Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2010) **77**, 414–424
doi:10.1111/j.1095-8649.2010.02715.x, available online at www.interscience.wiley.com

Molecular and morphological evidence supports the species status of the Mahachai fighter *Betta* sp. Mahachai and reveals new species of *Betta* from Thailand

N. Sriwattanarothai*, D. Steinke†, P. Ruenwongsa*, R. Hanner† and B. Panijpan*‡

*Institute for Innovative Learning, Mahidol University, 999 Phuttamonthon 4 Road, Salaya, Nakhon Pathom 73170, Thailand and †Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, 50 Stone Road E, Guelph, Ontario, N1G2W1 Canada

(Received 15 September 2009, Accepted 17 May 2010)

Two regions of mitochondrial (mt) DNA, cytochrome *c* oxidase subunit 1 (COI) and 16S rRNA, were sequenced in nine species of *Betta* from Thailand and Indonesia. Most species showed little intraspecific COI variation (adjusted mean = 0·48%) including the putative species *Betta* sp. Mahachai, but one species (*Betta smaragdina*) included three lineages showing much greater divergence (7·03–13·48%) that probably represent overlooked species. These findings were confirmed by maximum likelihood analysis and Bayesian inference, which revealed well-supported corresponding monophyletic clades. Based on these results and morphological differences, the putative species *Betta* sp. Mahachai from central Thailand is a species distinct from other members of the *B. splendens* group and represents a new and hitherto undescribed species. Furthermore, this study also demonstrated the probable existence of two overlooked *Betta* species found in the Khorat plateau basin, illustrating the utility of mitochondrial genetic markers in the revelation of overlooked diversity.

Key words: *Betta smaragdina* var.; *Betta* sp. Mahachai; *Betta splendens complex*; cytochrome *c* oxidase subunit 1; DNA barcoding; 16S rRNA.

INTRODUCTION

The Siamese fighting fish *Betta splendens* Regan native of south-east Asia, has been raised for its fighting and ornamental attributes. Combating males, especially of the *B. splendens* group (Witte & Schmidt, 1992; Rüber *et al*., 2004), are selected for large, strong bodies with hard scales and small fins. Fish fighting is a native sport of Thailand and has been ongoing since recorded history (Na-Ayudhya, 2001). In contrast, an appreciation of body features, such as colour pattern, scale iridescence, body shape and fin size, has resulted in a worldwide flourishing market for *Betta* species.

‡Author to whom correspondence should be addressed. Tel.: +66 2 4419020 ext. 1301; fax: +66 2 4410479; email: scbpn@mahidol.ac.th

With currently 66 nominal species, the genus *Betta* (Osphronemidae, Macropodusinae) is the largest in the perciform suborder of the Anabantoidei (Froese & Pauly, 2006; Rüber *et al*., 2006), which is further divided into several major clades (Britz, 2001; Rüber *et al*., 2004). One of these clades, the *B. splendens* group, builds bubble nests, the dominant reproductive style and plesiomorphic condition among osphronemid taxa (Tan & Kottelat, 1998; Britz & Cambray, 2001). Members of this group are *B. splendens*, *Betta smaragdina* Ladiges, *Betta imbellis* Ladiges, *Betta stiktos* Tan & Ng and an undescribed *Betta* species collected in Samut Sakhon and henceforth referred to as *Betta* sp. Mahachai. In Thailand, four members of this group occur in different areas. *Betta smaragdina* is found on the Khorat Plateau in the northeastern part of the country, while *B. imbellis* lives in the acidic swamps of southern Thailand. *Betta* sp. Mahachai has been found in coastal swamps of Samut Sakhon, Samut Songkhram and Samut Prakan (central Thailand), which exhibit more neutral (pH 7·8) but saline conditions. *Betta splendens*, with a distribution across Thailand, represents an exception (Goldstein, 2004), perhaps reinforced by the introduction of farm and aquarium-raised animals to the wild.

