Molecular phylogeny and species delimitation of the freshwater prawn *Macrobrachium pilimanus* species group, with descriptions of three new species from Thailand

Warut Siriwut [1] , **Ekgachai Jeratthitikul** [1] , **Somsak Panha** [2] , **Ratmanee Chanabun** [3] , **Chirasak Sutcharit** [Corresp. 2]

[1] Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand

[2] Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

[3] Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Sakhon Nakhon, Thailand

Corresponding Author: Chirasak Sutcharit
Email address: chirasak.s@chula.ac.th

Specific status and species boundaries of several freshwater prawns in the *Macrobrachium pilimanus* species group remain ambiguous, despite the taxonomic re-description of type materials and additional specimens collected to expand the boundaries of some species. In this study, the "*pilimanus*" species group of *Macrobrachium* sensu Johnson (1958) was studied using specimens collected from montane streams of Thailand. Molecular phylogenetic analyses based on sequences of three molecular markers (COI, 16S and 18S rRNA) were performed. The phylogenetic results agreed with morphological identifications, and indicated the presence of at least nine putative taxa. Of these, six morphospecies were recognised as *M. malayanum*, *M. forcipatum*, *M. dienbienphuense*, *M. hirsutimanus*, *M. eriocheirum*, and *M. sirindhorn*. Furthermore, three morphologically and genetically distinct linages were detected, and are described herein as **M. naiyanetri Siriwut sp. nov.**, **M. palmopilosum Siriwut sp. nov.** and **M. puberimanus Siriwut sp. nov.** The taxonomic comparison indicated wide morphological variation in several species and suggested additional diagnostic characters that are suitable for use in species diagnoses, such as the shape and orientation of fingers, the rostrum form, and the presence or absence of velvet pubescence hairs and tuberculated spinulation on each telopodite of the second pereiopods. The "*pilimanus*" species group was portrayed as non-monophyletic in both ML and BI analyses. The genetic structure of different geographical populations in Thailand was detected in some widespread species. The species delimitation based on the four delimitation methods (BIN, ABGD, PTP and GMYC) suggested high genetic diversity of the "*pilimanus*" species group and placed the candidate members much higher than in previous designations based on traditional morphology. This finding suggests that further investigation of morphological and genetic diversity of Southeast Asian freshwater prawns in the genus *Macrobrachium* is still required to provide a comprehensive species list to

guide efforts in conservation and resource management.

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5 Warut Siriwut[1], Ekgachai Jeratthitikul[1], Somsak Panha[2], Ratmanee Chanabun[3] and Chirasak
6 Sutcharit[2]*

8 *[1] Department of Biology, Faculty of Science, Mahidol University, Bangkok,*
9 *Thailand[2]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok,*
10 *Thailand*
11 *[3] Faculty of Agricultural Technology, Sakon Nakhon Rajabhat University, Sakhon Nakon,*
12 *Thailand*Corresponding author*
13 *E-mail: jirasak4@yahoo.com*
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Abstract

Specific status and species boundaries of several freshwater prawns in the *Macrobrachium pilimanus* species group remain ambiguous, despite the taxonomic re-description of type materials and additional specimens collected to expand the boundaries of some species. In this study, the "*pilimanus*" species group of *Macrobrachium* sensu Johnson (1958) was studied using specimens collected from montane streams of Thailand. Molecular phylogenetic analyses based on sequences of three molecular markers (COI, 16S and 18S rRNA) were performed. The phylogenetic results agreed with morphological identifications, and indicated the presence of at least nine putative taxa. Of these, six morphospecies were recognised as *M. malayanum*, *M. forcipatum*, *M. dienbienphuense*, *M. hirsutimanus*, *M. eriocheirum*, and *M. sirindhorn*. Furthermore, three morphologically and genetically distinct linages were detected, and are described herein as **M. naiyanetri Siriwut sp. nov.**, **M. palmopilosum Siriwut sp. nov.** and **M. puberimanus Siriwut sp. nov.** The taxonomic comparison indicated wide morphological variation in several species and suggested additional diagnostic characters that are suitable for use in species diagnoses, such as the shape and orientation of fingers, the rostrum form, and the presence or absence of velvet pubescence hairs and tuberculated spinulation on each telopodite of the second pereiopods. The "*pilimanus*" species group was portrayed as non-monophyletic in both ML and BI analyses. The genetic structure of different geographical populations in Thailand was detected in some widespread species. The species delimitation based on the four delimitation methods (BIN, ABGD, PTP and GMYC) suggested high genetic diversity of the "*pilimanus*" species group and placed the candidate members much higher than in previous designations based on traditional morphology. This finding suggests that further investigation of morphological and genetic diversity of Southeast Asian freshwater prawns in the genus *Macrobrachium* is still required to provide a comprehensive species list to guide efforts in conservation and resource management.

Introduction

Macrobrachium prawns have received particular attention worldwide because of their economic value and their use as model organisms for biogeographical study of evolutionary diversification (de Bruyn et al. 2014). Recently, evidence of high genetic diversity and species richness in some freshwater and terrestrial invertebrates in mainland Southeast Asia was revealed by integrating morphological and molecular systematic methods. Systematic studies of Asian shrimp and prawn species have been increasingly pursued due to evidence of unreported species and underestimation of genetic diversity (Bernardes et al. 2017; de Bruyn & Mather 2007; de Mazancourt et al. 2019; von Rintelen et al. 2007). New native species have been reported from several remote areas throughout both continental and insular Asia (Cai & Ng 2002; Chong 1989; Saengphan et al. 2018; Saengphan et al. 2019; Wowor & Short 2007; Xuân 2012).

In the past, Thai freshwater prawn and shrimp fauna were referred to in some taxonomic revisions among the oriental crustacean fauna (Holthuis 1950; Holthuis 1955; Johnson 1963).Twenty-eight described species of freshwater prawns of the genus *Macrobrachium* Spence Bate, 1868 have been reported in Thailand (Cai et al. 2004; Naiyanetr 2001; Naiyanetr 2007; Saengphan et al. 2018; Saengphan et al. 2019). All *Macrobrachium* species in Thailand are found abundantly within two major riverine systems, namely the Chaophraya and Greater Mekong Basins, as reported by previous taxonomic studies (Cai & Ng 2002; Hanamura et al. 2011). Cai et al. (2004) reported that the *M. pilimanus* species group sensu Johnson (1960) consisted of 12 species: *M. pilimanus* (De Man, 1879), *M. leptodactylus* (De Man, 1892), *M. hirsutimanus* (Tiwari, 1952), *M. dienbienphuense* Dang and Nguyen, 1972, *M. eriocheirum* Dai, 1984 (currently treated as a synonym of *M. dienbienphuense*), *M. ahkowi* Chong and Khoo, 1987, *M. gua* Chong, 1989, *M. forcipatum* Ng, 1995, *M. platycheles* Ou and Yeo, 1995, *M. pilosum* Cai and Dai, 1999, *M. amplimanus* Cai and Dai, 1999, and *M. sirindhorn* Naiyanetr, 2001. Later, five new species were added to this species group: *M. dalatense* Xuan, 2003 from southern Vietnam, three species from Indonesia, namely *M. urayang* Wowor and Short, 2007, *M. kelianense* Wowor and Short, 2007, *M. empulipke* Wowor, 2010 and one troglobitic species, *M. spelaeus* Cai and Vidthayanon, 2016 from Thailand. The diagnostic characters for this group were critically debated due to complicated morphological variation. However, several species exhibit compatible patterns by having a short blade-like rostrum, cupped or slightly elongated carpus, swollen merus of the second pereiopods, and the presence of velvet setae on the telopodites of the second pereiopods (Cai et al. 2004; Chong 1989; Holthuis 1979; Johnson 1960; Ng 1994).

Several species in the "*pilimanus*" species group exhibit widespread distribution, such as *M. dienbienphuense*, *M. amplimanus*, *M. hirsutimanus* and *M. forcipatum*. In contrast, there are also some species reported to be endemic and limited to a narrow territory, including *M. sirindhorn* and *M. spelaeus,* which are restricted to areas in northern Thailand (Cai and Vidthayanon 2016). Detailed information on the distribution range and type locality of all nominal taxa in "*pilimanus*" species group has been provided in Table S1. The limitation of using traditional taxonomic characters for species identification in the "*pilimanus*" group has been acknowledged, as several species exhibit similar morphological patterns and have few diagnostic characters (Holthuis 1950; Johnson 1960; Johnson 1963; Ou & Yeo 1995; Yeo 1999). The diagnosis of nominal taxa has usually been based on a combination of quantitative and qualitative characters such as the proportion of rostrum, podomeres of second pereiopods and the presence and absence of pubescence on fingers, palm and merus of second pereiopods. Because

118 of high morphological variation, the species diversity of *M. pilimanus* group has been debated
119 (Cai et al. 2004; Cai & Liang 1999; Hanamura et al. 2011; Holthuis 1952; Johnson 1960; Li
120 2007; Wowor 2010; Wowor & Short 2007). Previously, the phylogenetic position refered by
121 some *M. pilimanus* members also indicated the unclear relationship between congeneric species
122 in genus *Macrobrachium* such as *M. niphanae*, *M. yui* and *M. neglectum* (Liu et al. 2007; Wowor
123 et al. 2009).
124
125 Several taxonomic identifications of prawns in genus *Macrobrachium* were based on the
126 combination of traditional morphology. The re-examination of type specimens and additional
127 museum collections has been done in some *Macrobrachium* species (Cai et al. 2004; Cai &
128 Shokita 2006; Holthuis 1952). The comprehensive distribution and taxonomic status of several
129 species are questionable due to limited material available from different geographical areas and
130 their scattered distribution ranges (Cai & Ng 2002; Hanamura et al. 2011; Johnson 1963).
131 Although Thailand is located in the center of mainland Southeast-Asia, its freshwater fauna is
132 likely under-reported, including *Macrobrachium* prawns in both major river basins. The lack of
133 broad-scale specimen comparison and comprehensive data on geographical variation and genetic
134 composition are of critical concern, given the obscure justification for their taxonomic
135 boundaries (Castelin et al. 2017; Chen et al. 2015; Rossi & Mantelatto 2013). As a result,
136 classification and assignment of *Macrobrachium* species into a suitable species complex or
137 species groups has generally been problematic (Johnson 1960; Wowor & Ng 2007; Wowor &
138 Short 2007).
139 Molecular systematics based on DNA barcoding regions and species delimitation coupled
140 with DNA sequence variation has been widely used to screen for putative species identification
141 in some highly diversified decapod groups (Bernardes et al. 2017; de Mazancourt et al. 2019;
142 Venera-Pontón et al. 2020). In this study, we integrate traditional taxonomic examination and
143 molecular phylogeny using three molecular markers to delimit species boundaries and to
144 illustrate the phylogenetic relationships within the "*pilimanus*" species group collected from
145 Thailand, with further discussion of their distribution and phylogenetic position among mainland
146 Southeast Asian species.
147

148 ## Methodology
149
150 ### Field collecting and specimen preparation
151
152 Prawn specimens were collected from riverine systems throughout Thailand. Field
153 surveys were conducted to collect fresh specimens in some protected areas with permission from
154 the Department of National Parks, Wildlife and Plant Conservation, Thailand (DNP
155 0907.4/14262). Some species previously described with the type locality in Thailand were re-
156 collected and used as additional topotype material for species identity in morphological and
157 molecular examinations. The live habitus specimens were photographed in order to document
158 body colouration, and then euthanised by the two-step method following AVMA Guidelines for
159 the Euthanasia of Animals (AVMA 2013) before fixing in 95% ethanol for long-term
160 preservation. Animal use in this study strictly followed the protocols approved by Chulalongkorn
161 University (Protocol Review No. 1723018) and Mahidol University-Institute Animal Care and
162 Use Committee (MU-IACUC) under approval number MU-IACUC 2018/004.

163
164   Collected prawn specimens were registered and housed at Chulalongkorn University
165 Museum of Zoology, Bangkok, Thailand (CUMZ), and Mahidol University, Natural History
166 Museum (MUNHM). Species identifications were made by comparison with previous taxonomic
167 records of *Macrobrachium* prawns from Thailand and surrounding countries. Morphological
168 characteristics of each species were observed by using stereo-microscope. Traditional and
169 diagnostic characters for species identification were photographed with Cell'D imaging system.
170 In addition, the fine detail of some morphological characters were illustrated by free-hand
171 drawings to document their variation. For morphological variation analysis, constant characters
172 were selected for study using classical landmark-based geometric morphometrics. The protocols
173 used in this study followed Siriwut et al. (2015).
174
175   Species descriptions and technical terms used herein are based on previous taxonomic
176 studies of Southeast Asian *Macrobrachium* species (Cai & Dai 1999; Cai et al. 2004; Cai & Ng
177 2002; Hanamura et al. 2011; Holthuis 1950; Wowor & Short 2007; Xuân 2012). Abbreviations
178 for terms used in the comparison table are as follows: **Fin.**, fingers; **Pal.**, palm; **Carp.**, carpus;
179 **Mer.**, merus; **Dt.**, teeth on dactylus; **Pt.**, teeth on pollex. The rostrum teeth formula is the total
180 number of dorsal teeth/total number of ventral teeth. Total body length (**tl**) used in the species
181 description was measured from the end of the telson to the tip of the rostrum. Carapace length
182 (**cl**) was measured from the dorso-posterior margin of the carapace to the end of the post-
183 antennular margin of the carapace. Rostrum length (**rl**) was measured from the tip of the rostrum
184 to the posterior-most rostrum tooth. All characters are reported in millimeters.
185

186 ## Nomenclatural acts
187   The electronic version of this article in portable document format (PDF) will represent a
188 published work according to the International Commission on Zoological Nomenclature (ICZN),
189 and hence the new names contained in the electronic version are effectively published under that
190 Code from the electronic edition alone. This published work and the nomenclatural acts it
191 contains have been registered in ZooBank, the online registration system for the ICZN. The
192 ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed
193 through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The
194 LSID for this publication is: urn:lsid:zoobank.org:pub:F94C18CF-8E07-4D4B-94ED-
195 4153854B237E. The online version of this work is archived and available from the following
196 digital repositories: PeerJ, PubMed Central and CLOCKSS.
197

198 ## DNA extraction and PCR
199
200   All prawn samples used for molecular analysis in this study are listed in Table 1. Prawn
201 tissueThe genomic DNA was extracted from abdominal muscle tissue by using Commercial
202 DNA extraction kits (NucleoSpin Tissue kit; MACHEREY-NAGEL). The concentration of total
203 genomic DNA was measured and visualised by gel electrophoresis. Three standard molecular
204 loci for *Macrobrachium* were selected for phylogenetic study, including the barcode regions of
205 mitochondrial cytochrome c oxidase subunit I (COI), 16S rRNA (16S), and nuclear 18S rRNA
206 (18S). The criteria for DNA marker selection were 1) sequences of closely related taxa for
207 sequence comparison are available in a public database such as GenBank and BOLD ((Liu et al.
208 2007; Wowor et al. 2009), 2) marker is commonly used for phylogenetic tree reconstruction of

