DISCUSSION

Challenges of morphology based taxonomy

This study has highlighted the challenges faced when describing new species of *Macrobrachium* particularly when the general morphology of the nearest congeners to the species in question, is highly similar. A single character consisting of 3 components - pollex length, pollex width and total number of teeth within the pollex formed the major distinguishing character between *Macrobrachium* sp. nov. and *M. latidactylus*. In this work, this single character served to differentiate the two species as other characters utilised in this study overlapped between species and were of limited value and no other discernable differences could be found. In particular, body length, rostrum shape, teeth number and ratios of the segments of the second periopod were similar between the two species which in the absence of DNA data and the presence of literature highlighting the plasticity of the second periopod, may lead some to classify all specimens as *M. latidactylus*. Descriptions of *M. latidactylus* from the available literature were highly similar, if not identical to the morphological data obtained from *Macrobrachium* sp. nov. emphasizing the challenges faced by solely relying on traditional methods (Holthuis, 1950; Short, 1980; Jayachandran, 2001). *Macrobrachium* sp. nov. shares a number of morphological similarities with *M. latidactylus* as well as *M. grandimanus* including overall size, CL, RL, rostral shape and rostral teeth number. These represent some of the most commonly used characters in the classification of *Macrobrachium*, which may be used in the field when rapid identification is sought, potentially resulting in the misclassification of some species.

Morphological differences

Qualitative differences found involved the second periopod in terms of the general shape, location of spinules and setae. However, these characters were part of an appendage previously described as highly variable; exhibiting a degree of plasticity that may invalidate them as a lone means of differentiation (Holthuis, 1950; Short, 1980; Jayachandran, 2001). Pollex data aside, the two species could not be distinguished by reference to other characters assessed in this study, using the available material. The question of whether missing or broken appendages; underdeveloped males or juveniles of morphologically similar congeners exacerbate difficulties with morphology-based taxonomy of *Macrobrachium* in the future, as they did in this study, remains to be seen. Further, more comprehensive and detailed dissections conducted by a carcinologist are required to address this question. However, this could involve a substantial amount of time and effort as has been postulated for delimiting other cryptic species complexes such as *Astraptes fulgarator* (Hebert et al, 2004), *Catharus minimus* (Winker & Pruett, 2005) and the members of the family Formicarridae (Rice, 2005).

COI inference

All species had a different COI sequence with none shared between species. The 16 specimens of *Macrobrachium* sp. nov. inferred by COI analysis as a separate species group as indicated by the distinct clusters on both the 1120bp and 535bp based NJ-tree (Fig. 9 and Fig. 12)

Using an 1120bp fragment of COI an NJ-tree was constructed which indicated the presence of two distinct and genetically divergent congeneric groups with low intraspecific and high interspecific divergences. When the test sequences were reduced to the 535bp consensus sequence of the GenBank sequences and an NJ-tree constructed, clusters were formed that traditional morphology based taxonomy regard as species groups. Individuals hypothesized to be *Macrobrachium* sp. nov. formed a distinct cluster, separate from *M. latidactylus*, as was the case for the 1120bp-based NJ-tree.

DNA barcoding research often involves the use of COI fragments greater than 600bp (Hebert et al 2003, 2004; Hajibabaei et al, 2005; Ward et al, 2005). It has however, been shown that identical or highly similar results can been obtained using 500bp (Smith et al, 2005), or even 100bp fragments of COI (Hajibabei et al, 2006) in certain fauna. The construction of the two NJ-trees using only reference GenBank sequences tested whether a reduced sequence length contained sufficient data, and thus resolving power, to construct an NJ-tree consistent with the 608bp sequences for the genus *Macrobrachium*, which was the case (Fig. 7 and Fig. 8). The 535bp NJ-tree exhibited branch length and bootstrap support similar to the 608bp NJ-tree with monophyletic groups consistent with recognised species. It is therefore proposed that this 535bp fragment of COI is sufficient for resolving species complexes of *Macrobrachium* in the absence of longer sequences.

Intraspecific boundaries

There was an interesting case of deep intraspecific divergence in the species group *M. latidactylus* where the intraspecific sequence distance ranged from 0 – 9.4%. Excluding the sample from Thailand, intraspecific divergence for *M. latidactylus* ranged

from 0.0 – 2.3% (Fig. 10). The sample from Thailand had a sequence distance approximately 4-fold higher than that of the maximum intraspecific sequence divergence of *M. latidactylus* specimens from other locations. This indicates that the sample described may require further analysis in the form of a more detailed morphological review and additional COI analysis using a greater number of sequences from other species to rule out the possibility that it has been misidentified from a known species or that the GenBank sample represents a cryptic species. It may simply be the case that *M. latidactylus* represents a species group with unusually high levels of intraspecific variation which may reflect merged phylogeographic variants or retained ancestral polymorphisms.

