

Phylogeography of *Betta* and *Trichopsis* (Perciformes: Osphronemidae) Fighting Fishes in Thailand and Neighboring Countries

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ABSTRACT: Wild fighting fish species in the genera *Betta* and *Trichopsis* occupy freshwater habitats in Southeast Asian countries. The former's paternal care for the fertilized eggs and hatchlings differ: one type, the nest-builders, uses a bubble nest built by the male for protection of the eggs and the hatchlings, whereas the male of the other type puts the eggs and the young in its mouth. The latter share the same habitats with bubble-nester *Betta*, with similar morphology and behavioral patterns. A previous study showed that during their speciation, one type of paternal care could be succeeded by the other. Our in-depth study generally agrees with the overall conclusion of the above study but with some differences in ancestry. By fixing the first speciation event among the *Betta* to about 22 million years (Ma—megaannus) before present (BP), we found that the bubble nesters in Thailand appeared around 14 Ma BP, while the northeastern variety emerged about 7 Ma BP. The three recognized *Trichopsis* species separated from each other approximately 5–6 Ma BP. In addition, attempts were made to address the fish evolution in the context of present geography and more recent geological events.

KEY WORDS: *Betta*, *Trichopsis*, fighting fish, croaking gourami, phylogeography, Thailand, divergence time, descendant sequence likelihood

INTRODUCTION

Wild fighting fish of the genus *Betta* Bleeker, 1849 are native to a number of Southeast Asian countries such as Brunei, Cambodia, Indonesia, Laos, Malaysia, Thailand, and Vietnam (Panijpan *et al.*, 2017) (see Fig. 1). Species members can be divided into 2 groups according to paternal care of the fertilized eggs and newly hatched fry. The male of the bubble-nest building group builds a raft of bubbles for both the eggs and the fry to attach for oxygen and security until the fry

start swimming freely. The male of the mouthbrooding group cares for the fertilized eggs and fry by keeping them in the mouth until the fry become free swimmers (Rüber *et al.*, 2004).

At present, there are about 75 nominal species in the genus *Betta*; of these, 18 are bubble-nesters officially named so far in Southeast Asia (Panijpan *et al.*, 2017). The five bubble-nester species in Thailand occupy habitats in the northern, central-plain, west, and upper southern regions (*B. splendens* Regan,

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Figure 1. The geographical map of Southeast Asian countries wherein *Betta* and *Trichopsis* fishes in this study inhabit. Adapted from <http://www.freeworldmaps.net/asia/southeastasia/southeastasia-hd-map.jpg>

1910); northeastern region (*B. smaragdina* Ladiges, 1972); lower southern region (*B. imbellis* Ladiges, 1975); eastern region (*B. siamorientalis* Kowasupat, Panijpan, Ruenwongsa & Jeenthong, 2012); and west of Bangkok (*B. mahachaiensis* Kowasupat, Panijpan, Ruenwongsa & Sriwattanothai, 2012) (Panijpan *et al.*, 2017). Most of the mouthbrooding species inhabit locations in the southern peninsula of Thailand and further south around the equator in Malaysia and Indonesia (Panijpan *et al.*, 2017). Only *Betta prima* Kottelat, 1994, a brooder, inhabits some eastern provinces of Thailand and Southern Vietnam (Panijpan *et al.*, 2017).

Wild croaking gourami of the genus *Trichopsis* Canestrini, 1860 are morphologically similar to the *Betta* fighting fish and usually share the same habitats with the nest-building *Betta*; these gourami also build bubble nests and display courting and aggressive behavior (Panijpan *et al.*, 2015). There are three morphologically valid species of *Trichopsis*, namely *T. vittata* (Cuvier, 1831), *T. pumila* (Arnold, 1936), and *T. schalleri* Ladiges, 1962; all of which can generate loud croaks relative to their small sizes. Only in Thailand have all the three (not just one or two) species been found (Panijpan *et al.*, 2015).

Rüber *et al.* (2004, 2006) used DNA sequencing (based on mitochondrial cytochrome b, tRNA Val, 12s rRNA, 16s rRNA, and nuclear RAG1 genes) and analysis of a wide range of genetically related fishes, especially the *Betta* fishes, and found that ancestrally brooding and bubble-nesting paternal care can succeed each other throughout the calculated evolutionary timeline. They, however, did not report the habitat locations of the fishes studied.

Here we report our in-depth study based on the barcode region of the mitochondrial gene cytochrome c oxidase subunit I (COI) to

show ancestral relationship of *Betta* and *Trichopsis* species in Thailand and neighboring countries. With data on the present fish habitat locations and available geological data, we also try to put the evolutionary timeline of the two labyrinth fishes in the context of present-day geography and recent geological events, especially changes in the sea level.