It has been argued that *Betta* sp. Mahachai is a hybrid of *B. splendens* and *B. imbellis* or a hybrid of wild and domesticated *B. splendens* (Griffin, 2005). No other wild *Betta* species, however, seem to be able to survive in such an inhospitable environment. *Betta* sp. Mahachai is only found in tidal areas, subjected to a daily influx of saltwater. There is no record indicating that the *Betta* sp. Mahachai and *B. splendens* occur sympatrically. *Betta* sp. Mahachai is found in brackish water, while *B. splendens* prefers fresh water. Due to these specific water chemistry requirements and its restricted occurrence, *Betta* sp. Mahachai is especially endangered by recent human activities, such as urbanization and industrial development (Griffin, 2005; Monvises *et al*., 2009) potentially leading to extinction of wild populations before the species has been confirmed and formally described.

Several mitochondrial (mt) DNA marker systems have been used for species discrimination (Hwang & Kim, 1999; Hebert *et al*., 2003). A fragment of the cytochrome *c* oxidase subunit 1 gene (COI) has been extensively studied in fishes as an effective fragment of DNA-barcoding-based species identification (Ward *et al*., 2005; Hubert *et al*., 2008). Other studies report on the utility of barcoding to test species boundaries and to highlight potentially overlooked species (Smith *et al*., 2008; Ward *et al*., 2008; Steinke *et al*., 2009).

This study aims to examine molecular and morphological evidence to verify the validity of *Betta* sp. Mahachai, currently a subject of speculation about its taxonomic status. Two regions of mtDNA (COI and 16S rRNA), as well as morphometric and meristic data, were utilized. Furthermore, this study also examined sequence data from the same mitochondrial genes to highlight potential cryptic species of another member of the *B. splendens* group, *B. smaragdina* of various regions of Thailand.

MATERIALS AND METHODS

SAMPLE COLLECTION

This study examined 264 individuals representing three nominal species of the *B. splendens* group (*B. splendens*, *B. smaragdina* and *B. imbellis*) and specimens of *Betta* sp. Mahachai from Thailand as well as six other species (*Betta ocellata* de Beaufort, *Betta uberis* Tan

& Ng, *Betta albimarginata* Kottelat & Ng, *Betta falx* Tan & Kottelat, *Betta anabantoides* Bleeker, *Betta fusca* Regan) representing different *Betta* complexes according to Rüber *et al.* (2004). Specimens of *B. stiktos* were not included in the study because those are very rare and occur only in Cambodia. The samples (264 individuals) were collected in 2007–2008 across Thailand (Fig. 1): wild *B. splendens* are from the Phayao Province; domesticated (crown-tail, double-tail, delta-tail, veil-tail and short-finned) *B. splendens* from the pet trade in Bangkok; wild *B. smaragdina* were obtained in Kalasin, Khon Kaen, Nong Khai, Udon Thani and Nakhon Ratchasima (type locality); wild *B. imbellis* were from the Songkla Province and wild and stocked *Betta* sp. Mahachai have been collected in Samut Sakhon. The remaining 14 fish from the sister *Betta* groups were collected in Indonesia from East Kalimantan (*B. ocellata*), Kalimantan (*B. uberis*), Sebuku (*B. albimarginata*), Jambi (*B. falx*), Tangkiling (*B. anabantoides*) and Sumatra (*B. fusca*). Croaking gouramies *Trichopsis vittata* Cuvier from the Lampang Province, Thailand, were used as out-group in this study. The identification of

FIG. 1. Collection sites in Thailand and Indonesia for *Betta* sp. specimens examined in this study. Further details on the collection location for each specimen including GPS coordinates are provided in '*Betta* of Thailand' in the Published Projects section of the Barcode of Life Data System (BOLD, www.barcodinglife.org). A, *Betta splendens*; B, *Betta imbellis*; C, *Betta smaragdina*; D, *Betta* sp. Mahachai; E, *Betta ocellata*; F, *Betta uberis*; G, *Betta albimarginata*; H, *Betta falx*; I, *Betta anabantoides*; J, *Betta fusca*; K, *Trichopsis vittata*.

individuals in this study followed the taxonomic keys by Tan & Ng (2005). All specimens are stored as vouchers in the National Science Museum, Thailand. Collection details are recorded in the public project file '*Betta* of Thailand' on www.barcodinglife.org.

