interactions was already accounted for. Therefore, only a neglegible value of heritability was inferred for the control line. This also confirms that the value of heritability computed by offspring-parent regression was mainly attributed to maternal or common environmental effect and should rightly be deducted from the estimates from the selected lines.

However, the assumption of equal variance in successive generations generally implies that the computed value is at least 5-10% lower than the actual value (Falconer, 1981). Also, realised heritability, as estimated from the response to selection, relates more to the additive genetic variance exploited by the particular selection in a generation, and probably underestimate the true value. Values computed from the difference between selected and control line B as the selection response also showed a slightly higher values when compared to values computed from generation differences.

The value of 0.244, as estimated from response to selection of a threshold character, probably represent a lower limit to the actual value. The heritability of larval freshwater tolerance then ranged between 0.244 to 0.51 with an average of 0.377.

(4.8.2) Discussion in relation to aquaculture and evolution of the genus

Fitness traits generally have very low heritabilities. as most of the additive genetic variance have been utilised by natural selection (Gjerdrem,1983). Heritability of mortality before maturation for the copepod Eurytemora herdmanii was between 0.01-0.13, and mostly not significantly different from 0 (Mclaren,1976). The average value for early life stages of the lobster Homarus americanus was 0.1 (Fairful et al.,1981) but the estimation was from full-sib analysis which generally overestimates heritability. Therefore, the average value of 0.377 obtained by the present study was very high, considering the fact that larval survival is undoubtedly a fitness trait. The present population of M.nipponense was taken from Au Tau fish ponds, which has been increasingly freshened with tapwater in the last few years from the previous brackish regime. The present population was thus subjected to a process of genetic assimilation, resulting in the peculiar ability to survive in both fresh and brackishwater.

Doyle(1976) investigated the ecological, physiological and genetic effect of osmotic stress on the amphipod Gammarus lawrencianus. A realised heritability of 0.17 was inferred for the salinity tolerance trait TC (Time to Capture), computed from response to one generation of selection. However, the trait was found to relate to other fitness traits like TM(Time to mature) and ecological modelling, using the generated data, did not infer significant correlation between TM and fitness. The same author concluded that it would be invalid to assume a 'good'

physiological phenotype for an amphipod under salinity stress.

Mashiko(1983a) reported a reduction in larval duration in a freswater population of H.nipponense, as compated to its brackishwater counterpart in the same river. No apparent decrease in larval duration was observed in the present study, postlarvae generally metamorphosed between 14-17 days in the control lines and 17-20 days in the selected lines.

An experimental reduction in salinity to controlled populations of the copepod Tisbe holothuriae changed both life history as well as morphological traits (Fava and Battaglia, 1985). The same authors also suggested that salinity has served to promote a reduction in gene flow between natural population of another closely related species, T.clodiensis, which is very sensitive to the trait.

Recent reports also linked enzyme polymorphism, via this trait, to the fitness of individuals. Biochemical evidence have shown that polymorphism at the loci glutamate-pyruvate transferase (Gpt), which catalyzes the final step of alanine synthesis, has been responsible for physiological adaptation to salinity stress in the copepod Trigriopus californicus. Two common alleles exist, Gpt(s) and Gpt(f), the latter having a higher specific activity of enzymatic activity. Under hyperosmotic stress, adults of Gpt(f/f) and Gpt(f/s) genotypes accumulate alanine much faster than Gpt(s/s) individuals, resulting in higher mortality of the latter group of copepods. This genotypic difference in the ability to osmoregulate under hyperosmotic stress, coupled with microhabitat distribution, has produced a mosaic pattern of genetic differentiation among populations (even

a few hundred yards apart) of the copepod.

From the aforementioned studies, it is evident that salinity tolerance is probably one of the most important evolutionary traits, especially for species which inhabit the margin of the seas and have to face drastic fluctuation from freshwater to full-strength seawater.

M.nipponense is already harvested in freshwater bodies in China, Japan and Korea, implying that many populations have developed to LFT before the present one. Further studies should compare the salinity tolerance and production performance of inland populations. The genetic differentiation between some of these populations will be reported in the next chapter. The present strain of freshwater M.nipponense, being a recent departure from the brackishwater regime, should undoubtedly contribute to the genetic variability of the more inland populations.

In relation to artificial selection of this trait, the present study also confirmed that family selection, combined with small scale mass selection by progeny testing will be the best approach. Line B and Line C in the present selection study would thus be retained for further selection. As to other species of brackishwater Macrobrachium, the first stage larvae generally die in freshwater within several days after hatching. The results of the present study also point to the possibility of artificial genetic assimilation of these brackishwater larvae by a progressively freshened regime of larval rearing. However, the larval duration of most of these species, e.g. M.rosenbergii, are generally longer than M.nipponense, which are prone to more

environmental effect and possibly a lower value of heritability of larval viability. Also, as pointed out in this chapter, the 'rosenbergii' group may have evolved with special larval existence in brackishwater salinity. Nevertheless, Doyle and Hunte(1980,1981), observed a 1.7 fold increase in survivorship in a population of Gammarus lawrencianus which was linked to a 2.6 fold increase in yield, pointed out the importance of selection of survivorship in crustacean aquaculture. As Doyle et al.(1983) pointed out, a sizable increase in production of M.rosenbergii can be effected by indirect selection of collecting broodstock early in production cycle, fruitful results in freshwater tolerance may be obtained by progressive lowering of salinity for larval rearing (e.g. 2%o per generation initially to 5%o, and then 1%o per generation). However, as Doyle(1976) proved the possible related response in other fitness traits to selection for salinity tolerance, it is important to monitor simultaneously other possible responses like egg size and age to maturity.

"From the truth come one, from one come two, from two come three, from three come a multitude of things where the Ying and Yang of the Nature intermingled in a well balanced equilibrium."

-in 'Truth and Nature'
by Lao Tze (about 500 B.C.)

CHAPTER FIVE

INTRA-SPECIFIC VARIATION

(5.1) INTRODUCTION

(5.1.1) Population genetics

Darwin, in his masterpiece 'Origin of Species' referred to "protean or polymorphic" as "in which species present an inordinate amount of variation; and hardly two naturalists can agree which forms to rank as species and which as varieties." (in Chapter II, Variation under Nature). With the advent of neo-Darwinism, present-day 'naturalists' merely accept such phenomena as polymorphism in the same species.

The discovery of protein polymorphism in the early 1960s and the subsequent development of simple biochemical techniques have readily allowed a large scale study of such variation. These techniques have indeed revealed a greater level of genetic variation than has previously been anticipated. Apart from its major contribution to modern evolutionary theory, contemporary population geneticists rarely satisfied by mere description of such variation, have addressed themselves to problems of both theoretical and practical importance. Among the more well known:

are the selection vs neutral theory of molecular evolution and the possible association between protein heterozygosity and fitness (growth, fecundity, developmental homeostasis, etc).

The availability of recombinant DNA techniques have also added new methodologies of DNA sequence polymorphism, organelle (mitochondria and chloroplast) DNA sequence polymorphism and restriction enzyme digest polymorphism and have lead to new areas of interests (e.g. transposable elements). However, the conceptual development of modern population genetics has really been made possible by the ability to quantify gene frequency and the developments of mathematical models and indices to assess genetic variation, based upon protein electrophoretic data. It is also based upon this ability to quantify protein polymorphism that electrophoretic data have been used to address the original problem associated with polymorphism by Darwin - systematics and numerical classification. The main aim of the present and the next chapters is to investigate the inter- and intra-specific variation of Macrobrachium by electrophoretic analysis, supplemented by other data wherever possible, and relate to the evolution and aquaculture of the genus.

(5.1.2) The 'Ying and Yang' in population genetics

Population genetics is one of the few biological sciences that has both a theoretical and an observational aspect. "There is no other biological science in which observation are so often directly motivated by formal theory and in which formal theory is so often constructed in an explicit attempt to make sense of the observation" (Levinton, 1987). It is thus imperative to give a

brief introduction to the theory of population genetics before reporting on my observations.

The genetic structure of a population, and the frequency of a particular allele, is the manifestion of various natural processes. On the one hand, gene flow between sub-populations of a species tend to homogenise the genetic composition among the sub-populations. On the otherhand, the overall effect of mutation, random genetic drift and selection tends to increase the overall variability and differentiation between the populations. Apart from these forces, historical events and present-day changes in population size may also influence the direction and the rate of changes in genetic composition of populations.

(5.1.2.1) Random genetic drift

The proponents of the 'Neutral Theory' (e.g. Kimura, 1983a;1983b; Nei, 1983) proposed that much of the variation observed at the molecular level is neutral, and that much of the divergence among species and population has been caused by random genetic drift. In other words, for alleles that are neutral at a locus, new alleles would be substituted at a constant rate by mutation.

Thereby, at any moment in time, a large number of alleles at a polymorphic locus are drifting either to be lost or fixed. The process occurs in every finite population (i.e. every population), and its rate is determined by the effective population size, Ne, of the population concerned. Because of random genetic drift, the proportion of heterozygotes in a

population at a certain time would decrease:

$$H_T = H_t (1 - 1/2\ Ne)^t \quad \text{(equation 5.1)}$$

where H_T and H_t are the present and founding level of heterozygosity in the population. t is the number of generation since the founding of the population.

(5.1.2.2) Mutation in finite populations

Whenever there is a mutation from allele A to A', there is also a probability of the backward mutation from A' to A, Mutation, on its own, is generally regarded as a very weak force, at a rate (U) of about 10^{-5} or 10^{-6} per gamete per generation.

In a finite population of effective population size Ne, where alleles are lost by random genetic drift, mutation would be important in replenishing variation. The level of genetic variation (H), measured by heterozygosity, is

$$H = \frac{4\ Ne\ U}{4\ Ne\ U + 1} \quad \text{(equation 5.2)}$$

Under such conditions, H depends on the flux of new mutation, which in turn, is proportional to Ne.

(5.1.2.3) Gene flow in natural populations

The degree of differentiation between different populations depend on the gene flow between them. The rate of gene flow then depends upon the contribution of immigrants to the breeding population. Assuming negligible genetic drift and selectively neutral alleles, gene flow would cause the gene frequencies of all populations to converge.

One of the most widely used indices to measure genetic differentiation between populations is Wright's coefficient, F_{ST}.

$$F_{ST} = \frac{Vg}{q\ (1-q)} \qquad \text{(equation 5.3)}$$

where Vg is the variance of allele frequency and q is the mean allele frequency. The equilibrium variance among populations can be proved to be approximated to :

$$Vg = \frac{q(1-q)}{4Ne\ m + 1} \qquad \text{(equation 5.4)}$$

where m is the proportion of breeding population contributed from immigrant members. Therefore, variance between populations, and eventually between-population differentiation, decreases as the product of Ne and m increases.

Different mathematical models of gene flow have been proposed to account for the effect of gene flow on genetic differentiation, each generally differs from the other in their assumptions about geographic and demographic structure of populations. The 'Island model' was first suggested by Wright(1931) in which migration occurs at random among a group of small populations. Latter(1973) proposed a separate island model (later to be known as the n-island model; Slatkin,1984) in which there is a finite number of islands as opposed to the infinite number in the Wright's model. The island model represents a model of long distance gene flow. The 'stepping-stone' model of Malecot(1975)assumes that each sub-population receives its immigrants only from its neighbouring sub-population. The net amount of gene flow in both the island model

and the stepping-stone model depends upon the ratio of the mutation rate to that of the immigration (m/u). Many so called 'continuum' models (e.g. Wright, 1943) consider a continuously distributed population among local 'neighbourhoods' defined by a dispersal distance σ. The variance in dispersal distance affects the rate of decrease of probability of identity with distance in a population at equilibrium. The increase in differentiation with distance, between neighbourhoods at equilibrium, depends upon σ /uN.

In general, if immigrants arrive at random from all the sub-populations that make up the larger population, even a low amount of gene flow greatly reduces the divergence among populations caused by genetic drift. The average rate of gene flow among established sub-populations of a species is often quite low, and especially if immigrants have to compete with residents to survive and reproduce. On the otherhand, the effective rate of gene flow among sub-populations can be much higher than the average rate if local populations become frequently extinct and recolonised from several sub-populations.

(5.1.2.4) The effect of selection on the genetic structure in natural populations

Natural selection, in essence, is differential survival and reproduction, and operates whenever genotypes differ in 'fitness' (sensu Wright, 1931). The selection coefficient, as in the case of artificial selection (see Chapter 5), is represented by the difference in relative fitness of the most fit genotype and that of a less fit genotype. The average fitness, $\bar{w}$, of an individual in a population, is the sum over all the genotypes.

$$\bar{w} = \sum_{i} f_i \, w_i \qquad \text{(equation 5.5)}$$

where f is the frequency of the ith genotype. Fitness is essentially difficult to measure, but in asexually reproduced plants, it probably equals the abundance of population (Futuyma, 1986). The concept of average fitness also form the basis of many mathematical models of evolution, which generally account for the interaction of the various forces, including the 'adaptive landscape' model (Wright, 1931) and the 'shifting balance' model (Wright, 1977).

In relation to the effect on allele frequency, assuming no other forces acting on the population, three major types of selection can be distinguished. For a starting population with a normally distributed frequency of individuals at intermediate phenotypes: (a) a 'Stabilizing/balancing' selection implies that the intermediate phenotypes are the most fit; (b) a 'disruptive' selection applies when intermediate phenotypes are the least fit; (c) a 'directional' selection when one of the extreme phenotypes is the fittest. A disruptive selection then would split the populations fixed for the extreme phenotypes, probably forming the basis for allopatric speciation (see next chapter). A directional selection would then cause the fixing of the most fit phenotypes (and probably allele), but the loss of the less fit ones from the population. A balancing selection, on the otherhand, would favour intermediate phenotypes, and thus the original population would remain in a balanced equilibrium. The latter, while difficult to prove, has been suggested by some authors, as the basis to maintain polymorphism in nature

(Johnson, 1977).

Most studied examples on the effect of natural selection on enzyme polymorphism were reviewed in Koehn et al. (1983).

(5.1.2.5) The interaction of the various forces and factors affecting the effective population size, Ne

Selection, mutation and gene flow are 'deterministic' factors, i.e. if the values of these factors are the same for a number of populations with the same original population and allele frequency, all populations will attain the same equilibrium composition (assuming no drift). With random genetic drift, the allele frequency of a population then becomes a probabilistic event. As selection or the effective population size, Ne, becomes weaker, the probability of allele frequency would increasingly depart from the prediction of the deterministic models (Crow and Kimura, 1970; Wright, 1937). In a population with relatively large Ne, random genetic drift will generally increase the rate of change of allele frequency at their extremes, but has very little effect at their intermediate level, unless the selection differential is extremely high. On the otherhand, a regular strong gene flow from a large parental population can minimize the effect of random genetic drift, mutations, and even selection. In the case of strong selection and strong unidirectional gene flow, an apparent cline can form, as in the case of the LAP locus in Mytilus edulis (Koehn et al., 1983).

The effective population size, Ne, of a population then governs the way in which the forces of nature (selection,

mutation, random genetic drift and gene flow) affect the genetic composition and differentiation of populations. The effective population size, on the otherhand, is affected by various biological properties of the species concerned.

(5.1.2.5.1) Non-assortive mating

Any mating system in a species that departs from assortive mating may reduce the number of mature individuals in a population that participate in reproduction.

$$Ne = 4\ Nm\ Nf\ /\ (\ Nm + Nf\ )$$ (equation 5.7)

where Nm and Nf are the number of males and females participating in the breeding population.

In many species of Macrobrachium, the polygamous mating behaviour of dominant males (e.g. M.nipponense; Chow and Fujio, 1985a) may reduce the effective populations size.

(5.1.2.5.2) Inter-generation mating

Similar to non-assortive mating, inter-generation mating can cause the reduction of the effective population size, which in essence will increase the proportional contribution of a particular genome to the population.

(5.1.2.5.3) 'Bottlenecks' effect

Variation in the average reproductive output of the adult population will effectively cause an unequal genetic contribution to the next generation and thus reduce the effective population size. Intrabrood correlation in survival rate (e.g. selection experiment in Chapter 5)may contribute to the random variation already introduced by the winnowing of the larval population. In

such cases, the effective population size equals

$$Ne = \frac{4N - 4}{Vk + 2} \qquad \text{(equation 5.7)}$$

where N is the actual number of adults in the populationand Vk is the variance in progeny per parent contributing to the next generation.

Similarly, the bottleneck effect can also be caused by the fluctuation of the actual population size, especially if between-generation variation is high. The effective population size is equal to the harmonic mean of the population size:

$$\frac{1}{Ne} = \frac{1}{t} \left(\frac{1}{Ni} \right) \qquad \text{(equation 5.8)}$$

where t is the number of generations and Ni is population size of the ith generation.

Many natural and artificial phenomena could cause a bottleneck effect in natural populations. Ice-ages were the major cause of bottleneck effects that have lasted to the contempory populations. Similarly, severe weather and overfishing on isolated populations can have similar effects.

(5.1.2.5.4) Founder effect - a special 'bottleneck'

In the case of a new colonizing population, the initial population size can be extremely small (e.g. less than 10). In such cases, the rate of change of genetic variation would depend upon the rate of population growth. The slower the population growth, the faster the decline of heterozygosity as some of the founding members would die before contributing equally to the

genetic composition of the new population (i.e. Ne would be even smaller).

Initially, the level of heterozygosity would be almost the same as in the founding population as most of the loss of genetic variation would be of the rare alleles. The population would then retain similar capacity to response to selection but the rate to loss or fixation can be much faster than the original population. Mayr (1954,1963) suggested that rapid evolution is likely to caused by founder effects, which may even mark the origin of new genera. This has formed the basis for the proposal of 'punctuated equilibrium' by some palaeontologists (Eldredge and Gould, 1972; see next chapter). Most aspects of the effect of founders on speciation were reviewed in Carson and Templeton(1984).

(5.1.3) Genetics variation in Crustaceans

The first study of population genetics in crustaceans was concerned with the study of polychromatism in the intertidal copepod, Tisbe reticulata(Bocquet,1951) and the isopod, Sphaeroma serratum (Bocquet,1953). This and the subsequent study of colour and morphological polymorphisms in similar organisms has established a tradition of population genetic study in crustaceans, and has no doubt contributed to the "modern synthesis" of evolutionary theory. Many of the earlier studies have been tabulated and summarised in Hedgecock et al. (1982).

Since the early sixties, with the development of electrophoretic techniques and the subsequent search for genetic variation, a sizable number of works have been carried out in

crustaceans. Probably attributed to the historical tradition and their convenience to work with, population genetic studies have mostly been concentrated on the smaller crustaceans, with much less attention given to the Decapoda, and therefore the economically important species (Hedgecock, 1986). In the survey of Nevo(1978) of 243 species of plants and animals, invertebrates(less Drosophila) have the highest level of genetic variability (P=0.399; H=0.100) when compared to both plants and vertebrates.

In the survey of crustaceans by Hedgecock et al.(1982), a similar but slightly lower level of genetic variability (P=0.305; H=0.073) was inferred. The same authors also revealed distinct among-taxa variation of genetic variability within crustaceans. Kruskal-Wallis non-parametric analysis of variance on H and P over 10 major taxonomic groups was highly significant ($p << 0.001$). Diplotacea, copepoda, cirripedia and euphauseacea have much higher levels of variation ($P >> 0.38$; $H >> 0.12$)than the remaining groups of crustaceans ($P << 0.28$; $H << 0.07$). On the otherhand, within taxa variation in genetic variability also exist. In copepoda, species of the genus Tisbe and Tigriopus have values of H at 0.063 and 0.196 respectively (Battaglia et al.,1978). In Cirrepedia, species of Chthamalus and Balanus have average values of H of 0.094 and 0.149 respectively (Hedgecock,1979). A similar range of heterozygosity was also observed in three species of Euphausiacea (Ayala et al., 1979). Hedgecock(1982) suggested that a general trend towards higher genetic heterozygosity with relatively small body size or low mobility is evident. Such coarse-grained adaptation has also been

related to the high level of genetic variability in seed plants (Hamrick et al., 1979).

Table (5.1) summarised the overall data on the genetic variability of various crustacean groups while table (5.2) summarised data of individual studies in Decapoda. Data on Palaemonidae will be reviewed in the discussion of this chapter in relation to the new data of the present thesis. Within the Decapoda, Penaeid prawns generally have very low levels of genetic variability. Most species of Penaeid prawns have life cycles with both estuarine and oceanic phases, plus seasonal migration to various depths on the continental shelf. Therefore, it is unlikely that the low level is attributed to "bottleneck effect" or small population size. Mulley and Latter (1980) suggested the widespread nature of these prawns in various different habitats has caused the selective elimination of mutational variation, and thus accounts for the low values of heterozygosity (Cf. environmental heterogeneity-trophic diversity hypothesis, next section). Within the family, the genus Metapenaeus also has an apparently lower average value to that of the Penaeus, although further data is required to confirm such a trend.

In the Astacidae, the apparent average values seem to differ between the three genera being studied, Orconectes, Cambarus and Procambarus. However, the range of values overlap, with the possibility that Procambus may have a lower value of

Table(5.1): Summary of allozyme variation in various taxonomic groups of crustaceans

Taxonomic group	Number of species	Number of loci per species	Proportion of polymorphic loci	Expected proportion of heterozygous individuals
Anostraca(b)	5	22.1	0.44	0.13
(p)	1	22.0	0.32	0.138
Cladocera(b)	4	13	0.173	0.073
(p)	2	13	0.11	0.065
Copepoda	5	19	0.414	0.142
Cirripeda	15	19.2	0.552	0.120
Peracarida	4	18.5	0.214	0.073
Amphipoda *	6	19	0.27	0.064
Euphausiacea	3	31.3	0.625	0.137
Penaeoidea	20	29.8	0.248	0.0323
Palinura	2	28	0.249	0.050
Astacidae	11	18.5	0.130	0.0376
Anomura	19	23.3	0.271	0.068
Brachyura	23	24.0	0.200	0.039

(b) bisexual or cyclical parthenogenetic species
(p) parthenogenetic or asexual species

Data for Decapoda based on table(6.2);
Anostraca and Cladocera from Hedgecock(1986);
Amphipoda from Seigismund et al.(1985) and
others from Hedgecock et al.(1982)

Table (5.2) : Genetic diversity of Decapoda *

Species	Loci	P	H	Reference
		Penaeidae		
P.aztecus	24	0.33	0.089	Lester(1979)
P.duorarum	24	0.33	0.084	" "
P.setiferus	24	0.29	0.070	" "
P.stylirostris	24	0.25	0.06	" "
P.vannamei	24	0.16	0.02	Lester(1983)
P.kerathurus	18	0.169	0.067	Mattoccio et al.(1986)
P.latisulcatus	18	0.111	0.028	Richardson(1982)
P.kerathurus	34	0.265	0.055	De Matthaeis et al.(1983)
P.japonicus	31	0.387	0.121	" "
P.plebejus	40	0.24	0.022	Mulley and Latter (1980)
P.latisulcatus	40	0.32	0.032	" "
P.longistylus	40	0.08	0.006	" "
Metapenaeus macleayi	40	0.17	0.026	" "
M.bennettei	40	0.20	0.020	" "
M.endeavouri	40	0.20	0.030	" "
M.ensis	40	0.020	0.013	" "
M.insolitus	40	0.10	0.010	" "
M.enboracensis	40	0.17	0.019	" "
		Pandalidae		
Pandalus danae	28	0.107	0.020	Berthelemy(1978)
P.jordani	25	0.250	0.022	" "
P.platyceros	28	0.270	0.045	" "
Pandalopsis ampla	15	0.267	0.072	Gooch & Schopf(1973)
		Crangonidae		
Crangon franciscorum	30	0.267	0.057	(1)
C.nigricanda	30	0.231	0.053	(1)
		Nephrophidae		
Homarus americanus	43	0.191	0.038	Tracey et al.(1979)
H.gammarus	41	0.220	0.052	Hedgecock et al.(1977)
		Astacidae		
Orconectes virilis	19	0.111	0.029	Nemeth & Tracey(1979)
O.propinquis	18	0.182	0.060	" "
O.ommunis	19	0.138	0.042	" "

(continue)

Table (5.2) : (continued)

Species	Loci	P	H	Reference
Cambarus robustus	19	0.100	0.040	" "
C.bartoni	18	0.167	0.083	" "
C.latimanus	15	0.200	0.076	" "
C.latimanus	19	0.053	0.013	Brown(1981)
Procambarus pubesceus	19	0.105	0.015	" "
P.hirsutus	19	0.105	0.031	" "
P.trogladyles	19	0.105	0.012	" "
P.acutus	19	0.210	0.025	" "
P.raneyi	19	0	0	" "
		Palinuridae		
Panulirus interuptus	27	0.222	0.038	(1)
P.cygnus	29	0.276	0.061	(1)
Jasus edwardii	32	0.03	0.015	Smith et al.(1980)
J.novaehollandae	32	0.06	0.028	" "
		Xanthidae		
Eurypanopeus depressus	24	0.074	0.021	Turner & Lyerla(1980)
Panopeus herbstii obessa	24	0.042	0.025	" "
P.herbstii simpsonii	25	0.120	0.024	" "
P.purpureus	31	0.290	0.051	(1)
Rhithropanopeus harrisii	15	0	0	(1)
Xanthodeus sternbergii	20	0.200	0.034	(1)
Menippae caneria	38	0.260		Bert(1986)
Trapezia intermedia	25	0.175	0.020	Huber(1985)
T.digitalis	25	0.425	0.070	" "
T.ferregihnea	25	0.200	0.027	" "
T.wardii	25	0.200	0.039	" "
T.flavomaculata	25	0.150	0.038	" "
T.coralina	25	0.150	0.050	" "
T.formosa	25	0.175	0.039	" "
T.cymodace	25	0.200	0.053	" "

(continue)

Table (5.2) : (continued)

Species	Loci	P	H	Reference
		Ocypodidae		
Macrophthalmus hirtipes(marine)	22	0.091	0.0453	Sin & Jones 4(1983)
(estuarine)	24	0.083	0.0383	" "
Ocypode occidentalis	22	0.227	0.046	(1)
O.quadrata	24	0.167	0.011	(1)
Uca musica	23	0.261	0.097	(1)
U.princeps	24	0.167	0.028	(1)
U.speciosa	26	0.231	0.031	(1)
U.spinicarpa	26	0.115	0.029	Salmon et al(1979)
		Grapsidae		
Hemigrapes oregonensis	30	0.233	0.040	(1)
Pachygrapsus crassipes	22	0.174	0.023	(1)
P.transversus	27	0.259	0.032	(1)
		Gercarcinidae		
Gercarcinidus quadratus	23	0.304	0.033	(1)
Sesarma cinereum	13	0	0	Gooch(1977)
		Scyllaridae		
Thenus orientalis	28	0.214	0.034	(1)
		Callianassidae		
Callianassa californiensis	38	0.316	0.080	(1)
C.sp	24	0.375	0.099	(1)
Upogebia pugettensis	34	0.294	0.070	(1)
		Galatheidae		
Galatheia californensis	19	0.211	0.075	(1)
Munida hispida	25	0.320	0.073	(1)
Munidopsis diomedia	12	0.250	0.123	(1)
M.hamata	28	0.250	0.085	(1)

(continue)

Table (5.2) : (continued)

Species	Loci	P	H	Reference
		Porcellanidae		
Pachycheles rudis	30	0.200	0.049	(1)
Petrolisthes cinctipes	28	0.179	0.052	(1)
		Hippidae		
Emerita talpoida	25	0.200	0.07	Beckwitt(1985)
E.analoga	22	0.455	0.125	(1)
Hippa pacifica	19	0.105	0.009	(1)
		Paguridae		
Pagurus granosimanus	24	0.250	0.247	(1)
		Coenobitae		
Coenobita compressus	18	0.278	0.079	(1)
C.clypeatus	12	0.250	0.083	(1)
		Diogenidae		
Calcinus obscurus	23	0.304	0.060	(1)
C.tibicen	19	0.263	0.044	(1)
Cibananus panamensis	21	0.286	0.095	(1)
C.albidigitus	20	0.300	0.018	(1)
C.antillensis	26	0.269	0.035	(1)
		Calappidae		
Matuta lunaris	29	0.069	0.028	(1)
M.planipes	24	0.167	0.009	(1)
		Cancridae		
Cancer gracilis	23	0.217	0.050	(1)
C.magister	32	0.094	0.013	(1)
		Portunidae		
Callinectes arcuates	22	0.455	0.128	(1)
C.sapidus	25	0.320	0.081	(1)
C.sapidus	26	0.489	0.766	Cole(1978)
Potunus sanguinolentus	31	0.226	0.063	(1)
Charybdis callianssе	23	0.130	0.026	(1)
C.sp	17	0.294	0.053	(1)

* Palaemonidae is reviewed in table (5.18)
Loci - number of loci; P - frequency of polymorphic loci with the most common allele does not exceed a frequency 0.99;
H - frequency of heterozygosity observed
(1) Nelson and Hedgecock(1980), these authors only reported He

heterozygosity than Cambarus. Also, all the values for Procambarus came from one single study (Brown, 1981) and that his value for Cambarus latimanus (0.013) was several times lower than that observed in Nemeth and Tracey (1979; Ho = 0.076).

In the brachyurans, most of the values reported so far are He values (see 5.1.8) reported by Nelson and Hedgecock(1980) which are subsequently listed in Hedgecock(1982). Meanwhile, the average values of Ho of 7 species from other studies gives a value of 0.038, which is almost identical to the average value of He of Hedgecock(1982).

The amount of genetic variability in crustaceans was also related to the particular locus being studied. PGI, PGM, LAP, ALD and SDH tend to be more polymorphic than GDH, G3PDH, LDH, AMY, TO and PT (general proteins). Thus, 17% of average crustacean populations were heterozygous at PGI while GDH was unvaried in 17 different species.

(5.1.4) Gene diversity and environmental heterogeneity

With accumulating data on the genetic diversity of invertebrates and crustaceans, several authors have purported that allozymic variation may play a role in the adaptation of decapods. Several hypotheses have related gene diversity (heterozygosity) to environmental heterogeneity.

Nevo(1978) suggested that the amount of genetic polymorphism and heterozygosity vary non-randomly between loci, population, species, habitats and life zone and are correlated with ecological heterogeneity. The trophic resource-stability hypothesis of Ayala and Valentine (1974, 1978) suggested that in

trophically stable environments, where food resources are predictably invariable over time, interspecific competition leads to the coarse grain adaptation strategies (low homeostatic mechanisms, low behavourial plasticity, low mobility, etc) in response to spatial heterogeneity, resulting in a large number of specialised alleles and has increased heterozygosity. In environments where there is wide temporal variation in abundance of food resources with generation time, animals generalise their food needs in order to pursue fine grain adaptive strategies (good homeostatic mechanisms, behavourial plasticity and mobility, etc). This results in the fixation of a small number of highly adaptive alleles and hence lower heterozygosity. Selander and Kaufman(1973), by comparing heterozygosity of various animals, suggested that heterozygosity is generally inversely correlated with size, and thus mobility of animals. Gillespie and Kojima(1968), on the otherhand, related enzyme polymorphism to the specificity of the enzyme substrates involved.

Nelson and Hedgecock(1980) reviewed and measured the heterozygosity of 97 species of decapods and put forward the environmental heterogeneity-trophic diversity hypothesis. The hypothesis was based on observed correlation between heterozygosity and environmental parameters measured as well as between different groups of enzymes, probably representing a combination of most of the previous hypothesis mentioned. It purported that enzymes of basic energy metabolism (Group I enzymes) appear to be positively correlated with measures of environmental heterogeneity, while variation of enzymes having digestive or detoxifying functions (Group II enzymes)appears

positively correlated with phyletic diversity or trophic resources.

Increasingly, however, some doubts are expressed on the correlation of heterozygosity with environmental heterogeneity. Values of Ho seems to vary more than predicted from the various hypotheses. The major criticisms include:

(a) Different loci are associated with different degree of genetic variability. Some are more conserved than others;

(b) In many species of Decapoda (and in crustaceans), there may be insufficient variation along either genetic or ecological axes to permit statistical detection of such correlation;

(c) The heterozygosity of a population depends on the interaction of mutation, selection, gene flow and random genetic drift, as well as historical events like bottleneck effects and founder effects.

The measurement of heterozygosity on a population of a species is thus unlikely to relate to the environmental heterogeneity alone. Even one of the proposer of the theory acknowledged that: " even if the environmental heterogeneity-trophic diversity hypothesis is true on average, it has little predictive power at the level of individual species or populations" (Hedgecock,1986).

(5.1.5) Ontogenetic development of isozymes

Apart from intraspecific variation at the population and the individual level, different isozyme pattern have also been reported for different developmental stages of an organism. When Markert and Møller (1959) first put forward the isozyme concept, ontogenetic variation was already described, supplemented with a more detailed analysis in a later paper (Markert and Ursprung, 1962).

Apart from its immense value in the study of developmental biology itself(Markert,1975), the ontogenetic changes of isozyme pattern may also be related to special physiological function at different developmental stages. Furthermore, ontogenetic study may reveal additional enzyme loci, giving the full picture of interspecific variation, within the limit of electrophoretic techniques. However, while there is an extensive literature on the ontogenetic expression of isozymes in insects, fish and vertebrates, only a handful of reports have dealt with the subject in crustaceans.

The present study aims to carry out prelimnary investigations into the ontogenetic expression of isozymes in M.nipponense and M.rosenbergii.

(5.1.6) Application of biochemical population genetics to aquaculture

The potential for application of modern genetics in aquaculture has already been discussed in Chapter 1. The specific cases in which biochemical population genetics can be applied in aquaculture will be presented here.

(5.1.6.1) Genetic markers for broodstock management and selection programmes

Attributed to the ability to detect biochemical phenotypes in small biopsy samples, electrophoretic techniques can be used to characterise broodstocks from both hatchery and wild populations, which would be an invaluable tool for large scale selection programme.

More recently, genetic variation of trypsin-like isozymes have been observed to correlate directly to fish size of Atlantic

salmon (Torrissen, 1987). This would form an invaluable tool for selective breeding programme for size.

The direct examples of the association of enzyme polymorphism and physiological function have also been reported in the case of LAP locus in Mytilus edulis (Hilbish et al., 1982) and GPT in Trigriopus californicus (Burton and Feldman, 1983) (see Chapter 5). However, such examples are still comparatively rare.

(5.1.6.2) Choice of species and species identification

As pure biological entities are a prerequisite for modern production systems, systematic information or culture performance studies of the relevant species should preceed any large scale aquaculture project, especially in groups with a large number of cogenic species (e.g. Macrobrachium). Many ex-established species have been proved to consist of a number of sibling species or subspecies (e.g. Brown, 1973). One such example will be vividly provided in the next chapter. Modern systematic techniques (e.g. karyology, restriction enzyme digest, etc), including protein electrophoresis, not only delineate species, but can also generate quantitative data on the phlogenetic relationships between species. This, in turn, can provide valuable information on the likelihood of artificial hybridisation between species.

(5.1.6.3) Assessing genetic variability and the possible association of heterozygosity with fitness

The interpretation of biochemical phenotypes generates gene frequency data which can be used to compute various statistical indices to quantify the variability of population (e.g. Wright's

coefficient of inbreeding, Nei's coefficient of genetic variation). Not only can such indices be used to monitor the particular hatchery stock, but they can also be used readily to compare with published data of wild and other commercial stocks.

Empirical studies have reported the association of individual heterozygosity with growth rates, developmental stability and oxygen consumption among gymnosperms, angiosperms, invertebrates and vertebrates. Mitton and Grant(1984) inferred that "there is sufficient evidence on hand to state that individual organisms' level of heterozygosity is a major organising principle in natural population of plants and animals." However, opposing evidence exists to justify hesitation to accept such a generalised theory. Aquatic examples of positive correlation between growth rate and heterozygosity includes oyster Crassostrea gigas (Singh and Zouros, 1978), mussel Mytilus edulis (Koehn and Gaffney, 1984), Mulina lateralis(Garton et al.,1984). On the other hand, no such relatiohship was observed for the plaice Pleuronectes platessa (McAndrew et al.,1986) and scallops Pectin maximus (Beamont et al.,1985). A lower amount of developmental asymmetry was observed in heterozygous fish Peociliopsis monacha (Vrijenhoek and Lerman,1982), Fundulus heteroclitus (Mitton,1978) and Salmo trutta (Leary et al.,1983).