MATERIALS AND METHODS

Gene Choice

There are currently two sets of genes of the fish in the genera *Betta* and *Trichopsis* in GenBank that somewhat comprehensively represent the two congeneric species. The first set, from Rüber *et al.*'s (2004) study, consists of three gene regions: the mitochondrial cytochrome b and 12S rRNA + tRNA Val + 16S rRNA, and nuclear RAG1 genes. The genes were extracted from 32 specimens of 31 taxa. Rüber *et al.*, however, did not mention the habitat locations of the fishes in their GenBank DNA deposits. The other set is from numerous studies (Sriwattanothai *et al.*, 2010; Kowasupat *et al.*, 2012a, 2012b, 2014; Panijpan *et al.*, 2014, 2015), all of which contain the barcode region of the mitochondrial cytochrome c oxidase subunit I (COI) in their analyses. These studies involved several hundred specimens caught mostly in the wild from all over Thailand and locations in neighboring countries. Since we focus on the origins and geographical evolution of the two genera of loosely related fishes in Thailand, COI was selected for phylogeographical analysis.

Sequence Selection

Panijpan *et al.* (2014) provided the most extensive phylogeny of fighting fish based on the COI gene. Based on that phylogeny and the localities of the specimens, all the clearly

defined clades that either included a wildy caught taxon from Thailand or had an unambiguous basal or sister relationship with such a clade were considered. The clades with ambiguous relationship were excluded to facilitate the inference of ancestral sequences which were required to estimate the likelihoods of extant sequences. All the complete barcode regions (652 base pairs) of COI sequences of the wild fighting fish in those clades were obtained from GenBank. The sequences of all the fish in the same taxon were aligned to discern the most geographically prevalent sequence for that

taxon which was then selected as its representative COI sequence. If there were other sequences of the same taxon from different localities and with more than a few base-pair differences from the prevalent one, those sequences were also included in the analysis. On the contrary, we excluded the taxa whose sequences were too similar to that of an already included taxon. A similar selection procedure was also performed for the COI sequences of the fish in the genus *Trichopsis* wherein all the clades were considered. The selected sequences and their accession numbers are listed in Table 1.

Table 1. The COI sequences used in this study. For abbreviations, see Table 2 and Fig. 2

Specimens	Accession #
<i>B. macrostoma</i>	KM485437
<i>B. bellica</i> ID	KM485311
<i>B. bellica</i> MY	KM485408
<i>B. imbellis</i>	JQ818781
<i>B. siamorientalis</i>	JQ818698
<i>B. splendens</i>	GQ911737
<i>B. mahachaiensis</i>	GQ911746
<i>B. sp.</i> (cf. <i>smaragdina</i>) 1	GQ911777
<i>B. sp.</i> (cf. <i>smaragdina</i>) 4	KF278842
<i>B. sp.</i> (cf. <i>smaragdina</i>) 3	JQ818739
<i>B. smaragdina</i> KBn	GQ911838
<i>B. smaragdina</i> KBs	JQ818641
<i>B. stiktos</i>	KF278825
<i>B. ideii</i>	KM485316
<i>B. patoti</i>	KM485315
<i>B. ocellata</i>	KM485405
<i>B. compuncta</i>	KM485317
<i>B. unimaculata</i>	KM485312
<i>B. rubra</i>	KM485320
<i>B. persephone</i>	KM485407
<i>B. tussyae</i>	KM485462

Table 1. (Continued).

Specimens	Accession #
<i>B. coccina</i>	KM485461
<i>B. livida</i>	KM485460
<i>B. pallida</i>	KM485364
<i>B. prima</i>	KM485335
<i>B. sp. (cf. prima)</i>	KM485415
<i>B. chloropharynx</i>	KM485404
<i>B. hipposideros</i>	KM485457
<i>B. waseri</i>	KM485458
<i>B. pi</i>	KM485353
<i>B. pulchra</i>	KM485448
<i>B. sp. (cf. pulchra)</i>	KM485447
<i>B. sp. (cf. pugnax)</i>	KM485443
<i>B. pugnax</i>	KM485337
<i>B. stigmosa</i>	KM485451
<i>B. ferox</i>	KM485338
<i>B. sp. (cf. ferox)</i>	KM485390
<i>B. simplex</i>	KM485322
<i>B. sp. (cf. simplex)</i>	KM485431
<i>T. pumila</i> MK CP E TS	KP200384
<i>T. pumila</i> Chanthaburi	KP200379
<i>T. sp. (cf. schalleri)</i> 1	KP200437
<i>T. sp. (cf. schalleri)</i> 2	KP200497
<i>T. sp. (cf. schalleri)</i> 3	KP200371
<i>T. schalleri</i> KB	KP200391
<i>T. schalleri</i> Surin Ubon	KP200406
<i>T. sp. (cf. vittata)</i> 1	KP200396
<i>T. sp. (cf. vittata)</i> 2	KP200420
<i>T. sp. (cf. vittata)</i> 3	KP200372
<i>T. sp. (cf. vittata)</i> 4	KP200414
<i>T. vittata</i>	KU692944