DNA ANALYSIS

DNA was extracted from the muscle tissue of each specimen using an automated glass fibre protocol (Ivanova *et al*., 2006). The bar-code regions of COI and the 16S rRNA regions were subsequently amplified under the following identical thermal conditions: 2 min at 95° C; 35 cycles of 0·5 min at 94° C, 0·5 min at 52° C and 1 min at 72° C; 10 min at 72° C; held at 4° C. The 12·5 μl PCR reaction mixes included 6·25 μl of 10% trehalose, 2·00 μl of ultrapure water, 1·25 μl ×10 PCR buffer [200 mM Tris–HCl (pH 8·4), 500 mM KCl], 0·625 μl $MgCl_2$ (50 mM), 0·125 μl of each primer cocktail [0·01 mM, using primer cocktails C_FishF1t1/C_FishR1t1 from Ivanova *et al*. (2007) and 16Sar-5′–16Sbr-3′ from Palumbi (1996)], 0·0625 μl of each dNTP (10 mM), 0·0625 μl of platinum taq polymerase (Invitrogen; www.invitrogen.com) and 2·00 μl of DNA template. PCR amplicons were visualized on a 1·2% agarose gel E-Gel (Invitrogen) and bidirectionally sequenced using sequencing primers M13F or M13R (Ivanova *et al*., 2007) and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.; www.appliedbiosystems.com) on an ABI 3730 capillary sequencer following manufacturer's instructions. Bidirectional sequences were assembled in SEQUENCHER version 4.5 (Gene Codes Corporation; www.genecodes.com) and manually edited.

COI sequence data were submitted to the Barcode of Life Data system (BOLD, http://www.barcodinglife.org; Ratnasingham & Hebert, 2007) and both COI and 16S data to GenBank. Specimen and collection data, sequences, specimen images and trace files are provided in the project '*Betta* of Thailand' in BOLD.

PHYLOGENETIC ANALYSIS

A Kimura 2-parameter (K2P) distance metric was used for sequence comparisons (Kimura, 1980), and genetic distances and initial neighbour-joining (NJ) clustering used the BOLD management and analysis system. Maximum likelihood and Bayesian analyses were performed as multi-gene alignment including both sequence fragments. The total length of the concatenated data set was 1174 base pairs (bp; 652 bp COI and 522 bp 16S rRNA). Partitioned maximum likelihood analyses were performed with RAxML v.7.0.4 (Stamatakis, 2006). All searches were completed with the GTRMIX option and bootstraps were calculated with 1000 replicates. Partitioned Bayesian analyses were conducted using Mr Bayes (Ronquist & Huelsenbeck, 2003). Two independent Markov-chain Monte-Carlo analyses were run for each parameter set using a $GTR + \Gamma$ model estimated by Model Generator (Keane *et al*., 2006). Initial runs as well as a posterior inspection of the likelihoods in the final run showed that a burn-in phase of 10 000 generations was largely sufficient for the likelihood values to reach convergence. Each chain was run for 10 million generations and sampled every 100th generation. A majority consensus tree, rooted with *T. vittata* as out-group, was computed from the sampled trees, excluding the trees sampled in the burn-in phase.

MORPHOMETRIC AND MERISTIC ANALYSIS

Five random representatives of each of four *Betta* species (*B. splendens*, *B. smaragdina*, *B. imbellis* and *Betta* sp. Mahachai) were freshly caught and fixed in 10% formalin solution (Tan & Ng, 2005). After 1–2 weeks, the fixed specimens were leached with water followed by prolonged treatment in 95% ethanol. Morphometric measurements followed Witte & Schmidt (1992) and meristic counts were done according to Schindler & Schmidt (2006). All measurements and counts were taken as straight lines between two landmarks, which represent a modification of the cited methods. Measurements were taken from point to point from the left side of the specimens with a vernier calliper allowing readings to the nearest 0·02 mm.