209 genus *Macrobrachium* (Rossi et al. 2020; Saengphan et al. 2018; Saengphan et al. 2019) and 3) a
210 sufficient amount of variation, conserved and parsimony informative sites for multi-locus
211 phylogenetic study (Liu et al. 2017; Matzen da Silva et al. 2011; Pileggi & Mantelatto 2010).
212 The PCR primers used in amplification and sequencing are presented in Table 2. PCR reactions
213 were incubated using T100™ thermal cycler (BIO-RAD) with gradient temperature function.
214 The components of the PCR mixture followed Siriwut et al. (2015). Reaction conditions for each
215 molecular locus were based on previous phylogenetic studies of shrimp and prawns (Pileggi &
216 Mantelatto (2010); Rossi & Mantelatto (2013); von Rintelen et al. (2007); Wowor et al. (2009).
217 Successfully amplified PCR products were checked by using fluorescence-enhanced agarose gel
218 electrophoresis.
219   The PEG precipitation method was used to purify the PCR products. The purified PCR
220 products were sequenced at Bioneer Inc. (Korea). Raw sequences were aligned with libraries in
221 GenBank using the BLASTn algorithm to verify the organism's identity. Sequence configuration
222 was done in Sequence Navigator (Parker 1997). Sequence annotation and trimming were carried
223 out in MEGA 7 (Kumar et al. 2016) using MUSCLE (Edgar 2004). Sequence format was
224 constructed using MEGA 7 and Mesquite (Maddison & Maddison 2017). All newly obtained
225 nucleotide sequences were deposited in the GenBank database under GenBank submission
226 numbers MT235929-MT235968 for COI, MT248221-MT248260 for 16S, and MT248181-
227 MT248220 for 18S (in Table 1).
228
229 **Phylogenetic reconstruction and species delimitation**
230
231   For our phylogenetic study, the dataset of each partial gene was compiled from the newly
232 amplified sequences from fresh material and available sequences from public databases (NCBI
233 and BOLD). The number of sequences used per marker are as follows: 57 sequences for COI, 79
234 sequences for 16S, and 53 sequences for 18S. For the concatenated dataset, the number of
235 sequences used for each marker was optimized in order to average individual sequence length of
236 sample. Samples from public databases were included in the concatenated dataset when at least
237 two of the three marker sequences were available. In total, 54 sequenced samples were used in
238 phylogenetic tree reconstruction based on the concatenated dataset. A list of outgroups and other
239 *Macrobrachium* taxa in this study is provided in Table S2.
240   Maximum likelihood (ML) and Bayesian inference (BI) methods were applied to
241 reconstruct phylogenetic trees. Thehe concatenated dataset of three genetic markers with the
242 partitioned file for nucleotide substitution model fit was prepared using Kakusan 4 (Tanabe
243 2007). The alternative substitution model for phylogenetic tree reconstruction was tested by
244 using JModelTest v.1.7 (Posada 2008). For ML analysis, RAxML 8.0.0v (Stamatakis 2006) with
245 default parameter set was used to reconstruct phylogenetic tree. The ML tree topology was
246 confidential tested under 1,000 bootstrap replicates. Bayesian inference tree was sampled in
247 MrBayes, ver. 3.2.6. (Ronquist et al. 2012). Markov chain Monte Carlo (MCMC) chains were
248 configured to run for 10 million generations, and trees were saved each 500 generations. Twenty-
249 five percent of tree samples were discarded under burn-in fragment parameter settings. The
250 consensus tree was generated from a 50% majority rule. The annotation and illustration of clade
251 and branch length were configured by FigTree (Rambaut 2009). Node creditable values,
252 Bootstrap (ML) and posterior probablility (BI), are labelled on the clade based on the acceptance
253 criteria as follow: bootstrap values exceed 70% (Larget & Simon 1999)and posterior
254 probabilities exceed 0.95 (Huelsenbeck & Hillis 1993). A p-distance method was used to

255 calculate the genetic distance of all gene fragments in MEGA 7.. The nMDS plot of pairwise
256 sequnce results was constructed for COI and 16S by using PAST program (Hammer et al. 2001).
257   Species delimitation was performed using four standardised methods for automatic
258 species delimitation to detect the Molecular Operational Taxonomic Units (MOTUs): automated
259 barcode gap (ABGD by Puillandre et al. (2012)), Bayesian implementation of Poisson Tree
260 Processes model (bPTP by Zhang et al. (2013)), the multi-rate Poisson Tree Processes (mPTP by
261 Kapli et al. (2017)) and the Generalized Mixed Yule Coalescent model (GMYC by Pons et al.
262 (2006)). Each gene dataset was tested separately as a single partition. For the COI dataset, the
263 sequence analysis function in BOLD including BIN clustering was implemented to designate the
264 possible putative species in sequence dataset. For the ABGD method, the intra-specific variation
265 obtained from each molecular marker dataset was calculated in MEGA7 and the optimised
266 barcode relative gap was calculated using the ABGD online server
267 (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). The PTP analysis was conducted
268 under the Maximum likelihood algorithm using a web server (https://species.h-its.org/gmyc/;by
269 Zhang et al. (2013)). The best-scoring tree dataset was estimated under 95% confidence of
270 statistical probability. In the GMYC method, the starting tree was randomly sampled and
271 manually calculated under a suitable model for the construction of an ultra-metric tree using
272 BEAST package v1.10.4 (Drummond & Rambaut 2007; Suchard et al. 2018) or implemented in
273 CIPRES (Miller et al. 2010). The maximum clade credibility tree from each gene analysis was
274 summarised in TreeAnnotator v1.10.4 and was analysed under the GMYC species delimitation
275 approach using an online server. The results of automatic delimitation methods were compared
276 1) with the morphological identification of genus *Macrobrachium* species based on their original
277 descriptions and with recent taxonomic reviews of nominal taxa to match each clade under
278 biological species and 2) with molecular phylogenetic partional analysis based on the three
279 concatenated gene datasets.
280

281 ## Results

282

283 **Phylogenetic relationship and species delimitation of Thai "*pilimanus*" species**
284 **group**

285

286   Thirty-nine sequences from three partial genes were successfully amplified and
287 comparatively aligned. The sampling locality of each species is illustrated in Figure 1. The
288 annotation of each partial gene sequence is described in Table 3. The genetic distance of each
289 mitochondrial DNA dataset (COI and 16S) and nuclear 18S dataset was calculated with 1,000
290 bootstrap replicates. The estimates of inter- and intra-specific variation of all representative taxa,
291 are listed together with standard deviation in Table S3. Interspecific variation between members
292 of the "*pilimanus*" species group found in Thailand was 9.8-23.3% for COI, 2.3-7.7% for 16S
293 and 0.2-11% for 18S. Intraspecific variation was 0.45-8.36% for COI, 0-3.5% for 16S and 0-
294 2.1% for 18S. Non-metric multidimensional scaling (nMDS) plots representing pairwise
295 comparison of COI and 16S sequences used for single gene analysis (including sequences from
296 NCBI and BOLD) were generated (see Fig. S1 and table S4-5).
297

298   The phylogenetic tree based on the concatenated dataset of three partial genes indicated
299   the non-monophyletic relationship of genus *Macrobrachium* because two outgroups
300   (*Coralliocaris superba* and *Exopalaemon styliferus*) were nested inside and represented
301   polytomy (clade A in Figure 2). This result was also found in single-locus phylogenetic analyses
302   (see Fig. S2-3). After being rooted by outgroups and additional "*pilimanus*" members (*M.*
303   *pilimanus* and *M. urayang*), most "*pilimanus*" members except an OUT of *M. urayang* from
304   Indonesia showed a monophyletic relationship and nested with *M. niphanae*, with values
305   showing support in both BI and ML analyses (clade B). The monophyletic relationship of most
306   "*pilimanus*" members was indicated in clade C, and they were separated into two linages. The
307   clade D linage comprised six species: *M. malayanum*, *M. naiyanetri* sp. nov., *M. forcipatum*, *M.*
308   *sirindhorn*, *M. pilimanus*, and *M. palmopilosum* sp. nov. The monophyly of *M. malayanum* was
309   detected and it was positioned as a basal clade to other congeneric species within this linage. The
310   phylogenetic tree also indicated the nesting of *M. sirindhorn* with two other species, namely *M.*
311   *pilimanus* and *M. palmopilosum* sp. nov., although this clade was not supported by statistical
312   tests. In clade F, specimens of *M. forcipatum*, *M. naiyanetri* sp. nov. and one sample referred to
313   as *M.* aff. *pilimanus* formed a monophyletic group, with statistical support from both ML and BI
314   analyses. *Macrobrachium naiyanetri* sp. nov. and *M.* aff. *pilimanus* formed a monophyletic
315   group, while *M. forcipatum* was placed at the base of the clade. The monophyly of *M. naiyanetri*
316   sp. nov. was further separated into two distinct geographical clades: a clade including samples
317   from the southern peninsulaof Thailand plus *M.* aff. *pilimanus* from Khammouane, Laos, and a
318   second clade of two samples from eastern Thailand.
319

320   In clade E, *Macrobrachium hirsutimanus*, *M. eriocheirum*, *M. dienbienphuense* and *M.*
321   *puberimanus* sp. nov. were nested as a monophyletic group with statistical support in both ML
322   and BI. Within this clade, the phylogenetic positions of *M. hirsutimanus* and *M. eriocheirum*
323   were uncertain due to low support of clade composition; however, the monophyletic relationship
324   of representative OTUs was indicated consistently in ML and BI for both taxa. Clade G included
325   two species with similar morphology, *M. dienbienphuense* and *M. puberimanus* sp. nov.; the
326   monophyly of each species is questionable due to two sequences of *M. dienbienphuense* from the
327   public database nested with *M. puberimanus* sp. nov. In the major clade of *M. dienbienphuense*,
328   two genetically distinct subclades were found with statistical support.
329

330   Species delimitation of each partial sequence dataset indicated a different number of
331   candidate taxa, and there was also variation by calculation approach (Figure 2). The BIN
332   clustering method in BOLD indicated 29 putative species for the COI dataset. The ABGD
333   method indicated 19 species in COI, 19 species in 16S and 9 species in 18S. In the Bayesian
334   Poisson Tree Process (bPTP), the clustering result indicated 20 species in COI, 19 species in 16S
335   and 15 species in 18S. The multi-rate Poisson Tree Process (mPTP) indicated 14 species in COI,
336   2 species in 16S and 1 species in 18S. In the GMYC analysis, the clustering method indicated 18
337   species in COI, 21 species in 16S and 3 species in 18S, based on the ultrametric tree. The
338   separation evidence (red box) detected eight taxa while lumping evidence (blue box) was found
339   mainly in the clade of *M. puberimanus* sp. nov. and from two samples of *M. dienbienphuense*
340   from the public database.
341

342   Systematic diversity of the "*pilimanus*" species group in Thailand

343

344 In this study, field collection and taxonomic identification of Thai *Macrobrachium* indicated
345 nine morphological species, three of which are totally distinct from the others by both
346 morphology and molecular delimitation. Six described species, namely *M. hirsutimanus*, *M.*
347 *eriocheirum*, *M. dienbienphuense*, *M. forcipatum*, *M. malayanum* and *M. sirindhorn* were re-
348 confirmed with previous taxonomic studies. The distribution of these six species mainly included
349 montane tributary streams, while some species such as *M. dienbienphuense* also occupied larger
350 rivers. The geographical distribution of "*pilimanus*" members is illustrated in Figure 1. Based on
351 this study and previous taxonomic records of *Macrobrachium* prawns in the "*pilimanus*" group,
352 Thailand hosts eleven species. However, only the three new species found in this study will be
353 described here, along with their phylogenetic placement, genetic relationship and geographical
354 distribution.
355
356 Taxon names declaration: The proposed three new species herein are attributed to Warut Siriwut;
357 thus, the authorship of these new taxon names should be cited as **M. naiyanetri Siriwut** in
358 Siriwut et al., 2020, **M. palmopilosum Siriwut** in Siriwut et al., 2020 and **M. puberimanus**
359 **Siriwut** in Siriwut et al., 2020.
360

361 ## Taxonomic account
362 Palaemonidae Rafinesque, 1815
363 *Macrobrachium* Spence Bate, 1868
364
365 ***Macrobrachium naiyanetri* Siriwut sp. nov.**
366 **ZooBank ID:** urn:lsid:zoobank.org:act:22EBCA17-2E29-4193-9D9E-87CABCD65D7D
367 **Figures** 3A and 4
368
369 **Type locality.** A large and shallow stream with large gravels at Hui Prik, Cha-wang District,
370 Nakhon Si Thammarat Province, Thailand.
371
372 **Type examined. Holotype:** CUMZ MP00001, one male spm. from Hui Prik, Cha-wang District,
373 Nakhon Si Thammarat Province (M128 in molecular analysis). **Paratype:** CUMZ MP00002,
374 four male spms from the same locality as holotype (M127, M154 and M155). CUMZ MP00003,
375 nineteen male and nine female spms from the same locality as holotype.
376
377 **Additional material.** CUMZ MP00004, two male spms from Khao Banchob Waterfall, Makham
378 District, Chanthaburi Province (M102). CUMZ MP00005, one male spm. from Klong
379 Rattaphum, Rattaphum District, Songkhla Province (M134). CUMZ MP00006, twenty-six male
380 and nine ovigerous female spms from Klong Krabiead, Hui Prik, Cha-wang District, Nakhon Si
381 Thammarat Province.
382
383 **Diagnosis.** Rostrum short and striate distally, not reach beyond the end of second telopodite of
384 antennular peduncle. Rostral formula: 8-14/ 2-4 teeth. Small spinulation presents on anterolateral
385 margin of carapace. Epistome trilobed. Second pereiopods slightly longer than body lenght,
386 similar in shape, unequal in size. Second pereiopods with long setae,present on finger, palm,
387 anterior inner part of carpus and merus. 10-18 teeth on figers. Carpus elongated or slightly
388 cupped, shorter than fingers, palm and merus. All telopodites of second pereiopods covered with
389 spinules. Thoracic sternites; T4 with postero submedial plate; T5 with transverse plate with

390   median process. Second and third abdominal sternites with moderate triangular median process.
391   Preanal carina present. Telson slightly short and stouth, surface glabrously, with long plumose
392   seta and posterior projection with two long inner and two short outer spines. Uropods glabrous;
393   uropodal diaeresis with inner moveable spine, equal to outer angle. Developed eggs large,
394   approximate diameter 0.7 mm, ovoid.

396   **Composite description** (type specimens in parentheses). A medium-sized *Macrobrachium*
397   species, tl 30.6-54.2 mm (41.5 mm in holotype), with pale or brownish body colouration (Figs
398   3A).