Reconstruction of a Clock Tree

Since there are numerous steps in the

reconstruction of a dated evolutionary tree and they vary according to the results up to a certain point in the reconstruction, this

section depicts a general guideline for Bayesian inference using MrBayes version 3.2.3 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist *et al.*, 2012). First, the best model for a non-clock tree should be identified because a clock tree analysis is much more computationally intensive than a non-clock one and, from our experience, the posterior distributions of the parameters in both kinds of analyses are similar. The two most important model choices are the partitioning of the data and the nucleotide substitution model for each partition. To get general information about them, a codon position-based partitioned Bayesian inference should be performed either with the general time reversible (GTR) model or by sampling across all the time-reversible models, referred to as mixed models (Huelsenbeck *et al.*, 2004). The sampled parameters, especially the substitution rates, should reveal whether other partitioning and/or other substitution models should be used. For example, if the sampled parameters for the first and second positions of codons are similar but distinct from those for the third position of codons, the first two codon positions should be partitioned together. Likewise, if all the transition rates are similar yet different from the transversion ones, Kimura's (1980) or Hasegawa, Kishino, and Yano's (1985) model should be used. If more than one model is likely, the marginal model likelihood for each model should be estimated using the stepping-stone sampling (SS) algorithm (Xie *et al.*, 2011) and the one with the highest model likelihood should be selected. The likelihoods for the GTR and mixed models should always be compared unless GTR is clearly a better choice.

Once the proper partitioning and nucleotide substitution models are identified, we should try to find the rates at which different sites vary. Usually, this can be done by estimating 2 parameters: the proportion of invariable

sites (+I) and the shape parameter of the distribution of the rates of the remaining sites (+G). These parameters are correlated (changing one parameter would greatly affect the other) and the latter is much more influential than the former. If their posterior distributions are sampled, their impacts could be gauged by examining the sample. For the parameter +G, a high value (say, double digits) would indicate that it should not be included in the model. As for +I, a high value would be preferred. For both parameters, a flat distribution would be a sign of a low-impact parameter. Again, the SS algorithm should be used to decide borderline cases. The usual choices for state frequencies—equal versus estimated frequencies—are in general no better than empirical frequencies when their model likelihoods are compared.

After the suitable model for a non-clock tree is identified, the model of the clock for each partition should be investigated while forcing the branch lengths of all the partitions to be the same. MrBayes provides five tree models and four clock models for analyses. A tree model should be selected before proceeding to a clock model. For our purpose, only the uniform branch length and the birth-death tree models should be compared. As for the clock models, each model should be tried for each partition and the SS algorithm should be used to select the best model. To complicate matters, the default priors for a clock model may result in a diverging Markov chain Monte Carlo run. Thus, the likelihoods and the posterior distributions should be investigated to ensure that each run converges; otherwise, the priors need to be modified. The last step in the reconstruction is to calibrate the tree. According to Rüber *et al.* (2006), the only fossil record of labyrinth fish that could be dated was that of an ancestor of the genus *Osphronemus* Lacépède, 1801, not an ancestor of fishes in the two genera. The time of the first speciation in the genus

Betta inferred using the fossil and DNA sequences was about 22 million years (Ma—megaannus) before present (BP) and that in the genus *Trichopsis* was about 7 Ma BP. The former was used to calibrate our tree due to its lower percentage estimation error. To gain some insight into the evolutionary rate of COI in fighting fish, we elected to use rate dating instead of node dating. That is, the clock rate was calibrated so that the first divergence time within the genus *Betta* in the inferred tree was about 22 Ma BP.

COI Sequence Likelihood Estimation

When reading a phylogenetic tree, without a conclusive fossil record or knowledge of geological changes, one cannot determine the likely origin of any clade. In particular, one cannot infer that the sole extant species that is sister to all the rest is the basal species in the sense of having evolved first or others evolving from it. Couple that with incomplete sampling of extant species, the tree contains no such information whatsoever. Since we were interested in the geographical pattern of evolution of different groups of fishes in both genera, being able to identify the likely origin of a clade would be a good starting point.

We began by separately inferring the ancestral COI sequence of each clade in the tree. Then the likelihood of obtaining each extant COI sequence given its ancestral state was estimated based on the DNA evolution model (base frequencies, rate multiplier, and nucleotide substitution matrix of each partition) obtained from the reconstruction of the clock tree. In particular, if any partition exhibited rate variation among sites, the rate of each site in that partition was also inferred and used in sequence likelihood estimation.

The COI sequence likelihood estimation employed all the information involved in the

inference of the tree. Since the estimation was based on a shared DNA evolution model, the sequence likelihoods could be interpreted as being obtained under hypothetical common environmental condition. Thus, comparing sequence likelihoods of sister taxa should be one of the better ways to indicate probable degrees of adaptation given the limited information. That is, a taxon with a more likely sequence probably went through less adaptation than that with a less likely one. In other words, the geographical origin of a clade was probably near and similar to the habitat of the taxon with the highest sequence likelihood.

To facilitate the upcoming discussion, the name of a species may be used to refer to the ancestor of that species or the concestor of that species and its “cf.” species right after speciation. In addition, a species name followed by the word ancestor may signify the most recent common ancestor (MRCA) of a clade containing that species, also right after speciation. For example, *B. ferox* Schindler & Schmidt, 2006 ancestor may refer to the MRCA of the clade that includes *B. ferox*, *B. stigmosa* Tan & Ng, 2005, and *B. simplex* Kottelat, 1994. For clarification of the meaning of a species name or one followed by the word ancestor, an inferred divergence time in Fig. 2 should be matched to the context being discussed. For example, *B. splendens* ancestor would refer to the MRCA of all the nest-builders in Thailand if the speciation time was around 7 Ma BP while it would refer to the MRCA of *B. splendens/mahachaiensis* clade if the speciation time was about 3 Ma BP.