RESULTS

PHYLOGENETIC INFERENCE

Unambiguously aligned sequences were obtained for 652 positions of COI and 522 positions of 16S rRNA from the same tissue samples of 264 *Betta* spp. Four hundred and ten nucleotide sites were variable and 347 were parsimoniously informative in the total data set. For the COI bar-code region, the mean K2P sequence distance between congeneric species (11·89%) was *c*. 8·5-fold higher than within-species variation (1·4%). For 16S, the distance was *c*. 10-fold higher (between congeners 5·13% and within species 0·5%). Both maximum likelihood and Bayesian phylogenetic analyses produced trees with similar topologies supported by high bootstrap values and high posterior probabilities (Fig. 2).

The reconstructed phylogeny strongly supports the monophyly of the *B. splendens* group by 100% of bootstrap replicates and a posterior probability of 100. This clade splits into six monophyletic groups representing *B. splendens*, *Betta* sp. Mahachai, *B. imbellis* and three distinct clades identified as *B. smaragdina*. *Betta imbellis* appears basal to the sister taxon *B. splendens*–*Betta* sp. Mahachai, although these relationships are only represented by high posterior probabilities and lack sufficient bootstrap support. Specimens identified as *B. smaragdina* appear to be paraphyletic with specimens from the type locality (Nakhon Ratchasima) clearly distinct from all other fishes collected in Thailand (within group COI K2P distance 13·05%). While one of these clades [*Betta* sp. (cf. *smaragdina*) 1 – collected in Nongkhai] seems more closely related to the *B. splendens*–*Betta* sp. Mahachai/*B. imbellis* group, the others [*Betta* sp. (cf. *smaragdina*) 2 – collected in Udon Thani, Kalasin and Khon Kaen] forms a sister group with *B. smaragdina* specimens from the type locality. Re-analysis of mean COI K2P distances treating the three clades as distinct species decreased the overall within-species variation to 0·48%, a value much closer to those reported in barcoding studies on fishes.

Wild and stocked *Betta* sp. Mahachai appear to be monophyletic. The same is true for wild and domesticated (crown-tail, double-tail, delta-tail, veil-tail and short-finned) *B. splendens*.

MERISTICS AND MORPHOMETRIC DATA

Meristic and morphometric data from five representatives of wild *B. splendens*, *B. imbellis*, *B. smaragdina* and *Betta* sp. Mahachai, respectively, are shown in Table I.

Two characters, the number of rays in the dorsal fin and the number of lateral scales below the anal fin origin, distinguished specimens of *Betta* sp. Mahachai from other species. In contrast to the molecular data, however, the remaining meristic data as well as the standardized morphometric results overlapped considerably and were within the range of species variability.

DISCUSSION

The limited morphological divergence among some *Betta* species has made identification difficult for field workers and taxonomists alike (Tan & Tan, 1996). In contrast, COI sequence divergence between congeneric taxa in this study was

