Introduction

River prawns of the genus *Macrobrachium* Bate, 1868, Class Malacostraca: Order: Decapoda: Suborder Pleocyemata: Infraorder Caridea: Superfamily Palaemonioidea: Family Palaemonidae: Subfamily Palaemoniae: Genus *Macrobrachium* are a common entity in estuarine and freshwater ecosystems throughout the tropical, warm and temperate areas of the world (Short, 2000). The economic importance of a number of species, mainly the Giant River Prawn, also known as *Macrobrachium rosenbergii* (De Man, 1879) and *Macrobrachium. nipponense* (De Haan, 1849), cannot be underestimated. They are farmed extensively in South East Asia providing employment and nutrition to a large number of people (Jayachandran, 2001). In 2003, these two species accounted for all farmed freshwater prawns, about two thirds *M. rosenbergii* and one third *M. nipponense*. China produces an estimated 180,000 tonnes of freshwater prawns per year followed by India and Thailand with around 35,000 tonnes each (Jayachandran, 2001).

In Taiwan there are currently 17 recognised species of *Macrobrachium* (Shy and Yu, 1998; Cai and Jeng, 2001) of the 210 plus members of the genus. Of these, 15 species are considered euryhaline, that is, they spend their larval cycle in salt or brackish (semi-salt) water, migrating up-stream as they mature into adults. *M. asperulum* (Von Martens, 1868) and *M. nipponense*, are also euryhaline but land-locked too, relating to the fact that they have adapted to be able to spend their entire life-cycle in fresh water.

Classification challenges

Members of the genus *Macrobrachium*, taken from Latin – *Macro* meaning large and *brachium* relating to the arm, can be easily identified. Their enlarged, elongated second periopods are a prominent feature, with male appendages of certain species often exceeding their total body length (Bate, 1868; Holthuis, 1950). Conversely, below the genus level, *Macrobrachium* is noted for being a considerable taxonomic challenge to many a carcinologist, and is widely viewed as one of the most difficult groups of decapod crustaceans to separate at the species level (Holthuis, 1950; Short 2000; Jayachandran 2001). This is confounded by the number of species, approximately 210 (Short, 2004), with over half of these described after the major revisionary work of Holthuis (1950). The creation of a new genus, *Allobrachium*, was proposed by Jayachandran (2001) to include species with unequal second periopods although at present the genus remains in its current form.

In the early fifties the first revisionary work on the Australian fauna which included a revised key as well as describing 4 new species and 6 new subspecies of *Macrobrachium* was performed (Riek, 1951). From this work, 4 sub-subspecies of *Macrobrachium. australiense* were re-evaluated using a combination of morphological and F1 progeny to conclude that the differences were not sufficiently large, or consistent enough, to justify dividing the species group (Lee, 1979). Riek's key was used by Fincham (1987) when describing a new species, *Macrobrachium bullatum*. The work showed inconsistencies when comparing the key with the material at hand, which raised the question of the validity of a number of species including the subspecies *M.*

australiense due to observed developmental variation in the second periopod. In addition to this, Fincham also noted that the holotype of *M. tolmerum* (Riek, 1951) was incorrectly classified as a male, highlighting the difficulties faced by even seasoned carcinologists when determining sex as well as species type. *M. latidactylus* (Thallwitz, 1891), found in Taiwan, is another example of a species that is difficult to classify, with others commenting on the variation exhibited with the larger periopod, which in contemporary times, persists as a common character for differentiating between species (Short, 2000; Jayachandran, 2001). The validity of using a character, such as the second periopod, as a taxonomic indicator has been questioned due to the observed plasticity of this appendage in which the appendage does not complete its final development (Holthuis, 1950). This was supported by reference to previous, incorrect descriptions of new species of *Macrobrachium* by De Man (1892). By careful analysis of the original material, along with the material at hand, it was concluded that the species showed greater morphological similarity to *M. latidactylus* (Holthuis, 1950). No observable morphological differences could be found between females and young males. Furthermore, the use of the second periopod as a primary character for distinguishing species of *M. latidactylus* was questioned as various transition states of the available material led to the conclusion that this character was of no taxonomic value.

Work has been conducted into the social growth of *Macrobrachium* suggesting that aggressive and dominant males will often deprive subordinates (smaller individuals) of food thus creating a situation whereby growth is disproportionate among members of a given population (Segal and Roe, 1975). This behaviour and its observable effects, are exacerbated when food is in a position to be defended, or is restricted by quantity or

location in some manner. Other research which draws parallels with these findings has noted that the presence of dominant males of *Homarus americanus* has a negative impact on the amount of food consumed by subordinates, even when the amount of food remained constant (Cobb et al, 1982). It has been postulated that due to the lower social status of subordinates, a perturbation in their metabolism may arise, or ability to digest food, leading to a decreased food-conversion efficiency (Karplus, 1992). Environmental pressures have also been thought to play a part in development as implied in research on *M. australiense* progeny suggesting that parameters, other than genotype, influence the phenotypic expression of taxonomically important morphological traits such as rostrum length and second periopod shape and size (Dimmock et al, 2004).

Taken together, these findings suggest that the variations in key morphological characters like the second periopod are likely to be multifactorial and thus time consuming and challenging to unravel as classification of certain members of the genus can be complicated by morphological similarity. Furthermore, it highlights the potential that some species of *Macrobrachium* may be incorrectly classified, with cryptic species going unnoticed due to their similarity and/or plasticity of key morphological features.