400   *Rostrum* (Fig. 5C, D). Anteriorly striate and angled downward distally, rl 7.3-11.4 mm (10.8 mm
401   in holotype) cl 6.7-13.0 mm (13.0 mm in holotype), and reaching not beyond the end of
402   antennular peduncle. Dorsal part of rostrum with 8-14 (14 in holotype) teeth in total, 2-7 (6 in
403   holotype) teeth present in postorbital area. Area with postorbital teeth covering nearly half of
404   carapace length. Ventral part of rostrum with 2-4 (3) teeth, located about half-way distally.

406   *Cephalon*. Eye well developed. Ocular beak moderately developed, without laterally expanded
407   tip. Postantennular carapace margin rounded. Cornea osculum longer than stalk. Antennular
408   peduncle longer than wide, lateral carina well developed, dorsal carina without sinuous. Antero-
409   lateral part of carapace with antennal (one side without antennal spine in holotype). Small
410   hepatic spines present lower than orbital angle; located behind ; branchiostegal suture present
411   starting from hepatic spine to carapace margin. Spinulation present on ventro-lateral part of
412   carapace (Fig. 5C). Epistome trilobed. Scaphocerite with margin concave laterally, distolateral
413   tooth minutes and not reaching the end of lamella. Third maxilliped not reaching beyond
414   antennal peduncle.

416   *First pereiopods*. Long and slender, reaching beyond the end of scaphocerite. Fingers about as
417   long as palm; carpus longer than merus. Carpus, merus and ischium covered with small spinules.
418   Scattered setae present on all segments but dense on finger and ischium.

420   *Second pereiopods*. Robust and longer than body length, similar in both shape and form; carpus
421   of both major and minor second pereiopods extending beyond the end of scaphocerite.

423   *Major second pereiopod* (Fig. 5E, G). Spinulation present on all segments except fingers and
424   palm. Fingers, palm, inner margins of carpus covered by fine setae. Dense, fine setae present on
425   proximal part of finger. Merus with setae in some specimens. Fingers slender and longer than
426   palm (17.6: 11.1 mm), finger bending with gap and tips crossed when closed in males. Dactylus
427   with 10-18 (15) prominent teeth, basal teeth larger than distal teeth, pollex with 10-18 (12) teeth
428   (Fig. 5F). Teeth sub-equally distributed and concealed by long velvety setae, without oblique
429   carina distally. Upper and lower margins of palm slightly expanded. Carpus elongated, shorter
430   than merus (7.6: 11.8 mm in holotype). Merus equal to palm (11.8 mm in holotype). Ischium
431   tapered, shorter than merus.

433   *Minor second pereiopod* (Fig. 5H). Similar in form but shorter than major cheliped, spinulation
434   present on all segments except fingers and palm. Fine setae densely covering proximal part of
435   fingers and palm. Dactylus with 6-18 small teeth, pollex with 8-15 small teeth. Teeth sub-equally

436 distributed, only half of finger length, concealed by long, fine setae. Oblique carina present on
437 distal part, about one-third of finger length. Carpus elongated, shorter than merus. Merus
438 subcylindrical and equal to palm. Ischium tapered, shorter than merus.
439
440 *Third pereiopods* (Fig. 5I). Long and slender, propodus extending to the end of scaphocerite.
441 Small spinulation present on all segments except ischium. A fine seta present on all segments.
442 Dactylus short (2.1 mm in holotype) and curved, with dorsolateral setae; ventral carina well
443 developed. Propodus long (4.6 mm in holotype), with 6-8 (7) ventral pairs of spines distributed
444 along length of propodus; carpus shorter than propodus (3.1 mm in holotype), with dorsal
445 projection on distal part. Merus longer than carpus (5.6 mm in holotype). Ischium shorter than
446 merus and carpus (2.8 mm in holotype).
447
448 *Fourth and fifth pereiopods*. Dactylus extending to the end of scaphocerite. Spinulation present
449 on all segments except ischium. Scattered fine setae present on all segments. Propodus with 5-7
450 pairs of ventral spines distributed along its length, 2 corner spines with grouped setae on distal
451 part. Carpus shorter than propodus and merus, with dorsal projection on distal part. Ischium
452 shorter than merus and carpus.
453
454 *Thoracic sternum*. T4 without median process. T5 with transverse plate without median process.
455 T8 with posteromedial lobes in males.
456
457 *Abdomen*. Usually smooth, with tiny spinules on pleural margins of first and second abdominal
458 segments. All abdominal sternites with transverse ridge. Second and third abdominal sternites
459 with moderate triangular median process, subsequent segment without process. The sixth sternite
460 with median obtuse process. Preanal carina present, with group of small setae at tip in males.
461
462 *Telson* (Fig. 5B). slightly short and stouth (5.9 mm in holotype), lateral margins straight. Cluster
463 of setae present on antero-median part. Dorsal surface with 2 pairs of dorsal spines. Projection
464 present on posterior margin, with two spines and plumose setae on each side, inner pair of
465 posterior spines longer than outer spines.
466
467 *Uropods* (Fig. 5B). Uropodal diaeresis with inner moveable spine, equal to outer angle. Exopod
468 longer than broad (5.5: 2.5 mm in holotype) and not reaching the end of endopods.
469
470 **Etymology.** The specific name *naiyanetri* is given in honor of Professor Phaibul Naiyanetr from
471 Chulalongkorn University for his extensive contributions to the knowledge of crustacean fauna
472 in Thailand.
473
474 **Size.** Males slightly larger than females; the largest male recorded being 54.2 mm tl, 13.0 mm cl;
475 the largest female 39.8 mm tl, 9.5 mm cl and egg size is 0.7 mm in diameter.
476
477 **Distribution.** Most populations are restricted to the southern part of Thailand; however, one
478 specimen collected from Chantaburi Province extends its recorded distribution range to include
479 the eastern part of Thailand.
480

481 **Remarks.** *Macrobrachium naiyanetri* sp. nov. resembles other members of the "*pilimanus*"
482 species group by having densely tufted setae on second pereiopods. The phylogenetic tree
483 suggests the position of this new species as nesting with *M. forcipatum*. However, the
484 distinguishing characteristics of *M. naiyanetri* sp. nov. used to separate it from the other
485 congener species in southern Thailand (e.g. *M. forcipatum*, *M. malayanum* and *M. hirsutimanus*)
486 are the carpus of the second major pereiopods that exhibit a slight cup-shape, the presence of
487 dense stiff setae on the antero-inferior part of merus, and fingers of the second pereiopods being
488 longer than palms. Moreover, the postorbital area contains more rostrum teeth (4-7 vs. 3-5 in *M.*
489 *forcipatum*; 3-4 in *M. malayanum*; 3-5 in *M. hirsutimanus*). The adult size of *M. naiyanetri* sp.
490 nov. is significantly larger and longer than the others (tl). The dactylus contains 12-13 prominent
491 teeth (vs. 13-14 in *M. forcipatum*; 4-6 in *M. malayanum*; 15 in *M. hirsutimanus*). The size of
492 major and minor second pereiopods is distinctly large in male specimens (vs. not distinct in other
493 species). The carpus of the second pereiopod is slightly cupped (vs. cupped and stout in other
494 species). The major second pereiopod in males is as long as tl. In addition, the species
495 delimitation methods suggest two distinct evolutionary lineages of *M. naiyanetri* sp. nov.
496 samples; the first lineage is composed of specimens from the western part of Khao Luang Range,
497 whereas the second lineage contains two samples from the eastern part of Khao Luang Range
498 (Songkhla Province) and from Chantaburi Province in eastern Thailand. Further investigation of
499 population structure between these two distinct lineages is necessary to test whether or not this is
500 the result of allopatric speciation.
501
502 *Macrobrachium palmopilosum* Siriwut sp. nov.
503 **ZooBank ID:** urn:lsid:zoobank.org:act:8065628A-4EDF-49EF-BA5D-91588F53D284
504 **Figures** 4B and 6
505
506 **Type locality.** A small and shallow stream with sand and gravel at Tat Man Waterfalls, Puea
507 Sub-district, Chiang Klang District, Nan Province, Thailand.
508
509 **Type examined. Holotype:** CUMZ MP00007, one male spm. from Tat Man Waterfalls, Puea
510 Sub-district, Chiang Klang District, Nan Province (M031). **Paratype:** CUMZ MP00008, twenty-
511 one male and twenty-seven female spms from the same locality as holotype.
512
513 **Additional material.** CUMZ MP00009, six male and two female spms from Sob-Pue, Sa-Iap
514 Sub-district, Song District, Phrae Province (M030). CUMZ MP00010, twelve male and ten
515 female spms from Mae Mang, Bo Kluea District, Nan Province (M011). CUMZ MP00011, one
516 male spm. from Ban Pha Lak, Mueang District, Nan Province.
517
518 **Diagnosis.** Rostrum short, anteriorly striate and upward distally, not reaching to the end of
519 second telopodite of antennular peduncle. Rostral formula: 10-12/ 2-3 teeth. Anterolateral
520 margin of carapace with small spines. Epistome bilobed. The robust pair of second pereiopod
521 longer than body length similar in shape, unequal in size. Densed andtufted setae present on both
522 side of second pereiopods. Anterior part of carpus with setae. Fingers with 10-12 teeth. Carpus
523 stoutand cupped, shorter than fingers, palm and merus. oSmall spinule present in posteriorpart of
524 palm, entirely in carpus and merus. Thoracic sternites: T4 with posterior submedial plate; T5-T7
525 with transverse plate without median process; T8 with contiguous posteromedially anterior lobes,
526 without median process. First to third abdominal sternites with moderate triangular median

527 process. Preanal carina present. Telson moderately long, with scaterred plumose setae on dorsal
528 surface. Two pairs of spines present. Posterior projection present with two long inner and short
529 outer spines. Uropodal diaeresis spine shorter than outer angle. Egg size 1.3 mm in diameter.
530
531 **Composite description** (type specimens in parentheses). A medium-sized *Macrobrachium*
532 species, tl 25.6-77.8 mm (57.3 mm in holotype), with pale or greenish-brown body colouration
533 (Figs 4B).
534
535 *Rostrum* (Fig. 6C, D). Anteriorly striate and turned upward distally, rl 4.1-16.7 mm (11.7 in
536 holotype) cl 5.9-20.4 mm (16.5 mm in holotype), and reaching not beyond the end of second
537 segment of antennular peduncle. Dorsal part of rostrum with 10-12 (12 in holotype) teeth in total,
538 4-6 (5) teeth present in postorbital area. Area with postorbital teeth covers one-third of carapace
539 length. Ventral part of rostrum with 2-3 (3) teeth, located about half-way to distal end.
540
541 *Cephalon*. Eye well developed. Ocular beak moderately developed, without laterally expanded
542 tip. Postantennular carapace margin rounded. Cornea osculum shorter than stalk. Antennular
543 peduncle longer than wide, lateral carina well developed, dorsal carina without sinuous. Antero-
544 lateral part of carapace with antennal spine. Small hepatic spines present lower than orbital
545 angle and antennal spine. Branchiostegal suture starting from hepatic spine to carapace margin.
546 A few scaterred spinules present on ventro-lateral part of carapace and branchiostegal regions of
547 carapace (Fig. 6C). Ocular beak moderately developed, without laterally expanded tip. Epistome
548 slightly bilobed. Scaphocerite with margin concave laterally, distolateral tooth minutes and not
549 reaching the end of lamella. Third maxilliped not reaching beyond antennal peduncle..
550
551
552 *First pereiopods*. Long and slender, reaching beyond the end of scaphocerite. Fingers about as
553 long as palm; carpus as long as merus. Small spinules present only on merus and ischium.
554 Scattered setae present on all segments but dense area on distal part of finger and on entire
555 ischium. The proximal part between palm and carpus with group of small setae.
556
557 *Second pereiopods*. Robust and longer than body length, similar in form; carpus of both major
558 and minor second pereiopods extending beyond the end of scaphocerite.
559
560 *Major second pereiopod* (Fig. 6E, G). Spinulation present in all segments except fingers and
561 anterior part of palm. Fingers, palm, inner margins of carpus covered by tufted setae. Merus
562 without setae. Fingers subcylindrical, shorter than palm in length (13.8: 15.9 mm.), closed
563 fingers with gap and crossing distally. Dactylus with 10-12 (10) prominent teeth, basal teeth
564 smaller than middle teeth, pollex with 10-11 (11) teeth (Fig. 5F). Teeth sub-equally distributed
565 and concealed by long tufted setae, without oblique carina distally. Upper and lower margins of
566 palm slightly expanded. Carpus cup-shaped, shorter than merus (7.1: 13.9 mm). Merus slightly
567 shorter than palm (13.9: 15.9 mm), stout and inflated laterally. Ischium tapered, shorter than
568 merus.
569
570 *Minor second pereiopod* (Fig. 6H). Similar in form to major cheliped but smaller in size,
571 spinulation present on all segments except fingers and anterior part of palm. Tufted setae
572 covering fingers, palm and anterior part of carpus. Dactylus with 6-8 (6) small teeth, pollex with

573 7-8 (8) small teeth. Teeth distributed only on basal half of finger length, concealed by long, fine
574 setae. Oblique carina present on distal part, about half of finger length. Carpus cup shaped,
575 shorter than merus. Merus subcylindrical and as long as palm. Ischium tapered, shorter than
576 merus.

577

578 *Third pereiopods* (Fig. 6I). Dactylus short (1.9 mm) and curved distally, with lateral short seta
579 and ventral carina well developed. Propodus extending to the end of scaphocerite. Small
580 spinulation present on all segments except ischium. A fine seta present on all segments.
581 Propodus longer than dactylus (6.5: 1.9 mm), with 5-6 (6) ventral pairs of spines distributed
582 along length of propodus. Carpus shorter than propodus (3.6 mm), with dorsal projection on
583 distal part. Merus longer than carpus (6.5 mm). Ischium shorter than merus and carpus (3.3 mm).

584

585 *Fourth and fifth pereiopods*. Dactylus extending to the end of scaphocerite. Spinulation present
586 on all segments except ischium. Scattered fine setae present on all segments. Propodus with 5-6
587 pairs of ventral spines distributed along length of propodus. Propodus of fifth pereiopods with
588 group of setae on distolateral part. Carpus shorter than propodus and merus, with dorsal
589 projection on distal part. Ischium shorter than merus and carpus.

590

591 *Thoracic sternum*. T4-T8 with transverse plate without median process. T8 with posteromedial
592 lobes in males.

593

594 *Abdomen*. Usually smooth, with tiny spinules on pleural margin of first to third abdominal
595 segments in some specimens. All abdominal sternites with transverse ridge. First to third
596 abdominal sternites with moderate triangular median process. Fifth sternite without median
597 obtuse process. Preanal carina present, without small setae in males.