RESULTS

Phylogeny

The number of COI sequences selected for the phylogenetic tree inference was 51,

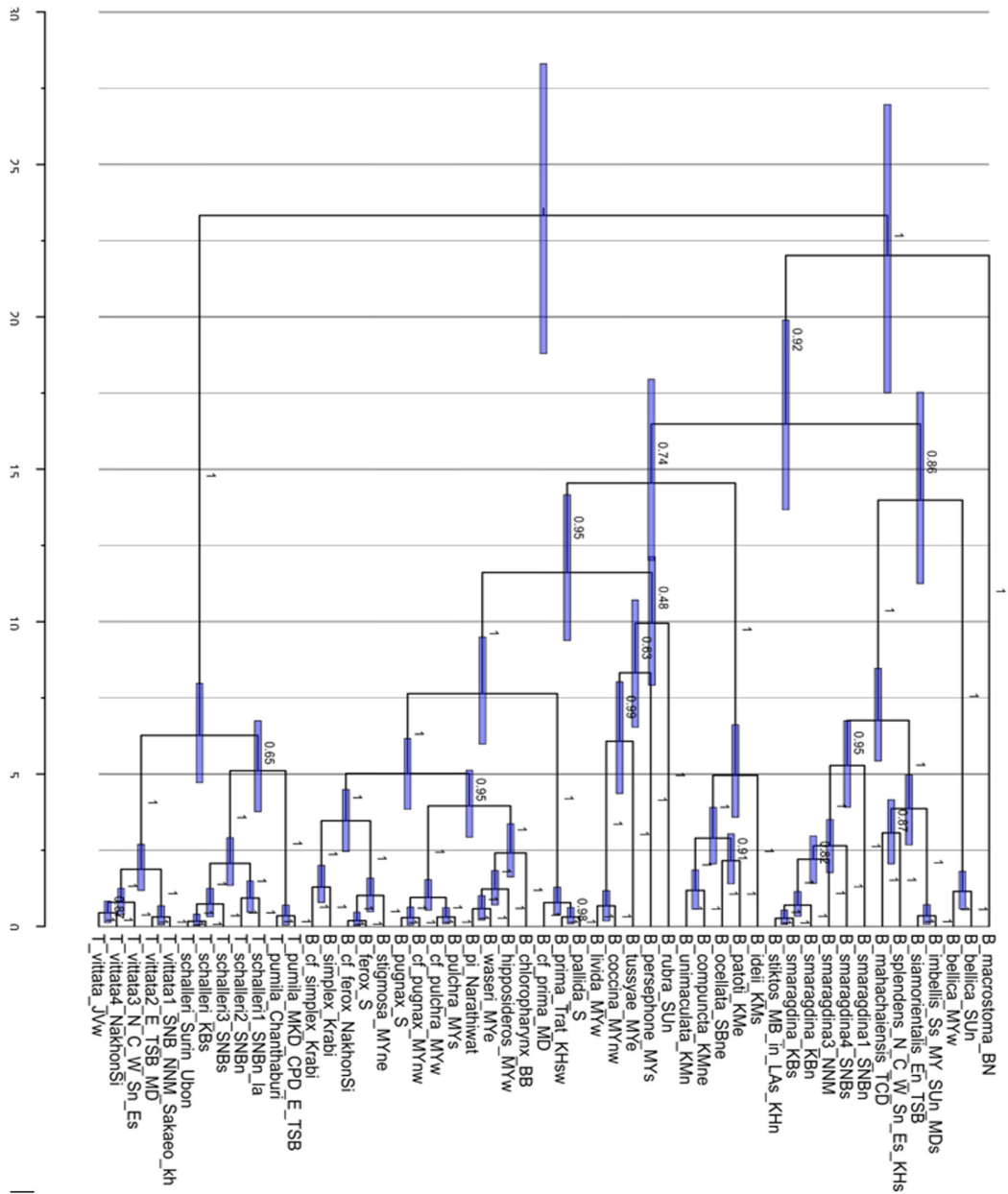


Figure 2. The rooted phylogenetic tree of the fishes in the genera *Betta* and *Trichopsis* reconstructed using their barcode region of the mitochondrial COI gene. The time scale at the bottom represents Ma BP. The clock rate was calibrated so that the last concestor of *Betta* lives around 22 Ma BP. A node bar indicates the 95% highest posterior density interval of the divergence time estimate. The number above a branch is the posterior probability of the corresponding clade. The number is omitted if the posterior probability is 1. The abbreviations for the locations of the taxa are shown in Table.