It is perhaps reasonable to assume that a significant increase in individual heterozygosity would have immense implication in the general physiology of the individual concerned. However, fitness trait like growth traits and

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It is perhaps reasonable to assume that a significant increase in individual heterozygosity would have immense implication in the general physiology of the individual concerned. However, fitness trait like growth traits and

developmental stability are complex traits, generally involving multi-gene families. The correlation of such traits with individual heterozygosity of general enzyme loci thus depends on the linkage between such loci with the loci concerned. It must be noted that while there exist various developmental genes (e.g. Homeobox genes) for the control of morphogenesis, the differentiation of antero-posterior asymmetry (at least in Drosophila melanogaster) was in fact resulted from a morphogenetic gradient of substances in the cytoplasm, rather than individual genetic switches (Lehmann and Nusslein-Volhard, 1987). It is possible that both environmental and genetic factors have contributed to the development of meristic symmetry.

Nevertheless, the existence of genetic variability is the basis for artificial selection and individual heterozygosity is undeniably a useful estimation.

(5.1.7) Genetic indices used

Mendelian inheritence has been assumed for the interpretation of allele frequency from electrophoretic phenotypes. Frequency of any allele, p, of a locus, is calculated from

$$p = (2HO + HE)/2N \qquad \text{(equation 5.9)}$$

where HO and HE are the observed frequency of homozygous and heterozygous individuals respectively while N is the total sample size.

The expected frequency of phenotypes for the Hardy-Weinberg equilibrium then is calculated from,

$$HO' = p_i^2 \qquad \text{(equation 5.10)}$$

$$HE' = 1 - \sum p_i^2 \qquad \text{(equation 5.11)}$$

where HO' and HE' are expected frequency of homozygous and heterozygous individuals according to Hardy-Weinberg equilibrium and p_i is the frequency of the ith allele. For a three allele locus, the HE' is calculated from two times the product of the frequency of the two particular alleles involved.

The Hardy-Weinberg equilibrium is then tested with X^2 tests or G-tests for goodness of fit between the observed and the calculated frequency of phenotypic distribution. Statistical indices are based on Sokal and Rohlf(1981). The G-test, whose distribution approximates to that of X^2, is defined as

$$G = 2 \sum \text{obs} \ln (\text{obs}/\text{exp}) \qquad \text{(equation 5.12)}$$

The X^2 test, and to a certain extent the G-test, require the expected frequency to have no single cell less than unity and no less than 20% of the the cell less than 5% (Siegel,1956). In such event, the cells with the smallest frequency would be combined for the test. This would be possible for loci with three alleles, for loci with 2 alleles, the result of the G-test has to be contended with.

P, the proportion of polymorphic loci, is the number of loci with the frequency of the most common alleles less than 0.99. In some other studies, the demarckation point of 0.95 has been used. In such cases (e.g. Chow and Fugio,1935a & b), the value of P would be recalculated for the purpose of discussion.

Ho, the observed heterozygosity is the average observed frequency of heterozygous individuals per locus. It would have the same value as the average heterozygous loci per individual,

but with a different variance.

He, the expected or the calculated heterozygosity, is calculated from the equation,

$$He = \sum (1 - \sum J_i^2) \qquad \text{(equation 5.13)}$$

where J_i is the frequency of the ith allele. He is also referred to as the 'genetic diversity' (Nei, 1975).

Genetic similarity, I, of Nei(1975) between 2 taxa x and y , is the average of the genetic similarity per locus I_l over all loci.

$$I_l = \frac{J_{xy}}{(J_x J_y)^{1/2}} \qquad \text{(equation 5.14)}$$

where J_x and J_y are the square of the frequency of the particular allele in taxa x and taxa y respectively and that J_{xy} is the product of the frequency of the alleles in taxa x and taxa y. A taxa, in this case, can be a sub-population of a species.

The standard genetic distance, D (Nei, 1975), is than defined as : $D = -\ln I$ (equation 5.15)

D is designed to relate to the number of codon substitution. The mathematical models are based on Nei(1971,1972) and are summarised in Nei(1975). It is related linearly to geographical distance on the stepping-stone model (Maruyama, 1970; in Nei, 1972) of gene flow of finite length.

The estimated value of D is theoretically equal to

$D = 2 c n_T @ t$ (equation 5.16).

where c is the proportion of amino acid substitution detectable be electrophoresis (see next section); n_T is the total number of

amino acid codons; @ is the rate of amino acid substitution per year and t is the time since genetic isolation. Therefore, with the knowledge or assumption of the first three parameters, it is possible to give an estimate of the time of genetic isolation between the two taxas involved. More detailed discussion on the molecular clock will be included in the next chapter.

Nei(1978) investigated the magnitudes of systematic bias involved in the estimation of Ho and D. It has been shown that the number of individuals to be used for estimating H can be very small if a large number of loci are used and that the values of H is low. The number of individuals to be used for estimating genetic distance can also be very small if the genetic distance is large and the average heterozygosities of the two species compared are low.

Gorman and Ranzi(1979) carried out an empirical study on the effect of the sample size on both D and Ho. Both parameters were relatively unaffected by sample size. In the extreme case of a sample size of 1(for inter-specific comparision), deviation of D from large sample size represented a D value of smaller or equal to 0.1 and did not influence the phylogenetic relationship. For Ho, a sample size of 8-12 individuals gave an estimate within 1% of the percentage of heterozygosity calculated for a large sample.

Deviation of D from real values can be great if intra-specific comparision of local populations (low D values) on a small number of loci is carried out (Nei,1975). However, the distance matrix generated still represents the relative values between the comparision involved. While other indices of genetic

distance and similarity are available (see next chapter), D would be used in this chapter as it is rather robust to small sample size and that it is related to biological entities. Also, some studies have proved that the few common indices available are correlated to each other to a various degree (e.g. Hedrick,1975). Furthermore, as the most common indices used, it generates values readily comparable to other studies.

The construction of phylogenetic trees using electophoretic data, on the otherhand, will be a subject of discussion in the Chapter 6. The various comparable studies (e.g. Prager and Wilson,1978; Nei et al.,1983) generally have different conclusion on the choice of methods. In the population studies in the present chapter, the UPGMA method, the Fitch-Margolaish method and the restricted maximum likelihood method would be used, representing a fair spectrum of all the methods available. For intra-specific phylogentic analysis, the assumption of relatively constant evolutionary rates of the UPGMA method using Nei's standard genetic distance (its main criticism) is probably justified.

To measure the genic diversity within and between subpopulations of a species, the coefficient of gene differentiation, G_{ST} is used (Nei,1975). In the presence of multiple allele loci (as in the present study), G_{ST} is equal to a weighted mean of F_{ST} (equation 5.3) for all alleles (Nei,1975) and should be regarded as an extension of F_{ST}.

It is based on a model to study gene diversities within and

$$H_T = 1 - (1/s^2) \sum_k \left(\sum_i q_{ik} \right)^2 \qquad \text{(equation 5.17)}$$

where q_{ik} is the kth allele frequency of the ith subpopulations and s is the number of subpopulations.

Gene diversity within subpopulation is estimated by the expected heterozygosity, He, and D_{ST}, the average gene diversity between subpopulations is defined by the equation:

$$H_T = He + D_{ST} \qquad \text{(equation 5.18)}$$

and

$$G_{ST} = D_{ST} / H_T \qquad \text{(equation 5.19)}$$

While G_{ST} is a good measure of relative differentiation between subpopulations, its value is highly dependent on the value of H_T (Nei, 1975). Thus, G_{ST} can be very high for small value of H_T, even if the actual differentiation is small.

An alternative measure, Dm, the absolute degree of gene differentiation, can be used and is defined by the formula:

$$Dm = s\, D_{ST} \quad (s - 1) \qquad \text{(equation 5.20)}$$

This is an estimate of the minimum net codon difference between different populations and individual gene diversity within subpopulations, and is referred to as the minimum genetic distance by Nei(1975).

The ratio of the inter-population to the intra-population gene diversity is then defined as :

$$R_{ST} = Dm/He \qquad \text{(equation 5.21)}$$

(5.1.8) The limitations of electrophoretic techniques

The genetic variation inferred from electrophoretic analysis is very dependent on the ability of the technique to differentiate different proteins with different amino acid sequences, in addition to the catalytic properties of the enzymes to react with the staining recipes. The mobility of a protein through a gel, which determine the ability of electrophoresis to differentiate proteins depends on :

(a) the size of the protein ;

(b) the assymetry or conformation of proteins ;

(c) the isoelectric point, reflecting the balance between positive and negative charges of the proteins;

(d) the net charge at a given pH, reflecting the absolute excess charge of the protein concerned.

(a) & (b) involve the protein-gel fibre interaction while (c) & (d) affect the rate of migration in an electric field. The latter is generally considered to contribute more significantly to the mobility (Nei,1975), especially for similar proteins (e.g. allozymes) at a certain set of running conditions.

Substitution of an amino acid in a protein may or may not change the net charge. The amino acid may be a neutral one or that the substitution involves an amino acid identical in charge to the original one. Also, the substitutions of two oppositely charged amino acids may constitute a 'neutral' event. It is attributed to the latter that the accuracy of D, in relation to evolution divergence (equation 5.16). may decrease as its value increase to more than unity (Nei,1975).

There are various estimate as to the number of substitution which would result in a net charge detectable by electrophoresis.

Powell(1975) gave values of 22%, Lewontin(1974) gave 32% while Nei(1975) suggested that a value of 25% should be adopted. The latter figure has been adopted by most authors (e.g. Hedgecock, 1982).

Ideally, each enzyme-protein should be characterise physically and biochemically for estimation of relative mobility (Johnson,1977). However, such tests require an immense manpower and resources.

On the otherhand, apparent differences in electrophoretic mobility between allozymes can be caused by non-genetic factors, either artefacts on the gel or some post-translational modification of the proteins, including prolonged storage. In a comparative study, especially for phylogenetic studies, provided that different samples are kept under relatively identical conditions, these problems probably would not arise. Furthermore, repeating runs and using fresh samples as controls would enable such problems to be realised.

Different electrophoretically detectable loci tend to have a different tendency for genetic variation (Hedgecock, et al.,1982; Cf. Resource-stability-trophic diversity hypothesis). As both D and H are estimates over a number of loci, the only counter-measure then is to survey a large number of loci.

The use of electrophoretic data in a genetic survey also requires the assumption of Mendelian inheritance of electrophoretic loci. Such assumptions can only be proved by actual breeding experiments involving individuals with known phenotypes/genotypes. Such experiments have so far been performed in Artemia, Daphnia, Tisbe,Trigriopus, Gammarus, Ascellus, Jaera,

Porcello, and Homarus (Hedgecock, et al.,1982). Such formal proof of Mendelian inheritance require established techniques for breeding and holding the species concerned, and the corresponding facilities. Most studies, however, have to be content with the χ^2 test for the goodness-of-fit to the Hardy-Weinberg equilibrium as partial indication of Mendelian inheritance.

(5.1.7) Morphological studies on inter-specific variation of Macrobrachium

Macrobrachium has been known for its morphological differentiation between developmental stages and sexes of the same species (Holthuis,1952; also see Chapter 6). However, considering the size of the genus, there have been relatively few studies on the differentiation of the same species into geographical races.

The study of Johnson(1973,1960) on the different races of M.rosenbergii have formed the basis for later electrophoretic investigation by Hedgecock et al.(1979) and Lindenfelser(1984) (see discussion). The M.malcolmsonii population in the Indian sub-continent were also separated by Johnson(1973) into sub-species with different geographical distributions. M.m.malcolmsonii was named as the subspecies in India and Ceylon, M.m.kotreeanum for sub-species of Kotree and M.m.choprai for sub-species in the Gangetic plain of Assam. In the same paper, Johnson also reported on the sub-division of M.javanicum into M.javanicum javanicum and M.j.neglectum in the south-east Asia region, with the possibility of a third race in Celebes.

For the M.pilimanus complex in south east Asia, however,

neither Holthuis(1950) nor Johnson(1960) had reached a satisfactory solution. The later author suggested that the species actually consisted of a series of sibling species. Evidence from larval development of M.pilimanus and M.malayanum (Chapter 3) confirmed this suggestion .

Other intra-specific variation in larval development includes M.rosenbergii (Sarver et al.,1979; Chen and Chang ,1980), M.niloticum (Williamson,1972) and M.nipponense (see Chapter 4).

Mashiko (1983,a,b,c) studied two populations of M.nipponense in the Sagami River (Japan) and showed inter-specific variation for egg size, growth pattern, maturation age and larval salinity tolerance. Similarly, Chow and Fujio(1985c) reported that upper and lower populations of Palaemon paucidens inhabiting the same river also differed in egg size and rostrum formula. A parallel electrophoretic study revealed that overlapping zones showed a seasonal fluctuation in the proportion of the two types and consequently resulted in deviation from Hardy-Weinberg equilibrium. Each type must have different genotypic frequencies.

Based on 13 morphological characters for females and 16 for males, Corrillo(1969) reported the differentiation of four populations of M.acanthurus in Mexico. In Australia, Riek(1950) recognised four sub-species of M.australiense and two sub-species of M.atactum. Most subspecies of the genus Macrobrachium have been mentioned in Table (1.1). It is likely that that more Macrobrachium at the sub-species level will be reported.

(5.2)Material and Methods

(5.2.1) Collection sites for prawns

Wild populations of M.nipponense and M.rosenbergii were collected from different parts of the world. The collection sites were as follows :

M.nipponense (Fig.5.1)

Hong Kong	fish ponds in Au Tau, New Territories, Hong Kong.
Wuhan	East Lake, Wuhan, Hubei Province, People's Republic of China.
Canton	Pearl River Estuaries
Japan	Inba Nama (estuarine marsh) in Chiba, near Tokyo.

M.rosenbergii(Fig. 5.2a&b)

Australia	(AUST)	hatchery stock in Queensland
Bangladesh	(BANG)	Khulna-Sunderban mangrove area
Malaysia	(MALAY)	near to Universiti Pertanian Malaysia, Selangor, Malaysia.
Sri Lanka	(SRILANK)	Estuarine area near to Columbo
Thailand	(THAI)	near to Asian Institute of Technology, near Bangkok.
Indonesia	(INDON)	Java
Sabah-1	(SABAH-1)	Lingkungan River, Sabah, Malaysia. (Fig. 5.2b)
Sabah-2	(SABAH-2)	Membakart River, Sabah, Malaysia. (Fig. 5.2b)

For the analysis of intra-specific variation between populations, it is obviously ideal to have as great a number of samples as possible. The policy of the present investigation is to keep the sample size above 30 if possible. However, This was

PLATE (I) :

Male and female *Macrobrachium nipponense*
(about actual size)

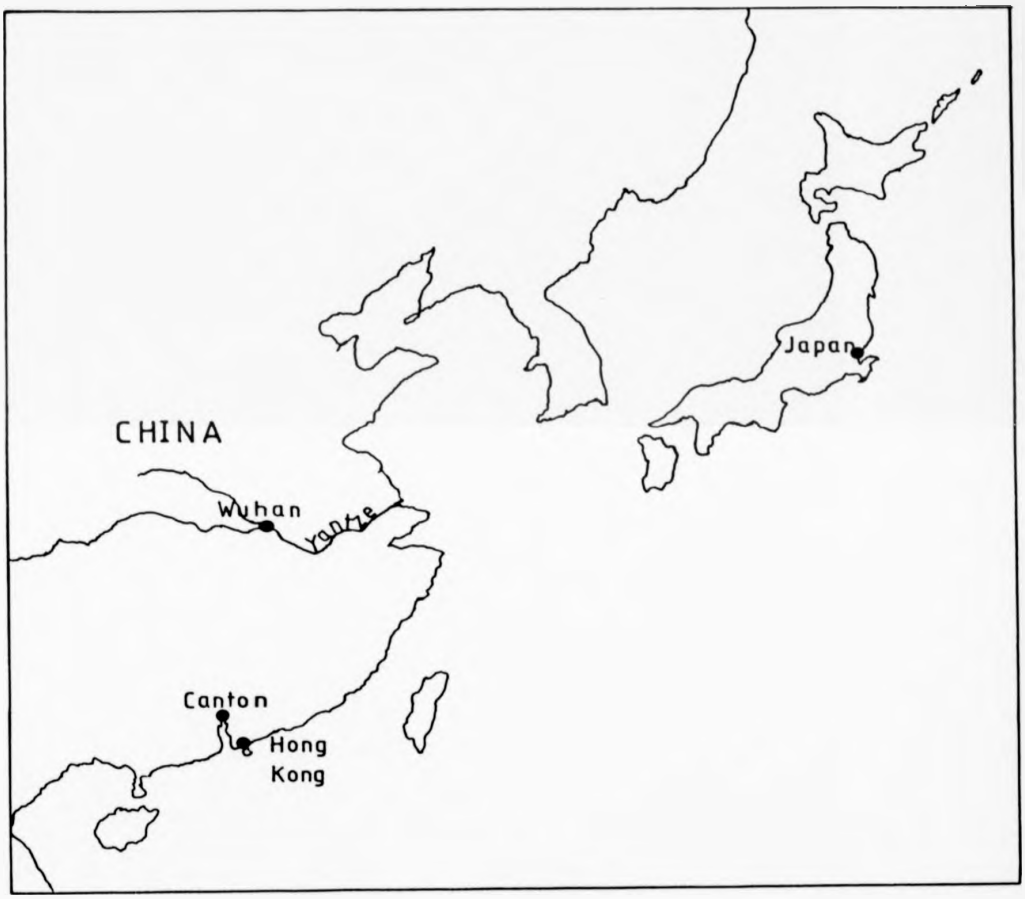
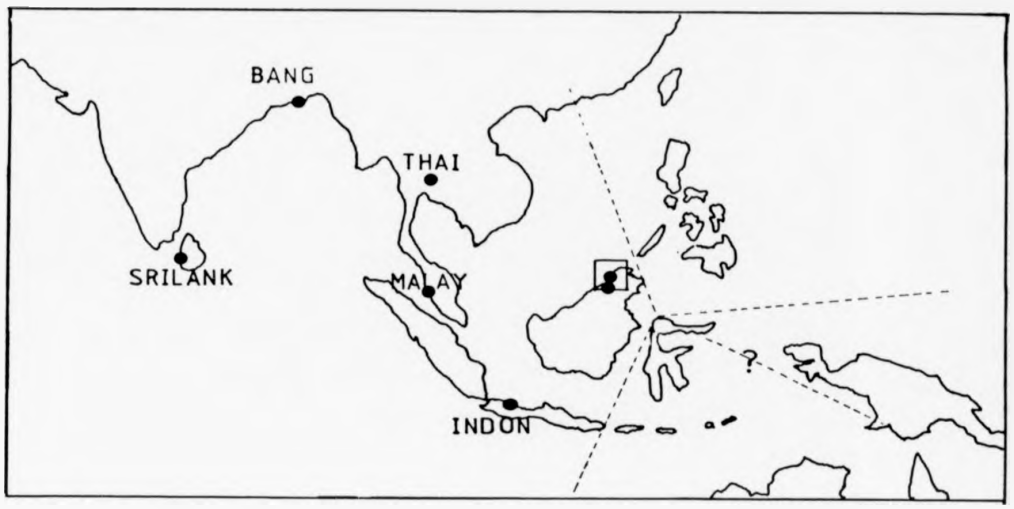
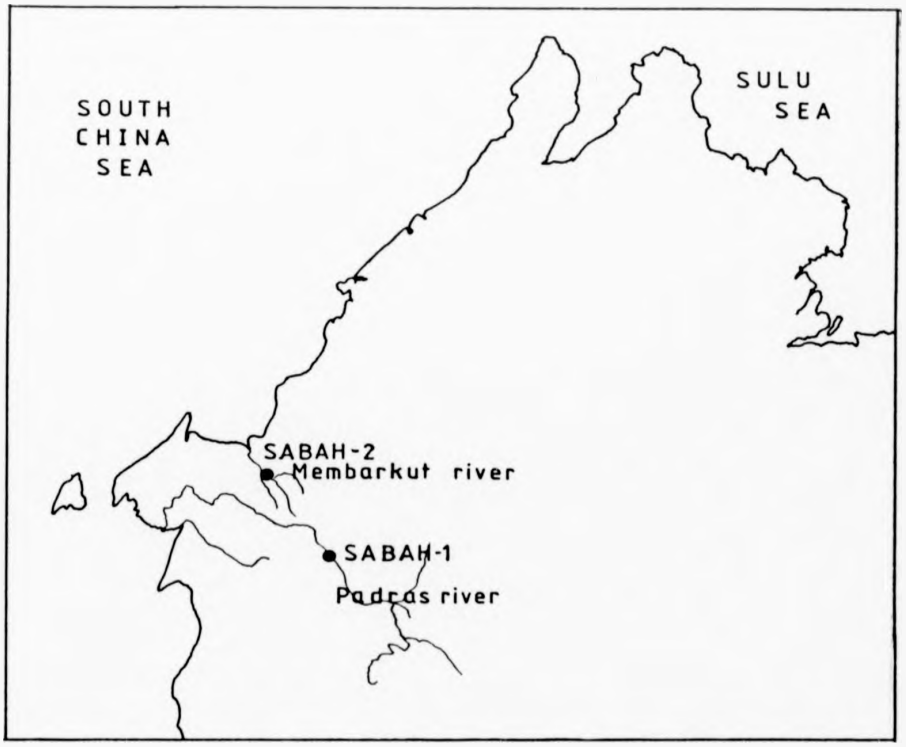


Fig. (5.1): Sampling sites for M. nipponense

Fig. 5.2

(a) Sampling sites for *M. rosenbergii*
(b) Sampling sites in Sabah for the two populations analysed (Sabah-1 and Sabah-2)


BANG
THAI
SRILANK
MALAY
?
INDON


SOUTH
CHINA
SEA
SULU
SEA
SABAH-2
Membarkut river
SABAH-1
Padas river

not always possible because of sampling difficulties.

(5.2.2) Sampling preparation

All prawns were sampled live, frozen, and transferred subsequently to Stirling with ice-packs or on dry-ice. Prawns were individually dissected for two tissues, the hepatopancreas and the abdominal muscle. All muscle blocks were dissected from the second and third abdominal segments. In smaller species, the fourth and the fifth segments were also used. The hepatopancreas extracted from the cephalothorax carefully, trying to avoid the gonadal tissue as far as possible. The eyes were also taken initially, but preliminary tests did not show any differentiation between the eyes and the abdominal tissues in the enzymes studied. The particular tissue utilised for each individual loci are listed in table(5.3).

Each tissue was homogenised in half volume of distilled water and kept frozen (-70°C) until use. Initially, samples were centrifuged lightly (about 1000g) before use. However, it did not improve the resolution in most enzymes and the step was not performed in later samples. Most enzymes were unaffected after storage of up to 6 months. However, there are several, including LAP-1, ODH, and all the kinases, in which resolution was drastically reduced after 2 weeks of storage after sample preparation.

(5.2.3) Electrophoretic systems

Electrophoresis were carried out with Shandon standard electophoretic trough and constant voltage/current power supplies. A starch gel slab (18cm X 15cm X 0.5cm) was prepared

from Connaught hydrolysed starch (12% w/v). The starch and buffer(Appendix I) solution was heated, degassed, and poured into a framed plate of the mentioned size. The gel was then covered by a second plate and allowed to cool before use. A vertical slit was then drawn at 4cm from the longer side of the gel slab. Sample solution were than transferred to the gel on filter paper strips (0.5-0.8cm). Care was taken not to transfer excessive solution, which would otherwise reduce resolution on staining. The gel was then connected to the reservoirs of buffer in the electrophoretic trough via cotton wicks. To prevent excessive dehydration, each gel was covered by a polystrene sheet, in addition to the trough cover. After the appropiate running time, the gel was immediately cut, with the help of the Shandon gel slicer, into three horizontal slices of about 2mm thickness. Each slice would then be stained for the appropiate enzymes.

All the enzymes tried are listed in table (5.3) with the numerical classification of the Enzyme Commission(E.C.). Macrobrachium, as a genus, has not been intensively studied electrophoretically . No standard protocols were available for enzyme electrophoresis and staining. Thereby, various buffer systems and enzyme stains were tried for the best results using an empirical approach. It would be impossible to list all the combinations of buffer systems and enzyme stains tried but the best resolving buffers are listed in table(5.4). Enzyme staining recipes were modified from Shaw and Prasad(1970), Harris and Hopkinson(1965) and Redfield and Salini(1980). The stains recipes with the best resolution are listed in Appendix (II).

Table (5.3) : Enzymes Investigated in the Present Study

Enzymes	Abbreviation	E.C. Class.	Subunit number *
Aspartate aminotransferase (Glutamat oxaloacetate)	AAT	2.6.1.1	1
Acid phosphatase	ACPH	3.1.3.2	1
Adenosine deaminase	ADA	3.5.4.4	1
Aconitase	ACON	4.2.1.3	1
Aldolase	ALD	4.1.2.13	4
Alkaline phosphatase	ALP	3.1.3.1	2
Adenylate kinase	AK	2.7.4.3	
Creatine kinase	CK	2.7.3.2	
Diaphorase	DIA	1.6.2.2	1
Enolase	Enol	4.2.1.11	
Esterase	EST	3.1.1.1	1
Fumerase (Fumarate hydratase)	FUM	4.2.1.2	
Guanine deaminase	GDA	3.5.4.3	
Glucose-6-phospate dehydrogenase	G6PDH	1.1.1.49	
@-Glycerophosphate dehydrogenase (Glycerol-phosphate dehydrogenase)	@-GPDH	1.1.1.8	2
L-Glutamate-dehydrogenase	GDH	1.4.1.3	1
Glutamate-pyruvate transferase	GPT	2.6.1.2	2
Hexokinase	HK	2.7.1.1.	1
Isocitrate dehydrogenase	IDH	1.1.1.42	2
Leucine aminopeptidase	LAP	3.4.11.	2
Lactate dehydrogenase	LDH	1.1.1.27	
Malic enzymes	ME	1.1.1.40	2
Malate dehydrogenase	MDH	1.1.1.37	2
Mannose phosphate isomerase	MPI	5.3.1.8	1
Octanol dehydrogenase	ODH	1.1.1.1?	
Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	
Phosphoglucose mutase	PGM	2.7.5.1	1
Phosphoglucose isomerase (Glucophosphate isomerase)	PGI	5.3.1.9	2
Superoxide dismutase	SOD	1.15.1.1	

* subunit structure inferred in the present investigation
blank places imply loci with poor resolution or lack of polymorphism

Table (5.5) : Buffers and tissue used for each enzyme loci

Enzyme loci	Tissue	Best buffer	Performance
AAT-1,-2	M	TCB	*
ACON	M	TEC	F
ACPH-1,-2	C	TCB	*
ACPH-3	M	TCB	F
ADA-1,-2	M	TEB	*
ALD	M	TEC	*
ALP	C	TCB	*
AK	M	CTC	F
CK	M	TEC	F
DIA	M	POULIK	VF
ENOL	M	TEC	VF
EST-1,-2,-3,-5	C	TCB	*
EST-4,-6	C	TCB	F
FUM	M	TCB	*
GDA	M	TCB	VF
G6PDH	M	TEB	*
@GPDH	M	TEC	*
GDH	M	TCB	*
GPT	M	TEC	*
HK	M	TEB	*
IDH	M	TEC	*
LAP-1	M	TCB	*
LAP-2	M	TCB	F
LAP-3	C	TCB	F
LDH	M	TEC	*
ME	M	TCB	*
MDH	M	TEC	*
MPI	M	TCB	*
NP	M	TCB	F
ODH	M	TEC	F
PK	M	TEB	VF
6PGDH	M	TCB	*
PGM	M	TCB	*
PGI	M	TEC	*
SOD	M	CTC	VF

(1) enzyme abbreviation as in table(6.1)
(2) M-abdominal tissue; C-cephalothorax
(3) buffer systems as in text
(4) * - good resolution which is used in the present study
F - faint resolution in some species, but resolvable in others
VF- very faint resolution in some species and unresolvable in most others.

(5.2.4) Sampling of larvae for ontogenetic studies

Larvae of M.nipponense and M.rosenbergii were hatched from broodstocks from Hong Kong and Malaysia respectively. The culture conditions were identical to the recirculation systems used in Chapter 4, only that a salinity of 10‰ was used. Several batches were tried to score for ontogenetic variation in isozymes. Stages of larvae were identified as in Kwon and Uno (1969, 1977). A number of larvae at each stage were pooled for each sampling. Care was taken to avoid sampling of Artemia and other debris from the culture media. The sample was then washed several times with distilled water before being homogenised with a half volume of distilled water. As the size of larvae increased with developmental stages, the sample size was reduced accordingly. The number of larvae sampled per stage were as follows:

Stage	no. of larvae
I	300-400
II	200
III-IV	100
V-VI	60
VII-IX	30
IX-PL	25

(5.3) RESULTS AND COMPUTATION

Most of the enzymes loci scored and the best conditions have been summarised in table (5.3). Phenotypes of some of the polymorphic loci would be described in Chapter 6.

(5.3.1) Population genetics of M.nipponense

32 enzyme loci in four populations of M.nipponense were studied for intra-specific variation. The inferred gene frequencies are summarised in table(5.5). 20 loci were monomorphic in all the populations studied.

(5.3.1.1) Hardy-Weinberg equilibrium

The observed genotypic frequency were tested for goodness of fit by both X^2 tests and G-tests. Most of the loci were at Hardy-Weinberg equilibrium. Those loci with X^2 values larger than p=0.05 are listed in table (5.6).

Most of the values have $p<0.05$ or $p<0.001$, and that the G-values are also substantially larger than the X^2 values, indicating that the effect was partly attributed to small values of the expected frequency. Of the 3 loci with $p<0.001$, 2 loci (AAT at Hong Kong, MPI at Japan) were loci of 3 alleles.The expected frequency of some phenotypes in these loci was considerably less than 0, and that pooling of the less common alleles (see section 5.1) substantially reduced the values of G. It is likely that most of the departure from H-W equilibrium were attributed to small sample sizes.

Table (5.5) :
Allele frequencies of M.nipponense populations

	HK	Wuhan	Canton	Japan	average
AAT-1A	0.170	0.000	0.000	0.000	0.043
AAT-1B	0.720	0.000	0.300	0.086	0.277
AAT-1C	0.110	1.000	0.525	0.879	0.629
AAT-1D	0.000	0.000	0.175	0.034	0.052
AAT-2A	1.000	1.000	1.000	1.000	1.000
ACPH-1B	1.000	1.000	1.000	1.000	1.000
ACPH-2B	1.000	1.000	1.000	1.000	1.000
ADA-1C	1.000	1.000	1.000	1.000	1.000
ADA-2B	1.000	1.000	1.000	1.000	1.000
ALD-B	0.670	0.610	0.688	0.613	0.645
ALD-C	0.330	0.390	0.313	0.387	0.355
ALP-A	0.650	0.390	0.500	0.850	0.598
ALP-B	0.350	0.610	0.500	0.150	0.403
EST-1B	1.000	1.000	1.000	1.000	1.000
EST-2B	1.000	1.000	1.000	1.000	1.000
EST-3A	1.000	0.950	0.663	0.475	0.772
EST-3B	0.000	0.050	0.337	0.525	0.228
EST-4A	1.000	1.000	1.000	1.000	1.000
FUM-1A	1.000	1.000	1.000	1.000	1.000
FUM-2B	1.000	1.000	1.000	1.000	1.000
G6PDH-A	1.000	1.000	1.000	1.000	1.000
GDH-B	1.000	1.000	1.000	1.000	1.000
@GPDH-1B	1.000	1.000	0.975	0.225	0.800
@GPDH-1C	0.000	0.000	0.025	0.775	0.200
@GPDH-2B	1.000	1.000	1.000	1.000	1.000
GPT-A	0.000	0.000	0.389	0.000	0.097
GPT-B	0.000	0.000	0.513	0.275	0.197
GPT-C	0.920	0.884	0.197	0.550	0.638
GPT-D	0.080	0.113	0.013	0.175	0.095

(to be continued)

Table (5.5) : (continue)

	H.K.	Wuhan	Canton	Japan	average
HK-1A	1.000	1.000	1.000	1.000	1.000
HK-2A	1.000	1.000	1.000	1.000	1.000
IDH-B	0.820	0.532	0.550	0.500	0.601
IDH-C	0.160	0.467	0.450	0.500	0.394
LAP-C	0.450	0.167	0.367	0.250	0.309
LAP-D	0.360	0.583	0.433	0.543	0.480
LAP-E	0.200	0.250	0.200	0.212	0.216
LDH-A	1.000	1.000	1.000	1.000	1.000
MDH-1A	0.200	0.060	0.000	0.850	0.278
MDH-1B	0.800	0.940	1.000	0.150	0.723
MDH-2B	1.000	1.000	1.000	1.000	1.000
ME-C	1.000	1.000	1.000	1.000	1.000
MPI-C	1.000	0.970	0.017	0.163	0.538
MPI-D	0.000	0.030	0.933	0.688	0.413
MPI-F	0.000	0.000	0.050	0.150	0.050
PGI-D	0.000	0.030	0.125	0.000	0.039
PGI-E	0.980	0.930	0.900	0.875	0.921
PGI-F	0.020	0.040	0.088	0.125	0.068
PGM-C	1.000	0.910	0.525	0.725	0.790
PGM-D	0.000	0.090	0.475	0.275	0.210
PGDH6-C	1.000	1.000	1.000	1.000	1.000
TO-B	1.000	1.000	1.000	1.000	1.000

H.K. - Hong Kong
-numerals of the same enzyme refer to different loci (isozymes: e.g. AAT-1, AAT-2).
-alphabets of the same loci refer to different alleles (allozymes; e.g. AAT-1A, AAT-1B) in

relation to all the species investigated

Table (5.6) : Summary of loci with significant departure from Hardy-Weinberg equilibrium in populations of M.nipponense

Population	loci	number of alleles	X^2	G (G')	p
Hong Kong	AAT-1	3	45.7	33.63(24.1)	<0.001
	GPT	2	10.4	5.88	<0.05
Canton	ALP	2	6.4	6.58	<0.05
	IDH	2	8.38	8.78	<0.05
Wuhan	ALP	2	10.28	10.45	<0.01
	PGM	2	7.59	4.70	<0.05
Japan	@GPDH	2	13.00	11.48	<0.001
	MPI	3	31.4	24.52(14.05)	<0.001
	6PGDH	2	9.24	7.43	<0.05

Table (5.7) : Indices of genetic variability of the different populations of M.nipponense

	Hong Kong	Wuhan	Canton	Japan	overall
P(%)	25.0	33.3	34.4	37.5	32.5
Ho(%)	7.4	7.5	11.5	13.1	9.86
He(%)	8.8	8.7	14.1	15.1	11.7
Ho/He	0.864	0.922	0.868	0.882	0.884
He(%)	8.8		14.6		
H_t	10.2		17.4	16.3	
G_{ST}	0.134		0.163	0.282	
Dm	0.028		0.056	0.061	

(5.3.1.2) Genetic diversity and population differentiation in M.nipponense

The various measures of genetic variation are summarised in table (5.7). The overall means for observed individual heterozgosity and percentage of polymorphic loci were 9.86% and 32.5% respectively. Both values increased in the direction of Hong Kong - Wuhan - Canton - Japan. The overall coefficient of genetic variation, G_{ST}, was 0.282. The overall calculated heterozygosity was 11.7%. This value, when compared to the observed value of heterozygosity, implied a slight deficiency of heterozygous individuals. The average ratio of H_o/H_e over all polymorphic loci for each populations ranged between 0.86 to 0.92. The freshwater groups (Wuhan/Hong Kong) having significantly smaller values of H_o, H_e and G_{ST} than the brackishwater group (Canton/Japan).

(5.3.1.3) Genetic distances and phylogenetic relationships between different populations of M.nipponense

Frequencies of Nei's genetic similarity , I, as calculated for each locus, are summarised in Fig. (5.3). Of the 32 loci, 20 were monomorphic (i.e. I=0) and 11 were less than 0.5. The overall genetic similarity and distance were 0.920 and 0.084 respectively. The matrix of genetic similarity, genetic distance and standard errors are summarised in table (5.8a&b) respectively.

The largest values of D were between Hong Kong/Japan and smallest were between Hong Kong and Wuhan. Canton, on the other hand, was as similar to the Hong Kong/Wuhan group as to the Japan population. A plot of D against relative geographical

distance (Fig.5.4) did not give significant values of r or r_s. However, a very significant value of r =0.99997 resulted if the Canton population was omitted from the analysis.