598

599 *Telson* (Fig. 6B). Moderately long (6.6 mm) Dorsal surface with 2 pairs of spines. Cluster of
600 setae present on antero-median part. Projection present on posterior margin, with two spines and
601 plumose setae on each side. The inner pair of posterior spines longer than outer spines.

602

603 *Uropods* (Fig. 6B). Uropodal diaeresis with inner moveable spine, shorter than outer angle.
604 Exopod longer than broad (7.4: 4.3 mm) and not reaching the end of endopods.

605

606 **Etymology.** The specific name "*palmopilosum*" is a compound Latin word with "*palma*"
607 meaning palm of the hand and "*pilosus*" meaning hairy. This name refers to the tuft of hairs
608 present on the palms of both second pereiopods.

609

610 **Size.** Males showing distinctly larger body size than females; the largest male recorded being
611 77.8 mm tl, 20.4 mm cl; the largest female 48.2 mm tl, 12.0 mm cl and egg size is 1.3 mm in
612 diameter.

613

614 **Distribution.** Their distribution is restricted to the northern part of Thailand, Nan Province.

615

616 **Remarks.** The population of this new species is dominant in the Nan River Basin, especially
617 living in clear, cool mountain streams. The colouration of this species varied from light pale to
618 dark brownish; the banding pattern on the dorso-lateral part of tergum was observed in some

619   individuals. *Macrobrachium palmopilosum* sp. nov. shares several characteristics with *M.*
620   *eriocheirum*, *M. amplimanus* and *M. hirsutimanus.* The character distinguishing *M.*
621   *palmopilosum* sp. nov. from *M. eriocheirum* and *M. hirsutimanus* is the presence of tufted setae
622   on the palms of the second pereiopods. *Macrobrachium hirsutimanus* and *M. eriocheirum*
623   exhibited tufted setae only on the anterior half of the palms, whereas *M. palmopilosum* sp. nov.
624   had setae present over the entire surface of palms. Moreover, the spinulation on the
625   anteromarginal surface of the carapace is always present in *M. palmopilosum* sp. nov. (absent in
626   *M. eriocheirum* and *M. hirsutimanus*). The epistome of *M. palmopilosum* sp. nov. is slightly
627   bilobed (trilobed in *M. eriocheirum* and *M. hirsutimanus*). The number of prominent teeth on
628   fingers of *M. palmopilosum* sp. nov. is 6-12, whereas *M. hirsutimanus* has 12-20 teeth and *M.*
629   *eriocheirum* has 12-15 teeth. *Macrobrachium palmopilosum* sp. nov. differs from *M.*
630   *amplimanus* by having more rostrum teeth on the postorbital area (4-6 vs. 2-4), slightly smaller
631   number of finger teeth on second pereiopods (10-12 vs. 11-15), the spinulation on palm surface
632   of second pereiopods (present vs. absent), the length of fingers shorter than palm (vs. longer or
633   as long as palm), and closed fingers with a gap (vs. without gap). The morphological
634   comparisons of *M. palmopilosum* sp. nov. and other species are presented in Table 4.
635
636   The results of phylogenetic tree construction suggested that *M. palmopilosum* sp. nov. is closely
637   related to *M. naiyanetri* sp. nov., as supported by all statistical tests. *Macrobrachium*
638   *palmopilosum* sp. nov. shows distinctive differences from *M. naiyanetri* sp. nov. by the stout cup
639   shaped carpus of the major second pereiopods (vs. slightly elongated carpus in *M. naiyanetri* sp.
640   nov.), the lack of setae on antero-inferior part of the merus of second pereiopods (vs. with dense
641   setae on merus in *M. naiyanetri* sp. nov.), the inflated form of merus in *M. palmopilosum* sp.
642   nov. (vs. subcylindrical in *M. naiyanetri* sp. nov.).
643
644   Tiwari (1952) described *M. hirsutimanus* based on specimens from northern Thailand (Doi
645   Chuang) and later the type locality was replaced by the neotype designation (Nan Province; in
646   Cai et al. (2004)). This taxonomic treatment advocates that the distribution of *M. hirsutimanus*
647   coexists with *M. palmopilosum* sp. nov. In this study, the coexistence of these two species of
648   prawns was confirmed in the Nan River Basin.
649
650   ***Macrobrachium puberimanus* Siriwut sp. nov.**
651   **ZooBank ID:** urn:lsid:zoobank.org:act:EE26BC6C-07F6-4C94-8B80-6F736B11F91A
652   **Figures** 4C and 7
653
654   **Type locality.** Mekong River at Wat Tha Khaek, Chiang Khan Sub-district, Chiang Khan
655   District, Loei Province
656
657   **Type examined. Holotype:** CUMZ MP00012, one male spm. from Wat Tha Khaek, Chiang
658   Khan Sub-district, Chiang Khan District, Loei Province (M099). **Paratype:** CUMZ MP00013,
659   two male spms from the same locality as holotype.
660
661   **Additional material.** CUMZ MP00014, one male spm. from Phu Ruea District, Loei Province
662   (M121). CUMZ MP00015, four male and twelve female spms from Nam Soam, Noan Thong
663   Sub-district, Na Yung District, Udon Thani Province (M049). CUMZ MP00016, four male spms

664   from Maekong River, Chiang Khan Sub-district, Chiang Khan District, Loei Province. CUMZ
665   MP00017, one male spm. from Maekong River, Pak Chom District, Loei Province.
666
667   **Diagnosis.** Rostrum moderately long, anteriorly striate and angled upward distally, reaching
668   beyond the end of second segment of antennular peduncle. Rostral formula: 12-15/3 teeth.
669   Carapace with small spinulation on anterolateral margin. Epistome trilobed. Second pereiopods
670   strong and robust, shorter than body length, similar in shape and unequal in size. Long-tufted
671   setae present on finger and palm of second pereiopods. Fingers of major second pereiopod with
672   11-16 teeth. Closded fingers with gap and crossing distally. Carpus elongated, shorter than palm.
673   Spinulation present on dorso-inferior surface of palm, carpus, merus and ischium. Minor second
674   pereiopod slight with tiny spines on each segment. Thoracic sternites: T4 with posterior
675   submedial plate; T4-T7 with basolateral median plate without median notch; male T8 with
676   posteromedially anterior lobes. Male and female without posteriorly medial process on T8. First
677   to third abdominal sternites with moderate triangular median process. Preanal carina present.
678   Telson moderately long, with long plumose setae on proximal part. Telson surface with two pairs
679   of dorsal spines, terminal projection with two long inner and short outer spines. Uropodal
680   diaeresis spine shorter than outer angle.
681
682
683
684   Rostrum short, anteriorly striate and upward distally, not reaching to the end of second telopodite
685   of antennular peduncle. Rostral formula: 10-12/ 2-3 teeth. Anterolateral margin of carapace with
686   small spines. Epistome bilobed. The robust pair of second pereiopod similar in shape, unequal in
687   size. Densed andtufted setae present on both side of second pereiopods. Anterior part of carpus
688   with setae. Fingers with 10-12 teeth. Carpus stoutand cupped, shorter than fingers, palm and
689   merus. oSmall spinule present in posteriorpart of palm, entirely in carpus and merus. Thoracic
690   sternites: T4 with posterior submedial plate; T5-T7 with transverse plate without median process;
691   T8 with contiguous posteromedially anterior lobes, without median process. First to third
692   abdominal sternites with moderate triangular median process. Preanal carina present. Telson
693   moderately long, with scaterred plumose setae on dorsal surface. Two pairs of spines present.
694   Posterior projection present with two long inner and short outer spines. Uropodal diaeresis spine
695   shorter than outer angle. Egg size 1.3 mm in diameter.
696
697   **Composite description** (type specimens in parentheses). A medium-sized *Macrobrachium*
698   species, tl 33.6-60.2 mm (60.2 mm in holotype), with pale or brownish-green body colouration
699   (Figs 4C).
700
701   *Rostrum* (Fig. 7C, D). Anteriorly striate and angled upward distally, rl 7.4-12.7 mm (12.7 mm in
702   holotype), cl 6.6-17.0 mm (17.0 mm in holotype), and reaching beyond the end second segment
703   of antennular peduncle. Dorsal part of rostrum with 12-15 (13) teeth in total, 5-6 (5) teeth present
704   in postorbital area. Area bearing postorbital teeth covering one-fourth of carapace length. Ventral
705   part of rostrum with 3 (3) teeth, located about half-way to distal end.
706
707   *Cephalon*. Eye well developed. Postantennular carapace margin rounded. Cornea osculum as
708   long as stalk. Antennular peduncle longer than wide, lateral carina slightly concave, dorsal carina
709   not sinuous. Sharp antennal and hepatic spines present at lower orbital angle; hepatic spine

710 smaller, situated behind and below antennal spine; branchiostegal suture running from hepatic
711 spine to anterior margin of carapace. Carapace without spinulation on ventro-lateral part and
712 branchiostegal regions (Fig. 6C). Ocular beak moderately developed, without laterally expanded
713 tip. Epistome trilobed. Scaphocerite, lateral margin slightly concave, distolateral tooth not
714 reaching the end of lamella. Third maxilliped reaching beyond antennal peduncle and covering
715 75-80% of length of scaphocerite; ultimate slightly shorter than penultimate.
716
717 *First pereiopods*. Long and slender, reaching beyond the end of scaphocerite. Fingers about as
718 long as palm; carpus as long as merus. Few setae scattered on all segments but dense on distal
719 part of finger and on lower margin of ischium. Proximal part between palm and carpus without
720 small setae.
721
722 *Second pereiopods*. Robust and slightly shorter than body length, similar in form but differing in
723 size. Carpus of major second pereiopods extending beyond the end of scaphocerite.
724
725 *Major second pereiopod* (Fig. 7E, G). Spinulation present on dorso-inferior surface of palm,
726 carpus, merus and ischium. Fingers, palm, inferior margins of carpus covered with few tufted
727 setae. Merus without tufted setae anteriorly. Fingers sharp and subcylindrical, longer than palm
728 in length (19.7: 15.3 mm), closed fingers with gap and crossing distally. Dactylus with 11-16
729 (16) prominent teeth, basal teeth slightly smaller than distal teeth, pollex with 10-14 (14) teeth
730 (Fig. 7F). Teeth sub-equally distributed and concealed by long tufted setae, with oblique carina
731 distally, about 15-20% of finger length. Upper and lower margins of palm not expanded. Carpus
732 slightly elongated, shorter than merus (9.2: 16.6 mm). Merus subcylindrical, as long as palm or
733 shorter (16.6 vs 15.3 mm). Ischium tapered, shorter than merus.
734
735 *Minor second pereiopod* (Fig. 7G). Short and smaller than major cheliped, spinulation absent in
736 all segments. Few tufted setae covering fingers and palm. Dactylus with 6-8 (6) small teeth,
737 pollex with 5-11 (7) small teeth. Teeth distributed only on basal half of finger length, concealed
738 by fine setae. Oblique carina present on distal two-thirds of finger length. Carpus elongated,
739 shorter than merus. Merus subcylindrical and as long as palm. Ischium tapered, shorter than
740 merus.
741
742 *Third pereiopods* (Fig. 7I). Long and slender; propodus extending to the end of scaphocerite.
743 Small spinulation absent in all segments. A fine seta present on all segments. Dactylus short and
744 curved (2.2 mm), with dorsolateral setae on distal part, ventral carina well developed. Propodus
745 longer than dactylus (6.5: 1.8 mm), with 5-7 ventral pairs of spines distributed along length of
746 propodus. Carpus shorter than propodus (3.5 mm), with dorsal projection on distal part. Merus
747 longer than carpus (8.6 mm). Ischium shorter than merus (3.2 mm).
748
749 *Fourth and fifth pereiopods*. Dactylus extending to the end of scaphocerite. Spinulation absent
750 on all segments. Few fine setae present, scattered on all segments. Propodus with 5-6 pairs of
751 ventral spines distributed along length of propodus. Propodus of fifth pereiopods with group of
752 setae on distolateral part. Carpus shorter than propodus and merus, with dorsal projection on
753 distal part. Ischium shorter than merus.
754

755 *Thoracic sternum*. T4-T7 with transverse plate without median process. T8 with posteromedial
756 lobes in males.

758 *Abdomen*. Smooth, without small spinules on pleural margin of abdominal segments. All
759 abdominal sternites with transverse ridge. First to third abdominal sternites with moderate
760 triangular median process. Fifth sternite with median obtuse process. Preanal carina present,
761 without small setae in males.

763 *Telson* (Fig. 7B). Moderately long (6.7 mm) andstraight. Dorsal surface with 2 pairs of spines.
764 Cluster of setae present on antero-median part. Projection present on posterior margin, with two
765 spines and plumose setae on each side. The inner pair of posterior spines longer than outer
766 spines.

768 *Uropods* (Fig. 7B). Uropodal diaeresis with inner moveable spine, shorter than outer angle (Fig.
769 4B). Exopod longer than broad (8.0: 3.7 mm) and not reaching beyond the end of endopods.

771 **Etymology.** The specific name "*puberimanus*" is derived from the compound Latin words
772 "puberis" for downy and "manus" for hand. It alludes to the long-tufted hairs on the second
773 pereiopods.

775 **Size.** Males with larger body size than females; the largest male recorded being tl 60.0 mm, cl
776 17.0 mm; the largest female tl 28.9 mm, cl 8.8 mm; egg size is 1.7 mm in diameter.

778 **Distribution.** Recent populations are restricted to the northeastern part of Thailand and possibly
779 occur in the Mekong River and its tributaries in Laos.

781 **Remarks.** This species is distributed commonly in tributaries of the middle Mekong River Basin
782 in northeastern Thailand. The molecular phylogeny and morphological characters of *M.*
783 *puberimanus* sp. nov. indicated close resemblance to *M. dienbienphuense*, which is commonly
784 found in the Mekong River Basin, including Thailand, Laos, Cambodia (?), Vietnam, and also
785 southern China (Hanamura et al. 2011). The characters distinguishing *M. puberimanus* sp. nov.
786 from *M. dienbienphuense* are the number of finger teeth on the cutting edge of the major second
787 pereiopod (11-16 vs. 18-32), spinulation on the anterior margin of carapace (absent vs. present),
788 the spinulation on merus surface (sparse vs. abundant), and the slightly elongated carpus of
789 second pereiopods (vs. highly elongated carpus).