representing 39 *Betta* and 12 *Trichopsis* taxa. Unconstrained reconstruction yielded a tree that placed the *Trichopsis* clade as a sister of a *Betta* clade containing 26 individual taxa, wherein *B. unimaculata* (Popta, 1905) species group was the most basal (not necessary the most ancient). This was probably due to fast evolving mtDNA which resulted in genetic saturation at the third positions of COI codons, while the first and second positions did not evolve enough to completely resolve the saturation. Using Rüber *et al.*'s (2004) set of genes which contained two longer segments of mtDNA and a relatively slowly evolving nuclear gene would eliminate this problem but would not cover the species of our interest. So, we had to force the *Trichopsis* taxa to be outside the *Betta* clade. Another unexpected problem was a small yet significant discrepancy between the trees reconstructed with and without reporting site rates. It seemed that requiring MrBayes to report site rates somehow interfered with its normal inference. Thus, the tree inferred without reporting site rates was the one reported in this study and the inferred site rates were proportionally adjusted so that the mean rate of each partition was the same as the rate multiplier estimated without reporting site rates.

The reconstructed phylogenetic tree in Fig. 2 is mostly consistent with those in Rüber *et al.*'s (2004) and Panijpan *et al.*'s (2014) studies with a few notable discrepancies. (For the meanings of the abbreviations in the three, see Table 2). Firstly, the relationship among *B. macrostoma* Regan, 1910, *B. bellica* Sauvage, 1884, *B. splendens* species group, *B. unimaculata* species group, and the remaining *Betta* contains a feature from both previous studies. Similar only to Rüber *et al.*'s, *B. bellica* (actually *B. simorum* Tan & Ng, 1996 from the same species group) is sister to *B. splendens* species group. Yet similar only to Panijpan *et al.*'s, *B. macrostoma*

is sister to all other *Betta*. (*B. unimaculata* species group played such a role in Rüber *et al.*'s). Next, the mouth brooder *B. rubra* Perugia, 1893 is sister to the bubble nesters in *B. coccina* Vierke, 1979 species group, but with low posterior probabilities (0.48–0.63), while it was inside *B. coccina* species group in Panijpan *et al.*'s. These discrepancies from Panijpan *et al.*'s are the results of inferring a clock tree instead of a non-clock one. (The non-clock tree [not shown] concurs with Panijpan *et al.*'s). Even though some of the posterior probabilities are not high, these relationships seem to be more logical than those in Panijpan *et al.*'s, indicating that a clock tree may yield a superior phylogeny compared to a non-clock tree. Lastly, the *B. pugnax* (Cantor, 1849) / *pulchra* Tan & Tan, 1996 group is a sister of *B. waseri* Krummenacher, 1986 species group instead of being a subgroup within the sister group of the latter as in Rüber *et al.*'s. Among the cryptic species of *T. vittata*, *T. sp.* (cf. *vittata*) 4 is closest to *T. vittata* in Java.

Clock Rate

The clock rate that matches the divergence time of the *Betta* clade to that in Rüber *et al.*'s (2006) study is 0.02 nucleotide substitutions per Ma. This rate is equivalent to the divergence rate of 0.04 nucleotides per million years which is twice the widely quoted universal animal mitochondrial DNA sequence divergence rate (Brown *et al.* 1979).

Divergence Time Estimates

The estimated divergence times of selected clades in Fig. 2 are shown in Table 3. By calibrating the clock rate so that the first divergence time estimate within the genus *Betta* coincides with the one in Rüber *et al.*'s (2006) study, the estimated confident

Table 2. The abbreviations for the geographical distributions/locations of the fishes in Fig. 2. The name of each taxon in Fig. 2 begins with an abbreviation for its genus (B for *Betta* or T for *Trichopsis*). Next comes its species name whose number ending indicates a potentially new species as suggested by the original research. The last part of each name refers to the location(s) that the fish were caught or the known geographical distribution of the taxon. Most locations (separated by an underscore) begin with one or two capital letters whose meaning appears in this table, optionally followed by a capital letter (B for basin, D for delta, or M for river mouth), and possibly ends in one or two lowercase letters indicating the relative position within the preceding location (the usual abbreviations for north, east, south, and west). Several Thailand province names, probably shortened, are used as locations. Two abbreviations for countries in the table appear in lower case (la and kh) to refer to only the parts of those countries neighboring their preceding locations. The location of *B. stiktos* (MB_in_LAs_KHn) is a special case and serves as an example of abbreviations: M stands for Mekong River, B for basin, LAs for the south of Laos, and KHn for the north of Cambodia. However, “in” is just a normal preposition. Thus the location is Mekong River basin in the south of Laos and north of Cambodia.

Thailand Region/ Province		Other Country/ Region/Province		River	
Abb.	Full name	Abb.	Full name	Abb.	Full name
C	Central	BB	Bangka-Belitung	CP	Chao Phraya
E	East		Islands	M	Mekong
K	Khorat	BN	Brunei	MK	Mae Klong
N	North	JV	Java	NN	Nam Ngum
S	South	KH	Cambodia	TC	Tha Chin
SN	Sakon Nakhon	KM	Kalimantan	TS	Tonlé Sap
W	West	LA	Laos		
		MY	Peninsular Malaysia		
		SB	Sabah		
		SU	Sumatra		

Table 3. The divergence time estimates in Ma BP of selected clades in Fig. 2 and their 95% highest posterior density (HPD) intervals.

