RESULTS

COI sequences were obtained for 21 specimens which subsequently underwent morphological and DNA-based analyses.

General morphology

Specimens were separated into two groups, *M. latidactylus* and *Macrobrachium* sp. nov. based on the former possessing a distinct and unique scissor-shaped chela which has been previously used to separate fully developed males from other species (Holthuis, 1950; Jayachandran, 2001; Short, 1980). Measurements of characters commonly assessed in *Macrobrachium* classification were taken from all specimens (Tables 2, 3, 4 and 5). A male specimen from *Macrobrachium* sp. nov. and *M. latidactylus* was selected as a representative type of their species group with their main appendages photographed and described (Fig. 3 and Fig. 4, below).

A number of these samples had missing or broken appendages, which included missing periopods (major and minor), a broken rostrum and numerous examples of fractured antennules. In the case of missing periopods, this confounded preliminary classification for several specimens. Therefore, the focus of the morphological part of this study turned to individuals that displayed typical, well developed second periopods, as the majority of other characters did not serve to differentiate between the two species (Tables 2, 3, 4, 5 and 6). The pollex was designated as a potential distinguishing character due to its general shape which contrasted sharply with that of *M. latidactylus* (Fig. 5 and Fig. 6) . This approach restricted the number of individuals that could be

analysed due to the presence of incomplete material; 3 of the 5 specimens of *M. latidactylus* which had undergone COI sequencing had missing major second periopods. Therefore, it was necessary to include morphological data for 3 additional samples of *M. latidactylus* that were initially excluded from further analysis after PCR failed to generate amplification product.

In total, 5 fully developed males were selected as representatives of each species group and subsequently underwent morphological analysis of the pollex width, height and total number of teeth. The length of the pollex was significantly greater in *Macrobrachium* sp. nov. than in *M. latidactylus* with mean values of 13.6 mm and 10.9 mm respectively (Table 7, Fig. 7a). Conversely, the width of the pollex was significantly shorter in *Macrobrachium* sp. nov. with a mean length of 2.7 mm compared to a 5 mm in *M. latidactylus* (Table 7, Fig. 7b). A greater number of teeth within the chela were recorded in samples of *Macrobrachium* sp. nov. compared to *M. latidactylus* with mean values of 21 and 16 respectively (Table 7, Fig 8).

Morphological diagnosis

Macrobrachium. latidactylus, **Thallwitz, 1891,** Five specimens. (Fig. 3, 4, 5 and 6; Tables 2, 3, 4, 5 and 6)

The rostrum was moderately long, extending up to the apex of scaphocerite. Maximum RL of 9.8 mm with the proximal upper margin straight or slightly concave with the tip occasionally marginally upturned. The proximal upper margin was either straight or slightly concave. The ratio of the CL to the RL ranged from 1.4 - 2.3. Dorsal

teeth totalled 14-16 and were visibly smaller than the ventral teeth, of which 3-4 were post-orbitally located with setose interspaces. The ventral margin possessed 3-4 teeth with setose interspaces. Percentage lengths of the ischium, merus, carpus, dactylus, and manus: 4, 18, 30, 17 and 30 respectively (NTHULS17 – designated fully developed male). The basal pollex was visibly broader than the basal dactylus. The cutting edge had a large incisor tooth proximally followed by poorly defined sub-equally spaced teeth along the length, diminishing in size distally. The minor second periopod had multidenticulate setae forming inwardly directed brushes on cutting edges of fingers.

***Macrobrachium* sp. nov.** Five specimens. (Fig 3, 4 and 5; Tables 2, 3, 4, 5, 6)

The rostrum was moderately long, extending up to the apex of scaphocerite. Maximum RL of 8.0 mm with the proximal upper margin straight or slightly concave with the distal tip occasionally slightly upturned. The ratio of CL to the RL ranged from 1.4–2.4. The general shape and length of the rostrum for the putative *M. latidactylus* and cryptic species were similar between the two groups. Dorsal teeth numbered 13-17 (smaller than ventral) with setose interspaces. Four dorsal teeth were located post-orbitally. The ventral margin possessed 3-4 teeth with setose interspaces. The second periopods were asymmetric and showed sexual dimorphism. Percentage lengths of the ischium, merus, carpus, dactylus, and manus: 5, 18, 27, 25 and 24 respectively (NTHULS1 – designated fully developed male). *Macrobrachium* sp. nov. possessed a narrower base at the proximal end of the pollex than that of *M. latidactylus*. The base of the pollex was slightly broader than the movable finger but was narrower than that of *M. latidactylus*. It contained a large angular incisor tooth located proximally with 8-12 sub-

equally spaced teeth. The dactylus was slender and curved inwards at the distal end with the tip sometimes slightly overreaching the distal end of the pollex. The proximal end exhibited 3 poorly produced crenulations followed by 8-12 well defined, angular, sub-equally spaced teeth diminishing in size distally. When the dactylus was closed, the teeth alignment appeared sub-parallel to the teeth in the pollex. Teeth were also sub-equally spaced, pronounced, angular and rounded, reducing in size distally.