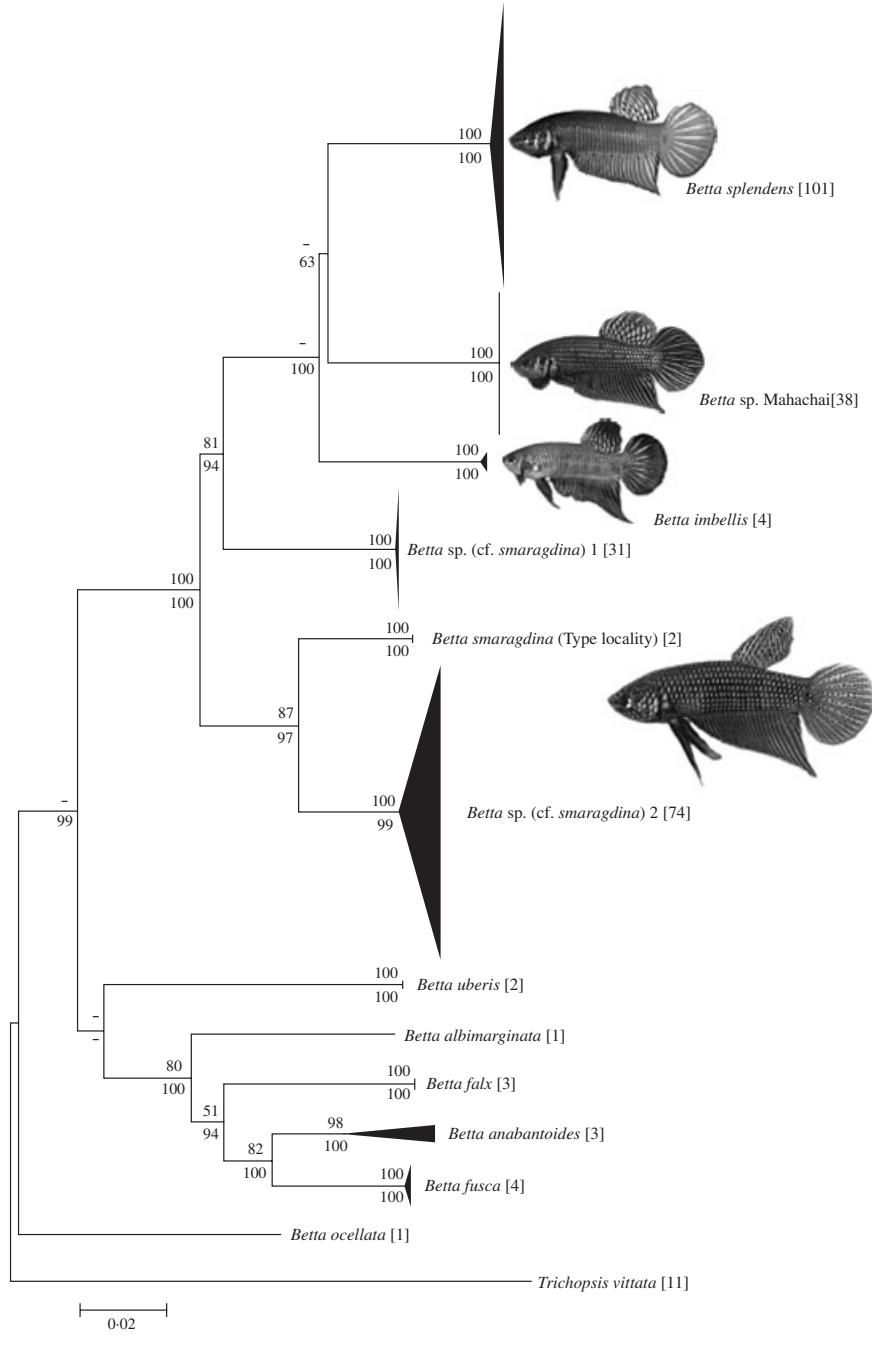

FIG. 2. Phylogenetic tree based on a combined data set of COI and 16S with a total of 1174 nucleotide sites using both maximum likelihood (ML) and Bayesian inference. Species and clade names are followed by the number of specimens analysed in square brackets. Upper values at each tree node represent ML bootstrap values (1000 replicates) and lower values represent posterior Bayesian probabilities. Bootstrap values below 60 are not shown. The tree has been rooted using *T. vittata*. Specimen images are of live males of the *B. splendens* group. Relative sizes are given in Table I.