Clade containing (see Fig. 2)	Median	95% HPD interval
<i>B. macrostoma</i> – <i>B. simplex</i>	22.02	17.51–26.97
<i>B. bellica</i> – <i>B. simplex</i>	16.49	13.67–19.90
<i>B. bellica</i> – <i>B. stiktos</i>	13.99	11.25–17.53
<i>B. imbellis</i> – <i>B. stiktos</i>	6.76	5.43–8.47
<i>B. imbellis</i> – <i>B. mahachaiensis</i>	3.86	2.68–4.97

Table 3. (Continued).

Clade containing (see Fig. 2)	Median	95% HPD interval
<i>B. imbellis</i> – <i>B. siamorientalis</i>	0.35	0.08–0.72
<i>B. splendens</i> – <i>B. mahachaiensis</i>	3.07	2.05–4.16
<i>B. sp.</i> (cf. <i>smaragdina</i>) 1– <i>B. stiktos</i>	5.28	3.91–6.75
<i>B. sp.</i> (cf. <i>smaragdina</i>) 3– <i>B. stiktos</i>	2.20	1.41–2.97
<i>B. smaragdina</i> KBs– <i>B. stiktos</i>	0.27	0.07–0.54
<i>B. ideii</i> – <i>B. simplex</i>	14.55	12.01–17.95
<i>B. rubra</i> – <i>B. simplex</i>	11.61	9.38–14.16
<i>B. rubra</i> – <i>B. livida</i>	9.95	7.91–12.13
<i>B. pallida</i> – <i>B. sp.</i> (cf. <i>prima</i>)	0.78	0.39–1.29
<i>B. pallida</i> – <i>B. prima</i>	0.31	0.08–0.62
<i>B. chloropharynx</i> – <i>B. pi</i>	2.41	1.62–3.37
<i>B. waseri</i> – <i>B. pi</i>	0.58	0.21–1.01
<i>B. pulchra</i> – <i>B. pugnax</i>	0.95	0.54–1.53
<i>B. stigmosa</i> – <i>B. ferox</i>	1.01	0.48–1.59
<i>B. simplex</i> – <i>B. sp.</i> (cf. <i>simplex</i>)	1.30	0.79–2.00
<i>T. pumila</i> – <i>T. vittata</i>	6.27	4.72–7.97
<i>T. pumila</i> – <i>T. schalleri</i>	5.11	3.77–6.75
<i>T. sp.</i> (cf. <i>schalleri</i>) 1– <i>T. schalleri</i>	2.07	1.35–2.91
<i>T. sp.</i> (cf. <i>schalleri</i>) 3– <i>T. schalleri</i>	0.73	0.32–1.25
<i>T. sp.</i> (cf. <i>vittata</i>) 1– <i>T. vittata</i>	1.88	1.18–2.69
<i>T. sp.</i> (cf. <i>vittata</i>) 4– <i>T. vittata</i>	0.45	0.13–0.83

interval also almost perfectly matches the corresponding interval. As for the first divergence time within the genus *Trichopsis*, the estimate is about 10% shorter than that in Rüber *et al.*'s; nonetheless, *T. pumila* and *T. schalleri* seemed to speciate from *T. vittata* at about the same time in both studies. It seems that using only a short segment of COI yields estimates that are in good agreement with those obtained using cytochrome b and 12S rRNA + tRNA Val + 16S rRNA. This might be due to the fact that Rüber *et al.* did not partition the mitochondrial DNA sequences while estimating divergence times; they tested

whether to partition into cytochrome b versus RNAs, but did not test whether to partition cytochrome b according to its codon positions.

Very early in the Aquitanian age (~22 Ma BP), *Betta* speciated into a clade that evolved into *B. macrostoma* and another that became the remaining *Betta*. Toward the end of the Burdigalian age (~16.5 Ma BP), the latter speciated to become the ancestor of bubble nesters (*B. bellica* and *B. splendens* ancestor) and the ancestor of mostly mouth brooders (remaining groups). The bubble nesters

separated into *B. bellica* and *B. splendens* ancestor at the end of the Langhian age (~14 Ma BP). Not until the Messinian age (~6.8 Ma BP) did the latter start to speciate into a clade on the Khorat Plateau (red shading in Fig. 1) (*B. smaragdina*) and another, the remaining bubble nesters in Thailand, on the lowlands. By the late Zanclean age (~3.9 Ma BP), the lowland clade further speciated northward (*B. splendens* and *B. mahachaiensis*) and southward (*B. imbellis* and *B. siamorientalis*) with the hypothetical Isthmus of Kra Seaway as a geographical barrier. Interestingly, *B. mahachaiensis* which inhabit a very small niche of brackish water near the shoreline to the south of Bangkok today evolved relatively shortly afterward (~3.1 Ma BP). For as long as they have developed a preference for brackish water, *B. mahachaiensis* must have been through several cycles of changes in sea level and moved northward and southward with the seashore.