Setae and spinules were located on the medial surface of the manus, merus and carpus as well as the proximal ends of the pollex and dactlyus being noticeably absent on the samples of *M. latidactylus* from Taiwan.

COI Sequence data

A total of 29 sequences from the genus *Macrobrachium* were obtained from GenBank representing 14 of the 17 species of *Macrobrachium* currently found in Taiwan (Shy & Yu, 1998) (Table 1). Of these, 21 COI sequences were obtained at a length of 1120bp. No gaps were detected and when the nucleotide sequence was translated into amino acids no stop codons or indels were detected, thus reducing the likelihood of pseudogene amplification (Williams and Knowlton, 2001). The number of transitions outnumbered transversions by 4.5.

NJ analysis was performed using 1120bp COI sequence data in which two distinct monophyletic groups were formed with pairwise distances between individuals ranging from 0.0 – 17.2% using MegAlign (Lasergene) (Table 8 and Fig. 9). Distances between individuals in the lower cluster contained 5 samples (NTHULS 11, 16, 26, 32 and 33) with pairwise divergence of 0.2 – 0.7% while the remaining 16 samples, forming

the upper group on the NJ tree, exhibited pairwise distances of 0.0 – 0.4%. The average distance between the two clusters was 17%.

A second NJ-tree was constructed from 29 *Macrobrachium* COI reference sequences of 608bp total length, obtained from GenBank to give an overall view of the resolution of species boundaries inferred by COI sequence data (Fig. 10). The groups at the terminal nodes of this NJ analysis were consistent with currently recognized species groups with high bootstrap support. This allowed for the construction of third NJ-tree, using the same reference sequences above, albeit, the sequence was reduced by 73bp in DNA Star (Lasergene) at the 5' end, resulting in a total length of 535bp to match the length of the overlap for the experimentally derived sequences. The resulting NJ-tree (Fig. 11) showed similar topology to the NJ-tree constructed with the 608bp fragments with high bootstrap support for all the monophyletic groups.

A fourth NJ-tree was constructed using the truncated GenBank sequences including those obtained from the experimental material. These sequences were aligned and trimmed to 535bp (Table 9 and Fig. 12). Five of the 21 test sequences formed a monophyletic group with *M. latidactylus.* The remaining 16 individuals formed a monophyletic group, containing 7 haplotypes, of which 3 were separate from other recognized species clusters with the remainder grouping with the group *M. latidactylus*. All monophyletic species groups had high bootstrap support of 99% with interspecific divergence ranging from 15.7 – 24.7%. The GenBank COI sequence of *M. latidactylus* from Thailand exhibited a noticeably deeper intraspecific distance between conspecific individuals from other localities (8.5 - 9.4%) which will be discussed below. Excluding the Thailand sequence of *M. latidactylus*, intraspecific variation ranged from 0.0 – 5.3%.

From the species complex 7 haplotypes were identified of which the largest group H1 contained 14 of the 16 specimens of *Macrobrachium* displaying either a distinctly curved and thin dactylus and / or those which formed a separate cluster away from recognized species groups on the NJ-tree accompanied with low intraspecific divergence values (Table 10.). H2-H5 comprised of the species group which displayed a typical *M. latidactylus* second periopod and / or formed a cluster with individuals of *M. latidactylus* on the NJ-tree with low intraspecific variation. H6 and H7 differed from H1 by 1 base substitution each over the entire 535bp sequence length.

When included in NJ analyses, the outgroup *C. pseudodenticulata* consistently formed a monophyletic clade with high bootstrap support, paraphyletic to *Macrobrachium*, and remained at the basal position of the tree. Maximum Parsimony (MP) analysis on *Macrobrachium* sp. nov. was unsuccessful due to the lack of parsimony informative sites within individuals for the 1120bp sequences, indicating the genetic similarity of the novel species group (data not shown).