The phylogenetic trees drawn from the UPGMA method, the Fitch-Margoliash method and the REML methods are shown in Fig.(5.5). The topologies of the various methods generally have good agreement with each other. It is inferred that the Hong Kong and Wuhan populations are phylogenetically more related to each other than either the Canton or the Japan populations.

Table (5.8) : Genetic distances and identity between different populations of *M.nipponense*

Genetic identities

	Hong Kong	Wuhan	Canton	Japan
Hong Kong		0.959	0.916	0.885
Wuhan			0.930	0.906
Canton				0.923

Genetic distances/standard errors (below diagonal)

	Hong Kong	Wuhan	Canton	Japan
Hong Kong		0.042	0.087	0.122
Wuhan	0.037		0.072	0.099
Canton	0.054	0.048		0.080
Japan	0.064	0.057	0.051	

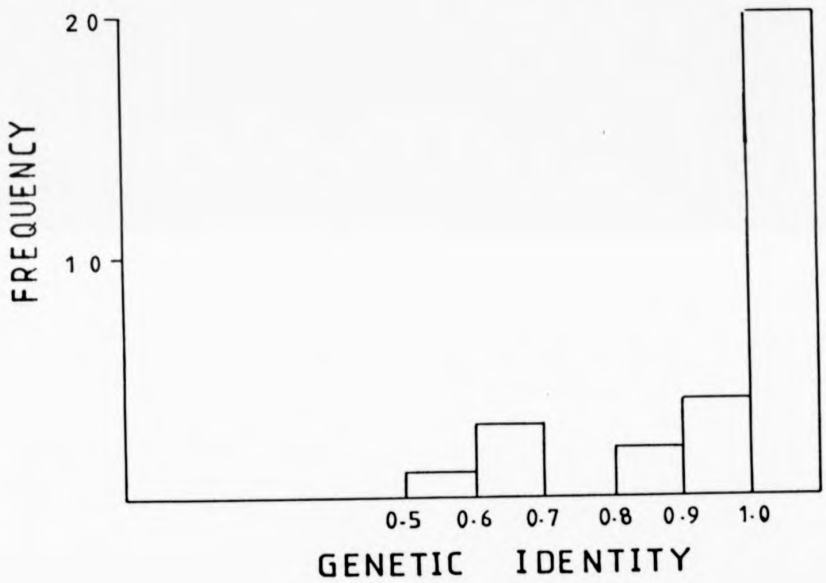


Fig. 5.3: Frequency distribution of average genetic distances at different loci for populations of M. nipponense

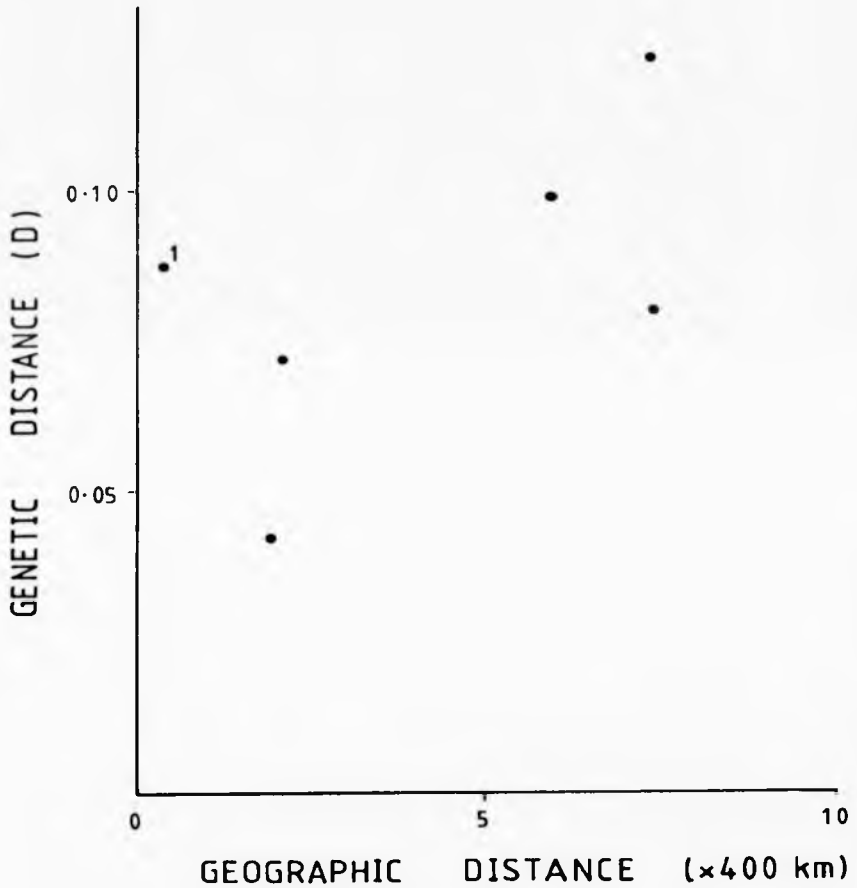


Fig. 5.4: Plot of genetic distances against corresponding geographic distances between the various populations of M.nipponense

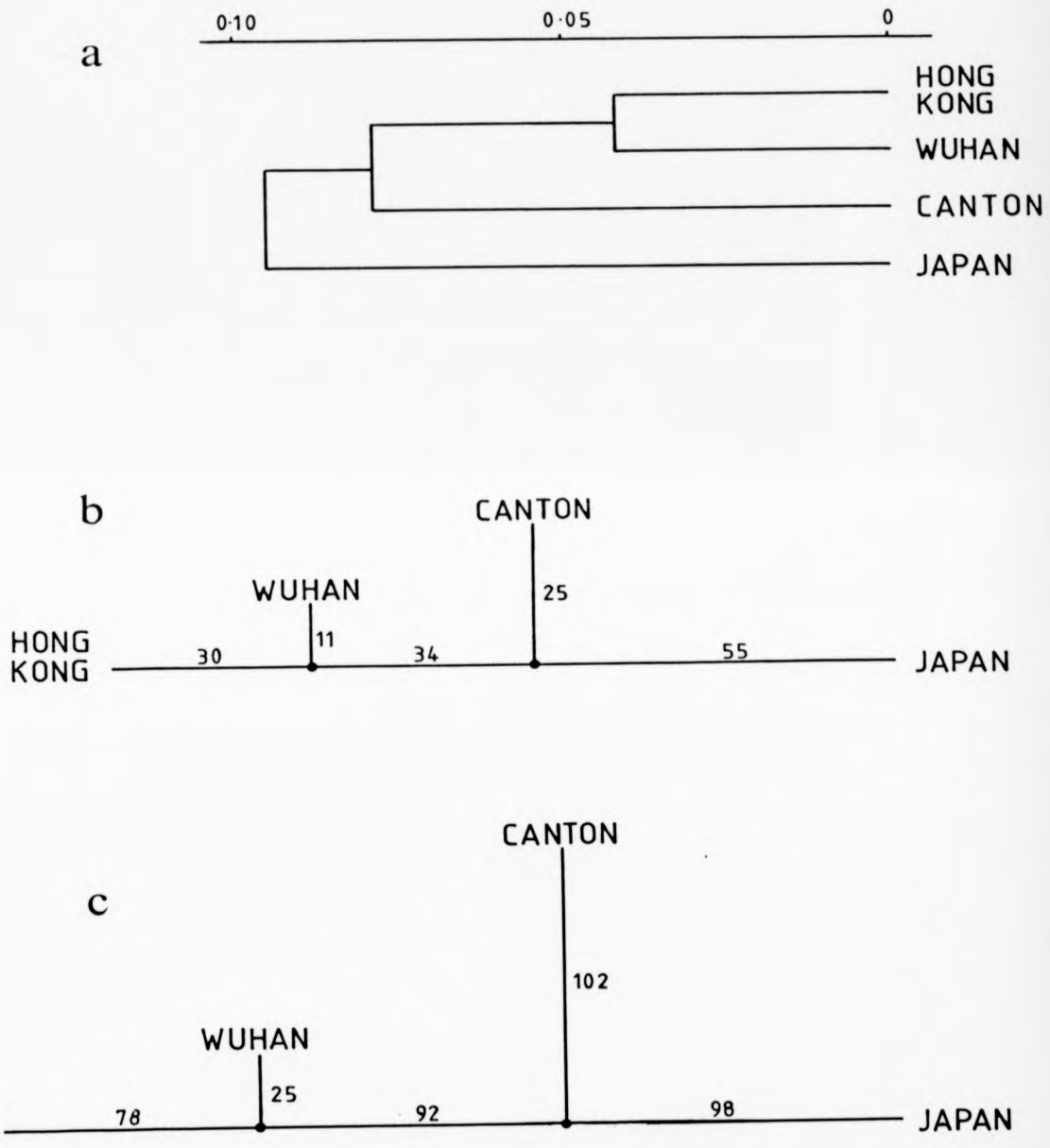
1 - comparision between Canton/Hong Kong populations, without which the correlation coefficient computed would be significant (p=0.05)

Fig. 5.5: Phylogenetic trees constructed for the populations of *M. nipponense* investigated

a. UPGMA method with Nei's genetic distance

b. Fitch-Margolaish method with Nei's genetic distance

c. Restricted maximum likelihood method, using allele frequency data of polymorphic loci


a
0·10
0·05
0
HONG KONG
WUHAN
CANTON
JAPAN
b
CANTON
WUHAN
25
HONG KONG
30
11
34
55
JAPAN
c
CANTON
102
WUHAN
25
HONG KONG
78
92
98
JAPAN

(5.3.2) Population genetics of M.rosenbergii

32 enzyme loci in eight populations of M.rosenbergii were studied for genetic variation. Phenotypes of some of the polymorphic loci will be described in chapter 6. The allele frequency data are summarised in table(5.9). Nine loci (ALD, ALP, EST-1, EST-2, GPT, LAP, MPI, PGI and PGM) were polymorphic in at least half of the populations while 20 loci were monomorphic in all the populations (Fig.5.6).

(5.3.2.1) Hardy-Weinberg equilbrium

All the observed phenotypic frequency agreed with the expected distribution at Hardy-Weinberg equilibrium. The only exception in all the populations is the MPI locus of the Bangladesh population with both X^2 and G values significant for $p<0.001$. The phenotypic frequency for this 3 allele loci were B/B 0.422, C/C 0.453, D/D 0.078, B/C 0.016, B/D 0, C/D 0.031 for the observed disribution and B/B 0.184, C/C 0.227, D/D 0.009, B/C 0.410, B/D 0.081, C/D 0.089 for the expected distribution (for n=60). Thus, there are very small values in the expected distribution that justify the pooling of the two less common alleles, B and D for further tests. However, the G values calculated for the pooled data at df=1 was still significant at $p<0.001$. The observed result was thus significantly deficient of heterozygous individuals. As the sample was obtained from commercial fisherman in a wide estuarine mangrove plain, it is possible that the sample actually consisted of two sub-populations from different tributaries with predominantly B and

Table (5.9) : Allele frequencies of M.rosenbergii populations

	AUST	BANG	INDON	MALAY	SABAH1	SABAH2	THAI	SRILANK
AAT-1A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
AAT-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ACPH-1B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ACPH-2A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADA-1A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADA-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ALD-A	0.500	0.588	0.833	0.875	0.800	1.000	0.670	1.000
ALD-B	0.500	0.413	0.167	0.125	0.200	0.000	0.330	0.000
ALP-A	0.500	1.000	0.500	0.775	0.600	1.000	0.330	1.000
ALP-B	0.500	0.000	0.500	0.225	0.400	0.000	0.670	0.000
EST-1A	0.500	0.000	0.300	0.280	0.333	1.000	1.000	0.417
EST-1B	0.500	1.000	0.700	0.720	0.667	0.000	0.000	0.583
EST-2A	0.500	1.000	0.211	0.140	0.321	0.500	1.000	0.750
EST-2B	0.500	0.000	0.789	0.860	0.679	0.500	0.000	0.250
EST-3B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
EST-4A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
FUM-1A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
FUM-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
G6PDH-A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GDH-B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GPDH-1B	1.000	0.975	1.000	1.000	0.967	1.000	1.000	1.000
GPDH-1C	0.000	0.025	0.000	0.000	0.033	0.000	0.000	0.000
GPDH-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GPT-C	0.000	0.300	0.025	0.000	0.000	0.000	0.000	0.500
GPT-D	0.500	0.467	0.925	1.000	1.000	0.000	1.000	0.500
GPT-E	0.500	0.233	0.050	0.000	0.000	1.000	0.000	0.000
HK-1A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

(to be continued)

Table (5.9) : (continue)

	AUST	BANG	INDON	MALAY	SABAH1	SABAH2	THAI	SRILANK
HK-2A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDH-A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LAP-A	0.500	0.389	0.800	0.600	0.700	0.000	0.833	0.500
LAP-B	0.250	0.611	0.100	0.200	0.150	0.000	0.000	0.500
LAP-C	0.250	0.000	0.100	0.200	0.150	0.000	0.167	0.000
LAP-D	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000
LDH-A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-1A	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000
MDH-1B	1.000	1.000	1.000	1.000	1.000	1.000	0.750	1.000
MDH-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ME-B	1.000	1.000	0.925	1.000	1.000	1.000	1.000	1.000
ME-C	0.000	0.000	0.075	0.000	0.000	0.000	0.000	0.000
MPI-B	1.000	0.430	0.925	0.030	0.933	0.000	0.833	1.000
MPI-C	0.000	0.477	0.075	0.970	0.067	0.000	0.167	0.000
MPI-D	0.000	0.094	0.000	0.000	0.000	1.000	0.000	0.000
PGI-D	0.000	0.000	0.975	0.000	0.933	0.000	0.000	0.000
PGI-E	0.750	1.000	0.025	0.970	0.067	0.500	1.000	1.000
PGI-F	0.250	0.000	0.000	0.030	0.000	0.500	0.000	0.000
PGM-B	0.000	0.067	0.000	0.030	0.000	0.167	0.000	0.000
PGM-C	0.000	0.900	0.000	0.000	0.067	0.833	0.000	0.920
PGM-D	0.750	0.033	0.975	0.970	0.833	0.000	0.917	0.080
PGM-E	0.250	0.000	0.025	0.000	0.100	0.000	0.083	0.000
PGDH6-B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
TO-B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

-abbreviations as described in text
-isozyme and allozyme coding as in teble(6.6)

C&D phenotypes. However, this assertions was not supported by other polymorphic loci.

(5.3.2.2) Genetic diversity and population differentiation

All the indices of inter- and intra-population genetic variation were summarised in table (5.10). The overall mean of P and Ho for 8 populations was 21.09% and 6.77% respectively. For the four populations with a sample size of 15 or more, the values were 25% and 6.7% respectively. Thus, the small sample size in some of the populations probably did not severely affect the overall mean of the populations. Ho and P increased in the direction of the Bangladesh, Malaysia, Indonesia and Sabah populations.

The coefficient of gene differentiation, G_{ST}, was 0.485 for all 8 populations and 0.395 for those populations with sample size of 15 or over. However, this relatively high value was attributed to the small values of Hs. The absolute degree of gene differentiation, Dm, was 0.064 for the four mentioned populations.

Table (5.10) : Indices of genetic variability of the various populations of M.rosenbergii

	AUST	BANG	INDON	MALAY	SAB-1	SAB-2	THAI	SRILAN
P (%)	25.0	18.8	28.1	25.0	28.1	9.38	18.8	15.6
Ho (%)	15.6	5.4	7.0	5.9	8.4	7.3	4.1	7.8
He (%)	12.1	7.5	7.5	6.1	8.6	4.0	6.2	6.3
Ho/He	1.27	0.78	0.97	0.98	1.01	1.73	0.85	1.22
H_o	overall = 0.073				4 pops* = 0.074			
H_T	overall = 0.141				4 pops = 0.122			
G_{ST}	overall = 0.485				4 pops = 0.394			
Dm	overall = 0.078				4 pops = 0.064			
R_{ST}	overall = 1.06				4 pops = 0.86			

* populations with n>/15 (i.e. BANG, INDON, MALAY & SAB-1)

(5.3.2.3) Genetic distances and phylogenetic relationships between populations

A matrix of Nei's standard genetic distance, D, genetic identity, I and standard errors of D between all the populations are shown in table (5.11). An average values of I=0.900 was between all the populations. Most of the genetic distance was contributed by the Sabah-2 population, which has an average value of D = 0.167 with the rest of the populations while the rest had an average values of 0.076. The lowest value observed was between the Indonesian and the Sabah-1 population with D approximating to zero.

Most of the differentiation of the Sabah-2 population was caused by the monomorphic alleles of LAP-D, GPT-E and MPI-D, which were either absent (LAP-D) or rarely present in the other populations. The small sample size probably did not contribute to the high values of D for this population as there was an excess of heterozygosity anyway. A plot of genetic distance (Fig. 5.7) with geographic distance generated significant value for the Spearman rank correlation coefficient ($p<0.05$) and linear correlation coefficient ($0.1>p>0.05$).

Phylogenetic trees constructed employed both the genetic distance matrix data as well as gene frquency data of polymorphic loci. The UPGMA, the Fitch-Magolaish, as well as the RFML trees all generally agree with each other (Fig.5.8). The following clusters can be recognised: (Bangladesh-SriLanka) - ((Malaysia-Indonesia-Sabah-1)-(Thailand-Australia)) with Sabah-2 forming an outgroup.

Table (5.11) : Nei's genetic distance and similarity matrix between the eight populations of *M.rosenbergii*

Genetic similarity (I) matrix

	SABAH2	AUST	BANG	INDON	MALAY	SABAH1	THAI	SRILANK
SABAH2		0.866	0.867	0.813	0.839	0.822	0.834	0.880
AUST			0.917	0.945	0.939	0.951	0.961	0.928
BANG				0.876	0.914	0.889	0.891	0.972
INDON					0.936	0.998	0.922	0.901
MALAY						0.939	0.915	0.908
SABAH1							0.928	0.914
SABAH2								0.915
SRILANK								

Genetic distance (D) matrix

	SABAH2	AUST	BANG	INDON	MALAY	SABAH1	THAI	SRILANK
SABAH2		0.143	0.142	0.207	0.175	0.195	0.181	0.128
AUST			0.086	0.056	0.063	0.050	0.040	0.075
BANG				0.133	0.090	0.118	0.115	0.028
INDON					0.066	0.002	0.081	0.104
MALAY						0.063	0.089	0.096
SABAH1							0.074	0.090
THAI								0.089
SRILANK								

Standard errors of D

	SABAH2	AUST	BANG	INDON	MALAY	SABAH1	THAI	SRILANK
SABAH		0.069	0.069	0.085	0.077	0.082	0.079	0.065
AUST			0.053	0.043	0.045	0.040	0.036	0.049
BANG				0.067	0.054	0.062	0.062	0.030
INDON					0.046	0.008	0.051	0.059
MALAY						0.045	0.054	0.056
SABAH1							0.049	0.054
THAI								0.054
SRILANK								

Abbreviations of populations as in the text

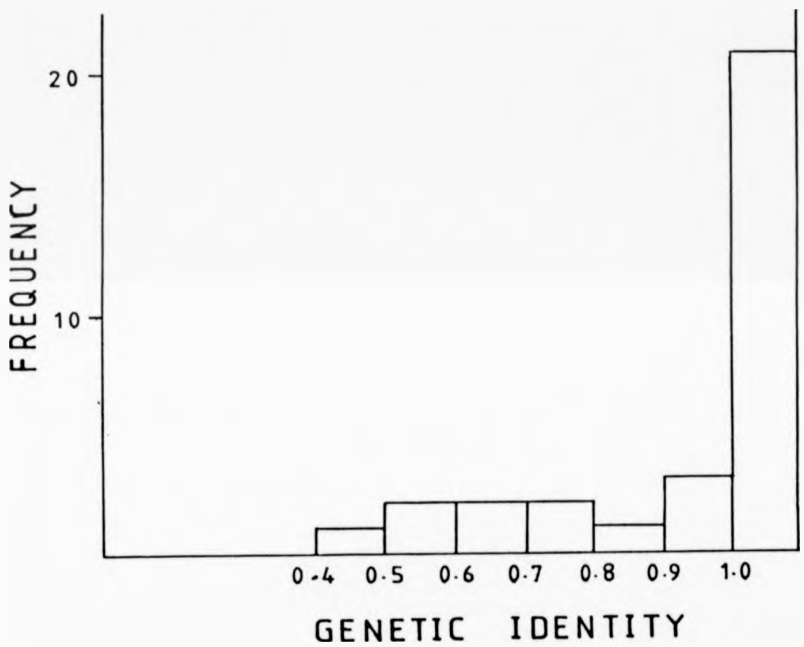


Fig. 5.6: Frequency distribution of average genetic distances at different loci for different populations of M. rosenbergii

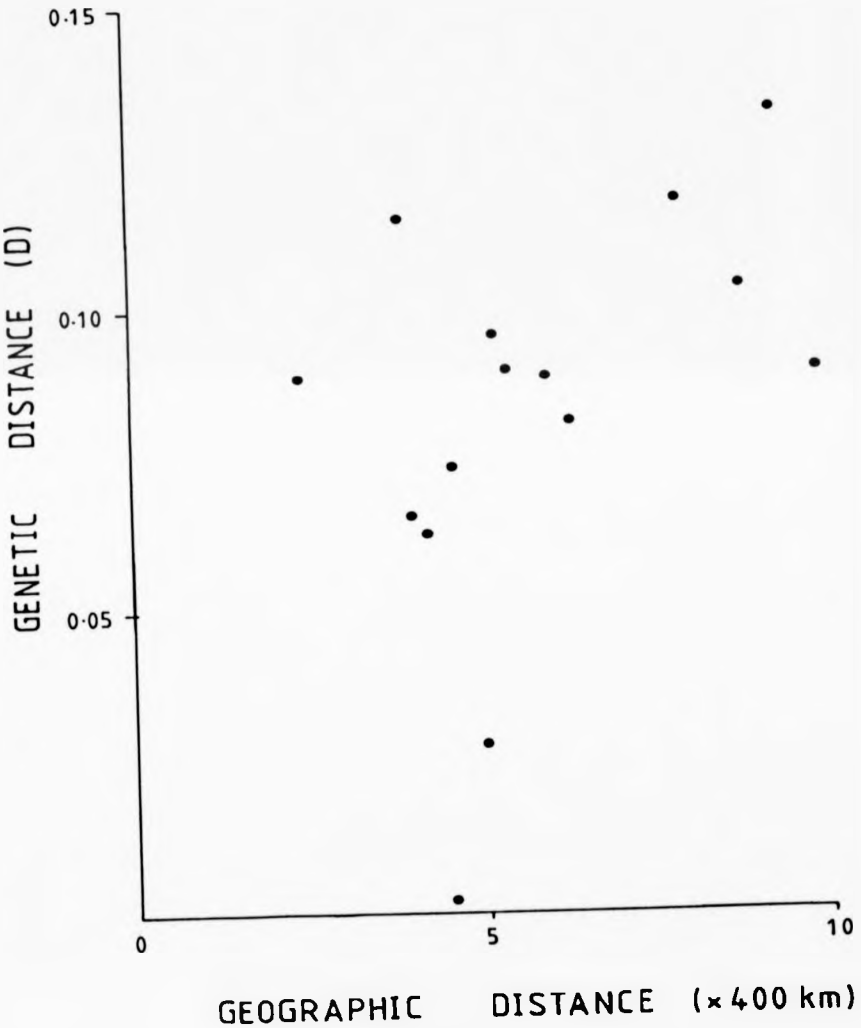


Fig. 5.7: Plot of genetic distances against corresponding geographic distances between the various populations of M.rosenbergii

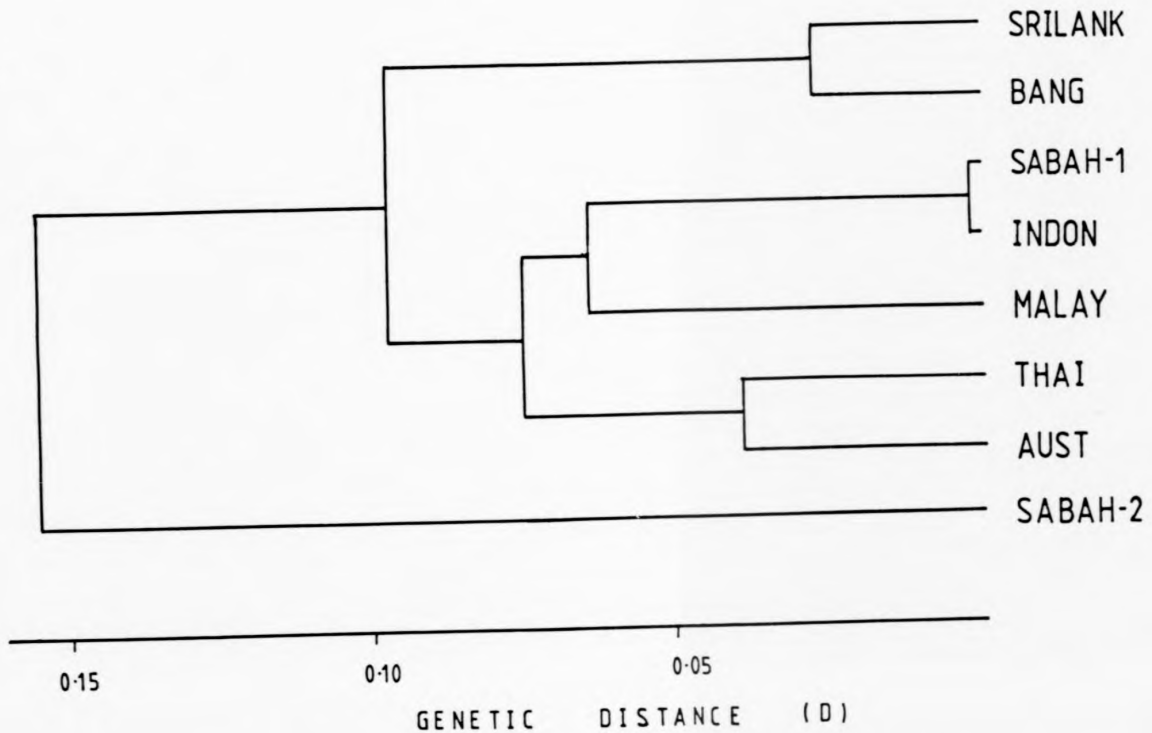
Fig. 5.8: Phylogenetic trees constructed for the populations of *M. rosenbergii* investigated

a. UPGMA method with Nei's genetic distance

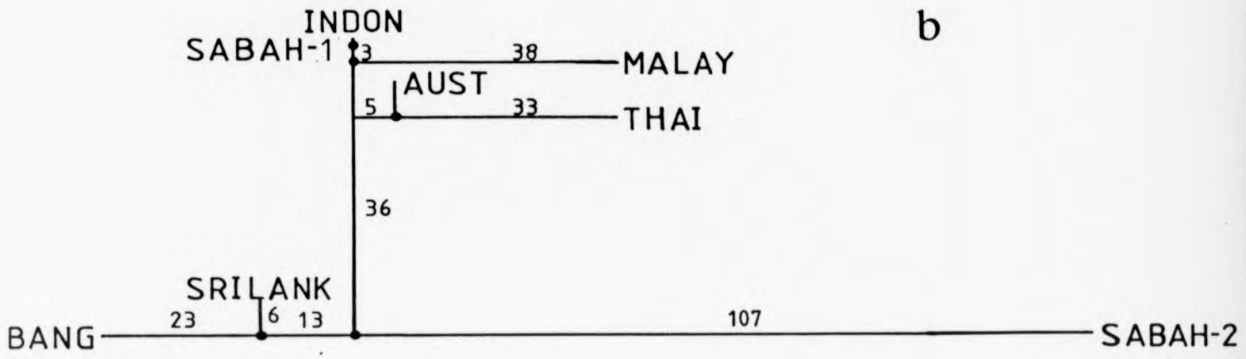
b. Fitch-Margolaish method with Nei's genetic distance

c. Restricted maximum likelihood method, using allele frequency data of polymorphic loci

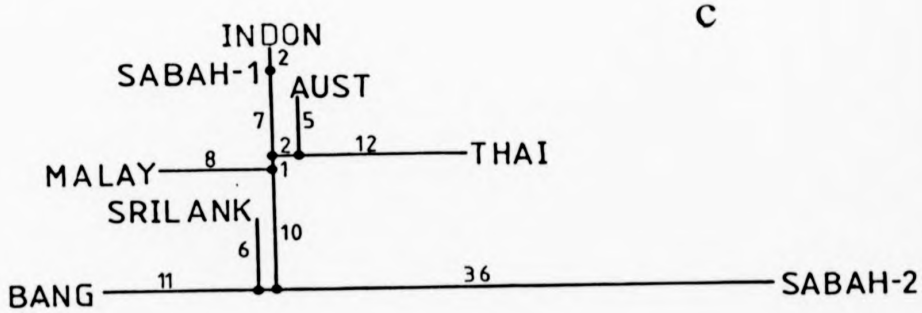
a


SRILANK
BANG
SABAH-1
INDON
MALAY
THAI
AUST
SABAH-2
0·15
0·10
0·05
GENETIC DISTANCE (D)

b


INDON
SABAH-1
3
38
MALAY
AUST
5
33
THAI
36
SRILANK
23
6
13
107
BANG
SABAH-2

c


INDON
2
SABAH-1
AUST
7
5
2
12
THAI
8
MALAY
1
SRILANK
6
10
11
36
BANG
SABAH-2

(5.3.3) Results of the ontogenetic expression of isozymes

Two batches of M.nipponense larvae and two batches of M.rosenbergii larvae were analysed for ontogenetic expression of isozymes. Most of the enzyme loci scored did not show any variability with different developmental stages.

(5.3.3.1) Results for M.rosenbergii

Almost no variability was observed in any of the enzyme loci studied in M.rosenbergii (MPI, LDH, ME, APH, MDH, ALD, @GPDH, PGI, GDH, FUM, PGM, 6PGDH, EST, AAT & APH). The only possible exception was IDH which did show a different band between zoeal IV to zoeal VII. However, only one batch of larvae was scored for this locus.

(5.3.3.2) Results for M.nipponense

Most of the loci scored showed no variability with ontogenetic development (MDH, ALD, MPI, LDH, ME, @GPDH, PGI, HK, GDH, PGM, 6PGDH, EST & FUM).

5 enzyme loci were observed to show ontogenetic changes (ACPH, LAP, IDH, GPT & APH). Both ACPH and APH were differentiated from stage VI onwards. For GPT and IDH, stage II zoea had a different band from the other stages. LAP-2 was not expressed until stage VIII.

(5.4) DISCUSSION

(5.4.1) Discussion on the population genetics of M.nipponense

The only other study of the population genetics of M.nipponense is that of Chow and Fujio(1985a). Their findings on genetic variability are summarised in table(5.12). The proportion of polymorphic loci, in which the most common allele had frequency of less than 0.99, ranged from 0 to 0.333 while He ranged from 0 to 0.092. In the present study, P ranged from 0.25 to 0.375 while He ranged from 0.087 to 0.15. The higher values of He were probably attributable to the much larger number of loci (more than double) investigated in the present study but could also be related to inter-laboratory differences. The EST observed by Chow and Fujio(1985a), using muscle tissue, showed activity in two zones, but the faster zone was poorly resolved and that both loci were monomorphic. In the present study, 6 zones (EST-1 to EST-6) could be recognised but only 2 (EST-1 and EST-5) could not be resolved in some individuals. It is possible that the two zones observed by Chow and Fujio(1985a) correspond to EST-5 and EST-6 in the present study. Two other polymorphic loci (LAP-2 and ODH) observed in some individuals have been precluded from the data due to poor resolution in some individuals. Therefore, the values of Ho reported in the present study should considered conservative.

Differences in genetic variation between lake&ponds/rivers populations were observed in Chow and Fujio's study and the present study. In both cases, the freshwater populations (ponds & lakes) had lower values of both P and Ho (see table 5.7 and

Table (5.12): Indices of genetic variability observed by Chow and Fujio(1985a)

Species	n	H_T	H_e	G_{ST}	D_m *
Macrobrachium nipponense					
(Lakes & ponds)	4	0.037	0.029	0.216	0.011
(rivers)	4	0.065	0.056	0.123	0.012
total		0.054	0.043	0.204	0.012
M. formosense	4	0.064	0.062	0.031	0.0027
M. japonicum	4	0.036	0.036	0.000	0.0000
P. paucidens	7	0.084	0.080	0.048	0.0047
P. macrodactylus	3	0.080	0.080	0.000	0.0000

* abbreviations as defined in text
Dm was recalculated from the data of Chow and Fujio(1985a)

table 5.12). Both studies had also reported high values of G_{ST} for populations of M.nipponense (0.204 and 0.282). The slightly larger values observed in the present study may be attributed to the larger geographical area covered in the present study.

Chow and Fujio(1985a) observed a higher value of G_{ST} (table 5.12) among the pond and lake populations (0.216) than that of the river populations (0.123) and related this to random genetic drift due to isolation in ponds and lakes. In the present study, G_{ST} values for within freshwater (0.134) and within brackishwater populations (0.163) were much smaller than the overall values (0.282), suggesting that a large proportion of the population differentiation was also attributed to pond/river (freshwater/brackishwater) differences. The slightly higher values for the brackishwater populations could probably be related to trans-oceanic distance involved between the Canton and the Japanese populations while all the populations in Chow and

Fujio's study were within Japan and that 4 of their 8 populations were in close proximity to each other on the Kanto plains. In the present study, Dm of the river populations, the minimum amount of genetic differentiation (that is unaffected by the small values of He), double that of the two pond populations. In Chow and Fujio's study, their Dm values for the river and for the pond populations were in fact identical (Table 5.12), implying that a large amount of their observed apparent differences in G_{ST} were attributed mainly to their low values of He. The small number of freshwater populations involved in the present study may also have contributed to the apparent low values of G_{ST}. Considering the allele frequencies of the populations, parts of the genetic differentiation between the river & the pond populations were attributed to the present of extra alleles in the river populations. Founder effects in the pond&lake populations, which contributed to the decrease of heterozygosity, might have also caused the apparent genetic differentiation, and thus similar values of Dm within each group.

Significant values of the linear correlation coefficient, r, was observed by Chow and Fujio(1985a) between genetic distances and geographical distances of the various populations in their study. Significant r values ($0.01<p<0.05$) were also observed for the river populations but not for the pond and lake populations. This led Chow and Fujio(1985a) to conclude that random genetic drift was responsible for the genetic differentiation between their populations of M.nipponense. In the present study, no significant linear nor Spearman rank correlation coefficients ($r=0.4$; $r_s=0.414$) were observed

between genetic and geographic distances (Fig. 5.4). A high value of r (0.99997) would have resulted if the Canton population was omitted from the calculation. Thus, for long distance comparision, random genetic drift may have contributed to much of the differentiation between populations.

The average standard genetic distance between all the Japanese populations was 0.0133 (Chow and Fujio, 1985a) and 0.084 for the present study were well within the values expected for interpopulation comparisions (Nei, 1975). Thus, despite the geographical separation, populations of M.nipponense have diverged relatively recently, bearing in mind that many populations exist in enclosed freshwater bodies. The ability to grow at tropical as well as temperate conditions (see Chapter 2), the mainly coastal distribution of natural populations, and the relatively high heritabilities of larval freshwater tolerance, many populations of M.nipponense have evolved relatively recently. Many populations in Japan, northern China and Korea have probably became established since the last ice-age.

Diagnostic alleles (MPI-F, GPDH-1C, GPT--B) are present in the Japan and Canton populations, but not in the Hong Kong nor the Wuhan populations (except AAT-1A in Hong Kong). This undoubtedly has caused the phylogenetic grouping of the Wuhan and Hong Kong populations, in relation to either the Canton or the Japan populations. This has been attributed to their coastal and estuarine existence, the Canton and Japan populations probably still have level of Ho similar to the ancestral marine population. If so, and assuming geographical isolation to be the

mechanism for the decrease of genetic diversity in the Hong Kong and the Wahan populations, it may be possible to estimate the time of isolation and the number of effective founding populations.