791 Recently, a cavern-dwelling species was found from the central part of Thailand, namely *M.*
792 *spelaeus* by Cai & Vidthayanon (2016). The morphological characters indicate similarity with *M.*
793 *dienbienphuense* in several aspects except for the form of the anterior rostrum, the reduced eye,
794 the bilobed epistome and the second pereiopod being as long as the body. In this study, *M.*
795 *puberimanus* sp. nov. shows morphological differences from the latter species by having less
796 elongated carpus, distal part of rostrum not upturned, and merus of second pereiopods with less
797 spinulation. The distribution of *M. puberimanus* sp. nov. seems associated with the open riverine
798 system of the Mekong River Basin, whereas the distribution of *M. spelaeus* is restricted to
799 subterranean limestone systems in the central part of Thailand. Two additional species that
800 resemble *M. dienbienphuense* and are co-distributed in the Mekong River Basin are *M.*

801 *amplimanus* and *M. eriocheirum*. Hanamura et al. (2011) reviewed the morphological characters
802 of these two species based on specimens from Laos and provided additional 16S rRNA
803 sequences for molecular phylogenetic analysis. In this study, the 16S rRNA sequences of *M.*
804 *puberimanus* sp. nov. were totally separated from Laotian *M. amplimanus* sequences, whereas
805 *M. eriocheirum* from Laos nested within *M. puberimanus* sp. nov. samples (see Fig. S2 in
806 supplement). However, the Laotian *M. eriocheirum* differs from *M. eriocheirum* sensu Dai
807 (1984) in some aspects such as the number of dorsal and ventral rostrum teeth (9-12/2-3 vs. 11-
808 14/2-3 in Laotian specimens) and the number of teeth on fingers of second pereiopods (10 teeth
809 vs. 11-17). For this reason, the samples called *M. eriocheirum* in Hanamura et al. (2011) herein
810 are excluded from this study; either they are *M. puberimanus* sp. nov. or a separate species.
811
812 Key to mainland SE-Asian species in *"pilimanus"* species group (after Cai et al., 2004)
813 1. a) A rudimentary appendix interna present on the first male pleopod……*M. dalatense*
814 b) A rudimentary appendix interna absent on the first male pleopod……………….2
815 2. a) Merus of second pereiopods with pubescence……………………………………….5
816 b) Merus of second pereiopods without pubescence………………………….…..3
817 3. a) Rostrum short and convex distally, second pereiopods with fingers shorter than
818 palm…………………………………………………………………………………..*4*
819 b) Rostrum short and straight distally, second pereiopods with fingers shorter than
820 palm…………………………………………………………….*M. naiyanetri* **sp. nov.**
821 4. a) 17 small teeth on fingers of second pereiopod………………………….*M. pilosum*
822 b) 8-10 blunt teeth on fingers of second pereiopod……………………..*M. sirindhorn*
823 5. a) Tuberculation present on palm surface of second pereiopods…………………….6
824 b) Tuberculation absent on palm surface of second pereiopods…………………….10
825 6. a) Elongated carpus of major second pereiopod…………………………………….7
826 b) Cupped carpus of major second pereiopod……………………………………….8
827 7. a) Cutting edges of fingers of second pereiopod with 23-32 teeth, closed fingers without
828 gap………………………………………….…………………*M. dienbienphuense*
829 b) Cutting edges of fingers of second pereiopod with 11-16 teeth, closed fingers with
830 gap…………………………………….….………………*M. puberimanus* **sp. nov.**
831 8. a) Carapace margin without spinulation ……………………………………………*9*
832 b) Carapace margin with spinulation…………………..………*M. palmopilosum* **sp. nov.**
833 9. a) Rostrum teeth arrangement 4+7/2, cutting edges of fingers of second pereiopod 7-
834 10……………………………………………………………..………*M. forcipatum*
835 b) Rostrum teeth arrangement 6+7/2, cutting edges of fingers of second pereiopod 11-
836 12……………………………………………………………………………*M. pilimanus*
837 10. a) Epistome bilobed………………………………………………*M. hirsutimanus*
838 b) Epistome trilobed……………………………………………………………11
839 11. a) Velvet pubescence on fingers and palm of second pereiopods……*M. eriocheirum*
840 b) Densely tufted pubescence on fingers and palm of second pereiopods
841 …………………………………………………………..………….*M. amplimanus*
842
843 Using geometric-morphometric measurements, shape variation among species was
844 detected by ten classical landmarks on the rostrum and carapace (Fig. S4). Canonical variates
845 analysis (CVA) displayed sharp variation among nine species in the *"pilimanus"* group (Fig. S5).
846 Shape variation between *M. palmopilosom-M. naiyanetri* was detected in both Procrustate and

847 Mahalanobis distance analyses. In terms of Mahalanobis distance, the comparison of shape
848 measurements resulted in seven paired species with statistical support (P<0.0001). A summary of
849 Procrustate and Mahalanobis distance analyses is given in Table S7.

Discussion

851

Phylogenetic relationship of "*pilimanus*" species group members in mainland Southeast Asia

854

855 The monophyletic status of genus *Macrobrachium* is still questionable based on samples
856 of "*pilimanus*" members and other *Macrobrachium* species used in this study. The insertion of
857 outgroups, *Coralliocaris superba* and *Exopalaemon styliferus*, within a clade of genus
858 *Macrobrachium* contradicted previous phylogenetic studies indicating the separation of these
859 two genera from genus *Macrobrachium* (Saengphan et al. 2018; Wowor et al. 2009). The
860 selection of outgroup rooting is critical in phylogenetic analysis in order to clarify the
861 evolutionary history of *Macrobrachium* species as indicated in previous reports (Murphy &
862 Austin 2005; Wowor et al. 2009). However, like this study, broad scale sampling of decapod
863 phylogeny has shown that within family Palaemonidae, the genus *Macrobrachium* can be either
864 nested with other genera such as *Cryhiop*, *Exopalaemon* and *Palaemon* or inserted within
865 another closely related genus i.e. *Leptopalaemon* (Bracken 2009).

866

867 Molecular phylogenetic analysis of three partial gene datasets indicated at least ten different
868 evolutionary lineages in the "*pilimanus*" species group. The two major clades (Fig 1) are usually
869 found in mainland Southeast Asia tributaries; clade E consists of *M. dienbienphuenses* + *M.*
870 *Puberimanus* sp. nov. + *M. eriocheirum* + *M. hirsutimanus,* and clade D consists of *M.*
871 *forcipatum* + *M. naiyanetri* sp. nov. + *M. palmopilosum* sp. nov. + *M. malayanum* + *M.*
872 *pilimanus* + *M. sirindohrn. Macrobrachium sirindhorn* is further grouped with *M. pilimanus*.
873 The morphological characters of *M. sirindhorn* are quite unique and distinct from the congeners
874 in this species group by having tufted setae on carpus and merus (except *M. naiyanetri* sp. nov.,
875 which has a group of stiff setae on the inner side of carpus and merus) and the distal downward
876 pattern of rostrum. The distribution range of *M. sirindhorn* is questionable due to scattered
877 records from northern Thailand and Laos. Another species that presents similar characters to *M.*
878 *sirindhorn* is *M. pilosum* Cai and Dai, 1999 from southern China (Yunnan) and possibly northern
879 Vietnam. Without genetic data of *M. pilosum*, we would keep these two as distinct valid species.

880

881 *Macrobrachium* species in clade E exhibited sympatric distribution in several river systems in
882 north-central and eastern Thailand. *Macrobrachium dienbienphuense* and *M. puberimanus* sp.
883 nov. exhibited elongated carpus of second pereiopods. However, the gap and slender shape of
884 pollex and dactylus, and fewer spinules on the merus of second pereiopods are morphologically
885 diagnostic characters of *M. puberimanus* sp. nov. The collected sample of *M. puberimanus* sp.
886 nov. included a smaller number of individuals than for *M. dienbienphuense* in every locality.
887 This finding might suggest a low population density of *M. puberimanus* sp. nov. in its natural
888 habitat.

889

890 In clade E, *Macrobrachium hirsutimanus* and *M. eriocheirum* are morphologically distinct from
891 each other by having incomplete covering of velvet setae on the palms of second pereiopods;
892 however, they typically co-exist in the Chaophraya River Basin of Thailand. *Macrobrachium*
893 *eriocheirum* Dai, 1984 was originally described from Yunnan and recently treated as a synonym
894 of *M. dienbienphuense*. In this study, specimens from Yunnan (M97-M98) indicated genetic
895 compatibility with Thai samples by forming a monophyletic relationship. This finding might
896 suggest the validity of *M. eriocheirum* as mentioned by previous taxonomic studies (Cai et al.
897 2004; Hanamura et al. 2011). In addition, the southern population of *M. eriocheirum* was
898 collected from peninsular Thailand, extending the known distribution range of this species. A
899 taxonomic review of *M. hirsutimanus* has been made and the neotype designation of this species
900 was described using specimens from Nan Province, in northern Thailand (Cai et al. 2004). In this
901 study, we sampled the northern riverine areas including the Nan River Basin to obtain a
902 representative collection of specimens. The phylogenetic tree indicated a monophyletic group
903 among molecular samples of *M. hirsutimanus*. However, there is another species from North-
904 Central Thailand that is similar in morphological characters to *M. hirsutimanus*, namely *M.*
905 *spelaeus*. The distribution of *M. spelaeus* is restricted to the underground freshwater system in a
906 limestone cave; however, there may be some connection with the Nan River.
907
908 The mainland Southeast Asia "*pilimanus*" species group includes species from the southern
909 peninsula of Thailand and a part of the Mekong River Basin (clade D). *Macrobrachium*
910 *forcipatum* and *M. malayanum* exhibited small body length and short second pereiopods.
911 Previously, two samples of *M. malayanum* were reported from Narathiwat, Southern Thailand
912 (Cai et al. 2004). In this study, two genetically diverse lineages of *M. Malayanum* were found
913 from the same locality. This might suggest the endemism of *M. malayanum*, which has restricted
914 distribution in some natural habitats in the southern part of Thailand. The two new species in this
915 study, *M. naiyanetri* sp. nov. and *M. palmopilosum* sp. nov. are grouped with *M. pilimanus* and
916 *M. sirindhorn*; however, the phylogenetic relationship between these two species is questionable
917 due to low statistical node support. Geographical differentiation in samples of *M. naiyanetri* sp.
918 nov. was detected, and some species delimitation methods (ABGD, PTP and GMYC with
919 mitochondrial loci) suggested the possibility of cryptic speciation for the two geographically
920 different populations.
921

Species boundary of "*pilimanus*" species group designated by morphological and molecular delimitation methods

924
925 The *Macrobrachium pilimanus* group was initially proposed by Johnson (1960) and by
926 the morphological concept, they shown the high morphologically complex group (Johnson
927 1963). The high phenotypical variation was previously observed by Holthuis (1950), who
928 reported morphologically complex forms of a single species, *Macrobrachium pilimanus*. In *M.*
929 *pilimanus* sensu stricto (Johnson 1963), the features used to diagnose this species from the two
930 conspecific species (*M. leptodactylus* and *M. malayanum*) are the short fingers without a gap,
931 inner edge of carpus of second pereiopods convex, and short rostrum. Geographical variation
932 was detected in Javanian *M. pilimanus* and Bornean *M. leptodactylus* by either having a small
933 number of teeth on rostrum or slightly different pattern of second pereiopods. The specimens
934 were later re-examined, and found to be either the same species (Javanian *M. pilimanus* = *M.*
935 *leptodactylus*; Ou & Yeo (1995) or two distinct species (Bornean *M. leptodactylus* = *M. urayang*;

936 (Wowor & Short 2007). The type re-examination of some members in the "*pilimanus*" species
937 group show inappropriate species boundaries applied previously, or even the co-existence of
938 unknown species within the type series, such as the specimens of *M. malayanum* in Johnson
939 (1960); Johnson (1963) was found morphologically differ from type of *M. malayanum* by Roux
940 (1934) (see taxonomic treatment in Chong & Khoo (1987b)), while in another case, four
941 paralectotype specimens of *M. leptodactylus* were found to be a distinct species, *M. empulipke*
942 (see Wowor (2010)). In this study, a geometric morphometric examination of the *M. pilimanus*
943 group was conducted for the first time. The use of measurable characters for species delimitation
944 has been successful in several taxa with statistical confirmation (see Fig S4-5 and Table S6-7).
945 Broad sampling, optimal specimens of each of the "*pilimanus*" members, and other landmark
946 methods are required in further study. This study result would be relieved the alternative
947 approach to delimit the species boundary of *M. pilimanus* group under morphological species
948 concept.
949
950 The phylogenetic relationships within the *M. pilimanus* group have never been
951 specifically investigated in order to verify the group's phylogenetic position and taxonomic
952 validity. However, some members, including *M. pilimanus*, *M. dienbienphuense*, *M.*
953 *eriochierum*, and *M. amplimanus* were previously included in several large-scale phylogenetic
954 studies of genus *Macrobrachium* or higher taxa (Bracken et al. 2010; Jose & Harikrishnan 2019;
955 Liu et al. 2007; Pileggi & Mantelatto 2010; Wowor et al. 2009). A taxonomic review of some
956 "*pilimanus*" members based on molecular delimitation was done by Hanamura et al. (2011), and
957 morphological identification was supported by the 16S sequences to confirm the biological
958 species concept. Using single genetic markers, the combination of available sequences from
959 previous literature and newly amplified sequences from this study indicated unresolved
960 phylogenetic relationships. The effect of long-branch attraction caused by gap insertion and short
961 sequence length has been found in several database sequences. However, the *M. pilimanus* group
962 shows a close evolutionary relationship with *M. niphanae*, another common species group found
963 in mainland SE-Asia, based on the concatenated dataset.
964 In this study, the molecular delimitation using the optimum sequence dataset agreed well
965 with traditional morphological classification despite the few diagnostic characters that have been
966 observed in some nominal species. The monophyletic clade of each representative taxon detected
967 from the concatenated dataset, including the three new species, was subsequently confirmed by
968 automatic delimitation approaches based on single gene datasets (see Fig. 2). The BIN algorithm
969 reflects the highest number of putative species in the COI dataset. According to the barcode gap
970 threshold, over-estimation might be caused by genetic divergence of the dataset used, including
971 the previously deposited sequences. The point of caution for BIN delimitation results in this
972 study seems to be obscurity onthe species identification concept in several deposited sequences,
973 especially in *M. dienbienphuense*. The genetic divergence among samples named *M.*
974 *dienbienphuense* raises warning (max intraspecific divergence higher than Nearest-neighbor
975 species; see Table S8) in barcode gap analysis. The BIN discordance also detects the non-
976 compatibility of sequence divergence and BIN assignment which agree with the barcode gap
977 threshold. In the case of mPTP and bPTP, the delimitation results showed moderate support for
978 the designation of three new species found in this study. The clustering reassigned some
979 "*pilimanus*" members to be a single species, as inferred in the 16S dataset under bPTP and
980 mPTP. In the case of GMYC, the delimitation using COI and 16S agreed with morphological
981 identification and phylogenetic clade composition. In this study, all delimitation methods also