The mean intraspecific variation was calculated for *M. formosense*, *M. japonicum*, *M. lar* and *M. latidactylus* as these groups were represented by three or more sequences from GenBank (Table 8). The average distance was calculated from these sequences and multiplied by a factor of 10, as described by Hebert et al (2004), which gave a maximum intraspecific limit of 13.9% including *M. latidactylus* from Thailand and 6.3% if excluding the sample. Taking the classification of the Thailand species to be correct, this value may represent a broad, yet conservative, threshold where clusters from the genus *Macrobrachium* exhibiting sequence distances < 13.9% could be regarded as the same species whereas clusters with sequence distances > 13.9% could be flagged for further, more detailed morphological analysis. On the other hand, taking 6.3% to be the threshold would flag the Thailand sample as potentially belonging to another species group while implying that the second largest intraspecific distance group, the North and South variants of *M. asperulum*, are a single species group.

This study highlights certain circumstances where incomplete specimens missing the major periopod, exhibiting particular environmentally, socially or chemically induced growth perturbations (Karplus et al, 2005; Dimmock et al, 2004), may be misidentified with existing species and subsequently go undescribed (Hebert et al, 2004). This work also implies that COI sequence data can be beneficial in terms of complementing morphological data when delimiting cryptic species complexes with particular reference to *Macrobrachium.*

A note on the comparison of *Macrobrachium* sp. nov. with *Macrobrachium grandimanus* (Randall, 1840)

Macrobrachium grandimanus is found in the Ryukyus, the Hawaiian Islands, New Caledonia, Fiji and Taiwan (Short, 2000; Holthuis, 1950). It shares a number of morphologically similar characters to *Macrobrachium* sp. nov. and *M. latidactylus*. The number of rostral teeth, the thin curved fingers on the major periopod including their inward orientation and location of setea within the fingers of the minor periopod are all

shaired phenotypical traits of *M. grandimanus* and *Macrobrachium* sp. nov., nevertheless, it is a different species. A description of *M. grandimanus* is given by Lin (2007) together with a photograph of what appears to be *Macrobrachium* sp. nov. From the photograph alone the distinct curved dactylus of *Macrobrachium* sp. nov. is evident and conflicts with other descriptions of *M. grandimanus* which describes a more prominent tooth on the cutting edges of the dactylus and pollex located both proximally and distally, as well as velvety setea on the inner surface of the palm (Holthuis, 1950; Cai & Jeng, 2001). The

work of Liu et al, 2007 has shown that, via NJ analyses, *M. grandimanus* forms a separate cluster from the single cryptic specimen analysed in the study, using both 16S rRNA and COI sequence data. This study supports Liu's findings with an additional 15 examples of the novel species which form a separate group from *Macrobrachium grandimanus* with COI sequence distances ranging from 20.3 – 21.2%.

As a relevant note, Holthuis (1950) commented on the misidentification of *M. grandimanus* with *M. latidactylus* by Von Martens (1868) while Cai and Jeng (2001) suggested that the records for *M. grandimanus* from various localities were inconsistent and in need of further revision. These works lend further support to the notion that certain species within the genus *Macrobrachium* can be morphologically similar and thus a substantial challenge to classify.

A note on the comparison of *Macrobrachium* sp. nov. with *Palaemon* (*Macrobrachium*) *lampropus* (De Man, 1892)

Palaemon (*Macrobrachium*) *lampropus* (De Man, 1892), an invalid species type mentioned above, exhibits the general shape of the chela of *Macrobrachium* sp. nov. (Fig. 13). Specimens of *P. lampropus* were obtained in Celebes, now known as Sulawesi, Indonesia and were subsequently dismissed as valid species types due to *M. latidactylus* displaying transition states in the second periopod that mirrored those in *P. lampropus* (Holthuis, 1950). The plasticity of this appendage made it an unfavourable character particularly in the absence of other distinguishing characters. In relation to *Macrobrachium* sp. nov., although the general shape of the chela is similar, it is unclear if the two species are the same from the available reference. Attempts to obtain the original

description were unsuccessful due to the age and condition of the original paper. The numbers of teeth visible in the chela of *P. lampropus* appear to be fewer, more spaced out and sub-equal to a greater extent than those in *Macrobrachium* sp. nov. In addition, the tips of the fingers of *M. lampropus* appear less curved. To confirm this invalid species type is different to *Macrobrachium* sp. nov., future studies may require the examination of the original material along with obtaining a partial COI sequence.

Further research

A global COI sequencing strategy encompassing all known species of *Macrobrachium* may yet yield interesting divergences and is likely to further validate the use of COI as a species barcode as a supplementary tool for traditional morphology based taxonomy, particularly for those who do not possess extensive knowledge in the relevant field. It may also facilitate the recognition of cryptic species as well as assisting in the demarcation of existing species boundaries.

PCR amplification was not possible for 12 specimens in this study and may be related to sample quality via inadequate preservation methods and poor handling techniques. Also, the high variability of the COI gene may affect the binding of the primer to the template strand. Further research needs to be initiated to determine the factors behind the failed amplification of the 12 specimens in this study.

Further field trips to Hsiukuluan River have failed to yield more specimens of *Macrobrachium* sp. nov. making it difficult to ascertain the species' habitat, ecology and ethology at the time of its known existence from its initial capture in August 2002. From the similarity between *M. latidactylus* and *Macrobrachium* sp. nov., it is possible that the

two species have undergone a sympatric speciation event in their history, but this has yet to be investigated. More extensive surveys may need to be performed to assess whether this species is still present in Hsiukuluan River or elsewhere in Taiwan.