The value of Ho of the two freshwater population represent about 60% of those of the Japan and the Canton populations. Using equation (5.2), a hypothetical relationship between the effective population size and the number of generations after divergence can be constructed (Table 5.13). The Hong Kong population, which was a pond population, probably evolved from an original number of 10 (small 500 m^2 ponds) or so adult prawns, this would then give a t of approximately 10 years, assuming an effective generation time of 1 year. The time of 10-15 years also corresponded well to the founding of the Au Tau Fisheries station from which the present stock was obtained.

The origin of the Wuhan population was probably related to flooding from the Yantze River. Major flooding of the Yantze occurred once every 9 years during the Ming Dynasty (about 1500 A.D.) and once every 3-4 years in the Ching Dynasty (about 1700 to 1900 A.D.; Wong, 1979). In August of 1931, the water of the Yantze at Wuhan rose to a height of 28.28m above average, flooding not only the whole city (a major city of China), but also an area of about 30 million acres of cultured fields along the range of the river, and, alas, causing the loss of 145,000 human lives (Wong, 1979). These periodic floods from the Yantze river, which generally increased the effective population size, have probably accounted for the higher values of P in the

Wuhan population of M.nipponense. The Wuhan population has probably become isolated since the major construction of artificial banks along the river in this region, after the founding of the P.R.China. In 1954, while the water of Yantze at Wuhan was at a record height of 29.73m above average, the city was unaffected (Wong,1979). Assuming the isolation time of 50 years and a reduction of genetic diversity to 60% of its original value, an effective founding population size of 40-50 would be inferred. The above hypothesis involves a large number of assumptions (including no selection and mutation !) and should be challenged with future findings.

Nei's standard genetic distance can also be used to estimate the time of divergence between populations (see Chapter 6.1 for discussion on molecular clock). However, the standard errors for small values of D would be comparatively large. Taking the Wuhan/Canton comparision of 0.072 (S.E.= 0.048) and taking Nei's own estimate of 5×10^6 years/D, the time of divergence estimated would be of the range of 120000 to 600000 years. Using the more popular value of 18×10^6 years/D, an even longer time range of 432,000 to 2,160,000 could result. The comparatively large values of D (0.087 $\pm$ S.E. 0.054) between the Hong Kong and Canton populations may also be affected by the comparatively large standard errors and the general low value of D observed in the study.

Putting total faith on the molecular clock, and assuming the sub-populations of Canton/Wuhan were diverged at the time suggested, the hypothesis would involve the divergence of a separate population from the marine ancestral population, before

the ice-ages, which migrated northwards to the Yantze region after the ice-age, and transferred subsequently (by flooding) to the present location.

Considering the long history of civilisation in China, many of the biogeographic distribution and population characteristics of M.nipponense might be caused by Man-made effect. The contruction of the Giant Canal from Hanchou to Beijing (2,000 km) 2,500 years ago, which joined the water of five major rivers (including the Yantze and the Yellow Rivers), has undoubtedly assisted in the spread of M.nipponense to northern parts of China.

(5.4.2) Discussion on the population genetics of M.rosenbergii

Among 8 populations of M.rosenbergii studied, Hedgecock et al. (1979) only reported values of 14% and 2.8% for P and H respectively, as opposed to values of 21.09% and 6.77% in the present study. The difference was mainly attributed to the different enzyme systems studied, as the monomorphic loci of general protein (7 loci), TPI (triosephosphate isomerase, 1 locus) and 2 of the ACPH (4 loci) of Hedgecock et al.(1979) were not included in the present study. On the otherhand, 6 of the highly polymorphic loci (ALD, ALP, EST-1, EST-2, GPT and LAP) observed in the present study is not studied by the previous workers.

It is also possible that different electrophoretic techniques have contributed to the difference in the observed P and H. Polymorphism at AAT and HK as observed by Hedgecock et al. (1979) was not observed in the present study. However, this would only further increase the values of variation in the present study. Assuming that the observed variation in both studies were comparable, the maximum number of polymorphic loci would be 17 (including occasional polymorphism) out of a total of 42 loci (P= 40.5%) while the number of more polymorphic loci (over 3-4 populations oberved in either study) would be 11 out of 42 loci (P = 26.2 %). The average minimum value of P, as estimated by half the number of the more polymorphic loci only (assuming no occasional polymorphism present), would be about 14.2%. Thus, the average value of P, as estimated from the two studies, would probably range from 14%-26%, corresponded well with the result of the present study.

Johnson(1960) was the first worker to report on the sub-division of natural populations of M.rosenbergii into an eastern race and a western race. The same paper and a later one (Johnson, 1973) described the morphological differentiation of the adult males in the two sub-species, mainly based on the rostrum and the carpus (see table 5.14). The western subspecies was named M.rosenbergii schenkeli and the eastern one as M.rosenbergii rosenbergii (Johnson, 1973).

Chen and Chang(1979) reported on the differentiation of juvenile and adult Taiwanese M.rosenbergii from the cultured western morph. The main characteristic was the presence of yellow spots between the horizontal banding pattern on the carapace which gave the local name of 'golden thread' to the prawn. However, the yellow spots were not retained in the F1 hybrids with the western morph (Lin, 1980). Chen and Chang(1979) also reported on the larval development of the Taiwanese morph, which was mainly characterised in having the rostral formula of 2/0 from stage III up to stage XI (with small ventral indunation though), just before the final metamorphosis, as opposed to the gradual development in the western morph (Kwon and Uno, 1969). However, this characteristic is also present occasionally in the hatchery stocks from the western morph (personal observations). Also, the larval developmental rate of the Australian morph was also reported to be different to that of the western morph (Sarver et al., 1979).

Table (5.14): Morphological differences between the different races of M. rosenbergii

(A) adapted from Johnson(1973) refering to adult males

M.r.schenkeli (western)	M.r.rosenbergii (Australian & New Guinean)
Comparatively slender;	Comparatively stout;
Carapace comparatively and not pubescent;	Carapace comparatively scabrous and with a short velvety pubescence;
Rostrum as long as carapace, exceed the antennal scale by 1/3 of its length;	Rostrum rarely exceed the antenal the antennal scale by 1/3 of its length;
Rostral crest moderate to low;	Rostral crest low to very low;
11-15 ventral rostral teeth;	8-12 (mostly 10) ventral rostral teeth
Slender second pereopods;	Comparatively stout second pereopods;
Fingers of second pereopods 3/4 as the palm or longer;	Fingers of second pereopods 2/3 of the palm or shorter;
Carpus distinctly longer than palm.	Carpus as long as or just longer than palm.

(to be continued)

Table(5.14): (continue)

(B) from Malecha(1980)

Character	Western	Eastern	
		Australian	North Eastern*
Adult rostral colour	Same as body	Same as body	Prominent red
Rostral shape	Long,curved	Short	Long,curved
Antennae colour	Reddish	Reddish	Blue
Rostral base spots	Diminished	Prominent	Prominent
Terminal chelae segment	Thin	Thick	Thin
Chelae colour [1]	Blue,dark blue	Purple	Blue,dark blue
Chelae joint colour	Same as chelae	Same as chelae	Reddish
Body markings [2]	Scattered juvenile spots	Prounced spottings in juveniles to subadults	Very pronounced mottling in juvenile to small adults

* New Guinea animals were not included, but may constitute another race (Malecha,1980); Taiwanese race fairly similar to North eastern race except for its yellow spottings (Chen and Chang,1977)

1 -except for "golden claw" western morph and "yellowish claw" Australian morph.

2 -All markings dimished in older, larger prawns. Rank order for persistence is North Eastern > Australian > Western

Hedgecock et al.(1979) then described the allozymic differentiation among 8 populations of M.rosenbergii. Other members of the same group then describe the morphological and colour differentiation of juvenile/sub-adult (Halecha,1980) as well as putting forward a possible evolutionary explanation of the observed population structure (Lindensfelser, 1984). The major conclusion was that the populations were subdivided into a western race, an Australian race and the north-eastern race, with possibility of an additional race in Papua New Guinea (Robertson,1983). Also, there was a congruence (78%; Lindenfelser,1984) between the inference from allozymic data and that from morphological data.

Lindenfelser (1984) explained that as the Australian plate (in conjunction with Papua New Guinea) moved into close proximity to the Celebes and Indonesia some 10 to 20 million years ago, 'an initial rafting over the Molucca Sea may have precipitated the New Guinea population, this sea ultimately acted as a barrier, preventing regular gene flow and perhaps allowing founder effects to influence the subsequent genetic make-up of the eastern population'.

Most of the populations in the present study were from the western race. The average values of genetic distance of 0.076 among them (except Sabah-2 and Australia) agreed well with the general values expected for the same species. The significant value of r_s between genetic distance and geographic distance indicated that while these populations originated from the same stock, a neighbourhood effect (Ch.5.1.2) has resulted in the observed genetic structure within this area.

The Australian population was purchased in Queensland fish market (by my father), and has probably originated from another south east Asian region, likely somewhere near Thailand.

Differentiation of the Bangladesh-Sri Lanka group from the Indonesia-Sabah-1-Malaysia-Thailand (I-S-M-T) group was evident, as inferred in the morphological data of Lindenfelser(1984). She related this to the the climatic change of the ice-ages when the advancing polar frontal system would have isolated the population on the Indian subcontinent. However, her India population was more related to her I-S-M-T (very similar sampling site) group and Lindenfelser(1984) explained this in relation to either 'expanding western population' or the isolating effect of rising sea level. The sampling site of the Bangladesh population in the present study, which was in the closest proximity to her Indian site, was not affiliated to the I-S-M-T group.

The morphology of the three individuals of the Sabah-2 population was quite distinguishable from those of the other populations. The three individuals were all sub-adults of about 10-12 cm in total length. The major characteristic, as compared to the other populations (or as described for the western population by Malecha,1980), was the presence of fairly pronounced mottling and red spots on both sides of the carapace (fairly similar to the eastern morph as described by Malecha,1980). The average genetic distance between Sabah-2 and the other populations doubled the values of genetic distance among themselves. The presence of 'diagnostic' alleles confirm

that the inference was not affected by the small sample size of the population. Three possible alternative hypothesis would be possible to account for this observation:

(1) The population was a genuine differentiated race, as reported for the eastern subspecies;

(2) The population was originated from one of the nearest population from the eastern race;

(3) The population was formed from introgression by individual of the eastern races into an established population of the western race.

The Sabah-2 population was sampled in the Membakut River, which is the next major river to Padras River, the sampling site of Sabah-1(Fig.6.2b). Unless there is any special hydrographic conditions (pending further investigations), it is inconceivable that no gene flow exist between the two populations, considering the correlation of genetic distance with geographic distance between the rest of the populations.

Lindenfelser's Philippine population , which was sampled 'along the entire length of the archipelago' (?), was placed in the eastern race in both her allozymic and morphometric trees. However, the Philippines actually lies west of the Wallace's line, which was proposed to form the barrier between the east-west races. She suggested that the Philippines population originated from New Guinea or Australia, but acknowledged the 'enigma' that as the Philippines are in close prioximity to Borneo, one would expect prawns from there to belong to the western race. Sabah-2 in the present study represented the closest population of M.rosenbergii to the Philippines studied in both investigations. With the abundance of land bridges from the Philippines, it is reasonable to expect population of the eastern

race to have reached northern Sabah. The island of Palawan literally forms a bridge across most of the Sulu Sea. The population of Sabah-2, then, could have originated from the Philippines.

However, assuming that the Eastern race has evolved in the direction of western-(New Guinea-Australia)-Philippines, populations of M.rosenbergii of the western race would have already existed in Sabah before the arrival of their eastern cousins, making hypothesis (2) unlikely. The values of genetic distances for within race comparision (0.0001-0.0546) correspond to the values in the present study (0.0000 - 0.133). However, the observed genetic distance of 0.167 (0.128-0.207) between Sabah-2 and the other populations is thus halfway between those values inferred for east-west comparisions (0.35-0.40) by Lindenfelser (1984). Thus, assuming the compatibility of data in the two studies, the Sabah-2 population may have been a hybrid population between the two races (hypothesis 3). However, the presence of the monomorphic allele LAP-D would pose problem for the introgression hypothesis, assuming no dominance of the allele. On the otherhand, the locus was not studied for the eastern race in Hedgecock et al.(1979) and that larger sample sizes may have revealed the presence of rare heterozygous individuals. Steep clines will persist for loci that have low fitness in the heterozygous conditions, and so contributing to hybrid inferiority. If the heterozygous individuals with LAP-D have a lower fitness than either homozygotes in a hypothetical hybrid zone, a steep cline would exist. However, this can only be confirmed by biochemical studies of the enzymes concerned.

Nevertheless, hypothesis 3 seems to require the least assumptions in the pattern of gene flow.

It remains to be explained why Wallace's line has successfully formed a barrier across New Guinea/Indonesia but failed to do so across the Philippines/Guinea boundary. The distance of ocean of the latter is actually longer than that of the former. One possible explanation may be the presence of landbridges across such ocean barriers. Tectonic activity has produced numerous deep-sea trenches between the various boundary of the east-west races: the Java trench between Australia/Java; the Makasar Strait between Celebes/Borneo; the Philippine trench between Philippine/New Guinea. While no obvious land bridges exist across the Java trench, and Makasar Strait, numerous islands existed across the Philippine trench (e.g. the Moluccas) and across the Sulu sea. This may partly account for the the 'eastern' character of the Philippine population of Lindenfelser(1984) and possibly the Sabah-2 population of the present study.

However, the presence of adequate land bridges between Philippines/Borneo poses the question 'why didn't M.rosenbergii in the Philippines belong to the Western race in the first place. One possible explanation is the climatic changes of the recent ice-ages. Trukada(1966) revealed that much of the island of Taiwan was under the influence of freezing winter temperatures as recent as 14,000 years ago. As M.rosenbergii can only survive in temperatures above 19°C, much of the Philippines would probably be precluded from colonisation from both the western and eastern races in the late Pleistocene (or that the

existed population became extinct).

This then generates the questions 'why the eastern race first ?' and 'what keep them from the influence of the western race?'. Successful dispersal of pelagic larvae and thus gene flow depends on the size of the parental population, the hydrographic conditions, the ability of the larvae to survive in the prevailing conditions and the distance involved (or the presence of land bridges). The abundance of landbridges across Borneo/ Philippines/New Guinea has already been mentioned, the most obvious hydrographic conditions that may influence larval dispersal and survival would be currents, salinity and temperature.

The east coast of Philippines is dominated by the North Equatorial current of the Pacific throughout the year, which when diverged nothwards, joins the Kurisho current. To the south-west and south of Philippines (Sulu Sea and Celebes Sea), there is a general net flow westwards all through the year (Fairbridge, 1966, p833), probably representing the the North Equatorial Current that flows through the Philippines. This then would not favour the flow of larvae from eastern Borneo to the southern Philippines.

The currents along the northern coast of Borneo and the Philippines (where Sabah-2 is situated), as part of the southern and eastern margins of the South China Sea, are mainly dominated by the seasonal monsoon winds. In February, while the main flow in the South China Sea was through the Formosa Strait, weak currents also flow through the Philippines, across the Sulu Sea into the southern South China Sea. The flow is generally

southwards (Fairbridge, 1966).

In August, while there is a general north-easterly current along the western border of the South China Sea (coastal Vietnam), a weak counter current exists along the eastern coast of the sea (Fairbridge, 1966). In summary, the prevailing current direction in the south-eastern borders of the South China Sea is mostly southwards throughout the year. This would thus favour the transport of larvae from northern Philippines, via Palawan island, to northern Sabah.

The salinity of the south-eastern border of the South China Sea generally ranges between 33-33.5‰ with a temperature of 28-30°C (Fairbridge, 1966). Uno and Yagi (1980) investigated the effect of various combinations of temperature and salinity on the metamorphosis rate of M. rosenbergii. At a chlorinity of 18.4‰ (about 33.2‰ salinity) and a temperature of 30°C, 62% of larval M. rosenbergii metamorphosed to post-larva with an average larval duration of 27.2±1.74 days. Any reduction in salinity further increased the survival rate. This data implied that the prevailing salinity and temperatures in south-eastern South China Sea also favoured the transport of larval M. rosenbergii and the establishment of a population. The higher rate of larval mortality in nature cannot be estimated but should be balanced by the high fecundity of M. rosenbergii and the size of the parental population.

Much of the northern coast of Australia and western coast of New Guinea has a water mass with high salinity (generally over 34‰) and a slightly colder temperature (24-28°C) (Bramwell, 1977). This would approach the physiological limits of

M.rosenbergii, as purported by Uno and Yagi(1980). Thus, while there is a stong eastward flow from the Java Sea to the Bada Sea in Winter and vice-versa in summer, the prevailing conditions of salinity and temperatures and salinity in the oceanic current may have precluded successful regular long-distance transport from Java to Australia.

On the other hand, the strong north-south current through the almost north-south Makasar Strait may serve to prevent regular transport between Borneo/Celebes. Similarly, the strong westward current in Celebes Sea may form a barrier to regular transport between New Guinea and Philippines, but occasional rafting should be possible via the abundance of land bridges in this area. The hydrographic conditions of the Formosa Strait probably account for a separate race in southern Taiwan (Chen and Chang,1979), affiliated to the eastern race, as reported.

Lindenfelser(1984) hypothesised that the eastern race was established from an initial rafting from the western race when the Eurasian plate collided with the Australian plate some 10 to 20 million years ago. The foregoing discussion suggested that the prevailing hydrographic conditions alone can account for the population structure of M.rosenbergii in south-east Asia. The present-day observation of the subdivision of western, eastern, Philippines and probably New Guinean and Taiwanese sub-populations implies that the mechanism of separation is actively in force. It is possible that the the divergence of the east-west races might have occurred much more recently than suggested by Lindenfelser(1984). If rafting occurred some 10-20 million years ago as suggested, it may have continued as the plates become much

closer in recent time. The existence of the Philippine race with eastern characters defintely requires the explanation of a more recent migration. Lowering of sea level in the last ice-ages may have encouraged the transport between the various islands, before the present-day conditions prevailed. The only possible explanation may be that the presence of established populations may have precluded the influx of long distance immigrants.

Hedgecock(1982) himself acknowledged that the molecular clock estimate of divergence would range from 3×10^{5} to 8×10^{6} (as average D between eastern and western races was about 0.4) and that the error variance associated with estimating absolute divergence times from D is substantial. The only 'fossil evidence' suggested by Lindenfelser was that of Rathbun(1918), which only consist of broken pieces of unidentified appendages (also, the status of the genus Macrobrachium was not fully established in 1918 !).

Further data are required on the detailed population structure at the possible hybrid zone between the east west boundary, probably in north-eastern Sabah, in relation to both the western and eastern genetic composition. The transport of larvae suggested in the present study can only be confirmed by actual study of inshore current flow, and probably by drift bottle study. Before such additional data, all the hypothesis presented remain only speculative.

(5.4.3) Discussion in relation to population differentiation in Decapoda

While few studies have addressed themselves to inter-population differentiation in crustaceans, there are sufficient data to make a brief review.

In the American lobsters H.americanus, morphological divergence between populations have been reported by Saila and Flowers(1969). Greater differences were observed between inshore/offshore comparision than geographical comparision, and supported data from tagging experiments. Similar inferences were concluded in the electrophoretic study of Tracey et al.(1975). However, the overall average values of D for 8 populations was only 0.006 ± 0.001. Furthermore, low values of D were observed between the two species of lobster H.americanus and H.gammarus (0.11; Hedgecock et al.,1976), values associated with intraspecific variation. Hybrids between the two species also seem to be sterile (Hedgecock,1986; Talbot et al.,1983)!

For the rock lobster, Jasus edwardii, Smith et al.(1980) reported an average D values of zero for several populations in New Zealand.

The genetic similarity (I) reported for inter-population comparision for three species of crayfish (Astacidae)ranged from 0.95-0.997, representing very little difference between populations.

In Penaeus, as in their genetic diversity, very low values of D were observed for inter-populational comparision. Mattoccio et al.(1986) reported an average D value of 0.005 for 4 populations of P.kerathurus in the Mediterranean. For the

Australian species, Nulley and Latter(1981a,b) reported values of genetic divergence,Δ (Latter,1973), of 0.051,0.014,0.07,0.127 and 0.214 respectively for Metapenaeus benettae, M.macleayi, Penaeus plebius, P.latissulcatus and P.endeavouri. Salini(1987) also reported low level of differentiation in 6 populations of M.bennettae along the east coast of Australia. Genetic heterogeneity was tested by contingency X^2 tests on allele frequencies at 8 polymorphic loci. The differences in the two most distant populations contributed mostly to the overall genetic heterogeneity. The inter-specific differences were mostly caused by populations in the Gulf of Carpentaria. In the Gulf of Mexico, Lester(1979) reported inter-population values of D of the range from 0.997-0.998 for inter-populational comparision in P.aztecus, P.duorarum and P.setiferus.

The values of D mentioned so far are very low even for inter-population comparision suggested by Thorpe(1982). It is thus tempting to suggest a low level of genetic differentiation for Decapoda. However, most of the species mentioned so far involved species with oceanic existence, and with a relatively large number of larval stages.

Among 5 species of Palaemonidae, Chow and Fujio(1985) reported D values of the range 0.0005 to 0.0133. For 6 populations of M.ohione, Trudeau(1978) reported an overall D value of 0.040. The average D values of the present study gave 0.076 and 0.084 for inter-population comparisions in M.rosenbergii (without Sabah-2) and M.nipponense respectively and correspond well with the values generally observed for inter-population comparision. Hedgecock et al.(1979) reported values of

up to 0.40 for inter-population comparision for M.rosenbergii, values generally associated with inter-specific variation.

Genetic differentiation was observed between estuarine and marine populations of the mud crab, Macrophthalmus hirtipes (Sin and Jones, 1983). In the few cases where both morphological and allozymic data were available, the two generally compliment each other. Nakajima and Masuda(1985) reported on the genetic differentiation among Japanese populations of the snow crab Geothelphusa dehaani and allozyme data related well to morphometric traits. Similarly, morphological separation of subspecies of the mud crab, Panopeus herstii was confirmed by electrophoretic data (Turner and Lyerla, 1980). Using both allozymic and morphological data, Bert(1985, 1986) reported on the existence of two sibling species in the mud crab, Menippe mercenario, and related the speciation process of the genus to past climatic and geological events. In one of the two hybrid zones, at northwestern Florida, Bert suggested the existence of ecological and possibly ethological isolating barriers.

It is interesting to note that the G_{ST} values of M.rosenbergii (0.495) were actually larger than that of M.nipponense (0.282). Chow and Fujio(1985a) reported that G_{ST} value for populations of M.nipponense (0.204) was actually the largest among 4 other species of Palaemonidae studied. It is possible that the smaller geographical area involved in the two studies has caused the difference in observation. As mentioned in (5.1.8), the value of G_{ST} is much affected by small values of H_T. The absolute minimum genetic distance, Dm, however, is more independent of the value of H_T. In the present study, the values

of Dm for M.rosenbergii (0.064 for 4 populations and 0.078 for 8 populations) and M.nipponense (0.061) are comparable to each other but are both relatively high compared to other organisms which generally have values less than 0.03 (Nei,1975). Thus, despite variation in larval developmental duration and salinity tolerance, the two species seem to have a comparable degree of differentiation, as to their value of D. Most of the above examples with population differentiation, including the present, involve either freshwater or estuarine species. It is thus tempting to suggest that larval retention and possibly larval development has contributed to such events.

Trans-oceanic distance dispersal of larvae has been demonstrated in many invertebrates (e.g. Scheltema,1971). Such long-distance dispersal of planktotrophic larvae should maintain gene flow between disjunct populations. Data from electrophoretic studies generally confirmed that species with planktotrophic larvae tend to have a lower level of differentiation than species with non-planktotrophic ones (Cameron,1986).

On the other hand, genetic differentiation in some species does occur, despite larval dispersal (Hedgecock,1986). Sandifer and Smith(1979), investigating the variation in larval development of palaemonid shrimp, suggested that such variation may increase the probability of recruitment to existing parental populations and the likelihood of successful colonistion of freshwater environments. Similarly, Burton and Feldman(1982) suggested that the strong swimming and depth regulatory ability has the 'obvious evolutionary advantage' of avoiding being transported away from their parental grounds. Hunt(1978),

purported that the larvae of Jamaican freshwater shrimps have the ability to maintain position when a habitat is suitable, without relinquishing the ability to disperse. Hunt even went on to suggest that the criterion of suitability of the larval habitat for a particular species is the salinity near the river mouth which is associated with a particular type of river.

The consensus is that the adaptive significance of pelagic larvae may not lie in the benefits of gene flow, but instead in the ability to migrate to nursery habitats (Hedgecock, 1986b) and that the apparent gene flow is to be considered as accidental (Scheltema, 1986b).

On the other hand, Hedgecock(1982b) pointed out the problems of interpreting gene flow from genetic similarity between populations (attributed to other forces):

(a) similarities among populations need not imply high rates of larval leakage;

(b) differences among populations need not imply definite larval retention mechanisms or diversifying selection overriding the effects of larval leakages;

(c) spatial patterns such as clines need not imply selection along an environmental gradient.

Population genetic studies should indeed relate more to ecological studies for unequivocal analysis. On the other hand, other forces in population genetics are perhaps even more difficult to verify than gene flow. Significant correlation between geographic and genetic distance is generally taken as the only circumstantial evidence for equilibrium between random genetic drift and gene flow. The present report on M.rosenbergii and Chow and Fujio(1985b)'s report on M.nipponense represent the

only examples with such a comparision in decapods.

In relation to 'natural' selection, the only case with sufficient evidence in decapods was that of the PGM polymorphism in *Palaemon elegans* in relation to heavy metal pollution (Pontecorvo *et al.*, 1983; Nevo *et al.*, 1981, 1986).

Corbin(1977) reported on the polymorphism of PGI locus (10 alleles!) in the sand crab, *Emerita talpoida* and the correlation of PGI^{a} and PGI^{b} with latitude and suggested the phenomenon as a result of environmental gradient.

The ecological and reproductive barrier between upper and lower populations of the same river in *M. nipponense* and *Palaemon paucidens* (Mashiko, 1983a,b,c; Chow and Fujio, 1985c) required an initial penetration of upper freshwater environment by individuals of lower estuarine populations. It probably involves the selection for larval freshwater tolerance and shortening of larval duration (Sandifer and Smith, 1979b). The high value of heritability of larval freshwater tolerance reported in Ch.5 and the difference in salinity tolerance in upper/lower populations of the Sagami River(Mashiko, 1983c) would form very strong support for such inference. The proven case of selection on the LAP and GPT loci in copepod and mussel in relation to salinity tolerance (see Chapter 4) indicated that salinity can be a very important parameter in population differentiation in aquatic species. However, after the change in larval freshwater tolerance and larval duration, the initial selection pressure may contribute much less to maintaining the population differentiation. The seasonal fluctuation of the hybrid zone of the upper/lower populations, as revealed by gene frequency data (Chow and

Fujio, 1985c) at the Natori and Hirose River also suggested that environmental factors (probably freshwater flow or saline intrusion) may still have strong role in maintaining the two populations.

(5.4.4) Gene diversity of Palaemonidae

13 species of Macrobrachium were investigated for genetic diversity (next chapter), the observed values of P, He and Ho are listed in table(5.15). However, the sample size of M.rude and M.lepitodactylus were small, 3 and 5 respectively. The average values of P=0.249(0.125-0.40), He=0.095(0.053-0.136) and Ho=0.103(0.053-0.162) are higher than previous values reviewed by Hedgecock et al.(1982). The average values for other species in the Palaemonidae (mainly Palaemon) was Ho = 0.0647.

Chow and Fujio(1985a) suggested that the differences in the mating system of Macrobrachium and Palaemon have contributed to their observed lower values of Ho for the former group. The average values of all Macrobrachium studied is in fact larger than that of Palaemon. This may be related to the small number of enzymes studied by Chow and Fujio and that no comparative values for Palaemon are available in the present study. The values reported by them for M.nipponense were about half of those reported in the present study. However, Hedgecock(1986) gave his doubts on the suggested mating system of Macrobrachium, based on the observation 'sneaky' runt males.

While it is acknowledged that a different set of enzymes studied can actually contribute to different values of genetic diversity, the values in the present study, using the same set of enzymes, can be grouped into 2 catagories. Group I species have

Table(5.15): Genetic diversity of Palaemonidae

Species	L	P	He	Ho	Reference
Palaemonetes pugio	16	0.243		0.683	Fuller & Lester (1980)
Palaemon adspersus	25	0.12		0.0495	Berglund & Lagerevanty(1983)
P.squilla	25	0.11		0.052	" "
P.paucidens(A)	15	0.333		0.109	Chow & Fujio (1985a)
" " (B)	15	0.229		0.080	" "
P.orientus	15	0.133		0.052	" "
P.macrodactylus	15	0.200		0.052	" "
Macrobrachium rosenbergii	20-28	0.14		0.028	Hedgecock *et al.* (1979)
		0.188		0.027	
M.nipponense	15	0.330	0.043	0.043	Chow & Fujio
M.formosense	15	0.400	0.055	0.062	" "
M.japonicum	15	0.133	0.036	0.036	" "
M.lar	15	0.200	0.010	0.011	" "
M.ohione	14	0.36		0.170	Trudeau(1978)
M.intermedium	24	0.186			Boulton & Knott (1984)
M.acanthurus	32	0.344	0.110	0.142	present study
M.dayanum	32	0.156	0.053	0.060	" "
M.equidens	32	0.281	0.125	0.137	" "
M.hainanense	32	0.281	0.109	0.112	" "
M.idella	32	0.156	0.063	0.071	" "
M.lanchesteri	32	0.313	0.132	0.148	" "
M.lammarei	32	0.281	0.136	0.162	" "
M.lepidactylus	32	0.219	0.103	0.106	" "*
M.nipponense	32	0.325	0.117	0.099	" "
M.rosenbergii	32	0.250	0.073	0.074	" "
M.rude	32	0.313	0.133	0.076	" "*
M.sintangense	32	0.125	0.064	0.076	" "
M.sintangense-2	32	0.188	0.058	0.055	" "

abbreviations as defined in text

* - sample size < 10

values ranging between 0.05 to 0.10, including M.dayanum, M.idella, M.rosenbergii, M.rude, M.sintangense and M.spp. Group two species have values of H ranged over 0.10, and include M.acanthurus, M.equidens, M.hainanense, M.lanchesteri, M.lammeri, and M.nipponense.

Trudeau(1977) suggested that the heterozygosity of 5 species of Macrobrachium was related to the size and thus the mobility of the prawns (coarse grain adaptation). However, M.acanthurus, the largest of Trudeau's species and with the lowest Ho has relatively high values of Ho (0.142) in the present study. Most of the other species in group I are relatively small, except of course M.rosenbergii.

Fuller and Lester(1980) reported a significant correlation (Spearman correlation coefficient) between measures of genetic variation (e.g. Ho) and important habitat characters (e.g. size of habitat, potential for immigrants and age of habitats) and concluded that small isolated populations are less genetically viable than large inter-connected populations. This seems to support the findings for the populations of M.nipponense in the present study.

(5.4.5) Discussion on the ontogenetic variation in isozyme expression

Studies on the ontogenetic expression of isozymes in Decapoda (even in Crustacea), have been extremely rare. Gooch(1977) observed no genetic variability among zoeal, megalopa and adult of 3 brachyurans (Rhithropanopeus harrisii, Sesarma cinereum & S.reticulatum) in 15 enzyme loci. Morgan et al.(1978) also investigated the developmental changes of isozymes in

R.harrisii and found a considerable amount of ontogenetic changes in 6 enzyme loci (EST, LDH, MDH, ALP, PGI & ACPH). The number of isozymes observed at various developmental stages varied from 5 in PGI to 11 in LDH ! Morgan et al.(1978) accounted for the differences because they used pooled samples, as opposed to individual crab larva used by Gooch(1977). The major isozyme changes of R.harrissii occurred at zoeal III and IV and Morgan et al(1978) related it to the dietary requirements of the brachyuran larvae. A third study on the same species, R.harrissii, was reported in Kannupandi(1981). Similar isozymes (EST, LDH, APH, & ACPH; MDH was not studied) were observed to be variable , in addition to a number of general proteins.

In a specific study on the esterases alone in developmental stages of the lobster Homarus americanus, Barlow and Ridgeway(1971) reported differential expression of different esterase group in the first 8 stages of larval development.

Recently, Lester and Cook(1987) reported the ontogenetic changes of isozyme expression in Penaeus setiferus, P.stylirostris, P.vannamei and P.aztecus. Variable isozyme patterns were observed across the four developmental phases (protozoea, mysis, postlarva, and adult) in all the loci studied (AO ,ACPH, GDH, PGI, PGM, MPI, EST, peptidase and general protein). In general, the complexity of banding increased with development. However, Lester and Cook(1987) acknowledged that absence of isozyme bands at a certain stage might also be the result of low concentration of that protein in the larval samples or its absence in the only 2 tissues sampled in the adult samples (muscle and hepatopancreas).

In the present study, the 5 loci observed to be variable included ACPH and APH, both reported to be variable in R.harissi. Kannupandi(1980) suggested that APH variability might be related to the synthesis of glyco- and lipoprotein matrix of the integument. Similarly, changes in LAP and GPT may be related to the physiological function (osmoregulation; see Chapter 4 & 5) of these two enzymes. Furthermore, most of the changes also took place either at late developmental stages (approaching final metamorphosis) or at stage II, the stage at which feeding and possible estuarine existence commence in M.nipponense and M.rosenbergii.

From this circumstantial evidence, it is tempting to suggest that ontogenetic variation in isozyme expression may be related to life-cycle heterogeniety. However, as in the case of R.harissii, results from different laboratories may reveal very different patterns of variation, in addition to the sampling problems of small-size larvae as acknowledged by Lester and Cook(1987). Further study with more batches of larvae and with other species have to be carried out to confirm the findings of the present studies. Bearing in mind that early developmental stages of these freshwater prawns is the phase in their life cycle facing the most heterogenous environmental conditions, much more attention is required in this area.

'And... the Lord God....brought them unto Adam to see what he would call them : and whatever Adam called every living creature, that was the name thereof. And Adam gave names to all the cattle, and fowl of the air, and to every beast of the field.'

(Genesis 2:19-20)

Chapter SIX :

PHYLOGENY IN THE GENUS *MACROBRACHIUM*

(6.1) INTRODUCTION

(6.1.1.1) Phylogeny, Taxonomy and Systematics

Taxonomy is the naming and assignment of organisms to taxas (the practice of classification). Phylogeny is the genealogical relationship between organisms, present or extinct. Systematics is the study of evolutionary relationships among organisms, as well as their phenotypic similarities and differences. However, taxonomy, which is based on the similarity between organisms, can also be related to phylogenetic relationships (except in the case of convergent evolution). On the otherhand, phylogeny is partially related to the similarities of characters.

The aim of this chapter is to study the phylogeny of the genus *Macrobrachium*, drawing information from allozyme, larval developmental, morphological, hybridization as well as biogeographical data.

(6.1.1.2) The Contending Schools of Taxonomy

While taxonomy is probably one of the oldest human preoccupation, its methodologies and working principles are probably in no greater agreement than those in any other new profession. Two major principles and three major schools of classification can be recognised.

The phenetic school of taxonomy (e.g. Sneath and Sokal, 1973) is based on overall similarity among species, measured by as many characters as possible, even if such a classification does not reflect common ancestry. Species are thus grouped on a phenogram. Apart from the construction of a dendrogram, a phenogram can also be in different formats, including three dimensional diagrams of canovariate analysis. Most of the techniques of phenetics can be found in Sneath and Sokal(1973).

The cladistic school of taxonomy (or systematics) expresses the branching (cladistic) relationships among species. As such a cladogram is not based on overall similarity of species, it may differ substantially from a phenogram.

In essence, the cladisists emphasised that classification should be strictly monophyletic in origin, each taxon should include species only from the same ancestor. A taxon classified phenetically may include species from different ancestors, forming a polyphyletic group.

In addition, a third school, the evolutionary taxonomy (sensu Ridley, 1985) can be recognised, which represents a mixing of the cladistic and phenetic principles, but does not allow convergent evolution. However, a faster-evolving lineage (e.g.

birds vs crocodiles and lizards) may be classified to separate clades (favouring the phenetic principle).

Similar to the various schools of taxonomy, phylogenetic analysis also has identical contending principles, relating to the construction of phylogenetic trees (see 6.1.6). In the present thesis, a phylogenetic tree implies a dendiogram constructed by a certain phylogenetic principle and does not refer to the full phylogenetic tree which would involve all the present and extinct species.