982   presented the warning of cryptic speciation in samples assigned as *M. malayanum*.
983   Unsurprisingly, low success in using 18S rRNA sequences was found with ABGD, PTP and
984   GMYC; clustering lumped members of the "*pilimanus*" group into one to three putative species.
985   Furthermore, the COI barcoding region seems to provide the fine resolution required for genus
986   *Macrobrachium*. This suggestion has also been reported in recent studies of DNA barcode
987   application on marine decapods, including *Macrobrachium* prawns (Hernawati et al. 2020;
988   Matzen da Silva et al. 2011).
989   The integrative approaches applied herein resolve the problems of morphological
990   concordance among "*pilimanus*" members. However, the species boundaries delimited by
991   traditional identification seem to be carefully interpreted when abundant samples were used for
992   comparison according to geographical variation. A combination of morphology and molecular
993   taxonomy approaches is recommended for future species delimitation in the *M. pilimanus* group
994   for the following reasons: first, the molecular operational taxonomic unit (MOTUs) is helpful to
995   accelerate the sample clustering process under traditional identification despite morphological
996   complexity; second, the phylogenetic species concept can be used to force the species
997   assignment and taxonomic validity when diagnostic characters of paired species are shown as
998   unclear; third, molecular taxonomy can provide supporting evidence of cryptic speciation.
999

Species diversity and distribution of the Thai "*pilimanus*" species group

1001
1002   Recent taxonomic reviews of Thai *Macrobrachium* species included nine species
1003   belonging to the "*pilimanus*" species group: *M. eriocheirum*, *M. hirsutimanus*, *M.
1004   dienbienphuense*, *M. forcipatum*, *M. amplimanus*, *M. forcipatum*, *M. malayanum M. sirindhorn*
1005   and *M. spelaeus* (Cai et al. 2004; Cai & Vidthayanon 2016). In this study, seven previously
1006   recognised species were studied along with three new species that morphological and molecular
1007   datasets suggest should be grouped in the "*pilimanus*" species group. However, there are two
1008   nominated species in the Thai freshwater fauna that were not included in this study: *M.
1009   amplimanus* and *M. spelaeus*. The distribution of *M. amplimanus* has been reported from
1010   Thailand in four provinces, namely Chiang Mai, Loei, Kanchanaburi and Narathiwat (Cai, 2004);
1011   it is also present in Laos (Hanamura et al. 2011). Cai et al. (2004) reported that the characteristics
1012   of *M. amplimanus* are very similar to *M. forcipatum* and *M. hirsutimanus* in several aspects. The
1013   distinguishing features that can be used to identify *M. amplimanus* are the short rostrum, stoutly-
1014   inflated second pereiopods, and the number of rostrum teeth. The collected specimens from the
1015   Mekong River in this study indicated only two morphological species: *M. dienbienphuense* and
1016   *M. puberimanus* sp. nov. However, the available 16S DNA sequences in GenBank of *M.
1017   amplimanus* used in Hanamura *et al.* (2008) were initially combined with the 16S dataset in this
1018   study (Table S2 in appendix). The results indicated that the Laotian sequences of *M. amplimanus*
1019   sensu Hanamura *et al.* (2008) resembled species within the *M. eriocheirum* clade. To confirm the
1020   true taxonomic identity of these samples, new analyses using a combination of molecular
1021   markers are required due to high variation of sites detected in the 16S rRNA gene.
1022
1023   *Macrobrachium spelaeus*, the only Thai cavern species, was reported from Phra Wang Dang
1024   Cave in Phitsanulok Province (Cai & Vidthayanon 2016). This species resembles *M.
1025   dienbienphuense* in morphology by having bilobed epistome, convex anterior rostrum, reduced
1026   eye, and by the length of the major second pereiopod being as long as the body. In this study, we
1027   could not find any specimens that resembled the morphology of *M. spelaeus* from central or

1028 northern Thailand. Moreover, fresh materials for DNA analysis of this species is limited, and
1029 gaining access to the exact location of the type locality is difficult due to conservation efforts.
1030 However, the samples from neighboring rivers and small streams indicated two species that
1031 possibly co-exist with this species: *M. eriocheirum* and *M. dienbienphuense*.

1033 Previously, the study of freshwater prawn genus *Macrobrachium* mainly focused on the
1034 commercial species due to their economic value both globally and at a local scale (New & Nair
1035 2012). Recently, two newly named species, *M. suphanense* and *M. chinatense* were described
1036 from freshwater tributaries in central Thailand (Saengphan et al. 2018; Saengphan et al. 2019).
1037 There is also some genetic evidence of Thai *Macrobrachium* species exhibiting distinct
1038 geographical populations (Khanarnpai et al. 2019; Saengphan et al. 2018). In total, thirty-one
1039 *Macrobrachium* species have been reported from Thailand, including the three new species
1040 found in this study. These findings suggest that the species diversity of freshwater fauna in
1041 Thailand has been under-reported and needs more attention. Furthermore, several native species
1042 of the genus *Macrobrachium* in Thailand and adjacent areas are of critical concern due to
1043 disturbance by anthropogenic activity, especially taxa in the "*pilimanus*" species group. The
1044 habitat preference of these prawn species is usually small streams or river systems connected to
1045 mountainous territory, which recently have been impacted by tourism and plantation
1046 development. The water quality and current flow of several riverine systems in mainland
1047 Southeast Asia are monitored under several environmental and ecological programs (Dudgeon
1048 2000; Hughes 2017; Todd et al. 2010). Changes of the tributary system may cause the ecosystem
1049 to collapse by the disruption in species composition and loss of native freshwater fauna
1050 (Fukushima et al. 2014). However, the baseline data on biology, taxonomy and ecology are still
1051 insufficient. For this reason, further studies on biology, systematics and ecology of native
1052 *Macrobrachium* species are still required, especially in the context of biogeographical
1053 distribution related to migration, and river tributaries and their flows (de Bruyn et al. 2004;
1054 Wowor et al. 2009). The integration of recent novel methods such as molecular phylogeny,
1055 species distribution modeling and ecological monitoring methods would be beneficial for
1056 database implementation in conservation management of freshwater prawns at both local and
1057 regional scales (De Grave et al. 2008; De Grave et al. 2015; Michael 1988).

Conclusion

1059
1060   In this study, the integrative approach provided additional three new species of *M.*
1061 *pilimanus* members found in montain stream of Thailand. The species delimitation method
1062 related to biological and phylogenetic species concepts provided an alternative scheme for the
1063 justification of species boundary in this *Macrobrachium* species group. The geographical
1064 variation, refered both in molecular and morphological characteristics was documented in some
1065 species of *M. pilimanus* and would suggest the differences of dispersal abilities among
1066 congeneric species. The phylogenetic relationship among *M. pilimanus* members still be
1067 controversy due to non-monophyly but at least the mainland SE-Asian species united as
1068 monophyletic clade. The genetic variation based on this study and deposited samples suggests
1069 the possible cryptic fauna in *Macrobrachium* prawns from mainland SE-Asia where the massive
1070 network river basin was recognized. The distribution area of mainland *M. pilimanus* indicated
1071 the trend of species composition and abundant related to water flows from two basins; Chao
1072 Phraya and Mekong.

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Figure 1

Sampling localities of *Macrobrachium pilimanus* group in this study

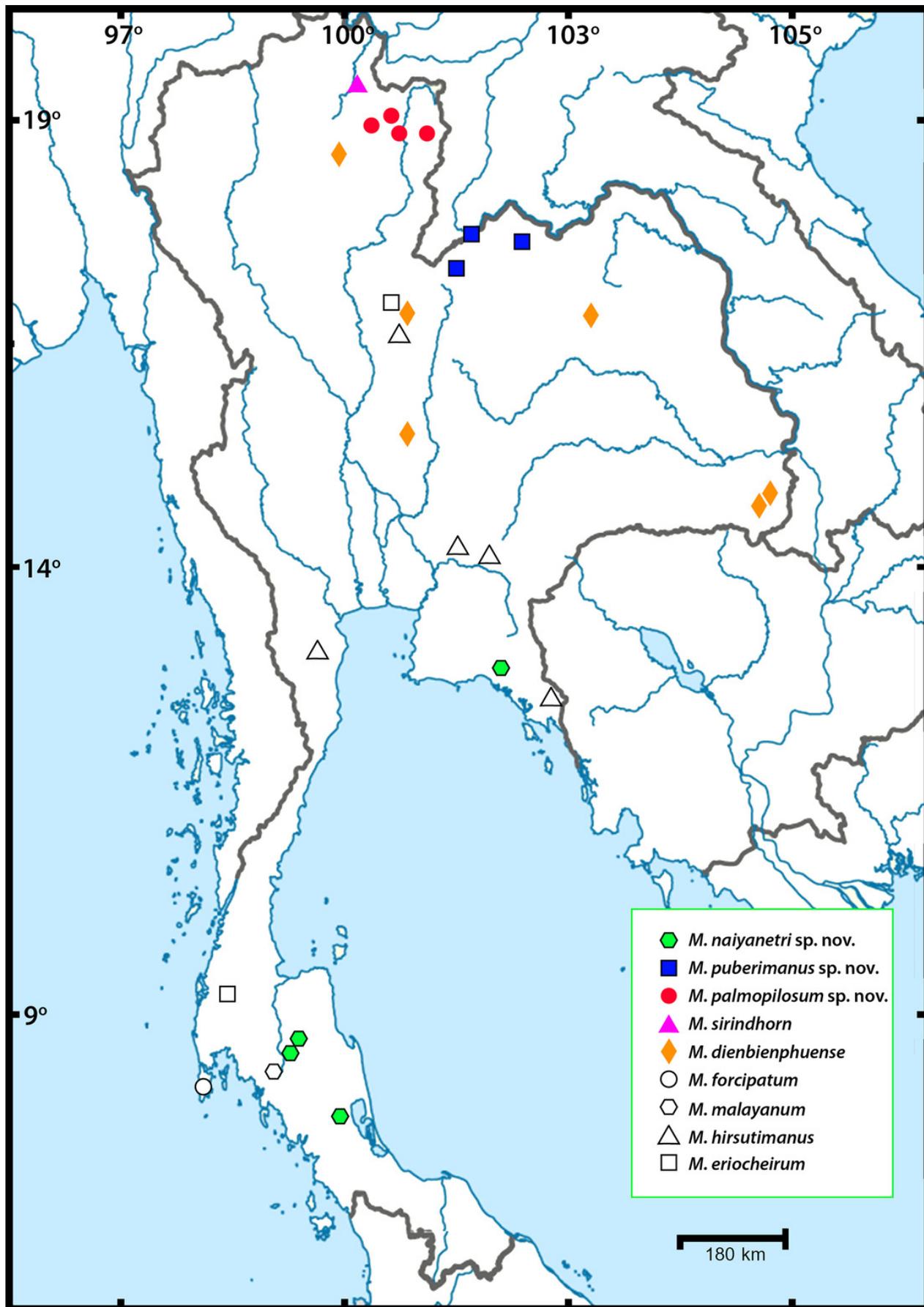



Figure 2

Phylogenetic tree based on concatenated dataset of three molecular genes (COI, 16S and 18S rRNA), geographical distribution and morphological characteristics of second pereiopods of *M. pilimanus* species group.

(A) Phylogenetic tree (B) Morphological character of second pereiopods (C) Distribution area. Nodes of phylogenetic tree marked with empty circles indicate statistical support from both ML and BI (>70 bootstrap value and >0.97 posterior probability score); grey circles indicate statistical support from only one (either ML or BI); asterisk indicates the sample obtained from NCBI.

A

- M155_*M_naiyanetri*_**n.sp.**
- MACR006_*M_aff pilimanus**
- M127_*M_naiyanetri*_**n.sp.**
- M154_*M_naiyanetri*_**n.sp.**
- M128_*M_naiyanetri*_**n.sp.**
- M102_*M_naiyanetri*_**n.sp.**
- M134_*M_naiyanetri*_**n.sp.**
- M130A_*M_forcipatum*
- M130B_*M_forcipatum*
- M130_*M_forcipatum*
- MACR035_*M_pilimanus**
- KC3110_*M_pilimanus**
- M009_*M_sirindhorn*
- M010_*M_sirindhorn*
- M011_*M_palmopilosum*_**n.sp.**
- M031_*M_palmopilosum*_**n.sp.**
- M030_*M_palmopilosum*_**n.sp.**
- M132_*M_malayanum*
- M151_*M_malayanum*
- M152_*M_malayanum*
- M153_*M_malayanum*
- M016_*M_dienbienphuense*
- M027_*M_dienbienphuense*
- M084_*M_dienbienphuense*
- M054_*M_dienbienphuense*
- M069_*M_dienbienphuense*
- M148_*M_dienbienphuense*
- M049_*M_pubersimanus*_**n.sp.**
- M099_*M_pubersimanus*_**n.sp.**
- M121_*M_pubersimanus*_**n.sp.**
- *M_dienbienphuense**
- *M_dienbienphuense**
- M051_*M_hirsutimanus*
- M053_*M_hirsutimanus*
- M083_*M_hirsutimanus*
- M052_*M_hirsutimanus*
- M140_*M_hirsutimanus*
- M050_*M_eriocheirum*
- M138_*M_eriocheirum*
- M097_*M_eriocheirum*
- M098_*M_eriocheirum*
- *M_niphanae**
- *M_niphanae**
- M023_*M_niphanae*
- MACR033_*M_niphanae**
- *M_lepidactylus**
- *M_lepidactylus**
- *Coralliocaris_superba**
- M115_*M_rosenbergii*
- M038_*M_sintangense*
- M060_*M_neglectum*
- MACR044_*M_urayang**
- MACR001_*Exopalaemon_styliferus**
- *Palaemon_debilis**

Clade F
Clade D
Clade C
Clade G
Clade B
Clade E
Clade A

MP5
MP4
MP6
MP1
MP3
MP2

0.5

B Morphology of second pereiopods

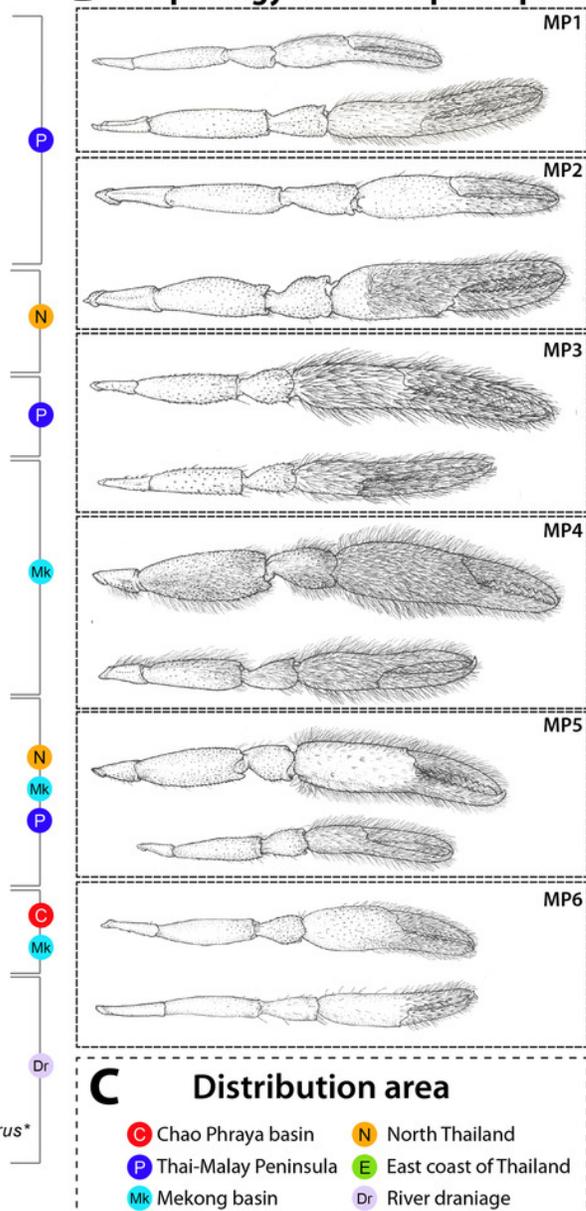
MP1

MP2

MP3

MP4

MP5

MP6

C Distribution area

- **C** Chao Phraya basin
- **P** Thai-Malay Peninsula
- **Mk** Mekong basin
- **N** North Thailand
- **E** East coast of Thailand
- **Dr** River draniage

Figure 3

Results of species delimitation based on multiple approaches.