During the late Langhian age (~14.5 Ma BP), the clade that consisted of mostly mouth brooders (*B. ideii* Tan & Ng, 2006 – *B. simplex* in Table 3) speciated to become the ancestor of mouth brooders on Borneo (*B. unimaculata* species group) and another *Betta* on Thai-Malay Peninsula, Sumatra and Java. Bubble nesting parental care resurfaced again as a clade separated to live on the Peninsular Malaysia (*B. coccina* species group) and Sumatra (*B. rubra*) around the beginning of the Tortonian age (~10 Ma BP). A very similar looking group of mouth brooders separated in the early Gelasian age (~2.4 Ma BP); again, one group would live on the mainland (*B. waseri* ancestor) while another on the Bangka-Belitung Islands (*B. chloropharynx* Kottelat & Ng, 1994). Surprisingly, two nearby (< 50 km apart) populations of small mouth brooders *B. simplex* separated quite a long time ago, early in the Calabrian age (~1.3 Ma BP).

Trichopsis has a much more recent history. The first speciation occurred in the Messinian age (~6.3 Ma BP). Both resulting clades managed to inhabit the highlands of the Khorat Plateau and the lowlands of central Thailand. Their timings, however, were quite different. *T. pumila* (a lower-land species) and *T. schalleri* (a higher-land one) separated at the beginning of the Zanclean age (~5.1 Ma BP) while the first separation within *T. vittata* complex happened at the end of the Gelasian age (~1.9 Ma BP).

Sequence Likelihood

Reading the tree of the *Betta* fishes in Fig. 2, one would usually infer that *B. macrostoma* is basal to all other clades. However, COI sequence likelihood estimates told us that *B. waseri* species group had the highest mean sequence likelihood given the inferred ancestral sequence of all *Betta* even though the group seems to be a derived one. In fact, the group always possessed the highest mean sequence likelihood no matter which node ancestral to it was used. Thus, one had to be extremely careful when making a conclusion concerning basal-derived relationship from a phylogenetic tree. By the same token, *B. splendens* species group had consistently and substantially higher mean sequence likelihood than that of *B. bellica*. However, within the former, relative sequence likelihoods would change as the ancestral node changes: *B. sp.* (cf. *smaragdina*) 4 seemed to have gone through the least amount of adaptation from older ancestors while *B. imbellis* and *B. siamorientalis* seemed to be most closely related to the group's most recent ancestor. This pattern of switching relative sequence likelihoods was rather common. Thus, from now on, only the conclusive relative sequence likelihoods are reported.

Betta splendens, the most widespread bubble nester in Thailand, consistently had higher

sequence likelihood than that of *B. mahachaiensis*, a highly specialized species. For *B. smaragdina* species group, next to *B. sp.* (cf. *smaragdina*) 4, *B. sp.* (cf. *smaragdina*) 1 and *B. sp.* (cf. *smaragdina*) 3 had consistently higher mean sequence likelihoods than those of *B. smaragdina* and *B. stiktos* Tan & Ng, 2005. In addition, the northern variety of *B. smaragdina* seemed to have conclusively gone through less adaptation than the southern variety and *B. stiktos*. As for the two varieties of *B. bellica*, the one on the island seemed to be more similar to their common ancestors.

The only conclusive result within *B. unimaculata* species group was that *B. ideii* had the lowest sequence likelihood. For *B. coccina* species group (nest-builders), *B. rubra* (a brooder) included, *B. persephone* Schaller, 1986 had gone through the least amount of adaptation while *B. tussya* Schaller, 1985 had gone through the most. For *B. prima/pallida* Schindler & Schmidt, 2004 clade, *B. sp.* (cf. *prima*), the variation found in Vietnam, had the highest sequence likelihood, followed by *B. prima* and then *B. pallida*. Within *B. waseri* species group, the sequence likelihoods in descending order belonged to *B. waseri*, *B. hipposideros* Ng & Kottelat, 1994, and *B. pi* (Tan, 1998) while within *B. pugnax/pulchra* clade, the order was *B. sp.* (cf. *pugnax*), *B. sp.* (cf. *pulchra*), *B. pugnax*, and *B. pulchra*. For the last clade of *Betta*, the order seemed to be *B. ferox*, *B. sp.* (cf. *ferox*), *B. stigmata*, *B. simplex*, and *B. sp.* (cf. *simplex*).

Among *Trichopsis*, *T. vittata* complex had the highest sequence likelihood, followed by *T. schalleri* complex and *T. pumila*. For *T. vittata* complex, *T. sp.* (cf. *vittata*) 2 had the highest sequence likelihood, followed by *T. sp.* (cf. *vittata*) 1, *T. sp.* (cf. *vittata*) 4, *T. sp.* (cf. *vittata*) 3, and *T. vittata* respectively. For *T. schalleri* complex, the order was *T. sp.* (cf.

schalleri) 3, *T. schalleri*, *T. sp.* (cf. *schalleri*) 2, and *T. sp.* (cf. *schalleri*) 1, with the more widespread variety of *T. schalleri* being more similar to the ancestor than the other variety. A similar conclusion was reached for the two varieties of *T. pumila*.