Early pheneticists (e.g. Sokal and Sneath,1963) had hoped that phenetic methods would result in stable classification (or phylogeny), by containing a maximum amount of information of the taxa concerned. This is, of course, related to the subjectiveness of the choice of algorithm and characters.

Different types of characters are now available to modern taxonomists, including allozymic, karyological, physiological, biochemical, as well as morphological characters. The availability of computers and easily quantifiable characters have facilitated a whole array of methods for the construction of phylogenetic trees (section 6.1.4).

(6.1.1.3) Difficulties in the taxonomy of *Macrobrachium*, using morphological characters

"*Macrobrachium* is a notoriously difficult genus (referring to taxonomy) as the animals change so much, and so gradually , during their growth " (Holthuis,1984; pers. com.). Indeed, as pointed out by the celebrated authority on the genus, the study of the genus *Macrobrachium* has been made very difficult by:

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"*Macrobrachium* is a notoriously difficult genus (referring to taxonomy) as the animals change so much, and so gradually , during their growth " (Holthuis,1984; pers. com.). Indeed, as pointed out by the celebrated authority on the genus, the study of the genus *Macrobrachium* has been made very difficult by:

(a) The general shape of the prawn and many other features are common to all the species in the genus. All identification are related to the morphology of the rostrum and the second pereopod only;

(b) Most of the characters, and especially the second pereopod, are extremely variable during the course of development. The rostrum generally becomes relatively shorter with growth;

(c) A big morphological difference generally exists between the male and the female of a species, especially in the morphology of the second pereopod. Even in the adult female, the size of the second pereopod probably resembles the juvenile stage;

(d) Great inter-specific variation does exist in many species, sufficient to the status of sub-species in many cases (e.g. M.rosenbergii);

(e) Early maturation in both females and males is known in many species, creating more confusion in an already confused situation.

Most, if not all, schemes of identification are generally constructed for use with adult male specimens only. For female specimens and juvenile Macrobrachium, species identification is generally very difficult, if not impossible.

(6.1.1.4) Phylogenetic studies of Macrobrachium, using morphological characters

Kubo(1940) investigated the possible phylogenetic relationship of some Japanese Palaemon (= Macrobrachium). However, his method was mainly phenetic in nature. Using 16 morphological characters, a table of 'coincidence of characters' was constructed. Kubo recognised two groups. The first group was characterised by slender second pereopods, including M.nipponense, M.formosense and M.longipes. The second group consisted of M.japonicus, M.grandimanus and M.latimanus, all of which had stout second pereopods. The third group, represented by M.equidens, consisted of species with small body size.

Johnson(1960) discussed the distribution of the M.pilimanus species complex. Even with more than 1000 specimens over the range of the complex, Johnson(1960) was unable to clarify the status of the species in the complex, especially the two species, M.pilimanus and M.malayanum. A recent study of the complex and the larval development of the species concerned has confirmed the specific status of both species (Chong and Khoo,1987a; 1986b; 1986c). M.pilimanus was also suggested to be synonymous with M.geron Holthuis,1950 (Chong and Khoo,1987a).

Johnson(1973), suggested that while the genus is taxonomically difficult, two groups can be recognised among some species of Macrobrachium. The first group, the M.rosenbergii group, consist of species with the following characters:

a. A pronounced basal crest;
b. Distal part of the dorsal rostral margin bears comparatively few or widely spaced teeth or is unarmed;
c. The ventral rostral teeth are also comparatively numerous to other species, varying from 4-16;
d. The second pereopods of males are very long and comparatively slender, spinulose and pubescent in parts, and symmetrical in size .

Young individuals retain juvenile characters until they have attained a considerable size and generally resemble species of the related genus Leptocarpus, Exopalaemon, and Palaemon. Johnson(1973) also suggested many characters of the group were comparatively 'primitive' (Cf. also larval development), and that it has probably originated independently of other species in the genus, from prawns closely related to the present members of Exopalaemon. This group, as suggested by Johnson(1973), consist of M.rosenbergii, M.malcolmsonii, M.villosimanus, M.weberii, M.palawanense, as well as the south American species of M.panamense and M.amazonicum. The affiliation of this group

was confirmed by the similarities in their larval development (see Ch.3) and by the success of hybridization between M.rosenbergii and M.malcolmsonii (Sankoli et al.,1982). M.lamarrei, which has many morphological attributes similar to M.rosenbergii (e.g. basal crest and curved rostrum) and also resembles juveniles of the group (Johnson,1973),may also be related to the group. However, Johnson expressed his doubt on account of the small size of the species (Cf. also greatly differ in larval development).

The second group recognised by Johnson(1973) was the M.equidens group. M.rude and M.equidens were treated as typical species of this group, with other 'possible' members including M.sintangense, M.idae,M.idella and M.mamillodactylus. These species generally have a long rostrum with fairly numerous ventral teeth . The second pereopod of adult males are long and cylindrical , with 1-2 proximal teeth at the fingers and the carpus of the full grown males are distinctly longer than that of the merus. The features of similarities in this group, as suggested by Johnson, seem to be less strong, as compared to the former group.

Figueroa(1969) compared the distribution and morphology of a group of species in central America, M.olfersii, M.fausitinum, M.hancocki, M.acanthochirus and M.digueti. Figueroa inferred that this group of spcies form a monophyletic group with an ancestor most related to M.olfersii. The major common character of all the species is the asymmetrical size of the second pereopods, and the strong dense spiculation and the cup-shaped second pereopods.

Tiwari (1952) described the Hendersoni group of species in northern India. This group of species, usually inhabiting streams in the hilly regions and at their bases, appeared to be closely related to the Eastern Himalayan form. The most important character is the presence of numerous longitudinal grooves in the fingers of the second cheliped and the presence of soft velvety pubescence. The group consists of 9 species and subspecies: M.hendersonii cacharensis, M.hendersonii platyrostris, M.hendersonii hendersonii, M.siwalikensis, M.assamensis penisularis, M.assamensis assamensis, M.hendersodayanus and M.dayanus. A phylogenetic tree was constructed, assuming that there was a progressive elongation of rostrum with a corresponding increase in the dentition of its upper and lower edges, elongation of the carpus in the second pereopod and a corresponding relative decrease in the proportion of the chela.

From the above review, it is evident that within the general difficulty in the taxonomy of the genus, several monophyletic groups can be recognised, mainly based on very few morphological characters (e.g. asymetrical second pereopods, basal crest and curved rostrum). However, most of the delimiting characters are related either to the morphology of the second pereopods or the rostrum. Because of this and developmental constraints, convergent evolution in some of the characters would be highly likely (e.g. increase in rostral dentition and in the case of M.lamarrei).

(6.1.3) Ontogeny and Phylogeny

The significance of ontogenetic information to phylogeny has long been realized. Haeckel's (in Gould, 1977) sentence of "Ontogeny recapitulates phylogeny" has been much quoted. Gould(1977) summarised most of the historical developments and conceptual issues in relation to the phylogenetic considerations of ontogeny.

Most of the examples given by Gould were related to the origin of higher taxas, or macroevolution by Paedomorphosis (e.g. neotony). The most famous case is, of course, the faculative paedomorphosis in several groups of salamanders (e.g. Typhlotriton, Ambystoma). Many juvenile features such as gills are retained in the sexually matured adults, in addition to species specific features (Tompkins, 1978). In crustaceans, the most famous case of paedomorphosis should be the dwarf males in several species of sand crab Emerita (Efford, 1967). Males of these species, which may amount to a mere 1/10 of the size of the females (e.g. in E.rathbunae and E.emeritus), mature precociously and are attached to the females under their third walking legs (!). In both of the mentioned species, many important larval characters are retained (e.g. stumps on the pleopods).

At the species level, inter-specific differences in larval characters have been used to delineate species of Cirripedi (see Chapter 3). In Brachyurans, Rice(1980) and Hines(1986) surveyed the inter-specific differences in the larval developmental pattern, especially in relation to the zoeal stages. Rice(1980) considered larval development with more zoeal instars and greater

variability in the number of zoeal instars to be primitive. Rice(1983) constructed a phylogenetic tree of several brachyuran groups, based entirely on the zoeal stages.

Adult characters have resulted from a series of development through larval forms, from the fertilised zygote. Complex changes in the geometric form of an organism (within developmental constraints) may arise from simple changes in developmental rates. A single gene substitution, which affects the concentration of thyroxin, can determine whether metamorphosis take place in axolotl (Tompkins,1978). It is revealed that most of the mechanism of segmental determination in Drosophila is polygenic (Davidson,1986) and generally related to the Homeobox gene (of which homologous sequences also exist in other vertebrates). However, the regulation of the expression of a particular gene (e.g. wingless gene wg) has resulted from interactions between several other genes (Ingham et al.,1988). Subtle changes in any of the related genes would thus result in profound changes in the morphology of the adult.

The Von Baer's Law stated that ' the early developmental stages of a character that differentiate between taxas are embryologically later accretions on a fundamentally similar developmental plan'. While it is expected that early developmental stages also preceed many species specific characters, the general characters of development have been suggested for the determination of the polarity of evolutionary changes (de Queiroz,1985; also next section).

Most of the larval developmental data have been reviewed in Chapter 3. While there are specific differences in a particular

appendage, most of the differencees are related to the developmental rate of an appendage, which generally develops from a 'bud' to an appendage that resembles the adult (still with larval characters in the early stages). The present study would also aim to study the phylogenetic relationships of the different species, in relation to their larval developmental stage data.

(6.1.4) Methodologies in the study of phylogeny

With the increased availability of computers and electrophoretic techniques, there is also a parallel increase in the technical approaches of phlogenetic analysis, generally related to the computation of data. This section aims to review briefly some of the methods, especially those related to the present study.

(6.1.4.1) The use of morphological data in phylogeny

A morphological character is a structural attribute of an organism. For a character to be taxonomically and phylogenetically useful, it has :

a. to vary between taxas being studied;

b. to vary in a correlated and related manner;

c. not to be related to the level of classification.

The overall similarity within the genus Macrobrachium implies difficulties in its study.

The taxonomy of different groups generally involves very different morphological characters. The choice of characters then is generally subjective, and also varies with the level of classification (species, genus, family). For phylogenetic

analysis, it would be desirable to know the ancestral state of characters, so that evolutionary polarity and path can be deduced. Species with the maximum number of derived states would then be grouped under the same clade.

However, the identification derived from ancestral states is generally difficult in many organisms (e.g. in Macrobrachium). Information can be available from fossils on the possible ancestral state from a hypothetical ancestor. However, it is also rare that such fossils exist in most studies. An outgroup criterea can be used, by relating characters of the interested group to a related but more primitive group. Also ontogeny of a character, as suggested in the last section can also be used to determine the polarity of an adult character (de Queirez, 1985). On the otherhand, many methods are available in constructing phylogenetic trees without prior knowledge of ancestral state, but the tree generated would be 'undirected ' (or unrooted; the direction of the ancestor is not shown). Morphological characters can either be expressed in continuous measurement (e.g. the length or size of an appendage) or coded in a binary format. Multi-state characters can also be recoded in binary forms (Sokal and Sneath, 1963). Morphological data can also be measured quantitatively for the construction of a phylogenetic tree, the methodologies involved are, in essence, very similar to the approaches used for the gene frequency data (Chapter 6.1.4.3).

Some of the particular problems in relation to the phylogeny of higher taxa in crustaceans are reviewed in Schram(1983).

(6.1.4.2) The use of karyotypic analysis in phylogeny and chromosome studies in crustaceans

Karyotype may be defined as the phenotypic appearance of somatic chromosomes in contrast to their genic content. The karyotype of different species can differ from each other in the following aspects:

a. The number of chromosomes;
b. The absolute size of chromosomes;
c. Size differences within a complement;
d. Arm ratios of chromosomes;
e. Number, size, position of secondary constriction and satellites;
f. Size of localised centromeres and number of microtubules attached;
g. Kind of centric activity;
h. Position, size, number and distribution of differentially stained heterochromatin segments;
i. Total amount of DNA; (adapted from Jackson, 1971)

In addition, there are now a large number of staining techniques (e.g. C-, G-, Q-, R-bandings, etc) which have very different implications to chromosomal structures (Comings, 1978) and would readily provide additional karyotypic characters.

One of the best examples of the use of karyotypes in phylogenetic analysis would undoubtedly be the proposed phylogeny of 103 Hawaiian species of Drosophila totally based on chromosome inversion data (Carson, 1983). In crustaceans, the most comprehensive single survey of karyotypes is still Niiyama (1959)'s study of 33 species of decapods, isopods and amphipods. Most other studies have been reviewed in Hedgecock et al.(1982) and White(1973).

In the Decapoda, although there occurs a wide range of chromosome numbers, the species investigated so far tend to fall into two distinct groups. One is characterised with over 200 chromosomes, the other with chromosome number of about 100.

Penaeus aztecus, P.setiferus, P.duorum and P.japonicus were reported to have 80, 90, 80 and 92 chromosomes (2n) respectively (Milligan,1976; Niiyama,1959). The lobster Homarus americanus and H.gammarus were reported to have chromosome numbers of 110 and 95.5 (average value reported) respectively (Hughes,1982). Niiyama(1966) then reported an unprecedented number of chromosome (2n = 376) for the crayfish Astacus trowbredgii, one of the largest numbers of chromosome known in animals !

Colombera and Lazzvetto-Colombera (1976) reviewed the chromosome data in several invertebrates taxas. In the Harpacticoid copepods, while there is a general uniformity in chromosome number, evolution has been characterised by reduction in chromosome number from 2n=24 to 10. Very closely related species of the genus Tisbe, even sibling species, can differ greatly in their chromosome numbers. A reduction in the chromosome number was also inferred for calanoids and cyclopoids in course of evolution.

The present chapter also aims to investigate the chromosome number of as many species of Macrobrachium as possible.

(6.1.4.3) The use of allozyme data in phylogenetic analysis

Allozyme data present readily quantifiable characters (different loci) for numerical analysis. However, because of its supposed relation to genotypes, its methodologies are different (at least conceptually) to those for other characters. The

different approaches adopted by different workers can generally be catagorised in the following approaches:

(6.1.4.3.1) Genetic distance/similarity approach

This approach generally involves the computation of similarity or dissimilarity between taxas(or populations) per locus, taking into account the frequency of different alleles, and then averaging over all the loci studied. The matrix of genetic distances/similarities generated would then be used to contruct a phylogenetic tree of the taxas/populations concerned.

This apparently standard procedure, however, has a whole array of different statistical indices available for the quantification of genetic distance/similarity.

Nei's standard genetic distance (D; Nei,1975) between populations, based upon his earlier models(Nei,1971;1972), was devised to measure the mean number of electrophoretically detectable substitutions per locus that have accumulated since divergence between the two populations. While this index has been used by most investigations, it is non-metric and does not satisfy the triangular inequality, which can occasionally led to negative arm length in phylogenetic trees.

Rogers(1972)'s similarity coefficient (S), however, estimates the mean geometric distance between allele frequencies and summarise over all loci. While it was not designed to measure biological properties, it satisfies the triangular inequality and would not give negative branch length in a phylogenetic tree.

While there are a whole list of other indices proposed, Nei's standard genetic distance, and Roger's similarity index which has been proved to be quite robust to small sample size

(Nei, 1978), will be adopted in the present study of population structure. The definition of Nei's index has already been treated in the last chapter.

A large number of similarity/dissimilarity indices are also available for quantitative morphological characters, most of which have been reviewed by Sneath and Sokal(1963).

(6.1.4.3.2) Presence/absence transformation

Most of the values computed by D would be accountable by those loci that have completely differentiated alleles in the two taxas concerned. This forms an argument to treat each allele as a separate discrete character with O/I states, depicting its absence/presence respectively. The resulting data set would then be used for the contruction of phylogenetic trees. However, such treatment has ignored the correlated nature of different alleles in polymorphic loci, as well as losing available information. In population studies of intra-specific variation, this method would probably overestimate the similarity between populations, especially in species with low intra-specific variation. For inter-specific study in species with high genetic distance, this method would probably generate very similar result to that of the first approach.

(6.1.4.3.3) Felsenstein's square root transformation for the restricted maximum likelihood contruction of phylogenetic trees

This method is mainly based on the model of Edwards and Cavalli-Sforza(1963), Cavalli-Sforza and Edwards(1967), Thompson(1973) and Felsenstein(1973). The gene frequency data is

first square-root transformed (similar to Cavalli-Sforza and Edward's Chord measure of genetic distance), rendering the variance of each alleles more or nearly equal. This would fulfil the assumption of evolutionary change of independently drifted characters. Phylogenetic trees would then be contructed using the maximum likelihood method of Felsenstein (1982).

(6.1.4.4) Molecular clock

According to neutral theory of molecular evolution, new alleles would be substituted at a locus by mutation, at a fairly constant rate. The comparision of the divergence between macromolecules then offer a molecular clock by which the time since common ancestry can be estimated. The results of the last 25 years have proved that the extent of divergence to be roughly proportional to the time elapsed, especially in a taxonomic group with many branch points (Wilson et al.,1987).

To calibrate the molecular clock, fossils would provide a fairly accurate source of reference. For those taxonomic groups with fossil records, the molecular clock has in fact proved to be proportional to the time elapsed (e.g. Carlson et al.,1978; Koop et al.,1986). However, it is more often that evolutionary studies do not have the good fortune of fossil evidence. In such cases, the study of evolution can still be pursued in several approaches:

a. Compare the relative rates from different genes and assume a consensus;

b. Compare the divergence in duplicate genes (e.g. the various globin genes);

c. Compare the divergence within a taxonomic group to those of a

reference species (outgroup) that lies phylogenetically outside the group;

d. Compare the several lineages of a group that diverge at roughly the same time (e.g. different orders of mammals).

The definiton of the Nei's standard genetic distance is related to the substituion rate per loci per year. It is, in effect, approach (a) which assumes a consensus over several genes. With different loci diverging at different time, the value of D would be highly affected by the different allozyme loci being studied. Proteins do differ extremely in their mean rates of electrophoretic divergence, which may relate to their biochemical structure (subunit numbers, subunit size, etc.; Thorpe,1982). It is generally established that enzymes not involved in the central metabolic pathways evolve at a faster rate. Sarvich(1977) suggested that protein loci can be split into 'fast' and 'slow' groups, differing in their substitution rates by an order of magnitude. Thorpe(1982), however, purported that the frequency distribution of average genetic distances approach more to a normal distribution than to a bimodal distribution as suggested by Sarvich(1977). Nei(1975) acknowledged the variation of D with different loci and put forward an average divergent time estimate of 5 million years per unit of genetic distance. However, the most commonly used value seems to be 18 million years per unit of D (Futuyma,1986; Thorpe,1982).

Apart from suggesting that most evolutionary substitutions are due to the fixation of neutral mutations, the following have to be assumed:

a. The mutation rate per year is higher in individuals from species with shorter generations;

b. The proportion of mutations that are neutral is lower in bigger populations ;

c. Species with shorter generations have bigger populations. (from Wilson et al.,1987)

For the genus Macrobrachium, which mostly consists of tropical prawns in tropical/subtropical regions in the world, it is thus assumed that their generation time are roughly the same.

(6.1.5) Construction of phylogenetic trees

The ultimate aim of a phylogenetic study is to elucidate the phylogenetic relationship between the various species, taxas or populations being studied. The different methods of transformation mentioned in section (6.1.4) present to the investigator many different types of data in the construction of phylogenetic trees. Numerous methods are also available for each type of these transformed data, as well as to other discrete, continuous and molecular(DNA) data.

(6.1.5.1) Methods for genetic distance data

For data in the form genetic distance matrix, e.g. immunological data, transformed electrophoretic data, continuous character matrix, a large number of methods are available to contruct the hypothetical phylogenetic trees. For most, it can be categorised into two groups, the clustering and the pairwise methods.

(6.1.5.1.1) Clustering methods for distance matrix data

One of the most used methods, the average-linkage clustering method or the unweighted pair-group method with arithmetic

averages (UPGMA), which is a simple hierarchical grouping of Operational Taxonomic Units (OTUs), assuming arithemetic mean of the already grouped OTUs. By such averaging, however, the method imposes an average constant evolutionary rate across all lineages. This would result in the clustering of non-monophyletic groups. The condition of relatively constant evolutionary rate would probably hold in intra-specific investigation of populations of the same species and for inter-specific analysis for closely related species.

(6.1.5.1.2) Pairwise methods for genetic distance matrix

Pairwise methods, in essence, are very similar to the clustering methods. Instead of the hierarchical manner of clustering and averaging OTUs, pairwise methods aim to account for, as much as possible, the observed distance.

Mathematically, pairwise methods aim to minimize a certain index, in order to fit the observed data. Fitch and Margoliash(1967)'s method, one of the more popular methods, minimize the sum over all observed distances of $(d-d')^2/d^2$, where d is the observed distance and d' the expected distance under additive hypothesis in the tree. Cavalli-Sforza and Edwards(1967), on the otherhand , proposed to minimize the term $(d-d')^2$ while Beyer et al.(1977) minimize the term (d'-d)/d.

Another widely used method, the distance Wagner method (Farris,1972), is not related to the Wagner parsimony(next section), despite its name (Felsenstein,1982), and does not minimize/maximize on any particular index, but merely present trees with d'>/ d.

(6.1.5.2) Methods for character state data

For most phylogenetic investigations of discrete characters, data are presented in the form of two state characters O/I (e.g. in the case of transformed allelic data). As for genetic distance data, a large number of methods have been developed for the construction of phylogenetic trees, which can generally be catagorised into the parsimony and the compatibility methods.

(6.1.5.2.1) Parsimony Methods for character state data

The main criteria of parsimony methods is to choose a phylogenetic tree with the smallest evolutionary change of character states (the most parsimonous), i.e. minimizing the number of convergent and reversal events. As in other methodologies, a large number of parsimony methods exists, the more widely used ones include the Wagner parsimony, the Camin-Sokal parsimony and the Dollo parsimony.

Different parsimony methods have different assumptions about the irreversibility of character state, especially in the presence/absence of hypothetical ancestral character state. Thus, the Wagner parsimony, which assumes the ancestral state to be unknown (say in the case of gene frequency data), the resulted trees obtained are undirectional or unrooted.

(6.1.5.2.2) Compatibility methods for character state data

Compatibility methods are devised to construct the most likely phylogeny that is fully compatible with the largest number of individual characters. It thus generates a cladogram rather than a phenogram, and is probably more related to the cladistic

rather than the phenetic school or the phylogenetic schools of classification.

Most practical methods have been developed by Estabrook and McMorris and their co-workers (e.g. Estabrook and McMorris, 1977). Attributed to its basic criteria, the compatibility methods imply that large cliques in a tree may contain two few characters and that information of many characters are totally ignored.

A partial solution of this is available by using all characters again within separate cliques, after the overall tree topology is found.

(6.1.5.2.3) Felsenstein's restricted maximum likelihood method

Felsenstein(1982) criticized both parsimony and compatibility methods in their lack of statistical justification: "most data require too many changes of state and have too many incompatible characters to allow us to believe that we are in a situation in which parsimony or compatibility are maximum likelihood methods."

He then proposed a Restricted Maximum Likelihood method(REFL) to deal with quantitative and gene frequency data. This method is mainly based on the model of Edwards and Cavalli-Sforza(1964) and Cavalli-Sforza and Edwards(1967), Thompson(1975) and Felsenstein(1973). For gene frequency, the data is firstly square-root transformed to obtain more equal variance of character.

(6.1.5.3) More considerations on character state data

While different versions of the above methods are available

to accomodate the varied nature of different data set (e.g. transformed data from molecular and continuous data), the data are still required in the form of two-state characters. For multi-state characters, different procedures are available to 'recode' into binary form.

Based on the understanding that some characters are more 'conservative' than others, some methods(e.g. Farris,1969) allow the individual weighting or even interactive weighting of the characters.

One important assumption of both parsimony and compatibility methods is that the characters considered have evolved independently. In most situations, correlation probably exist between the data set in a wide variety of continuous, discrete, as well as in gene frequency data. In some cases, it may be necessary to pool two correlated characters together, or delete some characters.

The criteria of the two methods also relate to the two schools of taxonomy, the compatibility method literally imply a phenetic classification while the parsimony method aims to produce a cladogram.

The assumption of the above methods implies examining a large number of possible trees for the best one. As the number of taxas/species increases in a particular investigation, the number of possible trees also increases exponentially. This obviously implies that the availability of particular computer softwares or hardwares disproportionately governs the use of a particular method. The phylogenetic trees constructed in the present thesis are drawn by the program PHYLIP written by Dr. Joseph Felsenstein

of Washington University.

(6.1.6) Allozyme studies of inter-specific variation in Decapods

Morphological and biochemical characters can differ greatly in their rate of evolution. Differentiation of species from morphological criteria may not necessarily imply the same amount of difference at biochemical level, and vice-versa. However, since the use of allozyme data in systematics, many sibling species, some belonging to established species, have been revealed.

Among 8 species of coral xanthid crabs, Huber(1985) reported an average value of 0.36 (0.15-0.52), values well within the expected values of differentiated species. This supports the specific status of the group which mainly differ in their colour. In previously suspected species, Metapenaeus bennettee and M.dalli were revealed to have 8 discriminant loci out of 44 (Salini and Moore,1985), confirming their specific status.

Similar to morphological keys for species identification, it is possible to construct keys based on allozyme bands. Johnson et al.(1974) contructed keys for 5 species of pandalid shrimp, using electrophoretic bands. Similarly, Kulkarni et al.(1980) reported different patterns of general proteins for Metapenaeus affinis, M.monoceros, Parapenaeopsis hardwickii and P.styliferus.

Within genus values of I, calculated from allozyme data, are generally higher than between genus comparision. Three genera of crayfish had within genus of I of 0.51-0.78 while between-genus values were 0.403-0.51 (Nemeth and Tracey,1979; Brown,1981

). Six species of Metapenaeus and 7 species of Penaeus had within-genus of I of 0.69 and 0.65 respectively. Between genus comparision was much lower (I = 0.39; Mulley and Latter, 1980). For two other species of Penaeus, P. japonicus and P. kerathurus, the value of genetic distance was high (D = 0.887; De Matthaeis, et al., 1983).

Small values of genetic distance were computed for some species. No genetic distance was calculated for two species of rock lobster (Smith, et al., 1980), but the morphological differentiation was also slight. For the two established species of lobsters, however, a small genetic distance (0.11) generally observed for intra-specific variation, was observed between H. americanus and H. gammarus.

Hedgecock et al., (1982), list values of 40 cogenic comparision in crustaceans, which when converted to genetic distance, amount to an average value of 0.58, with a range of 0.08 to 1,69.

(6.2) MATERIAL AND METHODS

(6.2.1) Electrophoretic study

All the protocols for electrophoretic analysis, including the enzyme loci, buffers and staining recipes, are identical to those of Chapter 5. 12 species were investigated originally, a thirteenth species was identified, which segregates at 3 loci with M.sintangense, but was morphologically indistinguishable from it.

The locations for sampling are summarised in table (6.2). The abbreviations listed in the same table will be used throughout the chapter for the construction of phylogenetic trees.

Table (6.2) : Sampling location and abbreviation used in the present study

Species	Abbreviation	Location
M.acanthurus	ACA	Veracruz, Mexico
M.dayanum	DAY	Bogra District, Bangladesh
M.equidens	EQI	Columbo, Sri Lanka
M.hainanense	HAI	Hong Kong
M.idella	IDE	Dodoma, Tanzania
M.lanchesteri	LAN	Malaysia
M.lamarrei	LAM	Bogra District, Bangladesh
M.lepidactylus	LEP	Mombasa, Kenya
M.nipponense	NIP	as in last chapter
M.rosenbergii	ROS	as in last chapter
M.rude	RUD	Columbo, Sri Lanka
M.sintangense	SIN-1	Bogor, Indonesia
M.spp	SIN-2	Bogor, Indonesia

All the species were identified by local scientists, reconfirmed subsequently in the laboratory, using published keys. Some of the species (M.hainanense, M.idella, M.equidens, M.nippoinense) were also confirmed by Prof. L.B.Holthuis.

In addition to computation of Nei's genetic distance

between all the species, Roger(1972)'s genetic similarity index was also calculated by the following formula:

$$SR = 1 - \{ 1/2 \sum_{i=1}^{m} (Pix - Piy) \}^{1/2} \qquad \text{(equation 6.1)}$$

where P_{ix} and P_{iy} are the allele frquency of the ith loci for the x and y populations respectively.

The distance matrix were then used for the construction of phylogenetic trees, using UPGMA, Fitch-Margolaish and Edwards and Cavalli-Sforza methods. The allele frequency data was also converted to presence/absence character state for the construction of phylogenetic tree using the Wagner parsimony method. This combination of methods should represent a fair spectrum of the methods available.

(6.2.2) Material and methods for the construction of phylogenetic trees using adult morphological characters

As mentioned in the introduction of this chapter, Macrobrachium is a notoriusly difficult genus for the taxonomists. The present analysis aims to construct a phylogenetic tree of some Macrobrachium species, using qualitative morphological characters. Reference were made to the previous study of phylogeny reviewed in section (6.1.1) in relation to the choice of characters. 24 different adult male characters were used, as summarised in table (6.3).

As expected, the majority of the characters were related to the morphology of the rostrum or the second pereopods. For three-state-characters, all were recoded into binary format (

00, 01 and 11), similar to the method suggested in Sokal and Sneath(1963). The result of this recoding would give more weight to the difference between state 00 and state 11. This would represent the real situation in all the 3-state characters considered here. An upward curved rostrum would logically be inferred to differ more from a straight rostrum than a slightly curved rostrum. To give equal weighting to all the others, the weighting of the the other two-state characters were thus doubled.

Both the parsimony and compatibility methods were used to construct phylogenetic trees. For the parsimony method, two approaches were used. The first one was based upon the Wagner parsimony related to Eck and Dayhoff(1966) and to Kluge and Farris(1969) with bi-directional changes of character state (0<---->1). In the second approach, a mix parsimony method was used which, in addition to the above Wagner parsimony, the Camin and Sokal parsimony method was used. In this, those characters in which the ancestral state can be reasonably assumed (e.g. increase in rostral dentition) were specified for the Camin and Sokal method, while those with no apparent delineation of ancestral/derived states were analysed by the Wagner parsimony method. In the Camin and Sokal parsimony, the change 0--->1 was much more likely than from 1--->0. Those characters with assumed ancestral states in this system are marked with a (*) in table (6.3). The sub-program in the main program PHYLIP used was 'MIX'. For the compatibility method, the program CLIQUE was used, which is based on Estabrook, et al.(1976) and no ancestral states were assumed.

Table (6.2) : Adult male characters used in phylogenetic analysis

(1)	0	Upper border of rostrum almost straight
	1	Upper border of rostrum a little convex
(2)	00	rostrum almost straight
*	01	rostrum curve slightly upwards
	11	rostrum curve distinctly upwards
(3)	00	base of rostrum has no elevated crest
*	01	base of rostrum has slightly elevated crest
	11	base of rostrum has distinctly elevated crest
(4)	00	number of upper rostral teeth < 11
*	01	number of upper rostral teeth > 10 and < 15
	11	number of upper rostral teeth > 15
(5)	0	number of lower rostral teeth < 6
*	1	number of lower rostral teeth > 5
(6)	00	no upper rostral teeth behind orbit
*	01	2-3 upper rostral teeth behind orbit
	11	4-6 upper rostral teeth behind orbit
(7)	0	rostrum do not pass or just reaching antennal scale
*	1	rostrum distinctly pass antennal scale
(8)	0	no naked space between apical and sub-apical upper
*		rostral teeth
	1	naked space between apical and sub-apical upper rostral teeth
(9)	0	upper rostral teeth regularly spread
*	1	rostrum with a distinct naked portion in the distal half of the upper margin
(10)	0	second pereopods symmetrical
*	1	second pereopods asymetrical

Character 11-22 refered to second pereopod; in the case of asymetrical second pereopods, the larger one.

(11)	00	merus < carpus
	01	merus = carpus
	11	merus < carpus
(12)	00	carpus < chela
	01	carpus = chela
	11	carpus > chela

Table(6.2): continue

(13)	00	carpus < palm
	01	carpus = palm
	11	carpus > palm
(14)	00	finger < palm
	01	finger = palm
	11	finger > palm
(15)	00	no teeth on the proximal portion on the cutting edge of fingers
	01	1-3 teeth on the proximal portion on the cutting edge of fingers
	11	> 3 teeth on the proximal portion on the cutting edge of fingers
(16)	0	merus/carpus elongate & cylindrical
*	1	merus/carpus swollen
(17)	0	palm elongate and cylindrical
*	1	palm swollen
(18)	00	merus/carpus surface smooth
*	01	merus/carpus surface sparsely covered with spinnules
	11	merus/carpus surface densely covered with spinnules
(19)	00	chela surface smooth
*	01	chela surface sparsely with spinnules
	11	chela surface densely with spinnules
(20)	0	palm without hair
	1	palm pubesense
(21)	0	finger without hair
	1	finger pubesense
(22)	0	carapace smooth
	1	carapace roughened with small tubercle
(23)	0	tip of telson pass its posterior dorsal teeth
*	1	tip of telson passed by its posterior dorsal teeth
(24)	00	maximum length (male) < 5 cm
	01	maximum length (male) =< 10 cm
	11	maximum length (male) > 10 cm

(*) characters with assumed ancestral state of 00 or 0

Table (6.3) : Character state of adult characters used in phylogenetic analysis

species	Character *																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
NIP	11	00	00	01	0	01	0	0	0	0	00	01	00	00	01	0	0	01	01	0	1	0	1	11
ACA	01	00	01	01	0	01	1	0	1	0	00	00	11	00	01	0	0	01	00	0	1	0	0	11
EQI	00	01	00	00	0	01	0	0	0	0	00	00	00	00	01	0	0	00	00	0	1	1	1	01
HAI	11	00	00	00	0	01	0	0	0	0	00	00	01	00	01	0	0	00	00	1	1	0	1	01
IDE	01	00	00	01	0	01	0	1	0	0	00	01	11	00	01	0	0	00	00	1	1	0	0	00
LAN	01	00	00	01	0	01	0	0	0	0	00	00	11	00	01	0	0	00	00	0	1	0	0	01
LAM	01	01	01	00	1	01	1	0	1	0	00	11	11	00	01	0	0	00	00	0	1	0	1	01
ROS	00	11	11	01	1	01	1	0	1	0	00	00	01	00	00	0	0	11	11	0	1	0	0	11

* - character number refered to table(6.2)

(6.2.3) Material and methods for phylogenetic analysis using larval developmental rate data

As reviewed in Chapter 3, a spectrum of larval developmental modes are represented in the genus Macrobrachium, from extended development (over 11 stages) to total direct development. It would thus be impossible to make a stage by stage comparision of the different species. Besides, most of the larval morphology was fairly similar. The major difference was attributed to the type of developmental modes, and in particular, the first appearance of a larval appendage and the rate of its subsequent development.

A numerical coding system was used in this analysis to describe the development of larval appendages. An appendage that first appears as a simple or biramous bud at stage III would score 3. If the same appendage were to become fully functional at stage VI, it would score 6 at another loci. However, it may be argued that the first appearance and first functional stage of a certain appendage is correlated, which would not comply with the

Table (6.3) : Character state of adult characters used in phylogenetic analysis

species	Character *																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
NIP	11	00	00	01	0	01	0	0	0	0	00	01	00	00	01	0	0	01	01	0	1	0	1	11
ACA	01	00	01	01	0	01	1	0	1	0	00	00	11	00	01	0	0	01	00	0	1	0	0	11
EQI	00	01	00	00	0	01	0	0	0	0	00	00	00	00	01	0	0	00	00	0	1	1	1	01
HAI	11	00	00	00	0	01	0	0	0	0	00	00	01	00	01	0	0	00	00	1	1	0	1	01
IDE	01	00	00	01	0	01	0	1	0	0	00	01	11	00	01	0	0	00	00	1	1	0	0	00
LAN	01	00	00	01	0	01	0	0	0	0	00	00	11	00	01	0	0	00	00	0	1	0	0	01
LAM	01	01	01	00	1	01	1	0	1	0	00	11	11	00	01	0	0	00	00	0	1	0	1	01
ROS	00	11	11	01	1	01	1	0	1	0	00	00	01	00	00	0	0	11	11	0	1	0	0	11

* - character number refered to table(6.2)

(6.2.3) Material and methods for phylogenetic analysis using larval developmental rate data

As reviewed in Chapter 3, a spectrum of larval developmental modes are represented in the genus Macrobrachium, from extended development (over 11 stages) to total direct development. It would thus be impossible to make a stage by stage comparision of the different species. Besides, most of the larval morphology was fairly similar. The major difference was attributed to the type of developmental modes, and in particular, the first appearance of a larval appendage and the rate of its subsequent development.

