MATERIALS AND METHODS

Collection of material

In total, 33 specimens of *Macrobrachium* were obtained from a number of field surveys conducted in Taiwan during the period from January 1999 to March 2004, in majority by M. Y. Liu, a former PhD student of Professor C. S. Teng's laboratory in National Tsing Hua University. The specimens were either stored frozen at -20^{o}C or preserved in 75-95% EtOH. The frozen material was subsequently thawed and immediately placed into separate 50ml sterile plastic centrifuge tubes containing 95% EtOH. The alcohol was changed after 1 day and repeated as necessary. All samples were allocated a formal ID from NTHULS1-33. Thirty COI reference sequences were obtained from GenBank (Table 1) consisting of 29 *Macrobrachium* sequences comprising of 13 different species and 1 outgroup (*Caridina pseudodenticulata*).

Morphological analysis

The specimens, where possible, were initially grouped into *M. latidactylus* and *Macrobrachium* sp. nov. by reference to the shape of the second periopod. Individuals that were missing, had sustained damage to, or were thought not to have fully developed the relevant appendage were classified as uncertain.

Specimens were measured using a vernier calliper for a number of morphological characters. Measurements and recordings were taken in millimetres (mm) and consisted of the number of dorsal teeth / number of ventral teeth, total length (TL), measured from

the tip of the rostrum to the tip of the telson; CL measured from the post orbital margin to the distal margin of the carapace; and the RL, measured from the tip of the rostrum to the post-orbital margin, as well as the length of all segments of the major periopod (ischium, merus, carpus, manus, pollex and dactylus). The number of teeth inside the pollex and dactylus were recorded, as a visual assessment of the major periopods indicated their potential as primary distinguishing character. Ratios of the following appendages were also obtained; carapace/rostrum, dactylus/manus, chela/manus, carpus/merus and merus/ischium. Where the second periopod was missing or underdeveloped, specimens were recorded as "uncertain" as a missing or underdeveloped second periopod could have lead to misidentification of both species type and sex, due to the possibility of the specimen being an adult female, juvenile or "male feminise" of another species (Ra'anan et al, 1991, Armand et al, 1987, Holthuis, 1950).

Width and number of teeth of the dactylus/pollex bar graphs were constructed for three data sets from the pollex with 5% standard error bars included. Photographs were taken with a mounted Nikon Coolpix 8700, or in the case of the close up of the rostrum, with a Zeiss Stemi 2000C.

DNA extraction

DNA extraction was performed from abdominal muscle using the Genomic DNA purification kit (Biokit, Taiwan) with 100µL elution solution. 5µL of each extracted sample of DNA was then run with 1µL loading dye on a 2% Agarose gel at 100V for 25mins, stained with Ethidium Bromide (EtBr) for 25 minutes and the gel subsequently viewed with a UVP BIODOC-It™ Transilluminator to confirm the presence of DNA.

Primer design

The primers LCO (GGTCAACAAATCATAAAGATATTGG), (Folmer et al, 1994) and COIfr (CGTCGTGGTATGCCDTTTARWCCTA) (Liu, 2005), were used to generate an 1120bp fragment of the COI gene (Fig. 2). These primers are part of different primer pairs which amplify different sections of the COI gene with an internal overlap of approximately 22 bases. Some samples did not amplify well with the primer pair LCO and COIfr. By using the primer pairs LCO/HCO (TAAACTTCAGGGTGACCAAAAAATCA) and COIfr / COIfc (CCTGCAGGAGGAGGAGACCC), it was noted that the latter pair produced little to no PCR product, thus indicating a lack of specificity of COIfr for the template strand in certain samples. The primer COIfr was modified in Primer Select (DNASTAR, Lasergene) by reference to a 608bp sequence of a putative cryptic species obtained previously by Liu (2006) in the following manner:

- The sequence of putative cryptic species was analyzed with Primer Select and the COIfr primer binding site located
- The bases AGA were added to the 3' end of the primer COIfr based on the reference sequence of the cryptic species sequence
- The modified primer sequence was sent to ProTech for synthesis

This procedure was carried out to increase the specificity of the primer to potential *Macrobrachium* sp. nov. samples sharing sequence similarity to the reference sequence. All successful amplifications involved the forward primer LCO. Conversely, three reverse primers, COIfr, COIfrAGA and COIa were employed in a sequential manner.

PCR amplification and sequencing

Pseudogenes have been shown to complicate PCR-based studies of mitochondrial gene diversity (Bensasson et al, 2001; Thalmann et al, 2004). To minimise the possibility of such amplification, DNA was extracted from mitochondria rich tissue. Primers with high universality (Soroenson and Quinn, 1998) were used to amplify a relatively long PCR product to reduce this risk as it has been indicated that most pseudogenes are relatively short (Pereira and Baker, 2004).

Amplifications were carried out using 2µL DNA, 5µL 10x PCR buffer, 1µL of a 10mM dNTP mix, 5µM of each primer , 1U *Taq* Polymerase (BioKit, Taiwan), and ddH_20 to a final volume of 50µL. *Positive and negative* controls were included in all PCR runs.

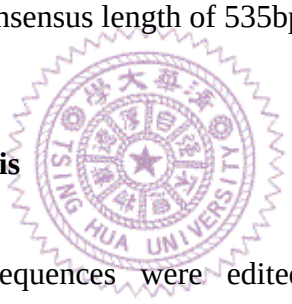
Not all samples could be amplified using the aforementioned primers. From the initial 33 samples, PCR product was obtained for 21 specimens. DNA integrity was thought to be a factor as samples varied in quality in their preserved states. When DNA was confirmed on agarose gels, post-extraction, smearing was visible on the lower section of the gels indicating the presence of fragmented DNA. Template sequence primer binding sites were also thought to be quite variable as combinations of primers failed to generate PCR product in 12 cases.

PCR was performed using the following thermal profile. An initial cycle of denaturation at 94^{o}C for 3 minutes followed by 40 cycles of denaturation for 30 seconds, annealing at 50 ^{o}C for 40 seconds, extension at 72 ^{o}C for 1.5 minutes and a final extension of 72 ^{o}C for 7 minutes. PCR products were verified on 1.5% Agarose gels by staining

with EtBr and purified using a BioKit purification and clean gel extraction kit (BioKit, Taiwan). Double-stranded PCR products were sequenced in both directions by BioKit using the aforementioned primer pairs.

Post sequence editing

The total sequence length for the 21 sequenced samples after trimming was 1120bp while the sequences obtained from GenBank were 608bp. In addition, the 5' end of the 608bp sequences from GenBank resided 73bp outside of the 5' end of the experimental sequences. SeqMan and MegAlign (DNASTAR, Lasergene) were used to edit and align all sequences to consensus length of 535bp.



Sequence alignment and analysis

Forward and reverse sequences were edited using SeqMan (DNASTAR, Lasergene) and aligned using MegAlign (DNASTAR, LaserGene). Analyses of aligned sequences were performed using Mega 3.1 (Kumar et al, 2004). Neighbour-joining analysis (NJ) (Saitou and Nei, 1987) was performed with branch support provided by bootstrap resampling (Felsenstein, 1985), with 1000 replicates, to assess the reliability of the inferred topologies. NJ-trees were rooted using *M. rosenbergii* or *C. pseudodenticulata* for the 1120bp and 535bp sequences respectively. For reference sequences, a total of 29 sequences were obtained from GenBank representing 14 of the 17 species of *Macrobrachium* currently recognised in Taiwan (Shy & Yu, 1998) (Table 1).