Mitochondrial DNA (mtDNA) as a marker

The use of nucleotide sequence variations to investigate evolutionary relationships is not a new concept. Sequence differences in ribosomal RNA (rRNA) were utilised to discover archaebacteria, which in turn led to the redrawing of the evolutionary tree (Woese & Fox, 1977). Brown et al (1979) published data that implied that the rate of animal mitochondrial DNA evolution was approximately 10 times that of the single copy

fraction of the nuclear genome and highlighted its potential for use in assessing relationships among species and populations that diverged relatively recently (within the past 5-10 million years) . Avise et al (1987) found that sequence divergence of mtDNA was much larger among species than within species implying its value in delineating the tree of life. In later work, Avise et al (1999) went further by postulating that current recognized species groups based on morphological and behavioral criteria may be constructed to a similar if not more accurate level by also including mtDNA sequence data.

COI as a "DNA Barcode"

The exact number of living species on Earth is unknown, although at present there are approximately 1.7 million named species with perhaps another 10 million (not counting bacteria and archae) that have yet to be described (Stoekle, 2003). In the last few years there has been increasing interest in DNA-based taxonomy with particular focus on a small fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Blaxter, 2004). The third-position nucleotides in COI have been shown to exhibit a high incidence of base substitutions which correlate to a rate of molecular evolution that is around three times greater than that of 12S or 16S rRNA (Knowlton & Weigt, 1998) which has been used to discriminate between phylogenetic groups within a single species as well as closely allied species (Cox & Hebert, 2001; Wares & Cunnigham, 2001; Hebert et al, 2003; Lambert et al, 2005; Clare et al, 2006). A fragment of the COI gene has been the subject of work primarily designed to evaluate its suitability as a species specific identifier, exhibiting high interspecific variation with relatively low intraspecific

variation (Hebert et al, 2003, 2004 ; Hajibabaei & Hebert, 2005; Smith et al, 2005; Clare et al, 2006; Witt et al, 2006). The use of COI as a broad taxonomic marker is further assisted due to the absence of indels (insertions and deletions), which generally lead to a frameshift in the animal mitochondrial genome and often complicate sequence alignments obtained from ribosomal (12S, 16S) DNA (Doyle & Gaut, 2000). DNA barcoding, as it is often referred to, uses a fragment of COI to provide a species specific marker which can be used primarily for confirming known species and implying the presence of cryptic species (Hebert et al, 2003).

Hebert et al. (2004) in an effort to study the correlation between traditional species boundaries established by taxonomy and those inferred by a DNA-based method, sequenced COI fragments of 260 of the 667 bird species that are known to breed in North America. They found that every one of the 260 species had a different COI sequence. 130 species were represented by two or more specimens. COI sequences were either identical, or were at most similar to sequences of the same species. COI variations between species averaged 7.93%, whereas variations within species averaged 0.43%. In four cases there were deep intraspecific divergences indicating possible new species. The work also proposed a standard sequence threshold to define new species; this threshold was defined as 10 times the mean intraspecific variation for the group under study.

DNA-based taxonomy has complemented traditional morphology based taxonomy in a number of cases across a range of diverse taxa in the animal kingdom including butterflies (Hebert et al, 2004), bats (Elizabeth et al, 2006), amphibians (Vences et al, 2005), flies (Smith et al, 2006) and snails (Pfenninger et al, 2006) assisting in delimiting species and identifying potential cryptic species for further analysis. It has

also been used to postulate the reduction in the number of certain fauna due to percieved morphological differences contrasting with low intraspecific variation (Simison & Lindberg, 1999).

It is within the scope of this study to address whether a fragment of the COI region can infer the presence of a new species of *Macrobrachium* from within a population containing a known species, *M. latidactylus*. Individuals within this group may express a high degree of plasticity in certain characters while retaining high morphological similarity which can make identification a challenging task using traditional taxonomic methods alone. More importantly it can allow for new, yet morphologically similar species to go undescribed. By using a standardised DNA marker, such as COI, to complement morphological data, it may be possible to confirm the existence of a new species of freshwater prawn. This is the first time that a 1kb+ fragment of COI will be used in Decapoda for the purpose of resolving a putative species complex.

Building on previous research

The work of Liu (2006), in relation to the first part of his doctoral thesis, and subsequent paper - Molecular Systematics of the Genus *Macrobrachium* (Liu et al, 2007), has laid the foundations for this study. It is due to the findings of a cryptic species of *Macrobrachium* in Hsiukuluan River, Eastern Taiwan and the subsequent phylogenetic analysis, that the proposed research can be initiated. Liu captured a particular specimen of the genus *Macrobrachium* that was found to be genetically distinct, with high boot-strap support, from other species groups. The cryptic species suggested by the research,

was obtained along with a number of putative *M. latidactylus* from a number of surveys and field studies in Taiwan. *M. latidactylus* is distributed across the Central Indo-West Pacific: Malaysia; Indonesia, the Philippines; New Guinea, Australia and Taiwan (Short, 2000)

This study will further investigate the cryptic species by analysis of all available material using morphological and COI sequence data to infer the presence of a new species of *Macrobrachium,* hereby referred to as *Macrobrachium* sp. nov., while distinguishing it from its nearest congener *M. latidactylus.* Issues pertaining to the challenges of classifying *Macrobrachium* at the species level using a single taxonomic method will also be addressed. As a direct consequence, this work should also further the emerging field of DNA barcoding as a fragment of the COI gene will be used to infer the presence of a new species of *Macrobrachium* in Taiwan.

Abbreviations used are TL for total length; CL for carapace length, measured from the postorbital margin to the posterior median margin of the carapace; RL for rostrum length, measured from the distal tip of the rostrum to the proximal most tooth situated on the carapace. Appendages can be found below on a general schematic diagram of a typical *Macrobrachium* (Fig. 1).