A numerical coding system was used in this analysis to describe the development of larval appendages. An appendage that first appears as a simple or biramous bud at stage III would score 3. If the same appendage were to become fully functional at stage VI, it would score 6 at another loci. However, it may be argued that the first appearance and first functional stage of a certain appendage is correlated, which would not comply with the

assumption of phylogenetic analysis. For a second analysis, a combined developmental coding system is used, the hypothetical appendage in the above example would thus score 4.5 at a single character, rather than 3 and 6 in two separate characters. On the other hand, it can also be argued that, as the set of characters also include species specific characters, the first method merely give more weight to the developmental rate data. Also, as can be seen from species with the same number of zoeal stages, the onset of an appendage merely limit it becoming functional after the onset, but a large variation exists in the eventual timing. An average coding then may result in the loss of information.

To include species specific data, the following characters of the first zoea was used: the number of plumose setae on telson, the number of plumose setae on maxilla and total zoeal size. The first appearance of the caridean lobe (exopodal lobe of the first maxilleped), the number of setae on its first appearance was also found to be variable. The first appearance of the various spines on the carapace was also found to be characteristic of larval development (Chapter 3). The characters used are listed in table (6.4).

A table of quantitative characters was then formed and Felsenstein(1981)'s restricted maximum likelihood method was then used to construct a phylogenetic tree. Both sets of data, original and combined (as explained earlier), were used separately. To see the contribution of the developmental rate data, a third set of data, which included only the developmental data on the original set (i.e. minus species specific data) was

Table (6.4) : Larval developmental characters used for phylogenetic analysis

1. Number of zoeal stages

The following characters referred to the first appearance of the corresponding appendages

2. first and second pereopods
4. chelipeds on first and second pereopods
6. third pereopod
8. fourth pereopod
10. fifth pereopod
12. pleopods
15. antennal spine
16. branchiostegal spine
17. epistatic spine
18. dorsal-medial knob of carapace
19. caridean lobe (exopodal lobe of first maxilliped)

The following characters refer to the zoeal stage when the corresponding larval appendages become functional

3. first and second pereopods
5. chelipeds on first and second pereopods
7. third pereopod
9. fourth pereopod
11. fifth pereopod

13. the zoeal stage when the setae bud on pleopods first developed
14. the zoeal stage when setae on the pleopods are developed

20. the number of setae on the caridean lobe when the later first developed

The following characters refer to the first post-embryonic stage

21. the number of setae on the posterior margin of telson
22. the number of setae on maxilla
23. the number of setae on the exopod of the antenna
24. the total size (measured from post-orbital margin to tip of telson)

also used, values of the characters have already been listed in Chapter 3.

(6.2.4) Chromosome study

The general colchicine-Giemsa method was used for chromosome preparation. Post-larvae of Macrobrachium were kept in aerated 0.0026% colchicine solution (Sigma) solution for 4 hours. This concentration of colchicine was used after initial test of 0.0013% gave no metaphase spreads. The hepatopancreas of each prawn was then dissected out and submerged in a 0.56% potassium chloride solution for 15 minutes. They were then washed twice and fixed in a mixture of methanol:acetic acid (3:1 by volume respectively) for 5 minutes. This was followed by dissolution and mechanical teasing of the fixed material on a drop of 5% acetic acid on a glass slide over a 55°C hot plate. One or two drops of methanol was then dropped onto the slide to facilitate evaporation and spreading of cells.

After the slide was air dried and cooled, it was stained in Giemsa stain (1.5 ml methanol + 1.5 ml 0.2M Na_2HPO_4 , 3ml Giemsa (Clin-tech) + 50 ml distilled water. After staining for 30 minutes, the slides were removed, washed twice in distilled water and air-dried. Each slide was then scanned for metaphase spreads. Counting was carried out under oil immersion, or on photographs.

The species used for chromosome study were M.nipponense, M.rosenbergii, M.hainanense, M.lanchesteri and M.acanthurus.

(6.2.5) Material and methods for artificial hybridization

Valuable information on phylogeny can also be revealed by the ability/inability of species to hybridize.

Sandifer and Smith(1979) first reported the artificial intra-specific insemination in M.rosenbergii and in M.acanthurus, using spermatophores dissected from the males and their subsequent placing into the cephalothorax of a female. Sandifer and Lynn(1980) then reported another method using low voltage electric shock to stimulate ejaculation of spermatophores, without sacrificing the male prawns involved.

A similar device was constructed and used in the present study, consisting of a variable resistor and a transformer, to generate an adjustable a.c. output (0-6 V). Spermatophores were readily available by applying the two electrodes in the vicinity of the two gonophores and increasing the voltage slowly to 6 volt (about 10-12 mA). Newly prespawning-moulted females were then captured swiftly, restrained and dried, and the spermatophore transferred carefully to the thoracic sterna.

Different combinations of inter- and intra-specific crosses were tried.

(6.3) RESULTS AND DISCUSSION

(6.3.1) Allozyme study

32 enzyme loci were scored in all the 13 species of *Macrobrachium* studied. The allele frequency are summarised in table(6.5). The overall percentage of polymorphic loci and heterozygosity has already been discussed in the last chapter. All the inferred phenotypic frequencies agree with the expected frequency of the Hardy-Weinberg equilibrium (except in the cases mentioned in the last chapter. Phenotypes of the more polymorphic loci (with 5 alleles or more in all the species) are shown in Fig.(6.1).

The Nei's standard genetic distance (D) and Roger's similarity index were calculated between the thirteen species and listed in table (6.6) and table (6.7) respectively. The average values of D for all the comparisions was 0.514 $\pm$ S.D. 0.138, which is well within the normal range of D observed for inter-specific comparision (Nei,1975). A highly significant value of linear correlation coefficient ($r=0.95$; $p < 0.001$) was computed between the two indices (Nei vs $1-S_R$).

The value of D calculated for 5 species of *Macrobrachium* surveyed by Chow and Fujio(1985b) was 1.286 $\pm$ 0.117 (15 loci), doubled the value estimated in the present study. It is likely that the difference was partly attributed to the different number of loci being studied. Also, their *M.rosenbergii* was obtained from a hatchery stock with no genetic variation detectable in the 15 loci studies. This would undoubtedly increase the possible value of D. For the comparision between *M.nipponense* and

Table(6.5): Allele frequencies of *Macrobrachium* species (A)

	ACA	DAY	EQI	HAI	IDE	LAN	LAM
AAT-1A	0.000	0.200	0.000	0.000	0.000	0.000	0.000
AAT-1B	1.000	0.400	0.607	0.000	1.000	0.000	0.333
AAT-1C	0.000	0.400	0.393	0.600	0.000	0.550	0.667
AAT-1D	0.000	0.000	0.000	0.400	0.000	0.450	0.000
AAT-2A	1.000	0.900	1.000	1.000	1.000	0.850	0.500
AAT-2B	0.000	0.100	0.000	0.000	0.000	0.150	0.500
ACPH-1A	0.000	0.000	0.000	0.967	0.000	0.000	0.000
ACPH-1B	1.000	1.000	1.000	0.033	1.000	1.000	1.000
ACPH-2A	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ACPH-2B	0.517	1.000	1.000	1.000	1.000	1.000	1.000
ACPH-2C	0.483	0.000	0.000	0.000	0.000	0.000	0.000
ADA-1A	0.000	0.000	0.000	0.000	0.000	1.000	0.000
ADA-1B	1.000	1.000	0.000	1.000	1.000	0.000	1.000
ADA-1C	0.000	0.000	1.000	0.000	0.000	0.000	0.000
ADA-2A	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ADA-2B	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ALD-A	0.000	0.000	0.000	0.500	0.000	0.000	0.000
ALD-B	0.750	0.967	1.000	0.500	1.000	0.533	0.800
ALD-C	0.250	0.033	0.000	0.000	0.000	0.467	0.200
ALP-A	0.000	1.000	0.000	0.000	0.000	0.000	0.333
ALP-B	1.000	0.000	1.000	0.950	1.000	1.000	0.667
ALP-C	0.000	0.000	0.000	0.033	0.000	0.000	0.000
ALP-D	0.000	0.000	0.000	0.017	0.000	0.000	0.000
EST-1A	0.767	0.000	0.000	0.000	1.000	0.000	0.000
EST-1B	0.233	1.000	1.000	0.700	0.000	1.000	1.000
EST-1C	0.000	0.000	0.000	0.300	0.000	0.000	0.000
EST-2A	0.000	0.000	0.000	1.000	0.000	0.476	0.467
EST-2B	1.000	1.000	1.000	0.000	1.000	0.524	0.533
EST-2C	0.000	0.000	0.000	0.000	0.000	0.000	0.000
EST-3A	0.317	0.000	0.643	1.000	0.350	0.000	0.000
EST-3B	0.682	1.000	0.357	0.000	0.651	1.000	1.000
EST-3C	0.000	0.000	0.000	0.000	0.000	0.000	0.000
EST-4A	1.000	1.000	1.000	1.000	0.000	1.000	1.000
EST-4B	0.000	0.000	0.000	0.000	1.000	0.000	0.000
FUM-1A	0.000	1.000	1.000	1.000	0.433	1.000	0.533
FUM-1B	1.000	0.000	0.000	0.000	0.567	0.000	0.467

(to be continued)

Table(6.5a): continue (A)

	ACA	DAY	EQI	HAI	IDE	LAN	LAM
FUM-2A	0.000	0.000	0.000	1.000	1.000	0.000	0.000
FUM-2B	1.000	1.000	1.000	0.000	0.000	1.000	1.000
G6PDH-A	1.000	0.000	1.000	1.000	1.000	1.000	1.000
G6PDH-B	0.000	1.000	0.000	0.000	0.000	0.000	0.000
GDH-A	1.000	0.000	0.000	0.000	0.000	1.000	0.000
GDH-B	0.000	1.000	1.000	1.000	1.000	0.000	1.000
GPDH-1A	0.000	0.000	0.000	1.000	0.000	0.000	0.700
GPDH-1B	1.000	0.000	0.964	0.000	1.000	0.516	0.300
GPDH-1C	0.000	1.000	0.036	0.000	0.000	0.483	0.000
GPDH-2A	0.000	0.000	0.000	1.000	1.000	0.000	0.000
GPDH-2B	1.000	1.000	1.000	0.000	0.000	1.000	1.000
GPT-A	0.000	0.000	0.000	0.000	0.000	0.000	0.300
GPT-B	0.600	0.000	0.393	0.000	0.000	0.000	0.500
GPT-C	0.400	0.900	0.607	0.000	0.200	0.617	0.200
GPT-D	0.000	0.100	0.000	1.000	0.800	0.383	0.000
GPT-E	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HK-1A	1.000	0.000	0.000	0.000	1.000	0.000	0.000
HK-1B	0.000	0.000	1.000	1.000	0.000	1.000	1.000
HK-1C	0.000	1.000	0.000	0.000	0.000	0.000	0.000
HK-2A	1.000	0.000	1.000	1.000	1.000	1.000	1.000
HK-2B	0.000	1.000	0.000	0.000	0.000	0.000	0.000
IDH-A	0.000	0.000	0.000	0.000	0.000	0.000	0.000
IDH-B	0.000	0.000	0.000	0.433	0.000	0.000	0.000
IDH-C	1.000	1.000	1.000	0.567	1.000	0.750	1.000
IDH-D	0.000	0.000	0.000	0.000	0.000	0.250	0.000
LAP-A	0.317	0.467	0.000	0.000	0.367	0.000	0.000
LAP-B	0.633	0.300	0.214	0.000	0.233	0.000	0.000
LAP-C	0.050	0.233	0.429	0.150	0.400	0.483	0.500
LAP-D	0.000	0.000	0.357	0.417	0.000	0.517	0.300
LAP-E	0.000	0.000	0.000	0.433	0.000	0.000	0.200
LDH-A	1.000	1.000	1.000	1.000	1.000	1.000	0.000
LDH-B	0.000	0.000	0.000	0.000	0.000	0.000	1.000
MDH-1A	1.000	0.000	0.000	0.000	0.000	0.950	0.000
MDH-1B	0.000	1.000	0.600	1.000	0.000	0.050	1.000
MDH-1C	0.000	0.000	0.400	0.000	1.000	0.000	0.000

(to be continued)

Table(6.5): continue (A)

	ACA	DAY	EQI	HAI	IDE	LAN	LAM
MDH-2A	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MDH-2B	1.000	1.000	1.000	1.000	1.000	0.000	1.000
MDH-2C	0.000	0.000	0.000	0.000	0.000	1.000	0.000
ME-A	0.000	1.000	1.000	0.000	0.000	0.000	0.000
ME-B	0.883	0.000	0.000	1.000	0.000	1.000	1.000
ME-C	0.117	0.000	0.000	0.000	0.000	0.000	0.000
ME-D	0.000	0.000	0.000	0.000	1.000	0.000	0.000
MPI-A	0.000	1.000	0.143	0.000	0.000	0.000	0.000
MPI-B	0.000	0.000	0.429	0.000	0.000	0.000	0.000
MPI-C	0.900	0.000	0.250	0.000	0.950	0.000	0.000
MPI-D	0.100	0.000	0.071	1.000	0.050	1.000	1.000
MPI-E	0.000	0.000	0.071	0.000	0.000	0.000	0.000
MPI-F	0.000	0.000	0.036	0.000	0.000	0.000	0.000
MPI-H	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MPI-I	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MPI-J	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGI-A	0.000	1.000	0.000	0.000	0.000	0.000	0.000
PGI-B	0.000	0.000	0.000	0.233	0.000	0.000	0.000
PGI-C	0.000	0.000	0.000	0.767	0.000	0.000	0.000
PGI-D	0.000	0.000	0.036	0.000	0.000	0.000	0.000
PGI-E	0.817	0.000	0.357	0.000	0.000	0.983	1.000
PGI-F	0.183	0.000	0.500	0.000	1.000	0.017	0.000
PGI-G	0.000	0.000	0.107	0.000	0.000	0.000	0.000
PGI-H	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGI-I	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGI-J	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGI-K	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM-A	0.000	0.000	0.000	0.650	0.000	0.000	0.000
PGM-B	0.000	1.000	0.000	0.350	0.000	0.000	0.000
PGM-C	0.000	0.000	0.967	0.000	0.000	0.000	0.000
PGM-D	0.083	0.000	0.033	0.000	0.000	1.000	1.000
PGM-E	0.900	0.000	0.000	0.000	1.000	0.000	0.000
PGM-F	0.017	0.000	0.000	0.000	0.000	0.000	0.000
PGDH6-A	1.000	1.000	1.000	0.000	0.000	0.000	1.000
PGDH6-B	0.000	0.000	0.000	1.000	1.000	0.000	0.000
PGDH6-C	0.000	0.000	0.000	0.000	0.000	1.000	0.000
PGDH6-D	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TO-A	0.000	1.000	0.000	0.000	0.000	0.000	0.000
TO-B	1.000	0.000	0.000	1.000	1.000	0.468	0.000
TO-C	0.000	0.000	1.000	0.000	0.000	0.532	1.000
TO-D	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TO-E	0.000	0.000	0.000	0.000	0.000	0.000	0.000

(to be continued)

Table (6.5): Allele frequencies of *Macrobrachium* species (B)

	Mlep	MN	MR	MRu	MS-1	MS-2
AAT-1A	0.000	0.000	1.000	0.000	0.000	0.000
AAT-1B	0.700	0.277	0.000	0.500	0.000	0.000
AAT-1C	0.300	0.644	0.000	0.500	1.000	1.000
AAT-1D	0.000	0.079	0.000	0.000	0.000	0.000
AAT-2A	1.000	1.000	1.000	1.000	1.000	1.000
AAT-2B	0.000	0.000	0.000	0.000	0.000	0.000
ACPH-1A	0.000	0.000	0.000	0.000	0.000	0.000
ACPH-1B	1.000	1.000	1.000	1.000	1.000	1.000
ACPH-2A	0.000	0.000	1.000	0.000	0.000	0.000
ACPH-2B	1.000	1.000	0.000	1.000	1.000	1.000
ACPH-2C	0.000	0.000	0.000	0.000	0.000	0.000
ADA-1A	0.000	0.000	1.000	0.000	0.000	0.000
ADA-1B	0.000	0.000	0.000	0.000	1.000	1.000
ADA-1C	1.000	1.000	0.000	1.000	0.000	0.000
ADA-2A	0.000	0.000	0.000	0.000	0.429	0.429
ADA-2B	1.000	1.000	1.000	1.000	0.571	0.571
ALD-A	0.000	0.000	0.823	0.000	0.000	0.000
ALD-B	0.400	0.645	0.176	0.500	0.000	0.929
ALD-C	0.600	0.355	0.000	0.500	1.000	0.071
ALP-A	0.000	0.598	0.744	0.000	0.536	0.719
ALP-B	1.000	0.402	0.256	1.000	0.464	0.281
ALP-C	0.000	0.000	0.000	0.000	0.000	0.000
ALP-D	0.000	0.000	0.000	0.000	0.000	0.000
EST-1A	0.000	0.000	0.476	0.000	0.000	0.000
EST-1B	1.000	1.000	0.524	1.000	0.000	0.000
EST-1C	0.000	0.000	0.000	0.000	1.000	1.000
EST-2A	0.000	0.772	0.560	0.000	0.000	0.000
EST-2B	1.000	0.228	0.440	1.000	0.000	1.000
EST-2C	0.000	0.000	0.000	0.000	1.000	0.000
EST-3A	0.000	1.000	0.000	0.500	0.000	1.000
EST-3B	0.300	0.000	1.000	0.500	1.000	0.000
EST-3C	0.700	0.000	0.000	0.000	0.000	0.000
EST-4A	1.000	1.000	1.000	1.000	1.000	1.000
EST-4B	0.000	0.000	0.000	0.000	0.000	0.000
FUM-1A	1.000	1.000	1.000	1.000	1.000	1.000
FUM-1B	0.000	0.000	0.000	0.000	0.000	0.000

(to be continued)

Table (6.5b): continue (B)

	Mlep	MN	MR	MRu	MS-1	MS-2
FUM-2A	0.000	0.000	0.000	0.000	0.000	0.000
FUM-2B	1.000	1.000	1.000	1.000	1.000	1.000
G6PDH-A	0.000	1.000	1.000	1.000	1.000	1.000
G6PDH-B	1.000	0.000	0.000	0.000	0.000	0.000
GDH-A	0.000	0.000	0.000	0.000	0.000	0.000
GDH-B	1.000	1.000	1.000	1.000	1.000	1.000
GPDH-1A	0.000	0.000	0.000	0.000	0.000	0.000
GPDH-1B	1.000	0.800	0.992	1.000	1.000	1.000
GPDH-1C	0.000	0.200	0.008	0.000	0.000	0.000
GPDH-2A	0.000	0.000	0.000	0.000	0.000	0.000
GPDH-2B	1.000	1.000	1.000	1.000	1.000	1.000
GPT-A	0.000	0.072	0.000	0.000	0.000	0.000
GPT-B	0.000	0.197	0.000	0.000	0.000	0.000
GPT-C	0.000	0.638	0.117	0.883	0.000	1.000
GPT-D	0.500	0.095	0.697	0.117	1.000	0.000
GPT-E	0.500	0.000	0.176	0.000	0.000	0.000
HK-1A	0.000	1.000	1.000	1.000	0.000	0.000
HK-1B	1.000	0.000	0.000	0.000	1.000	1.000
HK-1C	0.000	0.000	0.000	0.000	0.000	0.000
HK-2A	1.000	1.000	1.000	1.000	1.000	1.000
HK-2B	0.000	0.000	0.000	0.000	0.000	0.000
IDH-A	0.000	0.000	1.000	0.000	0.000	0.000
IDH-B	0.000	0.601	0.000	0.500	0.643	0.157
IDH-C	0.000	0.394	0.000	0.500	0.357	0.844
IDH-D	1.000	0.000	0.000	0.000	0.000	0.000
LAP-A	0.000	0.000	0.546	0.000	0.000	0.000
LAP-B	0.500	0.000	0.223	0.000	0.000	0.000
LAP-C	0.500	0.309	0.088	0.333	0.214	0.469
LAP-D	0.000	0.480	0.143	0.667	0.536	0.531
LAP-E	0.000	0.211	0.000	0.000	0.250	0.000
LDH-A	1.000	1.000	1.000	1.000	1.000	1.000
LDH-B	0.000	0.000	0.000	0.000	0.000	0.000
MDH-1A	0.000	0.277	0.036	0.000	0.000	0.000
MDH-1B	1.000	0.723	0.964	1.000	1.000	1.000
MDH-1C	0.000	0.000	0.000	0.000	0.000	0.000

(to be continued)

Table (6.5) : continue

	Mlep	MN	MR	MRu	MS-1	MS-2
MDH-2A	0.000	0.000	0.000	1.000	0.000	0.000
MDH-2B	1.000	1.000	1.000	0.000	1.000	1.000
MDH-2C	0.000	0.000	0.000	0.000	0.000	0.000
ME-A	0.000	0.000	0.000	0.333	1.000	0.000
ME-B	1.000	0.000	0.990	0.667	0.000	1.000
ME-C	0.000	1.000	0.010	0.000	0.000	0.000
ME-D	0.000	0.000	0.000	0.000	0.000	0.000
MPI-A	0.000	0.000	0.000	0.000	0.000	0.000
MPI-B	0.000	0.000	0.589	0.000	0.000	0.000
MPI-C	0.000	0.560	0.126	0.167	1.000	0.000
MPI-D	0.000	0.400	0.227	0.833	0.000	1.000
MPI-E	0.000	0.040	0.000	0.000	0.000	0.000
MPI-F	0.000	0.000	0.000	0.000	0.000	0.000
MPI-H	0.100	0.000	0.000	0.000	0.000	0.000
MPI-I	0.800	0.000	0.000	0.000	0.000	0.000
MPI-J	0.100	0.000	0.000	0.000	0.000	0.000
PGI-A	0.000	0.000	0.000	0.000	1.000	0.031
PGI-B	0.000	0.000	0.000	0.000	0.000	0.969
PGI-C	0.000	0.000	0.000	0.000	0.000	0.000
PGI-D	0.000	0.039	0.273	0.000	0.000	0.000
PGI-E	0.000	0.921	0.651	0.167	0.000	0.000
PGI-F	0.000	0.068	0.076	0.500	0.000	0.000
PGI-G	0.000	0.000	0.000	0.333	0.000	0.000
PGI-H	0.300	0.000	0.000	0.000	0.000	0.000
PGI-I	0.500	0.000	0.000	0.000	0.000	0.000
PGI-J	0.200	0.000	0.000	0.000	0.000	0.000
PGI-K	0.000	0.000	0.000	0.000	0.000	0.000
PGM-A	0.000	0.000	0.000	0.000	0.000	1.000
PGM-B	0.000	0.000	0.038	0.167	1.000	0.000
PGM-C	0.000	0.790	0.389	0.833	0.000	0.000
PGM-D	1.000	0.210	0.543	0.000	0.000	0.000
PGM-E	0.000	0.000	0.030	0.000	0.000	0.000
PGM-F	0.000	0.000	0.000	0.000	0.000	0.000
PGDH6-A	0.000	0.000	0.000	0.000	1.000	1.000
PGDH6-B	0.000	0.000	1.000	1.000	0.000	0.000
PGDH6-C	0.000	1.000	0.000	0.000	0.000	0.000
PGDH6-D	1.000	0.000	0.000	0.000	0.000	0.000
TO-A	0.000	0.000	0.000	0.000	1.000	1.000
TO-B	0.000	1.000	1.000	0.000	0.000	0.000
TO-C	0.000	0.000	0.000	0.000	0.000	0.000
TO-D	1.000	0.000	0.000	0.000	0.000	0.000
TO-E	0.000	0.000	0.000	1.000	0.000	0.000

Table (6.6): Nei's genetic distance and genetic similarity calculated between species

Genetic Similarity (I)

	HAI	ACA	DAY	EQI	IDE	LAN	LAM	LEP	NIP	ROS	RUD	SIN-1	SIN-2
HAI		0.456	0.428	0.541	0.556	0.556	0.621	0.486	0.606	0.530	0.601	0.564	0.652
ACA			0.513	0.640	0.711	0.639	0.632	0.511	0.647	0.645	0.585	0.582	0.515
DAY				0.651	0.451	0.496	0.601	0.548	0.554	0.518	0.549	0.656	0.645
EQI					0.592	0.665	0.749	0.667	0.768	0.602	0.783	0.657	0.728
IDE						0.455	0.491	0.465	0.542	0.542	0.593	0.488	0.499
LAN							0.727	0.604	0.695	0.624	0.671	0.547	0.626
LAM								0.624	0.667	0.594	0.652	0.626	0.726
LEP									0.596	0.563	0.655	0.563	0.583
NIP										0.681	0.778	0.626	0.685
ROS											0.656	0.530	0.560
RUD												0.628	0.674
SIN-1													0.736
SIN-2													

Genetic Distance (D)

	HAI	ACA	DAY	EQI	IDE	LAN	LAM	LEP	NIP	ROS	RUD	SIN-1	SIN-2
HAI		0.786	0.849	0.614	0.586	0.587	0.477	0.722	0.500	0.595	0.576	0.573	0.428
ACA			0.668	0.446	0.341	0.448	0.459	0.672	0.435	0.499	0.527	0.669	0.554
DAY				0.429	0.796	0.701	0.509	0.601	0.591	0.718	0.612	0.422	0.439
EQI					0.524	0.408	0.289	0.404	0.264	0.560	0.261	0.420	0.318
IDE						0.788	0.712	0.766	0.613	0.582	0.584	0.717	0.695
LAN							0.319	0.504	0.364	0.494	0.430	0.603	0.468
LAM								0.472	0.405	0.560	0.453	0.468	0.320
LEP									0.518	0.595	0.477	0.574	0.540
NIP										0.389	0.270	0.468	0.378
ROS											0.487	0.653	0.640
RUD												0.530	0.406
SIN-1													0.307
SIN-2													

Standard errors of genetic distance

	HAI	ACA	DAY	EQI	IDE	LAN	LAM	LEP	NIP	ROS	RUD	SIN-1	SIN-2
HAI		0.080	0.081	0.080	0.081	0.079	0.076	0.083	0.078	0.080	0.078	0.081	0.074
ACA			0.080	0.074	0.071	0.074	0.070	0.081	0.070	0.074	0.073	0.080	0.078
DAY				0.078	0.083	0.081	0.079	0.083	0.081	0.081	0.080	0.078	0.080
EQI					0.078	0.071	0.061	0.079	0.061	0.073	0.062	0.073	0.071
IDE						0.078	0.080	0.081	0.075	0.078	0.078	0.082	0.080
LAN							0.069	0.077	0.068	0.075	0.073	0.078	0.077
LAM								0.075	0.071	0.075	0.071	0.076	0.068
LEP									0.079	0.078	0.079	0.080	0.081
NIP										0.074	0.062	0.080	0.075
ROS											0.071	0.084	0.081
RUD												0.074	0.073
SIN-1													0.075
SIN-2													

Table(6.7): Roger(1972)'s Similarity Indices calculated between each species

	HAI	ACA	DAY	EQI	IDE	LAN	LAM	LEP	NIP	ROS	RUD	SIN-1	SIN-2
HAI		0.444	0.414	0.527	0.550	0.530	0.577	0.505	0.581	0.543	0.560	0.537	0.612
ACA			0.499	0.634	0.674	0.597	0.599	0.524	0.601	0.584	0.581	0.515	0.571
DAY				0.636	0.425	0.474	0.580	0.545	0.530	0.490	0.543	0.637	0.620
EQI					0.564	0.621	0.692	0.684	0.717	0.567	0.751	0.628	0.690
IDE						0.608	0.457	0.451	0.512	0.520	0.547	0.459	0.475
LAN							0.690	0.596	0.644	0.584	0.627	0.510	0.578
LAM								0.615	0.564	0.592	0.593	0.673	0.601
LEP									0.601	0.569	0.660	0.548	0.584
NIP										0.657	0.739	0.592	0.632
ROS											0.624	0.548	0.549
RUD												0.566	0.643
SIN-1													0.724
SIN-2													

Fig. (6.1): Observed phenotypes of loci with 5 alleles or more in the 13 species of *Macrobrachium*

GPT - Glutamate pyruvate transaminase

LAP - Leucine amino peptidase

MPI - Mannose phosphate isomerase

PGI - Glucose phosphate isomerase

PGM - Glucose phosphate mutase

-A
B
C
D
E
+
0
1
ACA
DAY
EQI
HAI
IDE
LAN
LAM
LEP
NIP
ROS
RUD
SIN-1
SIN-2
GPT

LAP
-A
-B
-C
-D
-E
+O
ACA
DAY
EQI
HAI
IDE
LAN
LAM
LEP
NIP
ROS
RUD
SIN-1
SIN-2

+
0
-
ACA
DAY
EQI
HAI
IDE
LAN
LAM
LEP
NIP
ROS
RUD
SIN-1
SIN-2
MPI
A
B
C
D
E
F
G
H
I

PGI
A
B
C
D
E
F
G
H
I
J
SIN -2
SIN -1
RUD
ROS
NIP
LEP
LAM
LAN
IDE
HAI
EQI
DAY
ACA
-
0
+

+
0
-
ACA
DAY
EQI
HAI
IDE
LAN
LAM
LEP
NIP
ROS
RUD
SIN-2
SIN-1
PGM
A
B
C
D
E
F

M.formosense, two species with a high degree of morphological similarity and one of the few successful artificial crosses (sterile progeny though ; Uno and Fugita, 1972), a relatively high value of D (0.625) was inferred. Phylogenetic trees were contructed from the allozyme data from the following methods:

a. The UPGMA method with Nei's standard genetic distance, (Fig. 6.2);

b. Fitch-Margolaish method with Nei's standard genetic distance, (Fig. 6.3);

c. Edward and Cavalli-Sforza method with Nei's standard genetic distance, (Fig. 6.3; identical to method b);

d. The UPGMA method with Roger's similarity index,(Fig. 6.4);

e. Fitch-Margolaish method with Roger's similarity indice, (Fig. 6.5);

f. Edward and Cavalli-Sforza method with Roger's similarity indice, (Fig. 6.6);

g. Wagner parsimony with presence/absence transformation, (Fig. 6.7).

For method (g), 7 trees with an identical number of steps required for the Wagner parsimony were found.

In all these methods, the following clade can be recognised:

1. M.acanthurus, M.idella and M.rosenbergii;
2. M.rude, M.nipponense and possibly M.equidens;
3. M.lanchesteri and M.lamarrei;
4. M.dayanum, M.sintangense and M.spp., and
5. M.hainanense which is generally attached to clade (1).

M.lepidactylus, however, varied in its relative position in the various trees, but branched off between clade 3,4 or 5. The various clades seem to be related in their mode of larval development. M.hainanense has an abbreviated type of development. Species of clade 4 also have a large egg size and a certain

degree of abbreviation (Sabar, 1979), at least for M. sintangense. Clade (1) consists of species with the more extended type of development. Species of clade (2) also have an extended type of larval development, but all with a smaller number of larval stages than species of clade (1). In clade (3), while M. lamarrei has an advanced type of development, M. lanchesteri also has an accelerated type of development, as described in the present thesis.

The association of species from three geographic areas (Africa, South America, and South East Asia) in clade (1) and the presence of a supposedly 'primitive ' species (M. rosenbergii; Johnson, 1973) does support the inference from biogeographical analyis that species of the tropical Americas and Africa originated from ancestors in the Indo-west Pacific region. Clade(1) may thus constitute a phylogenetically ancient group, as suggested by Johnson(1973) and relates to species of related genus. The association of M. hainanense, a species with abbreviated larval development, with clade (1) would support the suggestion of Tiwari(1952) that the genus may have invaded the freshwater in more than one wave. The species with the more abbreviated type of development may have originated from different ancestral species, so that their evolutionary time in the freshwater environment was much longer than species with the advanced type of development.

Morphological similarity of species was also present in some clades. In clade(2), M. equidens and M. rude were the type species

Fig.(6.2): Phylogenetic tree of thirteen species of *Macrobrachium* contructed from allozyme data, using the UPGMA method with Nei's genetic distance

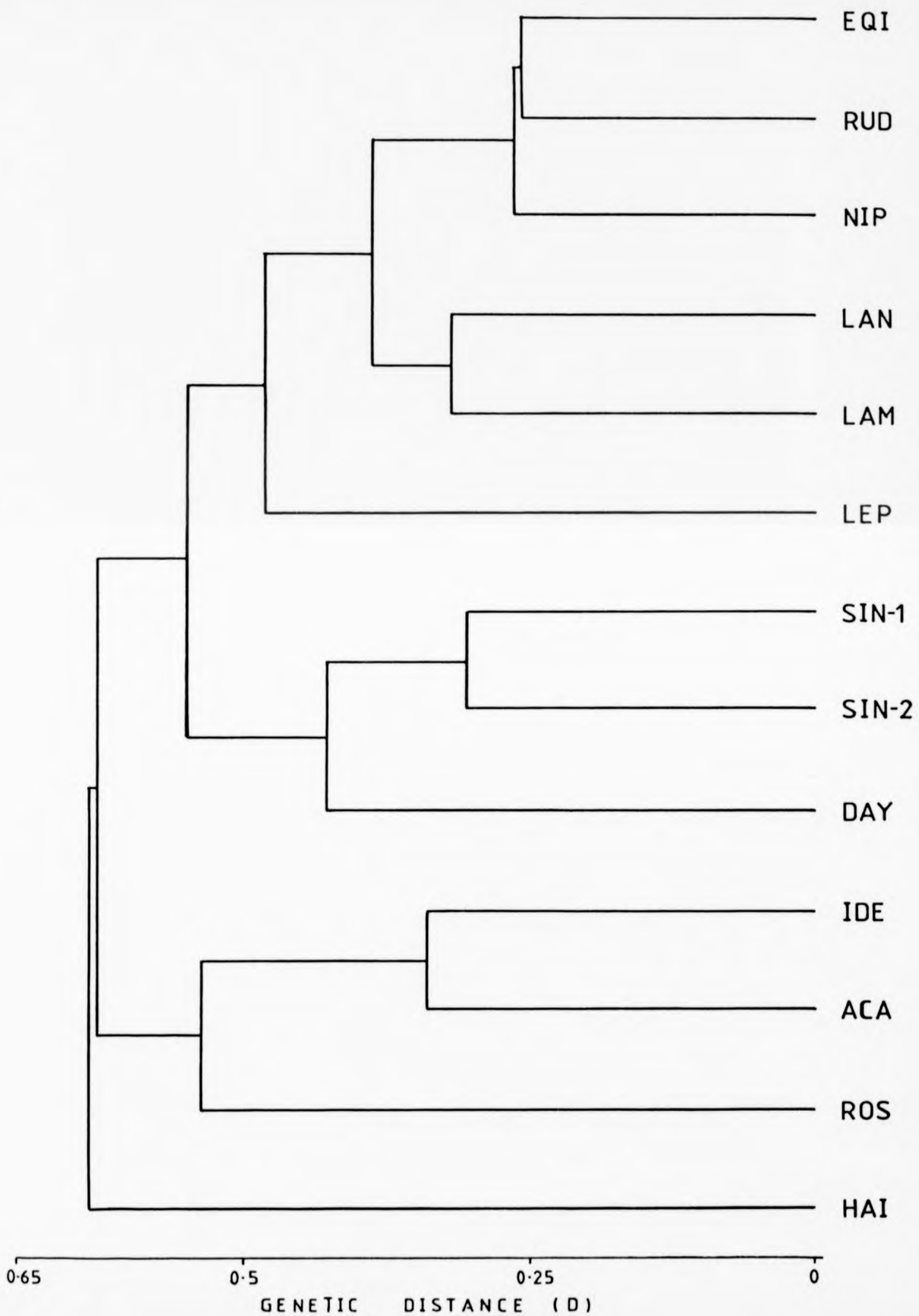
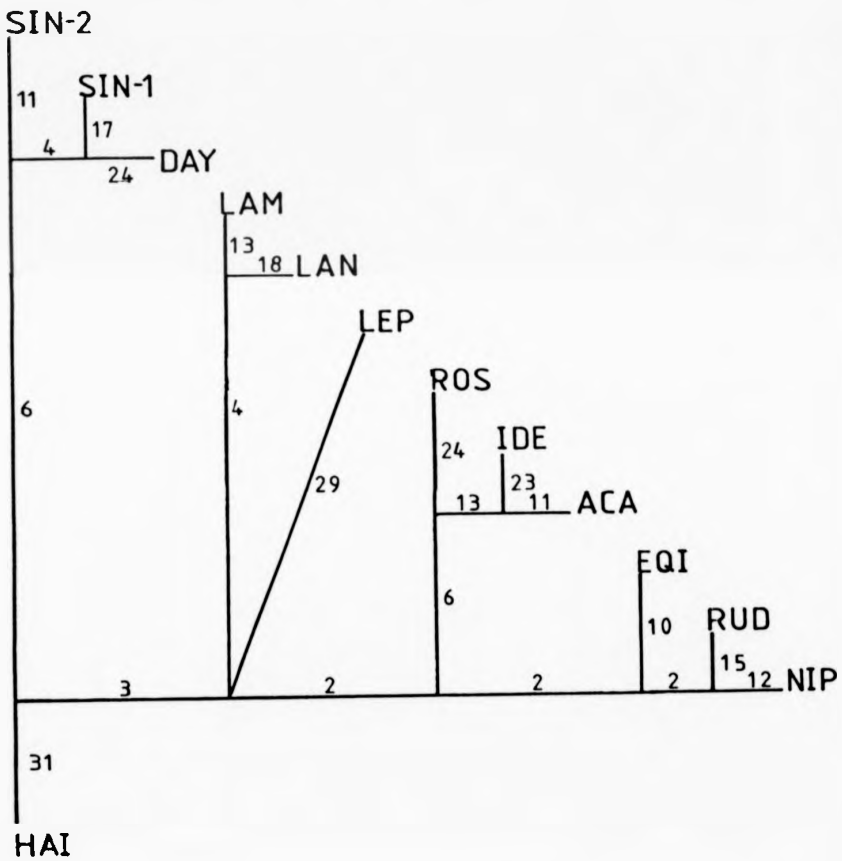

EQI
RUD
NIP
LAN
LAM
LEP
SIN-1
SIN-2
DAY
IDE
ACA
ROS
HAI
0·65
0·5
0·25
0
GENETIC DISTANCE (D)

Fig.(6.3): Phylogenetic tree of thirteen species of Macrobrachium constructed from electrophoretic data, using the Fitch-Margolaish method with Nei's genetic distance (numbers representing arm lengths, zero arm length not shown; an unrooted tree)

and

Phylogenetic tree of thirteen species of Macrobrachium constructed from electrophoretic data, using the Edwards and Cavalli-Sforza method with Nei's genetic distance (an unrooted tree)

both trees were identical in their topologies and arm lengths !