Abbreviations used on phylogenetic tree are as follow: Morpho, morphological identification; PHYLO, phylogenetic analysis; BIN, BIN clustering in BOLD; ABGD, automated barcode gap; bPTP, Bayesian Poisson tree processes; mPTP, multi-rate Poisson Tree Processes; GMYC, Generalized Mixed Yule Coalescent model. Box colours indicate the split (red) and lumped (blue) species recognized by each species delimitation method. Grey boxes indicate non-monophyly in phylogenetic analysis and missing sequences from dataset in each delimitation method; asterisk indicates the sample obtained from NCBI.

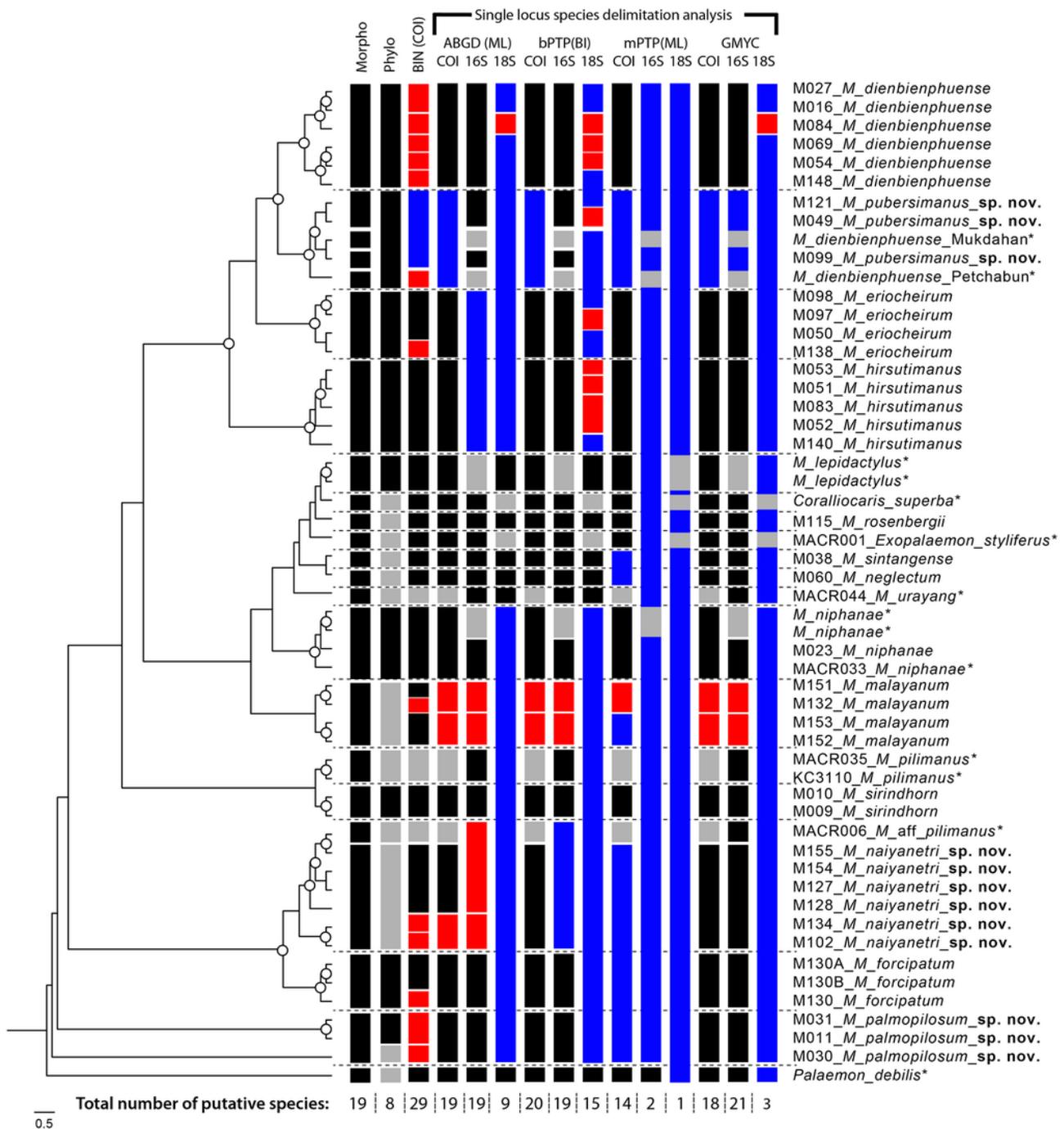



Figure 4

Live habitus specimens of three new *Macrobrachium* species in the *M. pilimanus* group from Thailand:

(A) *Macrobrachium naiyanetri* sp. nov. (B) *Macrobrachium palmopilosum* sp. nov. (C) *Macrobrachium puberimanus* sp. nov.

Figure 5

Morphological characters of *Macrobrachium naiyanetri* sp. nov. (A-G, I from holotype, H from paratype; CUMZ MP00003).

(A) Lateral view (B) Uropods (C) Carapace (D) Rostrum form and teeth (E) Major second pereiopod (F) Teeth on finger of major second pereiopod (G) Major second pereiopod length (H) Second pereiopods in female (I) Third pereiopod

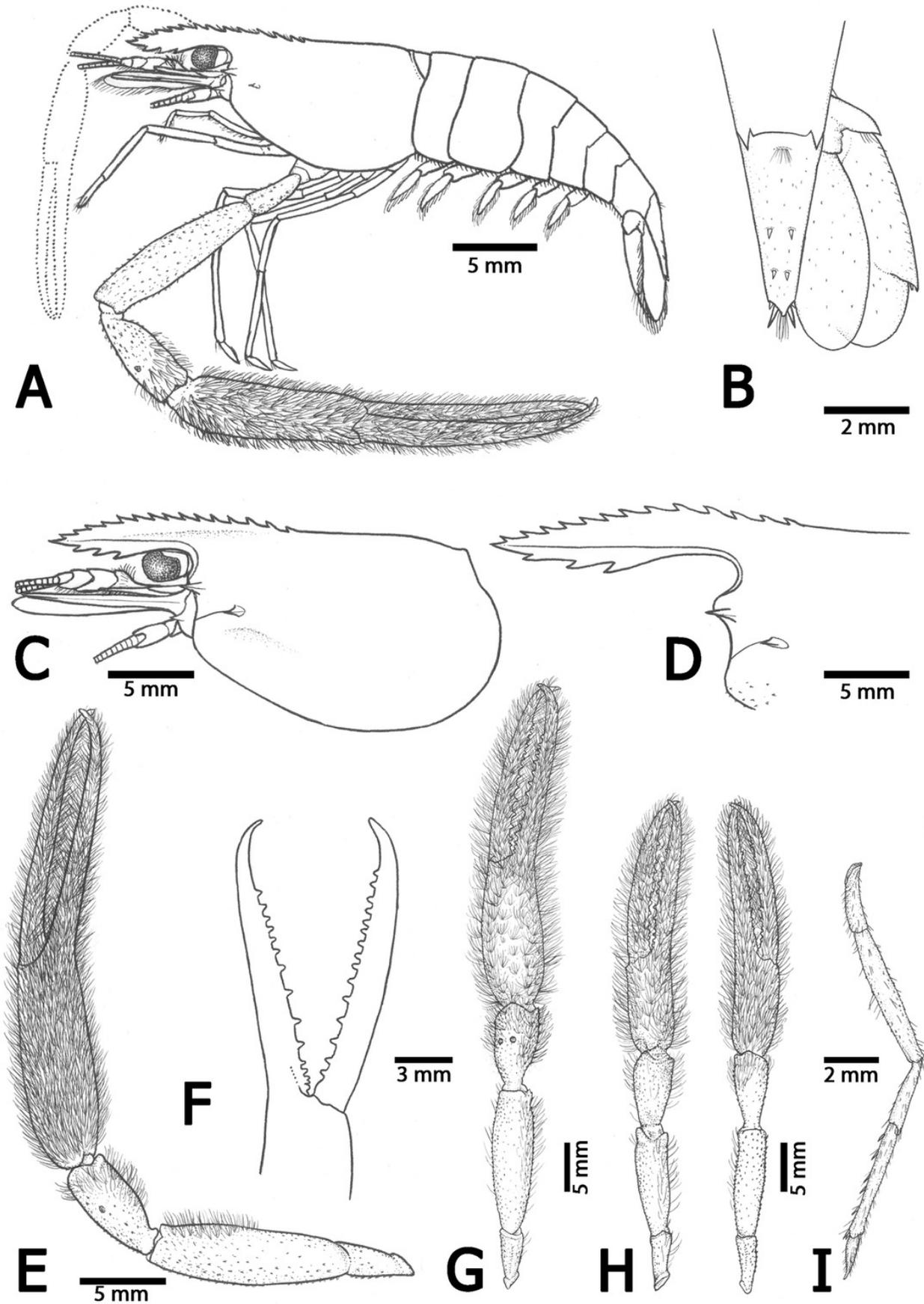



Figure 6

Morphological characters of *Macrobrachium palmopilosum* sp. nov. (A-G, I from holotype, H from paratype; CUMZ MP00008).

(A) Lateral view (B) Uropods (C) Carapace (D) Rostrum form and teeth (E) Major second pereiopod (F) Teeth on finger of major second pereiopod (G) Major second pereiopod length (H) Second pereiopods in female (I) Third pereiopod

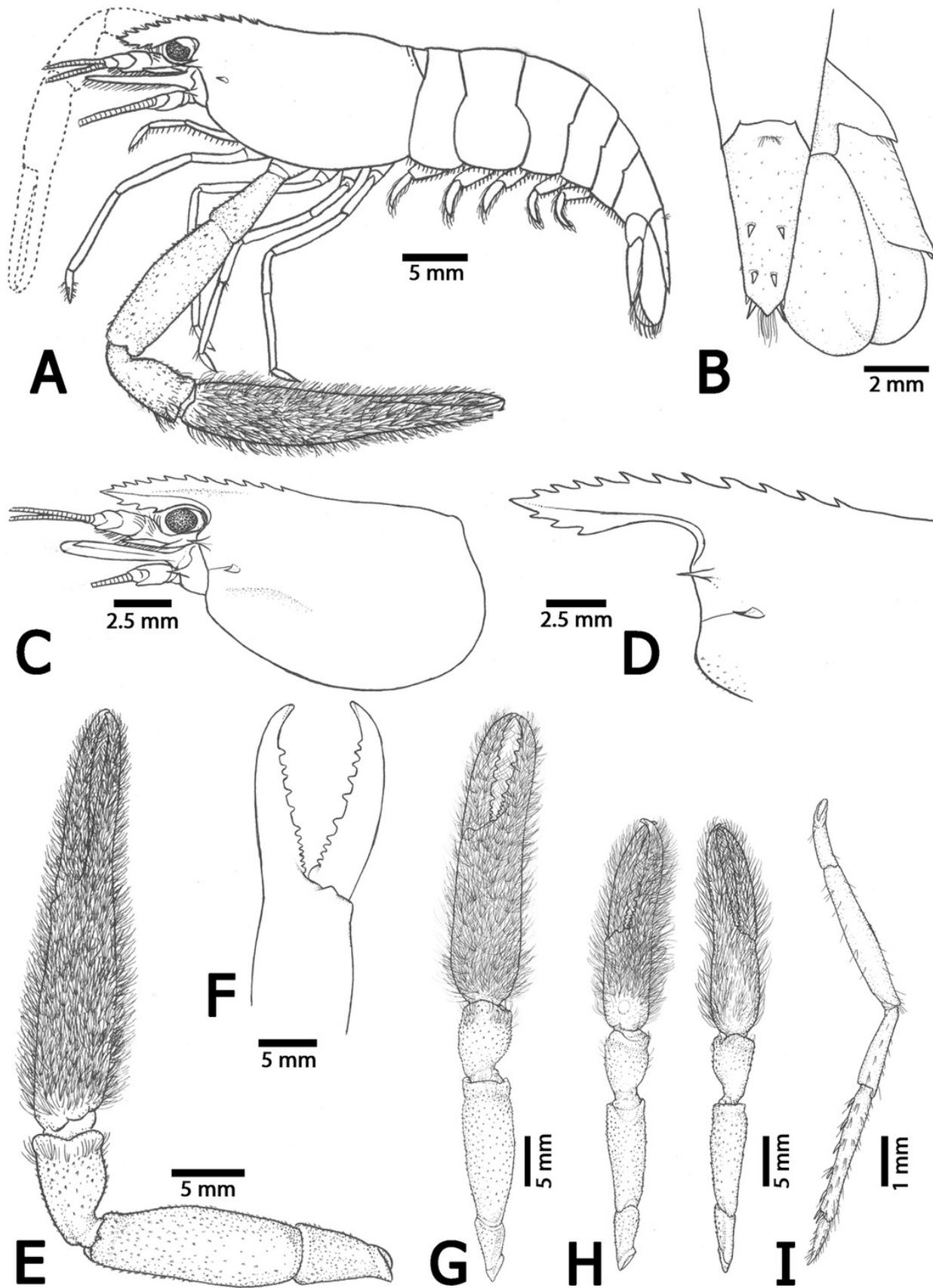
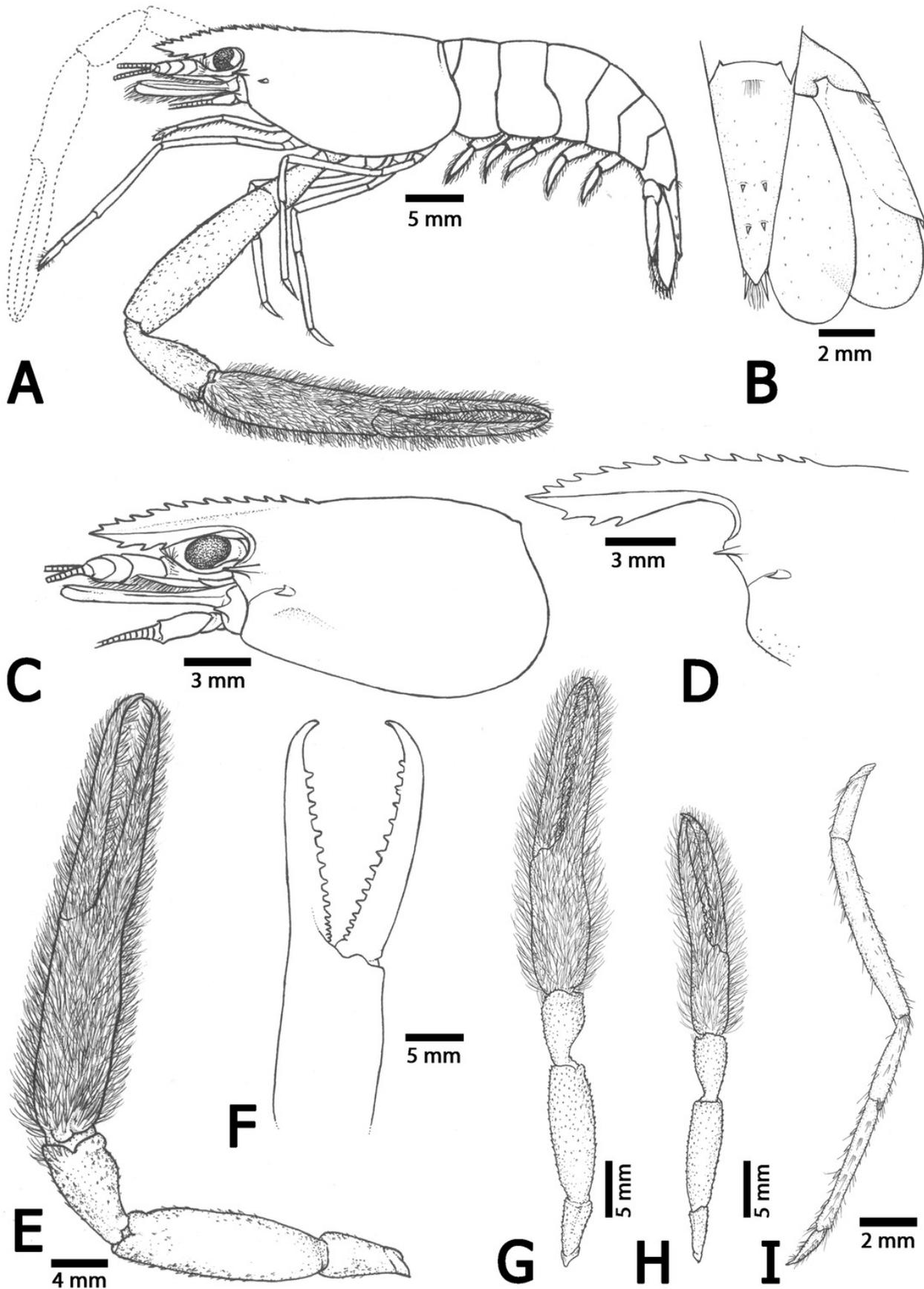