TABLE I. Comparison of meristic and morphometric data of *B. splendens*, *B. imbellis*, *B. smaragdina* and *Betta* sp. Mahachai

	B. splendens	*B. imbellis*	*B. smaragdina*	*B.* sp Mahachai
No of specimens	5	5	5	5
Meristics (mode)				
Anal fin rays	II, 24	II, 23	II, 27	II, 24
Dorsal fin rays	I, 7	I, 7	I, 7	I, 9
Pectoral fin rays	11	10	11	11
Subdorsal scales	7	7	6	7
Transverse scales	9	9	9	9
Lateral scales	28	29	29	27
Predorsal scales	22	17	19	22
Postdorsal scales	9	11	11	9
Lateral scales below dorsal-fin origin	16	16	16	16
Lateral scales below anal-fin origin	4	6	4	5
Morphometrics in $\%L_S$				
Total length	133·78–137·94	126·96–135·22	118·81–130·69	123·66–133·96
Predorsal length	62·79–69·80	60·83–64·72	53·88–62·12	63·09–66·88
Postdorsal length	13·11–21·75	15·14–25·78	15·85–27·84	19·08–24·48
Preanal length	37·42–66·50	25·61–41·58	39·85–46·20	42·75–47·46
Head length	16·27–19·05	22·33–28·44	21·82–26·32	25·78–30·32
Body depth	25·71–28·66	23·94–27·19	18·98–25·38	23·94–30·66
Caudal peduncle length	16·27–19·05	16·05–18·69	12·69–17·17	15·71–18·66
Pelvic fin length	26·84–42·86	41·15–56·73	17·24–34·19	35·41–41·70
Anal-fin base length	55·29–62·68	55·33–65·38	44·30–59·23	48·20–54·62
Dorsal-fin base length	12·05–15·63	14·72–17·58	8·46–13·22	14·36–23·01
Pectoral-fin length	11·49–16·71	11·59–19·00	9·49–20·54	17·38–19·50
($\%$ L_H)				
Orbit diameter	28·87–32·64	26·68–29·35	25·57–35·93	26·80–30·29
Postorbital length	45·57–54·71	44·47–50·25	45·14–64·07	51·44–60·59
Interorbital length	8·86–11·26	7·46–11·06	5·75–17·24	9·05–15·40
Snout length	16·77–20·45	16·67–21·04	6·71–16·78	12·06–22·94

L_H, head length; L_S, standard length.

typically high, averaging 11·89%. Conversely, within-species variation for most taxa was very low (adjusted mean = 0·48%), matching the lowest levels of conspecific variation reported in barcoding studies on fishes (Ward *et al*., 2005; Hubert *et al*., 2008; Steinke *et al*., 2009; Zemlak *et al*., 2009). This allows their separation from any other taxon included in this study (or any of the other 7240 fish species on BOLD).

Based on the phylogenetic results (Fig. 2) and the sequence divergences observed among the species included in this study (16S: 5·13%; COI: 11·89%), it was concluded that the specimens collected from Samut Sakhon and provisionally assigned

to *Betta* sp. Mahachai are indeed distinct from other species of the *B. splendens* group (Britz, 2001; Rüber *et al*., 2004) that occur in Thailand and represent a new and undescribed species that should be officially referred to as *Betta* sp. Mahachai, until it is formally described and named.

Differences in the number of dorsal fin rays and the number of lateral scales below the anal fin origin distinguish specimens from *Betta* sp. Mahachai (Table I). These counts of five specimens of each species provide meristic evidence that supports the conclusions from the DNA sequence data. Further morphometric measurements, however, overlapped considerably and were within the range of species variability (Table I).

It has been argued that *Betta* sp. Mahachai is a hybrid of *B. splendens* and *B. imbellis* or a hybrid of wild and domesticated *B. splendens* (Griffin, 2005; Monvises *et al*., 2009). Although both 16S and COI are maternally inherited and alone cannot be used to detect hybrids, the high divergence between *B. splendens*, *B. smaragdina* and *Betta* sp. Mahachai with no evidence for shared haplotypes, as well as the high sequence similarity in both markers (>99%) among wild and domesticated members of *B. splendens* suggests that *Betta* sp. Mahachai is not a hybrid of *B. splendens*, *B. smaragdina* or even *B. imbellis*, which is in contrast to previous hypotheses (Panitvong, 2002). This is congruent with specific habitat conditions under which these animals occur, such as specific pH value and water type (brackish swamp). *Betta* sp. Mahachai is only found in tidal areas, subjected to a daily influx of saltwater (Griffin, 2005). Furthermore, the fish live commensally with Nipa Palms *Nypa fruticans* building their bubble nests in the palm branch pockets (Panitvong, 2002). Single males together with two or more females can be found in those pockets during spawning season (Panitvong, 2002). Furthermore, males are less aggressive than *B. splendens* even during courtship (Griffin, 2005).