SIN-2
11
SIN-1
17
4
DAY
24
LAM
13
18
LAN
LEP
ROS
6
4
24
IDE
29
23
13
11
ACA
EQI
6
10
RUD
15
12
NIP
3
2
2
2
31
HAI

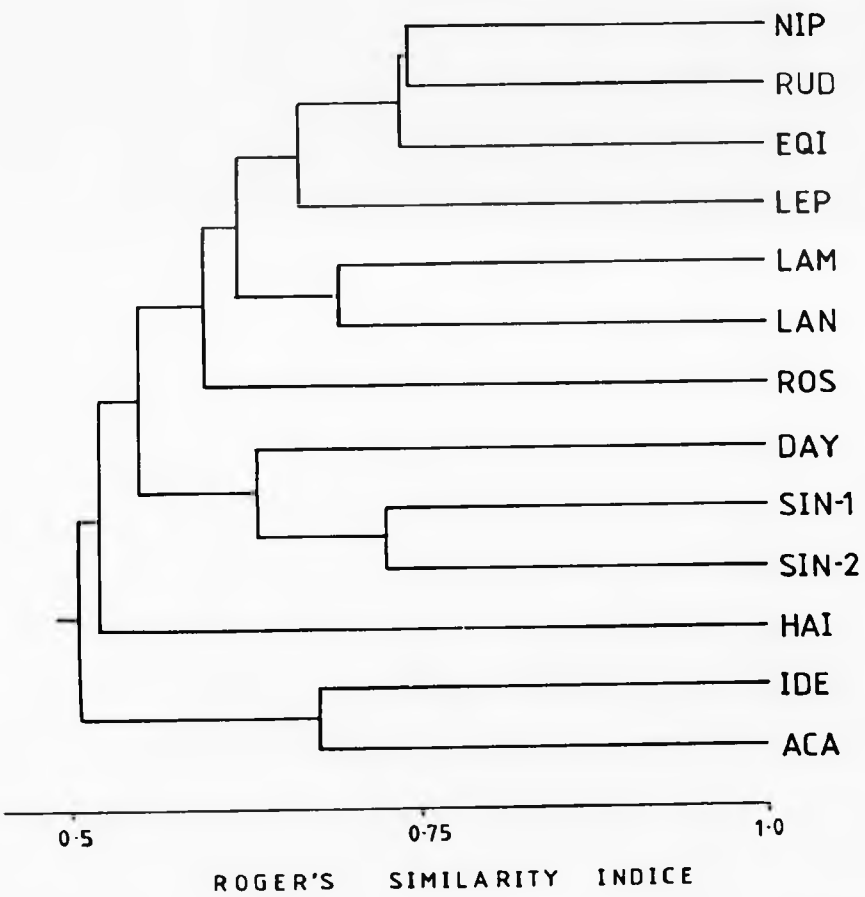


Fig.(6.4): Phylogenetic tree of thirteen species of Macrobrachium constructed from electrophoretic data, using the UPGMA method with Roger's similarity index (converted to distance matrix by $1 - S_R$)

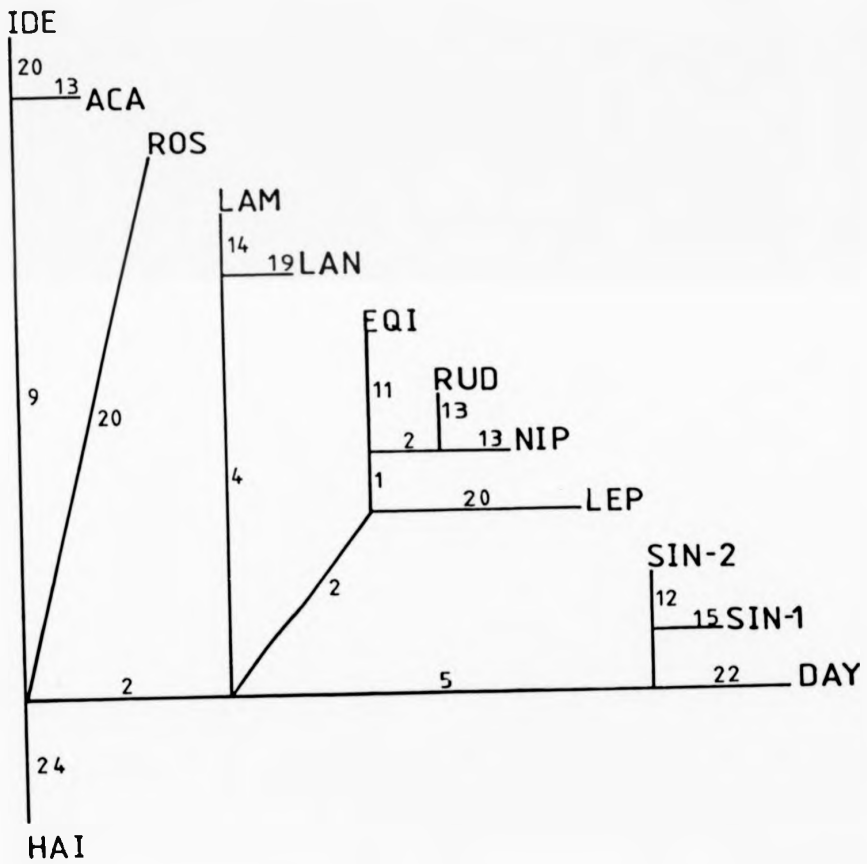


Fig.(6.5): Phylogenetic trees of thirteen species of Macrobrachium constructed from electrophoretic data, using the Fitch-Margolaish method with Roger's similarity index (converted to distance matrix by 1 - S ; an unrooted tree)
R

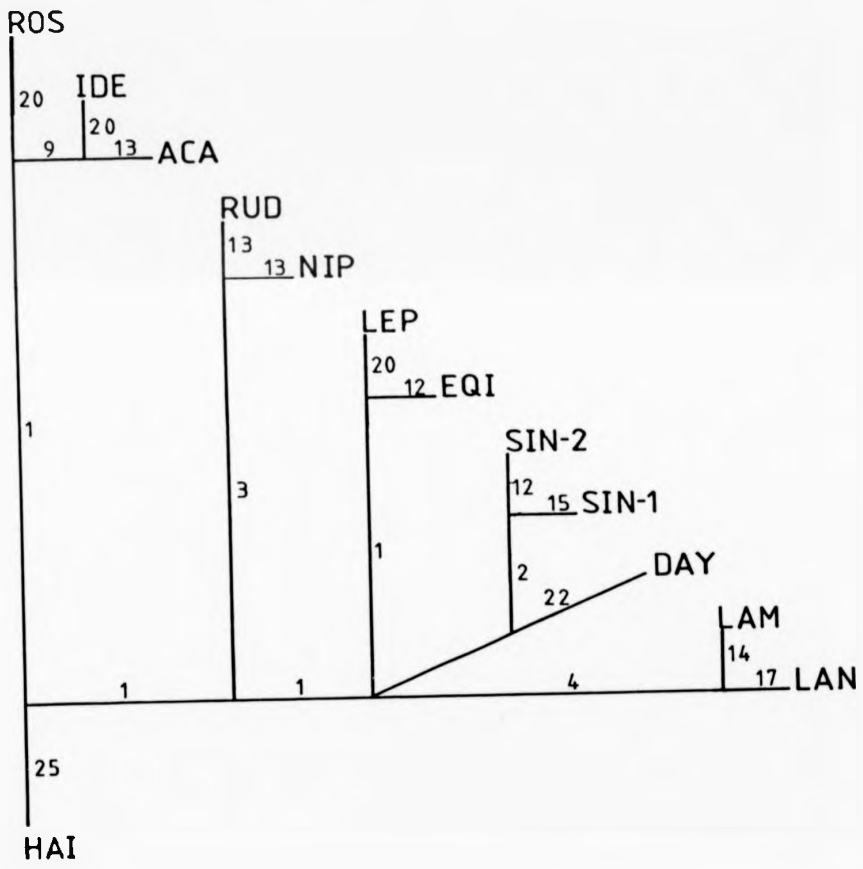


Fig.(6.6): Phylogenetic trees of thirteen species of Macrobrachium constructed from electrophoretic data, using the Edwards and Cavalli-Sforza method with Roger's similarity index (converted to distance matrix by 1 - S ; an unrooted tree)
R

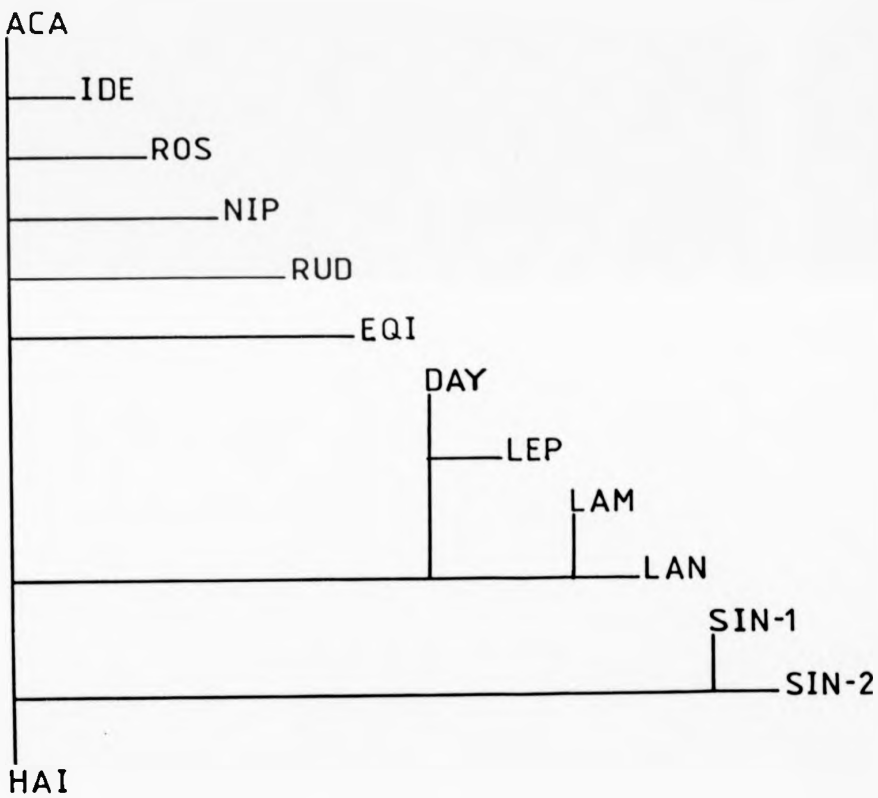


Fig.(6.7): Phylogenetic trees of thirteen species of Macrobrachium constructed from electrophoretic data, using the Wagner parsimony method with presence/absence transformation (This is a strict consensus tree of the 7 trees found; an unrooted tree).

suggested by Johnson(1973) for the 'equiden' group of species. M.acanthurus and M.rosenbergii, apart from their large size, do share similar morphology of their second pereopods. The varying position of M.lepidactylus may also related to the asymmetrical size of its second pereopods, which was inferred to be an important phylogenetic character of some American species (Figueroa,1969). On the other hand, the genetic distance of 0.25 between M.sintangense and its sibling species fall within the value expected for inter-specific comparision. M.lamarrei, a species with many similarities in rostral morphology, and which resembles juveniles of M.rosenbergii (Johnson,1973), was grouped in different clades.

Another inference from the various trees is that clade (2), (3) and (4) may constitute a larger lineage of species, probably of Indo-Pacific distribution.

In order to investigate these inferences in relation to larval development and adult morphologies. The next two sections aim to compare the phylogenetic trees inferred from allozyme data, adult morphological data and larval developmental data. As only 8 out of the 13 species have published (or reported in the present thesis) accounts of larval development, only these eight species would be investigated.

(6.3.2) Phylogenetic trees constructed from larval developmental data

Using the Felsenstein(1982) restricted maximum likelihood method, three trees were drawn :

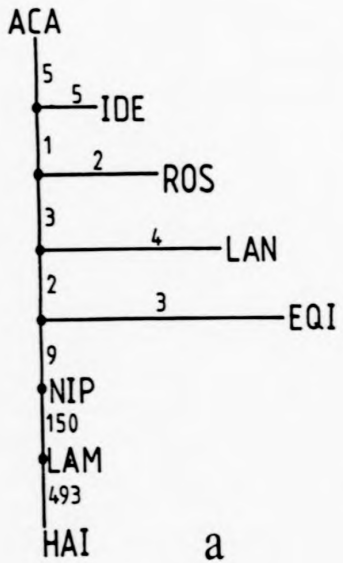
a. with 24 characters of developmental rate + species specific meristic characters; (Fig. 6.8a)

Fig.(6.8): Phylogenetic trees constructed from larval developmental data, using Felsenstein's restricted maximum likelihood method (numbers representing arm lengths, zero length not shown; all unrooted trees)

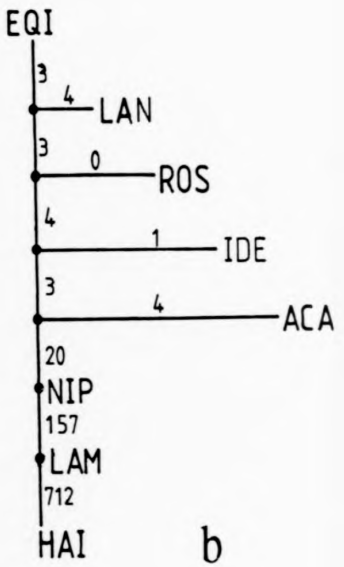
(a.) using 24 characters, including both developmental rate data and larval meristic data;

(b.) using combined developmental rate data and larval meristic data;

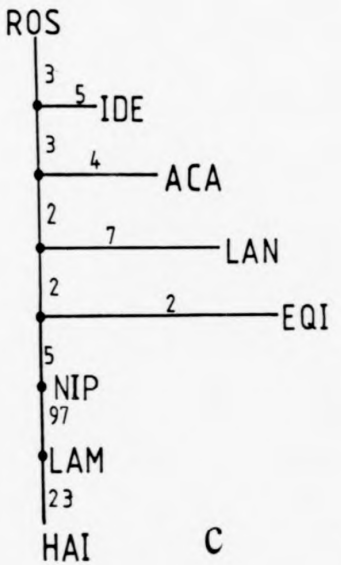
(c.) using developmental rate data only


ACA
5
5
IDE
1
2
ROS
3
4
LAN
2
3
EQI
9
NIP
150
LAM
493
HAI

a


EQI
3
4
LAN
3
0
ROS
4
1
IDE
3
4
ACA
20
NIP
157
LAM
712
HAI

b


ROS
3
5
IDE
3
4
ACA
2
7
LAN
2
2
EQI
5
NIP
97
LAM
23
HAI

c

a. with 24 characters of developmental rate + species specific meristic characters; (Fig. 6.8a)

b. with combined developmental rate characters + species specific meristic characters; (Fig. 6.8b)

c. with original developmental rate characters only. (Fig. 6.8c)

In all three trees, the following clades can be recognised :

(1) M.hainanense forms the longest branch in an unrooted tree, with M.lamarrei at the nearest node;

(2) M.acanthurus, M.rosenbergii and M.idella;

In the three trees, M.equidens, M.nipponense and M.lanchesteri varied in their position in relation to clade (2). This is probably related to their overall similarity in developmental pattern. As the three trees were drawn from different sets of data, the three trees would be compared separately in the following analysis.

(6.3.3) Phylogenetic trees constructed from adult morphological data

Three methods were employed to construct phylogenetic trees, using adult morphological data as listed in table (6.3) and table (6.4) :

a. Wagner parsimony method, without assumptions of ancestral state (Fig. 6.8a);

b. Wagner parsimony method, with assumptions of ancestral states in some characters (Fig. 6.8b);

c. Compatibility method, without assumptions of ancestral states (Fig 6.8c).

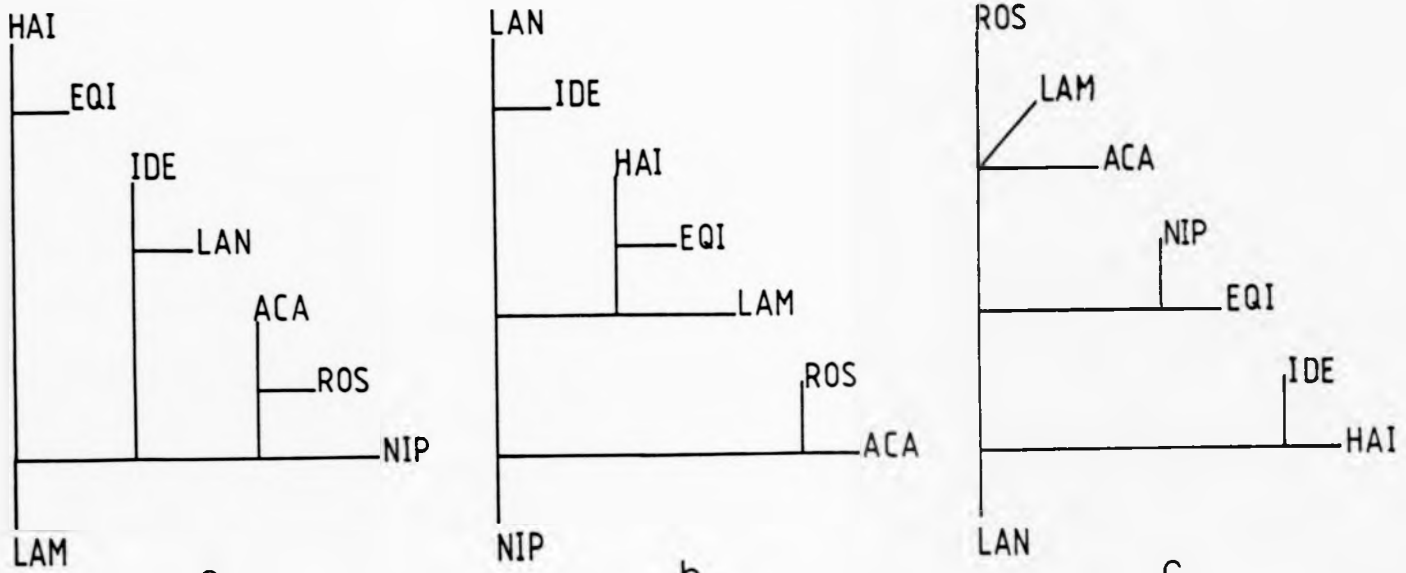
In method (a) and (b), 4 and 10 trees were found respectively with the same number of required steps. Consenus trees, using strict consensus (majority rule) criteria, were

Fig.(6.9): Phylogenetic trees of eight species of *Macrobrachium* constructed from adult morphological data,

(a.) using Wagner parsimony method without assumption of ancestral state (this is a strict consensus tree of the 4 trees found, each requiring 49 steps);

(b.) using a mixed Wagner parsimony and Camin-Sokal method, the later with the assumption of ancestral state (this is a strict consensus tree of the 10 trees found, each requiring 48 steps);

(c.) using compatibility method (CLIQUE) without assumptions on ancestral state.


HAI
EQI
IDE
LAN
ACA
ROS
NIP
LAM
a
LAN
IDE
HAI
EQI
LAM
ROS
ACA
NIP
b
ROS
LAM
ACA
NIP
EQI
IDE
HAI
LAN
c

then constructed for these resulting trees from a. and b. (Fig. 6.8a and Fig. 6.8b respectively).In these two trees, two clades, (NIP, (EQI-HAI),(IDE,LAN)) and (ACA,ROS) can be recognised. M.lamarrei was attached to either clade .

In method (c), different clades were formed but the (ACA,ROS) was joined with M.lamarrei which branched off from M.rosenbergii. The association of these two species in method (a) and (c), but not in (b) (in which the ancestral states were inputed) probably suggested that their similarity was due to convergent evolution in the rostral morphology, as implied by Johnson(1973).

A strict consensus tree of these three trees was constructed. The instability of the different trees probably related to the overall morphological similarity of forms in the genus.

(6.3.4) Comparision of trees constructed from different types of data

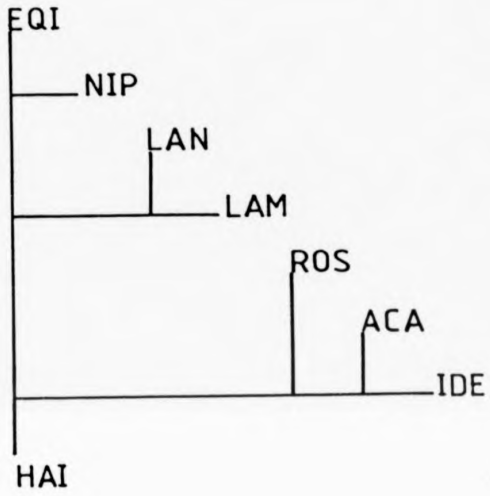
To facilitate comparision between the trees from different types of data, a strict consensus tree of 7 trees drawn by the various methods for the allozyme data was constructed, using only the 8 species employed in the last two sections. The consensus trees for trees drawn from the electrophoretic and adult morphological data are shown in Fig. (6.9).

The approach of Mickevich(1978) was adopted to measure the degree of congruence between the three trees. An Adam's consensus tree, using the criteria of Adam(1972) was first constructed between the corresponding trees (allozyme-larval and allozyme-adult) (Fig. 6.10). This is carried out by the 'least upper

bound" (LUB) criterion, which mainly combine trees by branching a set of terminals (species in this case) under the nearest common ancestor in both case (including nodes which are hypothetical ancestors).

The coefficient of similarity (CI), was then computed, mainly based on the procedures of Mickevich(1978) and Lindenfelser(1984). The Information (F) of each tree (including the Adam's consensus trees) was first calculated as the sum of extra steps required for a certain tree to evolve from its hypothetical root value, as compared to an undirected tree. For this, all trees are thus rooted at the branch of M.bainanense, which constituted the longest branch in the allozyme and larval developmental trees (disproportionately longer, in fact). Terminal species of each clade were assigned state values of 1 and those outside the clade, a state value of 0. The median value of state values (either 0 or 1) is regarded as the root value. The number of extra steps (X) is then the number of non-rooted values minus one. This one step is the step required by the root value to evolve into a different state. For a hypothetical clade of 6 out of 8 species in a tree, the root value is 1, X = 8 - 6 - 1 = 1 . This procedure is then repeated for each clade on the tree. The total number of extra steps X amount to the value of information (F) of that tree. In an Adam's consensus tree, a loss of nodes (hypothetical ancestors) would thus contribute to the loss of F. The amount of decrease of F

Fig.(6.10): Strict consensus trees of eight species of Macrobrachium, using electrophoretic data(a) and adult morphological data(b) (unrooted trees).


EQI
NIP
LAN
LAM
ROS
ACA
IDE
HAI

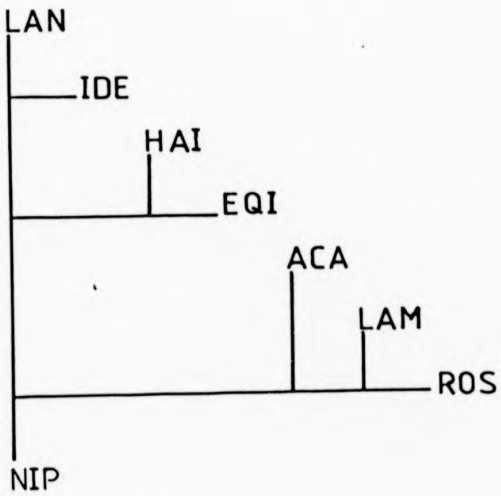
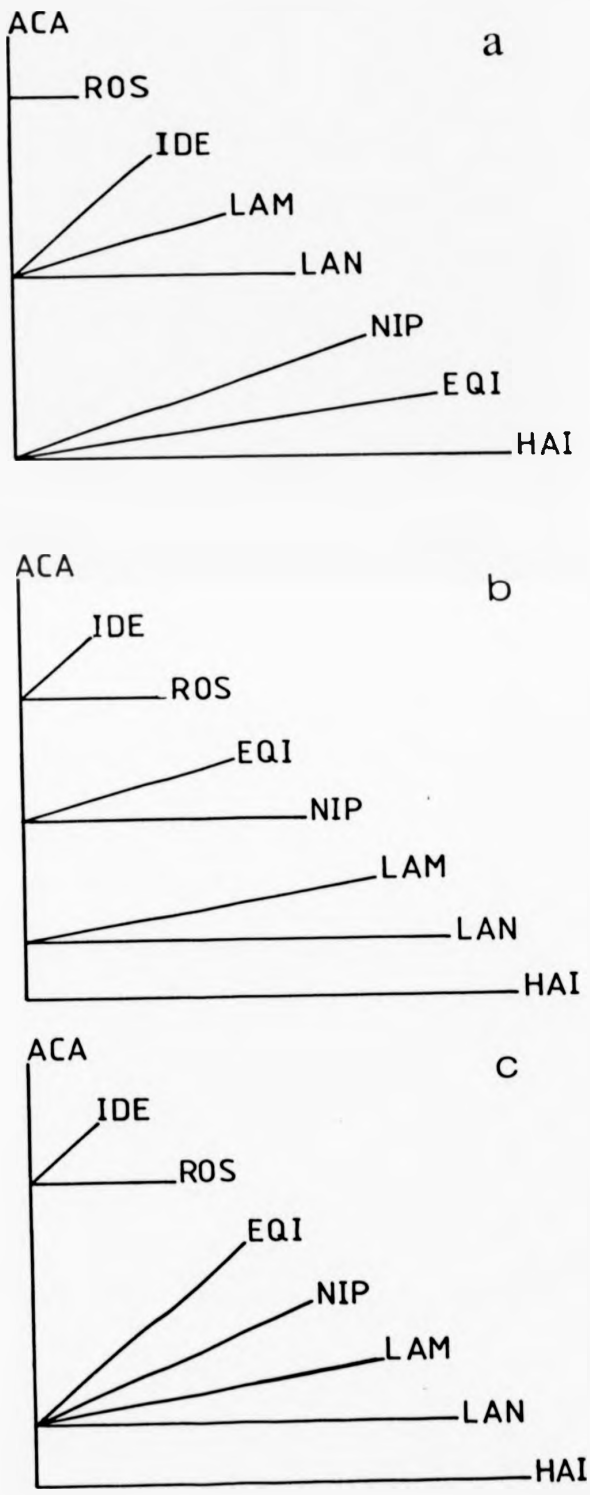

LAN
IDE
HAI
EQI
ACA
LAM
ROS
NIP

Fig.(6.11): Adam's consensus trees contructed between trees from different data sets, using the algorithm of Mickevich(1978), Adam(1972) and Lindenfelser(1984).

(a) Adam's consensus tree between electrophoretic data and adult morphological data;

(b) Adam's consensus tree between electrophoretic data and larval developmental data of Fig. (6.8a) and Fig. (6.8c);

(c) Adam's consensus tree between electrophoretic data and larval developmental data of Fig. (6.8b)


ACA
a
ROS
IDE
LAM
LAN
NIP
EQI
HAI
ACA
b
IDE
ROS
EQI
NIP
LAM
LAN
HAI
ACA
c
IDE
ROS
EQI
NIP
LAM
LAN
HAI

Table(6.9): Computation of congruence between trees constructed from different data sets (based on Mickevich, 1978)

Data set	Information (F)	% of congruence (CI)
1. Electrophoretic data	10	
2. Adult morphological data	11	
3. Larval developmental data		
a.	10	
b.	10	
c.	10	
Adam's consensus tree between		
(1.) and (2.)	4	38 %
(1.) and (3a.; Fig.6.8a)	6	60 %
(1.) and (3b.; Fig.6.8b)	7	70 %
(1.) and (3c.; Fig.6.8c)	6	60 %

then depends on the degree of congruence between the trees used to contruct the Adam's tree.

In the present study, the percentage of congruence (CI) is defined as the ratio of information of the consensus tree (F_A) to the average values ($\underline{F}$)of the original trees.

$$CI = F_A / \underline{F} \qquad \text{(equation 6.2)}$$

The results of comparision were listed in table (6.8). For the three trees of larval developmental data, which themselves consisted of different data sets, were compared separately.

The computed values suggested a higher degree of congruence between the allozyme data and the larval data than between the allozyme data and the adult morphological data. Values of the former comparision ranged between 60 to 70%, while that of the later was only 40%.

While this result demonstrated the importance of larval developmental characters in the phylogeny of the genus, the small number of species employed in the comparision may have constituted a loss in data. The small number of species with advanced or abbreviated larval development in the 8 species employed would undoubtedly reduce the possible contribution of larval-developmental data. The observed clade of SIN-1,SIN-2,DAY in the original allozyme trees did not contribute to the comparision.

(6.3.5) Results and disscussion on chromosomal study

Chromosome preparation was attempted in five species, M.hainanense, M.nipponense, M.rosenbergii, M.lanchesteri and M.acanthurus. However, due to lack of live specimens in 3 of the

species, resolvable metaphase spreads were only obtained for M.nipponense and M.rosenbergii.

For M.nipponense, over 160 prawns were sacrificed, 78 metaphase spreads were observed in 39 specimens. The median value (68%) of chromosome number was 104 (Plate III).

For M.rosenbergii, over 100 prawns were sacrificed, 34 prawns had metaphase spreads. The median value (57 %) of chromosome number was 115 (Plate IV).

It is thus inferred that the diploid number of chromosomes in M.rosenbergii and M.nipponense was 104 and 115 respectively. It confirmed the preliminary findings of Malecha(1977) that the chromosome number of M.rosenbergii was 115.

The only other published report of chromosome number in the genus was that of M.siwalikensis, with 2n = 100 (Mittal and Dhall,1977). For Palaemon lamarrei (= M.lamarrei ?), Vishnoi(1972) reported a chromosome number of 59.

While it is premature for conclusive statement on the evolutionary trend in the genus, of the three species reported so far, there is a reduction in the chromosome number from the more 'primitive' form (M.rosenbergii) to the more 'advanced' form (M.siwalikansis). The latter belongs to the hendersonii group of species investigated by Tiwari(1952) and probably has large size eggs. The general number of chromosomes also seems to fall within the '100' group suggested by Niiyama(1959).

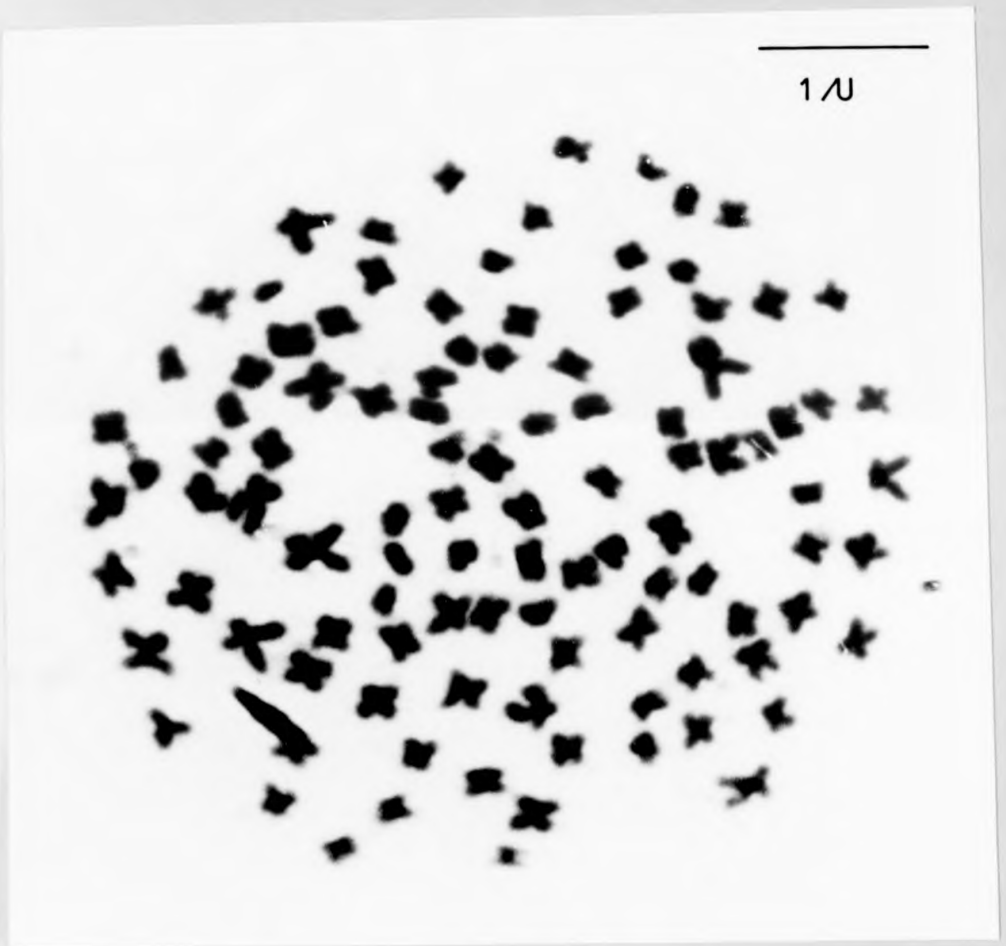


PLATE (III): Metaphase spread of Macrobrachium nipponense

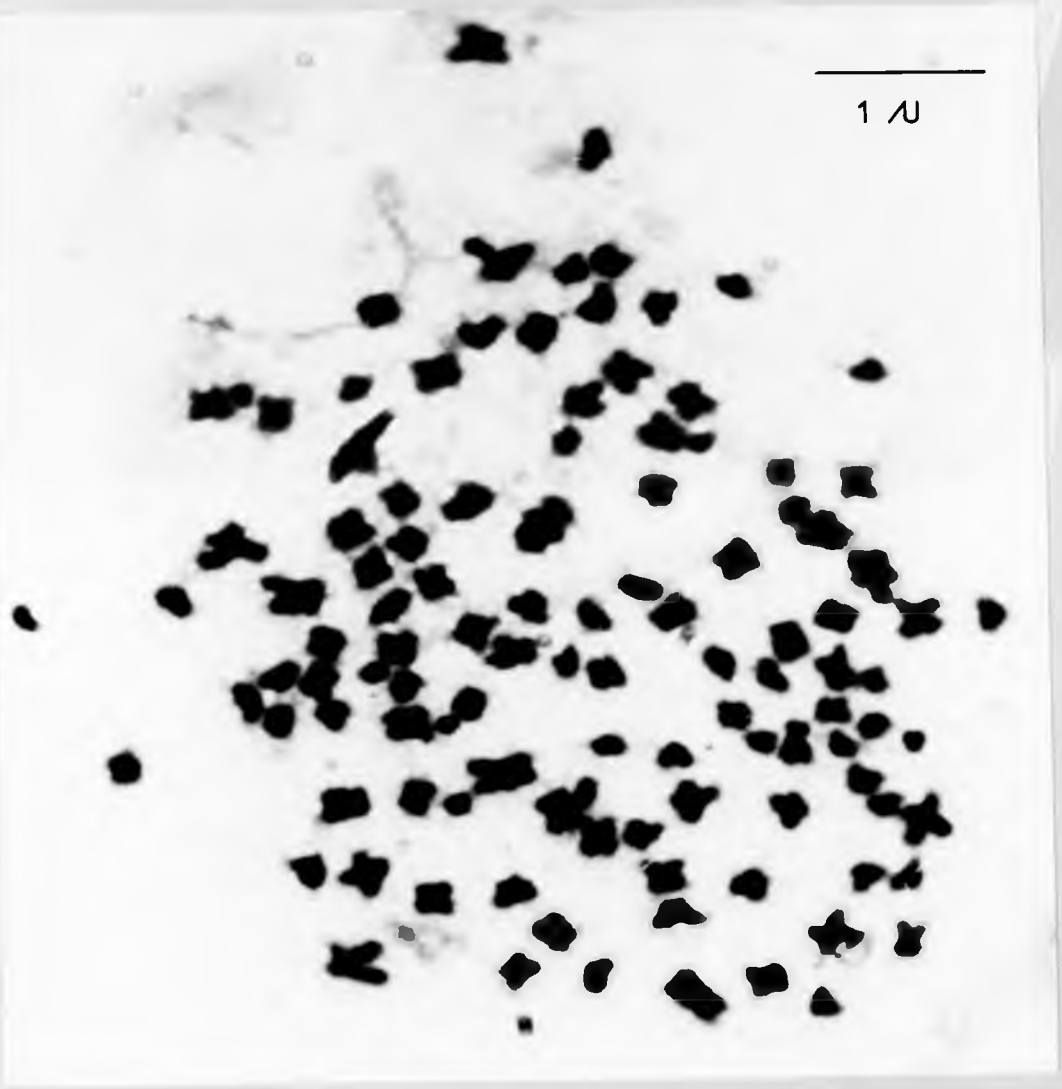


PLATE (IV): Metaphase spread of Macrobrachium rosenbergii

(6.3.6) Results and discussion on hybridization trials

Using the artificial insemination method described, the following bidirectional crosses have been tried : M.lanchesteri x M.nipponense, M.nipponense x M.rosenbergii, M.nipponense x M.acanthurus and M.hainanense x M.nipponense.