Figure 7

Morphological characters of *Macrobrachium puberimanus* sp. nov. (A-G, I from holotype, H from CUMZ MP00015).

(A) Lateral view (B) Uropods (C) Carapace (D) Rostrum form and teeth (E) Major second pereiopod (F) Teeth on finger of major second pereiopod (G) Major second pereiopod length (H) Second pereiopods in female (I) Third pereiopod

Locality with geographic coordinates and GenBank accession numbers for specimens used for molecular phylogenetic analyses.

1   **Table 1.** Locality with geographic coordinates and GenBank accession numbers for specimens used for
2   molecular phylogenetic analyses.

Taxon	CUMZ-Voucher ID	Locality	Coordinates	GenBank accession NO.		
				COI	16S	18S
Macrobrachium sirindhorn Naiyanetr, 2001	CUMZ MP00018-M009 CUMZ MP00019-M010	Namtok Nam Min, Mae Lao, Chiang Kham, Phayao	19°26'46.2"N 100°26'26.3"E	MT235929 MT235930	MT248221 MT248222	MT248181 MT248182
Macrobrachium dienbienphuense Dang and Nguyen, 1972	CUMZ MP00020-M016	Khek River, Wangthong, Phitsanulok	16°52'26.9"N 100°38'25.8"E	MT235932	MT248224	MT248184
	CUMZ MP00021-M027	Due Bridge, Yom River, Pong, Phayao	19°06'24.3"N 100°15'58.5"E	MT235934	MT248226	MT248186
	CUMZ MP00022-M054	Kaeng Lamduan, Dom Pradit, Nam Yuen, Ubon Ratchathani	14°26'46.2"N 105°07'16.3"E	MT235943	MT248235	MT248195
	CUMZ MP00023-M069	Hui Yang, Wang Sam Mo, Udon Thani	16°56'46.3"N 103°21'56.0"E	MT235945	MT248237	MT248197
	CUMZ MP00024-M084	Bueng Sam Phan, Phetchabun	15°49'58.5"N 101°02'07.3"E	MT235947	MT248239	MT248199
	CUMZ MP00025-M148	Dom Yai, Det Udom, Ubon Ratchathani	14°49'43.9"N 105°04'48.5"E	MT235963	MT248255	MT248215
Macrobrachium eriocheirum Dai, 1984	CUMZ MP00026-M050	Khao Sok National Park, Phanom, Surat Thani	8°54'47.2"N 98°31'28.2"E	MT235939	MT248231	MT248191
	CUMZ MP00027-M097 CUMZ MP00028-M098	Xishuangbanna, Yunnan, China	21°56'01.5"N 101°15'04.7"E	MT235948 MT235949	MT248240 MT248241	MT248200 MT248201
	CUMZ MP00029-M138	Kaeng Sopha, Wang Thong, Phitsanulok	16°52'37.7"N 100°38'28.1"E	MT235961	MT248253	MT248213
Macrobrachium forcipatum Ng, 1995	CUMZ MP00035-M130 CUMZ MP00036-M130A CUMZ MP00037-M130B	Kathu Waterfall, Kathu, Phuket	7°55'56.1"N 98°19'23.5"E	MT235956 MT235957 MT235958	MT248248 MT248249 MT248250	MT248208 MT248209 MT248210
Macrobrachium hirsutimanus (Tiwari, 1952)	CUMZ MP00030-M051	Petch Rimtarn Resort, Kaeng Krachan, Tayang, Phetchaburi	12°49'45.0"N 99°43'39.0"E	MT235940	MT248232	MT248192
	CUMZ MP00031-M052	Wang Ta Krai Waterfall, Hin Tung, Mueang, Nakhon Nayok	14°19'17.6"N 101°18'22.1"E	MT235941	MT248233	MT248193
	CUMZ MP00032-M053	Klong Soan Reservoir, Bo Rai, Trat	12°31'38.0"N 102°36'14.0"E	MT235942	MT248234	MT248194
	CUMZ MP00033-M083	Chomphu Bridge, Noen Maprang, Phitsanulok	16°41'32.1"N 100°40'15.2"E	MT235946	MT248238	MT248198
	CUMZ MP00034-M140	Hui Phra Prong, Kabin Buri, Prachin Buri	13°54'33.8"N 101°50'16.9"E	MT235962	MT248254	MT248214
Macrobrachium	CUMZ MP00038-M132	Roi Chan Phan	7°53'16.1"N	MT235959	MT248251	MT248211

malayanum (Roux, 1934)	CUMZ MP00039-M151 CUMZ MP00040-M152 CUMZ MP00041-M153	Wang Waterfall, Wang Wiset, Trang	99°19'54.4"E	MT235964 MT235965 MT235966	MT248256 MT248257 MT248258	MT248216 MT248217 MT248218
Macrobrachium naiyanetri sp. nov.	CUMZ MP00004-M102	Khao Banchob Waterfall, Makham, Chanthaburi	12°51'04.5"N 102°12'10.6"E	MT235951	MT248243	MT248203
	CUMZ MP00002-M127 CUMZ MP00001-M128 CUMZ MP00002-M154 CUMZ MP00002-M155	Hui Prik, Cha-wang, Nakhon Si Thammarat	8°35'41.2"N 99°27'55.6"E	MT235954 MT235955 MT235967 MT235968	MT248246 MT248247 MT248259 MT248260	MT248206 MT248207 MT248219 MT248220
Macrobrachium niphanae Shokita and Takeda, 1989	CUMZ MP00042-M023	Nam Ko, Lom Sak, Phetchabun	16°47'34.8"N 101°10'34.8"E	MT235933	MT248225	MT248185
Macrobrachium neglectum (De Man, 1905)	CUMZ MP00044-M060	Klong Chalung, Mueang, Satun	6°43'13.3"N 100°03'49.6"E	MT235944	MT248236	MT248196
Macrobrachium palmopilosum sp. nov.	CUMZ MP00010-M011	Mae Mang, Bo Kluea, Nan	19°08'12.7"N 101°09'01.2"E	MT235931	MT248223	MT248183
	CUMZ MP00009-M030	Sob-Pue, Sa-Iap, Song, Phrae	18°40'20.6"N 100°13'26.1"E	MT235935	MT248227	MT248187
	CUMZ MP00007-M031	Tat Man Waterfalls, Puea, Chiang Klang, Nan	19°17'11.9"N 100°47'20.0"E	MT235936	MT248228	MT248188
Macrobrachium puberimanus sp. nov.	CUMZ MP00015-M049	Nam Soam, Noan Thong, Na Yung, Udon Thani	18°00'30.5"N 102°14'42.8"E	MT235938	MT248230	MT248190
	CUMZ MP00012-M099	Wat Tha Khaek, Chiang Khan, Loei	17°54'17.7"N 101°40'58.4"E	MT235950	MT248242	MT248202
	CUMZ MP00014-M121	Phu Ruea, Loei	17°26'11.0"N 101°19'30.8"E	MT235953	MT248245	MT248205
Macrobrachium rosenbergii (De Man, 1879)	CUMZ MP00045-M115	Klong Phon Rang, Mueang, Ranong	9°53'12.5"N 98°38'00.6"E	MT235952	MT248244	MT248204
Macrobrachium sirindhorn Naiyanetr, 2001	CUMZ MP00018-M009 CUMZ MP00019-M010	Namtok Nam Min, Mae Lao, Chiang Kham, Phayao	19°26'46.2"N 100°26'26.3"E	MT235929 MT235930	MT248221 MT248222	MT248181 MT248182
Macrobrachium sintangense (De Man, 1898)	CUMZ MP00043-M038	Bang Ban, Phra Nakhon Si Ayutthaya	14°22'20.5"N 100°28'55.8"E	MT235937	MT248229	MT248189

Details of primers used in this study (F = Forward, R = Reverse)

1   **Table 2** Details of primers used in this study (F = Forward, R = Reverse)

Gene	Primer name	Sequence (5' to 3')	Reference
COI	LCO1490 (F)	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer et al. (1994)
	MacroNancy (R)	GCG GGT AGR ATT AAR ATR TAT ACT TC	This study
16S	16Sa-L (F)	CGC CTG TTT ATC AAA AAC AT	Palumbi (1996)
	16Sbr-H2 (R)	CTC CGG TTT GAA CTC AGA TCA	Palumbi (1996)
18S	18S-ai (F)	CCT GAG AAA CGG CTA CCA CAT C	DeSalle et al. (1992)
	18S-bi (R)	GAG TCT CGT TCG TTA TCG GA	Whiting et al. (1997)

Sequence annotation and DNA substitution model of each partial molecular marker used in this study

1 **Table 3.** Sequence annotation and DNA substitution model of each partial molecular marker
2 used in this study.

Molecular marker	Sequence length	Conservative site	Variable site	Parsimony-informative site	Substitution model for DNA evolution
COI	678	428	250	228	TIM2+I+G
16S	529	397	132	93	TPM3uf+G
18S	678	428	250	228	TIM1+I

Morphological comparison of three new species and the closely related species in the *M. pilimanus* group recorded from Thailand.

1 **Table 4.** Morphological comparison of three new species and the closely related species in the
2 *M. pilimanus* species group recorded from Thailand. '*' indicates data were retrieved from
3 original description and "?" were data deficiency.

Characters	Species						
	M. niyanetri **sp. nov**	*M. palmopilosum* **sp. nov**	*M. puberimanus* **sp. nov**	*M. amplimanus**	*M. dienbienphuense*	*M. hirsutimanus**	*M. eriochierum*
Rostrum teeth	8-14/2-4	10-12/2-3	12-15/3	9-12/2	8-14/1-3	10/2	10-13/2-3
Rostrum reaching end of antenular peduncle	Not reaching to the end	Not reaching to the end	Reaching to the end	Not reaching to the end	Reaching to the end	Not reaching to the end	Not reaching to the end
Spinule on margin of carapace	present	present	absent	present	present/absent	absent	absent?
Epistome	trilobed	bilobed	trilobed	trilobed	trilobed	bilobed	trilobed
Tuberculation/spine on palm surface of second pereiopods	absent	present	present	present?	present	absent	absent
Length of male second pereiopods	unequal	unequal	unequal	unequal	unequal	unequal	unequal
Segment of major second pereiopod	Fing.>Pal. Pal>Carp. Carp<Mer. Pal. =Mer.	Fing.<Pal. Pal.>Carp. Carp.<Mer. Pal. ≤Mer.	Fing.>Pal. Pal>Carp. Carp.<Mer. Pal. =Mer.	Fing.=Pal. Pal>Carp. Carp.<Mer. Pal. ≥ Mer.	Fing.>Pal. Pal>Carp. Carp.<Mer. Pal. ≥Mer.	Fing.<Pal. Pal≥Carp. Carp.<Mer. Pal. ≥Mer.	Fing. ≥Pal. Pal>Carp. Carp.<Mer. Pal. =Mer.
Carpus shape	Slightly elongate/cup	cup	elongate	cup	elongate	cup	cup
Teeth on dactylus (Dt) and pollex (Pt)	Dt:10-18 Pt:10-18	Dt:10-12 Pt:10-11	Dt:11-16 Pt:10-14	Dt:13 Pt:13	Dt:20-32 Pt:20-32	Dt:15 Pt:15	Dt:12-15 Pt:12-15
Gap in closed fingers	gapping	gapping	gapping	Not gapping	Not gapping	Slightly gapping	Slightly gapping
Moveable spine on uropodal diaraesis	Equally to outer angle	Shorter than outer angle	Shorter than outer angle	Shorter than outer angle	Shorter than outer angle	Shorter than outer angle	Shorter than outer angle

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