Cases of deep genetic divergence in mitochondrial genes within single species often indicate overlooked cryptic species (Moritz, 1994; Meyer & Paulay, 2005). *Betta smaragdina* qualifies as one such example in the current data set as it displayed three cohesive clusters separated by a deep average intraspecific divergence (13·05%) with one clade [*B*. sp. (cf. *smaragdina*) 1] grouping more closely with *B. splendens*–*B. imbellis*–*Betta* sp. Mahachai than with its presumed conspecifics.

A second clade consists of specimens exclusively collected at the type locality of *B. smaragdina* (Nakhon Ratchasima, Khorat) and therefore probably represents the true species, as described by Ladiges (1972). Members of its sister group, here referred to as *Betta* sp. (cf. *smaragdina*) 2, group monophyletically with high support values.

While these groups probably represent overlooked species, they might alternatively reflect deep phylogeographic variants linked to female philopatry. In this case, divergence at nuclear markers should be minimal or absent between the groups. Although this possibility still needs testing, Zink and Barrowclough (2008) found that genetic structure at mitochondrial loci was rarely contradicted by nuclear markers. Moreover, while it was unable to find any meristic or morphometric differences between specimens of the three groups (Table I), there are some behavioural observations for one clade that might indicate the presence of a cryptic species. In comparison with other species of the *B. splendens* group, *Betta* sp. (cf. *smaragdina*) 1 exhibits a different aggressive display. Males show scissoring movements of the pectoral (subopercular paired) fins when they display to other males (N.S., pers. obs.). This

scissoring movement of the fins is somewhat similar to the movement of the human hand when playing the guitar and the variety is recognized and named locally as guitar, short for guitar playing. This type of flickering movement has been observed before (Simpson, 1968), albeit in supposedly domesticated *B. splendens*.

A possible reason for this unexpected diversity among *B. smaragdina* specimens might be the geological history of the Khorat Plateau basin with several uplift and fault-slip events since the Tertiary (Smith & Stokes, 1997), leaving considerable salt deposits in the soil. Various *Betta* species in this region probably live in habitats with different saline concentrations, which would require specific adaptations.

It was shown that although *Betta* sp. Mahachai can interbreed with *B. splendens* in captivity with proper recognition of behavioural clues (Panitvong, 2002), it is genetically and morphologically different enough from *B. splendens* and *B. smaragdina* to qualify as a new species. Clearly these results have implications for the conservation and management of *Betta* sp. Mahachai in the coastal swamps of central Thailand in close proximity to the rapidly growing city of Bangkok. Urbanization and rapid industrial development will potentially lead to the extinction of this unrecognized species.

This study also highlights the likelihood of two overlooked *Betta* species in the Khorat Plateau basin, illustrating the utility of mitochondrial genetic markers, especially the DNA barcoding gene COI, in the revelation of overlooked diversity. The *B. splendens* group (Britz, 2001; Rüber *et al*., 2004), originally proposed with four members, seems to consist of at least seven species.

We would like to sincerely thank O. Lucanus for providing fighting fish specimens as well as A. Phumchoosri for sharing the original photographs and wild fish collections. We also gratefully thank P. D. N. Hebert and the staff at the Biodiversity Institute of Ontario especially J. Hortie, C. Steinke, J.-B. Zhang and R. Turner for their support. This study was funded by the National Research Council of Thailand (NRCT) and the National Sciences and Engineering Research Council of Canada (NSERC) and by Genome Canada through the Ontario Genomics Institute. This manuscript was greatly improved by the constructive comments of two anonymous reviewers.

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