However, where intra-specific hybrids were successful within each species, all interspecific crosses did not give viable spawns. All eggs were dropped 2-3 days after spawning.

This result is similar to those obtained by other workers, the following artificial crosses were not viable:

a. M.rosenbergii x M.acanthurus (Sandifer and Smith,1979),

b. M.rosenbergii x M.ohione (Sandifer and Smith,1979),

c. M.rosenbergii x M.carcinus (Sandifer and Lynn, 1980),

d. M.asperulum x M.formosense (Shokita, 1978).

While crosses a. to c. involved species from different geographical regions, crosses d. and crosses in the present study concerned species which overlap in their distribution.

Three cases of inter-specific hybrids, however, were successful:

e. M.rosenbergii x M.malcolmsonii (Sankoli et al.,1982),

f. M.nipponense x M.formosense (Uno and Fugita, 1972; and in Shokita, 1978),

g. M.asperulum x M.shokita (Shokita, 1978).

All these crosses (e. to g.) involved species within the same phyletic group mentioned in earlier sections. Cross (e) involved species from the 'rosenbergii' group (Johnson,1973). Cross (f) involved species with the closest morphology, the two species differed slightly in the dentition in their fingers (

Holthuis, 1950) ! Both species probably also belong to the 'equiden' group (Cf. Johnson, 1973). Cross (g) belongs to the 'pilimanus' complex (Johnson, 1960) which share both similar adult morphology and larval developmental mode. All these crosses, however, gave viable but infertile progenies. The species involved are thus genuine species in accordance with the biological species concept.

All the hybrids also have intermediate forms of larval development (Uno and Fugita, 1978; Sankoli et al., 1982; Shokita, 1978). Compatible larval development may thus be a prerequsite for successful hybrids.

(6.3.7) Overall discussion on the phylogeny of Macrobrachium

The similarity of many species on both sides of the Atlantic (American vs West African species) as well as on both sides of Americas (see Chapter 2) suggested that the genus Macrobrachium originated in the area of the present-day Indo-Pacific region, and spread subsequently to other parts of the world. M.rosenbergii, and its related species, considering its larval development and its 'primitive' morphology (Johnson, 1973), probably represents a group that is related directly to the ancestral stock of Macrobrachium. The salinity tolerance of adult M.rosenbergii typifies true freshwater decapods, the HOC remains hyperosmotic at low salinities, but increasing just before the isosmotic point (see Chapter 4.1.3; Singh, 1980). This also confirms that the species, and probably the 'rosenbergii' group, was ancestral in character, and has evolved for the particular way of life (freshwater adult phase with a brackishwater larval

phase). The large size of the species in this group might have provided a constraint (when compared to other species of the genus) to the species of this group developing abbreviated larval development, relating to the surface/volume ratio of brooding chamber and reproductive strategy (Strathmann, 1986; see Chapter 3.1.1). The phylogenetic clade which group M.rosenbergii, with M.idella and M.acanthurus, species from Americas and East Africa, then confirmed the above hypothesis from biogeographical analysis.

As ancestral Macrobrachium migrated into the freshwater environment (see Chapter 2), there was a tendency for the development of freshwater tolerance and shortening of larval development, as described in chapter 3 and chapter 4. Among the species investigated so far, there is a whole spectrum of variation in the degree of abbreviation of development (but fewer species with intermediate numbers of larval stages). The timing of the first appearance and subsequent development of a certain larval appendage may be very different, even for species with the same total number of larval stages.

A group of species in south-east Asia have been reported to have extremely similar modes of abbreviated larval development, but with slightly different larval meristic characters described for M.hainanense in chapter 3 (e.g. number of plumose setae on posterior margin of telson). So far, other species observed to be in the group include M.pilimanus, M.malayanum, M.asperulum and M.shokitai. M.hendersondayanum also has a very similar mode of larval development to the present group, and the 'hendersonii' group of species (Tiwari, 1952, 1955) may constitute a sub-phyletic

group in the Indian sub-continent.

These species generally inhabit the upper reaches of rivers with torrential water flow (e.g. Dudgeon,1985) and also often in higher altitude. Considering their lack of dispersal phase (planktonic larvae), they cover a wide geographical area from M.asperulum in the Yangstze River (Zhang et al.,1979) to M.pilimanus in Indonesia (Chong and Khoo,1985a). This , and the fact that M.hainanense was phylogenetically most related to the M.rosenbergii, suggested that the ancestors of the 'pilimanus' group (sensu Johnson,1967) of species were among the first members of Macrobrachium species to have invaded the freshwater environment. It is highly unlikely, and difficult to envisage, that this group of species is the result of convergent evolution in different river systems all over south-east Asia. It is more likely that the various species represent an allopatric speciation from similar ancestral specie(s), upon tectonic movement of the region. Shokita(1979), in determining the speciation process of Macrobrachium in the Ryukyu Islands, considering the evidence from larval development, artificial hybridization (Chapter 6.3.6), biogeography, tectonics and historical glacial events, suggested that M.shokitai was evolved from M.asperulum since the Pliestocene.

Also, on a hypothetical stepping-stone mode of evolution along each river, it would be expected that species with different degrees of freshwater adaptation (e.g. abbreviated larval development), which occupy different reaches of a river, would be more related to each other than to species from a distant river. The fact that the 'pilimanus ' group of species

are more related to each other, and the fact that the group (at least M.hainanense) branches off from the 'primitive' group in the phylogenetic trees constructed (rather than other species of less extended type of development in south east Asia) probably confirms that both groups have evolved directly from the ancestral Macrobrachium. The presence of other groups with advanced larval development, but less abbreviated than the 'pilimanus' group, probably implies that the genus might have advanced to the freshwater environment in more than one wave. The grouping of M.dayanum with M.sintangense and its sibling species suggests that some of these species with the advanced type of development, at least, may have formed another wave of freshwater advancement (also see later discussion). The salinity tolerance of M.potuiuna (Moreira et al.,1983; Chapter 4.1.3), a species with advanced type development in South America, suggests that the species is comparatively recent in its evolutionary history in freshwater, supporting the different evolutionary history of this group in the freshwater. Tiwari(1952), in considering the species of Macrobrachium in the Indian sub-continent, and especially the 'hendersonii' group of species, was also drawn to the conclusion that the genus has attacked freshwater in more than one wave. Considering the small proportion of species with abbreviated development being analysed, the congruence between the phylogenetic trees of larval developmental data and allozyme data implies that phylogeny of the genus relates more to the evolutionary history in the freshwater environment (different degree of abbreviated larval development) than to the geographic association of species. The only successes of inter-specific

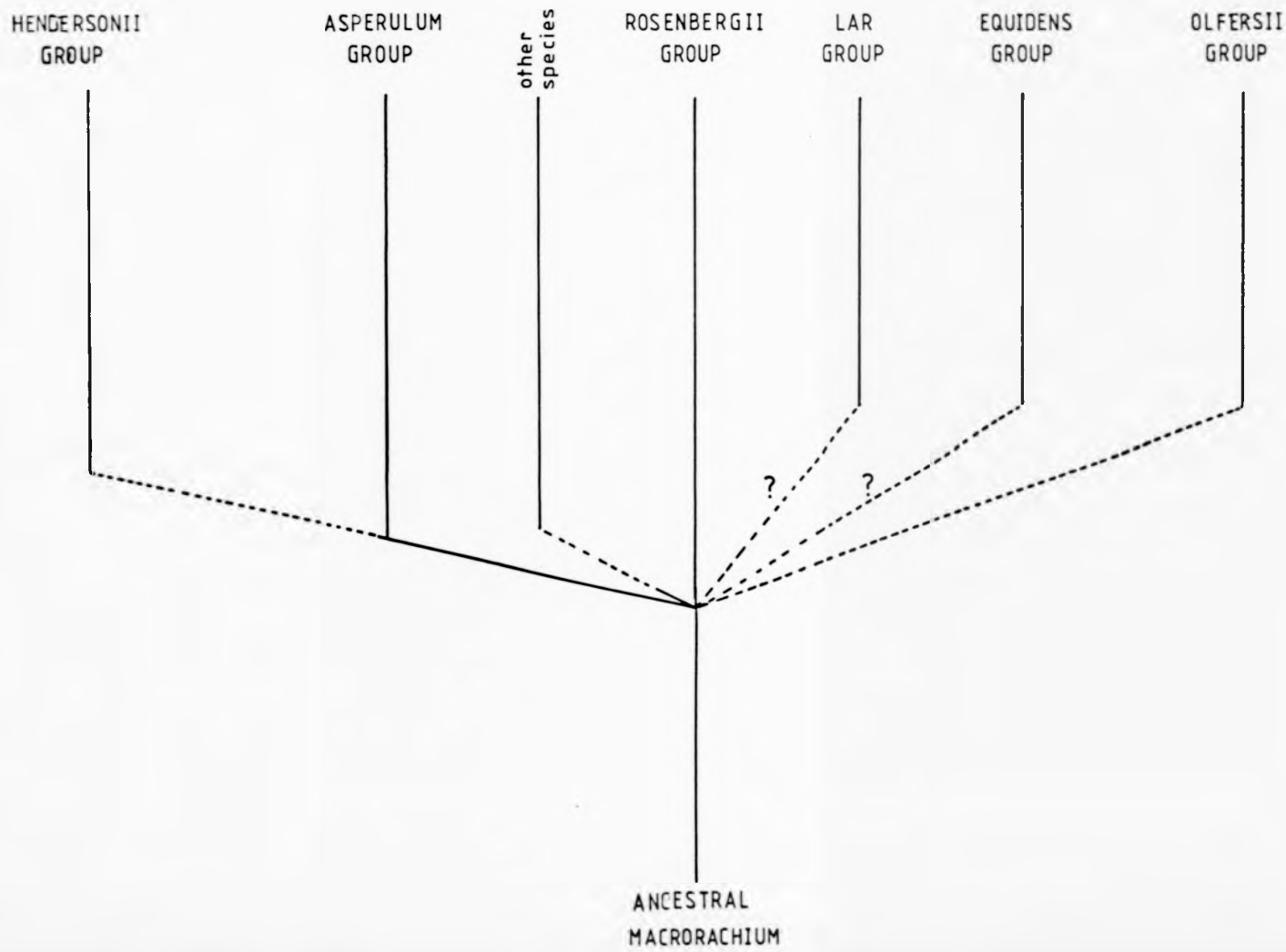
hybridization within each individual group of species ('rosenbergii', 'equidens' and 'pilimanus') and the inability of artificial crosses outside a group then also confirm the polyphyletic (arising from different time, from probably slightly different ancestors) nature of the genus.

Other possible clades in the evolutionary tree then would probably include species with asymetrical second pereopod which constitute the 'olfersii' group of species in the Americas. Apart from the type species of M.equidens and M.rude, which were confirmed by the allozyme data, other species of the 'equidens' group as suspected by Johnson(1973), may not have such a close phylogenetic relationship, especially in considerations of their larval development. M.nipponense, however, which was grouped with M.equidens and M.rude in the allozyme tree, and which has fairly similar mode of development to the former and shares a similar adult morphology (slender second pereopod and medium size), should be considered to be in this group. To this, its close relation, M.formosense, should also be included. M.nipponense, with its temperate distribution and vaious degrees of freshwater tolerance in different populations (chapter 4), would probably be a recent member of the group. The ability of some populations to adapt quickly to larval survival in freshwater (chapter 4) also indicates that it probably represents the present 'wave' of species attempting to complete their entire life cycle in freshwater. In the allozyme survey of Chow and Fujio (1985b), two species, M.lar and M.japonicum form a monophyletic group. Both species also have an extended type of development comparable to that of the 'rosenbergii' group, but having adult morphology

more similar to that of the 'equidens' group. M.intermedium may be another species of the group (lar group) with similar larval development and also larval tolerance for higher salinities than other species in the genus. These two species and any other related forms may constitute a group similar to the ancestral forms of the 'equiden' group. A hypothetical evolutionary tree of the different known clades was constructed in Fig. (6.12). Only the 'rosenbergii' group probably have representatives in all the biogeographical regions. The 'olfersii' group, if proved to be related to M.lepidactylus, as suggested from the asymetrical second pereopods, would constitute a second example. Considering the hypothesis of a first-wave invasion by the 'pilimanus' group, it would be unlikely that similar species would exist in other biogeographical regions. The abbreviated larval development of species in the Americas would thus be a most interesting subject for research. It is likely that they would share the same degree of variation in their developmental modes as in some of the species in south-east Asia with different degrees of advanced development (e.g. M.biramicus, M.lamarrei and M.australiense).

In an uncolonised river, the ancestral Macrobrachium would probably advance up the river in the suggested sequence of developing adult freshwater tolerance then larval freshwater tolerance and the shortening of larval development (Chapter 3,4,5; + other adaptations). The splitting of some M.nipponense populations into a freshwater and estuarine populations in the same river in Japan probably represent the first step in the process of advancement.

Fig.(6.12): A hypothetical phylogenetic tree of the known clade of *Macrobrachium* species


HENDERSONII GROUP
ASPERULUM GROUP
other species
ROSENBERGII GROUP
LAR GROUP
EQUIDENS GROUP
OLFERSII GROUP
?
?
ANCESTRAL MACRORACHIUM

With spawning migration, species with adult freshwater tolerance can occupy a fair length of the river (probably in the lower reaches, especially in a low gradient river). Indeed, many species with estuarine larvae can be found at great distances from the river mouth (Chapter 1). The shortening of larval development would enable the species to remain increasingly near to the parental ground, which would move increasingly up the river. Competition in the lower reach of the river would also cause the 'pioneer' species to move increasingly up the river. Similarly, newer species would also be precluded from advancement by the competition from the 'pioneer' group. On the other hand, as an abbreviated type of larval life cycle is developed, the species involved could occupy the most upper reach of rivers (with other adaptations, e.g. cold tolerance) and would subsequently form a secondary competition force to its ancestral stocks with intermediate type of larval development. The species with intermediate numbers of larval stage would then face competition from both the lower course and the upper course of the river, which may eventually cause its extinction. Therefore, species with an intermediate form of advanced larval development are generally found in relatively 'new' biogeogeographic area of the genus.

Examples would include M.australiense in Australia, and M.tenellum in south America. Also, such larval development can probably persist where competition from the two other groups is less, flat river plains with unconnected water bodies and rice paddies (e.g. M.lamarrei, M.sintangense) would be two

possibilities.

The suggestion of the two-wave invasion by Macrobrachium in the northern India can easily be explained by tectonics (Tiwari, 1955). The subsequent speciation of the 'pilimanus' group can also be explained (as in M.asperulum and M.shokitai; Shokita, 1978). Similarly ,species in the lower course of river can be displaced or become extinct by tectonic or geological displacement of rivers. In some cases, climatic processes can cause displacement of species of the lower course as species from the upper course are probably more adapted to colder temperatures. Recolonistion of of the lower course would thus involve different species to those already existing in the upper course. Thus, competitive exclusion of species in rivers with intermediate numbers of larval stages, coupled with tectonic and climatic processes can probably explain the observed polyphyletic nature of the genus. However, such a hypothesis involves many assumptions and definitely remains to be tested.

The fact that different species of the genus can be grouped into various clades characterised mainly by their larval development have profound implications in the interpretation of evolutionary modes. If the larval developmental characters were to be fossilised, a careful palaeotologist would probably draw the conclusion that larval development of the genus has evolved from the punctuated-equilibria mode. However, the inference would be a misinterpretation, as the hypothetical fossils would not reveal that the different groups were polyphyletic in origin, evolving to the freshwater environment in more than one wave. In fact the abbreviated type of development would have to go through

the intermediate stages (like M.biramicus, M.lamarrei, M.australiense). As mentioned earlier, there was a gradual variation in the appearance and subsequent development of each appendage, which overall resulted in the abbreviation of a certain stage.

The only fossil evidence claimed to be Macrobrachium was purported to be from early Miocene (Rathbun, 1918), some 12 to 25 million years ago. However, the 'fossil' mainly consisted of several broken species of unidentified appendages and its status should not be treated as unequivocal. Biogeographic analysis (Chapter 2) suggested that the genus has probably evolved some 6-17 million years ago, before the joining of the Americas, after the closure of the Mediterranean and widening apart of the Atlantic. A genetic distance of 0.6 - 0.7 between M.hainanense and the rest of the species would suggest that the ancestral species may have started to invade the freshwater environment some 12 million years ago (using the popular 18 million years per D). This also coincides with the spliting of the east-west race of M.rosenbergii, as suggested by Lindenfelser (1984; see Chapter 5). Assuming that the ancestral Macrobrachium started the freshwater migration fairly early after it was evolved, the genus may have been in existence for some 12-17 millon years.

"....But we are here not concerned with hopes or fears, only with the truth as far as our reason permits us to to discover it; and I have given the evidence to the best of my ability."

--Charles Darwin
in "The Descent of Man"
(1871 ; Chapter 21)

CHAPTER SEVEN :

CONCLUSIONS AND RECOMMENDATIONS

(7.1) Evolution and phylogeny of *Macrobrachium*

Macrobrachium, as a genus, has evolved comparatively recently in the freshwater environment. The larval development and evolutionary genetics of the genus are much characterised in relation to their history of advancement in freshwater.

Species of the genus can be found from the sublittoral area to torrential streams in high altitudes. Some species can spend their life cycle in the marine environment (*M.intermedium*), others have to perform spawning migrations to the saline/freshwater margin, where many can complete their entire life in freshwater. Correspondingly, species of the genus have a whole spectrum of larval developmental modes, adult salinity tolerance, as well as larval salinity tolerance. On advancement from the marine to the freshwater environment, there was a shortening of larval developmental stages and duration, accompanied by increasing tolerance to freshwater in both the adult and the larval phase. Evidence supports the hypothesis that adults acquired freshwater tolerance, prior to its development in the larval phase. Some species have also evolved towards the adult/freshwater- larval-marine life cycle, with large size and high fecundity ('rosenbergii' group).

While no species are circumpolar, many species have a wide geographic distribution with highly differentiated populations, in morphological, allozymic, as well as physiological traits. Different populations can also differ significantly in their larval salinity tolerance. The population of M.nipponense investigated has a high heritability of larval freshwater tolerance. This was probably resulted from the holding the population in low salinity enviroments (not complete freshwater), which constituted a slow process of genetic assimilation. However, this result implies that some species, at least, are still in the process of advancement into the freshwater regime. The differentiation of inland populations of M.nipponense could be explained by inbreeding due to small founding populations. The differentiation of the east/west races of M.rosenbergii could be explained by marine hydrographic conditions (surface currents) while population differentiation within the western race is related to geographic distances. A higher average level of genetic diversity compared to previously observed values for Palaemonidae was observed among the thirteen species of Macrobrachium being investigated.

Evidence from biogeography, larval developmental modes, larval developmental characters, adult salinity-freshwater tolerance, larval salinity-freshwater tolerance as well as inter- and intra-specific allozymic data in the present thesis have suggested that the genus is polyphyletic in origin. Congruence between the larval developmental data and the electrophoretic data also confirms that the phylogeny of the genus relates more to the evolutionary history of the species (different waves in

the freshwater advancement) rather than to the geographical association.

The construction of phylogenetic trees from larval developmental characters in the present thesis strengthens the significance of these rarely used data. The overall results also confirm the importance of considering evidence from different types of data for both systematics and evolutionary studies.

(7.2) Implication to aquaculture

Biogeographic analysis of Chapter 2 suggested that different species of the genus can have very different tolerances to cold temperature. M.nipponense can survive and grow (though slowly) even at 15'C(personal observations), implying that it can survive in many sub-tropical areas. While this has been realised in many parts of oriental Asia, where the species has been introduced into many inland water bodies, it has rarely been noticed outside China, Japan and Korea (with the exception of Israel).

Larval development of different species in the genus consists of a whole spectrum, from extended type of development to direct development. This would have great implication to the hatchery operation of any potential species, as planktonic larval stages requires both expensive diets and manpower. Similarly, different species differ greatly in their larval salinity tolerance, which would imply different suitability for culture in relation to the availability of seawater.

The relatively high value of heritability observed in the larval freshwater tolerance in M.nipponense suggest the possible utilisation of coastal populations of the species for inland

freshwater sites. The result also points to the possible success of larval freshwater tolerance in other species of the genus. However, potential genetic assimilation of this trait would probably take longer in the more saline species. However, the interbrood correlation of survival rate, even in the control line, strongly asserts the importance of selection for general larval survival in aquaculture.

High inter-specific and intra-specific variation in the genetic diversity exists in the genus. Some inland populations of M.nipponense (e.g. Wuhan) may have already had a significant decrease in genetic diversity when compared to coastal populations. In aquaculture, as well as in introduced fisheries (which is more popular at present), broodstocks or the founding populations should include individuals from different populations. Attention should also be given to the larval survival rate and temperature tolerance of the populations involved.

While the eastern and the western race of M.rosenbergii had a genetic distance of up to 0.40 (Hedgecock et al.,1979) and were characterised by completely different colour morphs, very little difference in aquacultural traits have been observed. Larvae of the Australian population, however, metamorphose at 15.5 days and 95% of all postlarvae would have metamorphosed by 20.5 days. This compars favorably with the larvae of the western morph which generally first metamorphose after 21-24 days and that 95% of its postlarvae appear before 32 days (Sarver,et al.1979). This intra-specific variation would thus contribute significant saving of both manpower and larval rearing resources in a commercial

hatchery.

Different strains of M.rosenbergii also differ slightly in their growth rate at 23-29'C(Sarver, et al.1979). This may be utilised for further selection for cold temperature tolerance of this species which has severely precluded the use of this species in the sub-tropics.

In Hawaii, the Anuenue stock which has been under captivity for over 20 years, has shown no evidence of inbreeding or randon genetic drift (Hedgecock, et al.,1979). Malecha (1986) suggested that successful techniques of larval rearing (> 50% survival) as compared to << 1 % in the wild has completely flushed the gene pool of the cultured stock.

On the otherhand, Sbordoni et al.(1986) present strong evidence that a steady decline in heterozygosity in a hatchery a hatchery stock of Penaeus japonicus has led to a parallel decrease in larval hatching rate from 50% to 10%. The observed decline approximated to a theoretical Ne of 2 ! Sbordoni et al(1986) suggested that the number of males contributing to the breeding is much smaller than the number of females (equation 6.6), and that offspring from different parents have probably contributed disproportionately to the next generation. Hatchery management should improve to record the pedigree of each batch of larvae and recruit equal broodstock from different spawning of the last generations.

Many desirable characters of aquaculture (e.g. size, temperature tolerance, larval developmental rate, larval salinity tolerance, direct development, large brood size) belong to different species of the genus. A theoretical solution will be

the artificial hybridization between some of these species. Although the techniques for artificial insemination have been established and have proved to be useful for intra-specific crosses (Hedgecock, 1986), most attempts at inter-specific crosses have failed. The phylogenetic analysis of the present thesis revealed the polyphyletic nature of the genus, explaining the successful hybrization only for those species within a phyletic group.

Nevertheless, useful hybridization can still be performed. Inter-specific hybrids between the different members of the 'rosenbergii' group should produce possible new candidates for aquaculture. More work should thus be done on the inter-specific variation in the aquacultural traits (e.g. larval development, temperature tolerance, etc.). Species with intermediate numbers of zoeal stages (rather than the 'pilimanus' group) would also be potential candidate for successful inter-specific hybridization with species with accelerated type of larval development (e.g. M.nipponense). Also, many species with extended type of development also differ in their larval salinity tolerance (e.g. M.lanchesteri). This suggests potential hybridization with species of larger size for the selection of larval freshwater tolerance in the later species.

-"..... one of the foremost genetic engineers..... his world was threatened by terrible invaders ..... had come up with a remarkable new breed of super-fly that could, unaided, figure out how to fly through the open half of a half-opened window.....the fearsome invaders.....joined in the celebrations..."

Douglas Adams
in 'So long, and thanks for all the fish' 1984

APPENDIX (I)

Buffer systems used for electrophoresis

(1) AM

Gel buffer	Electrode buffer
0.002M citric acid	0.004M citric acid

both adjusted to pH 6.1 with N-(3-amino-propyl)-morphine solution

(2) CTC (continuous tris-citric buffer) pH 8.0

Gel buffer	Electrode buffer
dilute electrode buffer 1:25	0.25M Tris
	0.075M citric acid

(3) TEC (from Redfield and Salini, 1980)

Electrode buffer (pH 7.0)	Gel buffer (pH 7.0)
Tris 16.35g/l	Tris 1.09g/l
Citric 0.63g/l	Citric 9.45g/l
$EDTA(Na_2)$ 0.47g	$EDTA(Na_2)$ 0.47g

(4) TCB

Electrode buffer (pH 9.3)	Gel buffer (pH 8.6)
LiOH 0.1M	Tris 0.76M
Boric acid 0.3M	Boric acid 0.015M
	Citric acid 0.005M
	Tris 0.76M

(5) TEB

Electrode buffer (pH 9.0)	Gel buffer (pH 9.0)
Tris 0.5M	
Boric acid 0.25M	dilute electrode buffer
$EDTA(Na_2)$ 0.02M	1 : 10

(6) Poulik

Electrode buffer (pH 8.2)	Gel buffer (pH 8.7)
Boric acid 18.55g/l	Tris 9.21g/l
NaOH 2.4g/l	Citric acid 1.05 g/l

Running conditions for all buffers were constant current at 40mA and the corresponding voltage (200V), for 5 hours.

Abbreviations as defined in Appendix (II).

APPENDIX (II) : STAINING RECIPES

All chemicals obtained from Sigma (U.K.) Ltd.
Abbreviation of enzyme loci as in table (5.4).
Unless otherwise stated, recipes are for 30ml of staining solution (i.e. + 30 ml of distilled water or staining buffer).
Formula for staining buffers listed in Appendix (III)

(1) AAT-1 and AAT-2

Tris	300 mg
L-aspartic acid	65 mg
@-ketoglutaric acid	20 mg
Pyridoxal-5-phosphate	10 mg
PVP	10 mg
Fast Blue R R	25 mg
distilled water	30ml

(2) ACON

cis-aconitic acid	50 mg
Magnesium chloride	10 mg
NADP	5 mg
Isocitric dehydrogenase	2 units
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer : Tris-HCl pH 8.0

(3) ACPH-1 and ACPH-2

soak gel in 0.05M acetate buffer (pH 5.0) for one hour before staining

Sodium @-napthyl phosphate	30 mg
PVP	125 mg
Fast blue R R salt	30 mg

staining buffer: 0.125M acetate buffer (pH 5.0)

(4) ADA-1 AND ADA-2

Adenosine	15 mg
Sodium arsenate	50 mg
NP	25 ul
Xanthine oxidase	50 ul
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer : 0.1M phosphate buffer (pH 7.5)

(5) ALD

Fructose-1,6-diphosphate	100 mg
NAD	20 mg
G3PDH	50 ul
Sodium arsenate	60 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer : Tris-HCl pH 7.0

(6) AK

Glucose	50 mg
MgCl	10 mg
NADP	5 mg
ADP	20 mg
Hexose kinase	50 ul
G6PDH	20 units
MTT	5 mg
PMS	2 mg

staining buffer : Tris-HCl pH 8.0

(7) ALP

B-naphthyl phosphate (sodium)	25 mg
Magnesium sulphate	60 mg
Fast blue R R	25 mg

staining buffer: 0.06M borate buffer pH 9.7

(8) CK

Creatine	30 mg
Magnesium acetate	40 mg
Potassium acetate	40 mg
Phospho-enol-pyruvate	15 mg
NADH	10 mg
LDH	200 units
PK	5 units

staining buffer : 0.5M Tris-HCl pH 8.0
observed under ultra-violet light

(9) DIA

Dithlerophenol-indophenol	2.5 mg

(filter before used in 2.5ml of distilled water)

NADH	10 mg
MTT	5 mg
agar	2 %

staining buffer : 0.5M Tris-HCl pH 8.0

(10) ENOL

3-phosphoglycerate (sodium)	50 mg
NADH	35 mg
MgCl	40 mg
ADP	40 mg
PGM	50 ul
PK	50 ul
LDH	200 units

staining buffer : 0.05M Tris-HCl pH 7.5
observed under ultra-violet light

(11) EST

@-napthyl-acetate	10 mg
acetone	1 ml
distilled water	1 ml

-the above mixed before adding to staining solution

Fast blue R R salt	20 mg

staining buffer : Tris-maleate pH 5.3

(12) FUM

Fumaric acid	100 mg
NAD	30 mg
MDH	20 units
MTT	5 mg
PMS	2 mg
sodium pyruvate	20 mg
agar	2 %

staining buffer : 0.5M Tris-HCl pH 8.0

(13) G6PDH

Glucose-6-phosphate	10 mg
MgCl	30 mg
NADP	5 mg
MTT	5 mg
PMS	2 mg

staining buffer: 0.5M Tris-HCl pH 8.0

(14) @-GPDH

@-glycerophosphate	50 mg
NAD	15 mg
EDTA	60 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(15) GDA

Guanine	50 mg
(dissolved in 10 ml of warm sodium	hydroxide)
Xanthine oxidase	25 ul
MTT	5 mg
PMS	2 mg
agar	2 %
staining buffer: 0.2M Tris-HCl pH 7.5	

(16) GDH

Sodium glutamate	70 mg
NAD	5 mg
MTT	5 mg
PMS	2 mg
agar	2 %
staining buffer: 0.5M Tris-HCl pH 7.5	

(17) GPT

L-alanine	500 mg
@-ketoglutarate	40 mg
NADH	10 mg
LDH	200 units
staining buffer: 0.5M Tris-HCl pH 8.0	

(18) HK

Glucose	60 mg
ATP	15 mg
NADP	4 mg
G6PDH	20 units
MgCl	10 mg
MTT	5 mg
PMS	2 mg
staining buffer: 0.5M Tris-HCl pH 7.0	

(19) LAP

L-Leucyl-glycine	12 mg
Peroxidase	8 mg
L-amino-oxidase	4 mg
O-Dianoside	4 mg
MnCl	4 mg
agar	2 %
staining buffer: 0.5M Tris-HCl pH 8.0	

(20) LDH

Sodium lactate solution	0.2ml
NAD	10 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(21) ME

L-Malic acid	150 mg
NADP	8 mg
MgCl	10 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(22) MDH

L-Malic acid	60 mg
NAD	15 mg
MTT	5 mg
PMS	2 mg
agar	2%

staining buffer: 0.5M Tris-HCl pH 8.0

(23) MPI

Mannose-6-phosphate	20 mg
NADP	5 mg
G6PDH	50ul
PGI	50ul
MTT	5 mg
PMS	2 mg
agar	2%

staining buffer: 0.5M Tris-HCl pH 8.0

(24) NP

Inosine	15 mg
MTT	5 mg
Xanthine oxidase	10 ul
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.1M phosphate buffer pH 7.5

(25) ODH

Octanol	1 ml
NAD	15 mg
MTT	5 mg
PMS	2 mg

staining buffer: 0.5M Tris-HCl pH 8.0

(26) 6PGDH

6-Phosphogluconate	10 mg
MgCl	20 mg
NADP	5 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(27) PGM

Glucose-1-phosphate (sodium)	50 mg
MgCl	70 mg
NADP	5 mg
G6PDH	50 ul
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(28) PGI

Fructose-6-phosphate	20 mg
NADP	4 mg
MgCl	20 mg
MTT	5 mg
PMS	2 mg
G6PDH	25 ul
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(29) SDH

Sorbitol	150 mg
NAD	15 mg
sodium pyruvate	50 mg
MgCl	10 mg
MTT	5 mg
PMS	2 mg
agar	2 %

staining buffer: 0.5M Tris-HCl pH 8.0

(30) IDH

Sodium isocitrate	35 mg
NADP	4 mg
MTT	5 mg
PMS	2 mg
MgCl	50 mg
agar	2 %
staining buffer : 0.5M Tris-HCl pH	8.0

Abbreviations:

PVP	polyvinylpyrolidone
MTT	3-[(4,5-Dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide
PMS	phenazine methosulphate
NAD	B-nicotinamide adenine dinucleotide
NADH	NAD reduced form
NADP	NAD phosphate
ADP	adenine diphosphate
ATP	adenine triophosphate
MgCl	Magnesium chloride
MnCl	Manganese chloride
Tris	Tris(hydroxymethyl)aminomethane

APPENDIX (III): STAINING BUFFERS

(1) Tris-HCl buffer — pH 7.0, 7.5, 8.0

Tris + 1N hydrochloric acid to make up to the required pH

(2) 0.1M Phosphate buffer — pH 7.4

Disodium hydrogen phosphate.$7H_2O$	17.2 g/l
Sodium dihydrogen phosphate.$2H_2O$	4.94 g/l

(3) 0.06M Borate buffer — pH 9.7

Boric acid 0.06M
adjusted to required pH with sodium hydroxide

(4) Acetate buffer — pH 5.2

Sodium acetate 27.2 g/l
adjusted to required pH with glacial acetic acid

(5) 0.1M Tris-maleate — pH 5.3

Tris	12.1 g/l
Maleic acid	11.6 g/l

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