DISCUSSION

Southeast Asia is one of the most biodiversified regions in the world (see, for example, de Bryun *et al.*, 2013). This has been the result of two main driving forces: tectonic plate activities and sea level variations. With regard to the two genera in this study, the former was more influential during their early speciation while the latter was more significant recently. The results in the previous section will be discussed in light of these changes.

The MRCA of all fighting fish (resembling fish in *B. waseri* species group, a brooder) probably lived around the present-day Peninsular Malaysia on the Sunda Shelf during the late Chattian age to early Aquitanian age (~23 Ma BP). Around that time, the Gulf of Thailand and Malay Basin (middle and south of the gulf, upper blue shading in Fig. 1) had already started forming (beginning ~32 Ma BP) as a result of the rifting among Indochina, Shino-Thai-Burma (SBT), and Sundaland blocks (Lee and Lawver, 1995). However, the Gulf of Thailand was still in the process of forming, with seawater in the Natuna Basin (lower blue shading in Fig. 1) and brackish water in the Malay Basin (Shoup *et al.*, 2012). On the other side (to the west) of Peninsular Malaysia, the Andaman Sea had probably just arisen from the thermal maximum at the end of the Chattian age (Shoup *et al.*, 2012) and the newly formed sea might transgress the central peninsula near the Isthmus of Kra as the sea level rose more than 100 m above the current level (Woodruff, 2003).

Around 22 Ma BP, a speciation event occurred: *B. macrostoma* separated from the remaining population. This event coincided with the one of the major changes in the rate and angle of convergence between the India and Eurasia plates. Prior to that, the India plate had been moving northward toward the Eurasia plate, more or less, at the rate of about 60 mm/yr (Lee and Lawver, 1995). Right around the speciation event, the India plate started to decelerate and turn clockwise. Within about 2 Ma, the India plate had slowed down to about 45 mm/yr and turned about 20 degrees. The slowdown perhaps resulted from collisions against higher-than-before frictional resistance from the Eurasia plate which, coupled with clockwise turning, would produce more significant impact upon the smaller plates east of the India plate. One such impact was the extrusion of the SBT and Indochina blocks to the east which, due to the subduction of the South China Sea, formed the central highland of Vietnam and tilted the Khorat Plateau westward (Rainboth *et al.*, 2012).

The sea level was high throughout the Aquitanian and Burdigalian ages (~23–16 Ma BP). During the latter, seawater transgressed the Malay Basin (Shoup *et al.*, 2012) before suddenly dropping by more than 50 m at the end of the age (Woodruff, 2003). This drop in sea level coincided with the speciation event that gave rise to most of today's nest-builders (~16.5 Ma BP). The seawater regression, which lasted about 100 thousand years (ka—kiloannus), might exposed low land areas with patches of shallow stagnant freshwater suitable for building bubble nests. Hence, given that an ancestor of *Betta* might be a bubble nester (Rüber *et al.*, 2004), some of the fighting fish might revert to the old parental care behavior and occupy this niche before migrating northward to the central plain of Thailand. After the regression, seawater transgressed the Gulf of Thailand

Basin (Shoup *et al.*, 2012). About 1 Ma after that, there was another 100-ka regression that might provide an opportunity for brooders to disperse to Borneo and for nest-builders to disperse to the other side of the peninsula along the river systems in the partially exposed Gulf of Thailand and Malacca Strait (Voris, 2000). The former would evolve to become *B. unimaculata* species group (and others not included in this study) while the latter to become *B. bellica*.

The sea level dropped three times during the Serravallian age (~14–11 Ma BP) and by the early Tortonian age (~10 Ma BP), it was about 90 m below the current sea level (Woodruff, 2003). At about the same time, the most recent major change in the angle of convergence between the India and Eurasia plates occurred as the former turned northward again, lessening the compression upon the smaller plates in Southeast Asia (Lee and Lawver, 1995). There were two speciation events involving *B. rubra* during this period. First, after the second drop in the sea level (~12.8 Ma BP) which might again provide more suitable habitats for nest-builders, *B. persephone* ancestor (nest-builders but for *B. rubra*) separated from other brooders. Then, during the last regression period (~11 Ma BP) where most of the Sunda Shelf was exposed, a part of the former probably dispersed into Sumatra via the river system along the Malacca Strait and evolved to become *B. rubra* while a part of the latter probably dispersed across the Gulf of Thailand to become *B. sp.* (cf. *prima*). This regression period might also facilitate the dispersal of *B. splendens* ancestor and across the gulf toward the central plain of Thailand and the lowland south of the Khorat Plateau. If the evolution of nest-building behavior in *B. persephone* ancestor happened before the speciation of *B. rubra*; this would be the only time after the speciation of and within the genus *Betta* that the parental-care switching

favored mouthbrooding.

The sea level rose again to finally fill the Gulf of Thailand. The sea level fluctuated between 20 m above to 30 m below the current sea level for about 5 Ma (Woodruff, 2003) during which the Gulf of Thailand stayed submerged (Shoup *et al.*, 2012; Voris, 2000). The nest-builders on the central plain of Thailand probably dispersed up the Chao Phraya River System. A major tributary of Chao Phraya is the Pa Sak River which probably joined the Loei River during that period, connecting Chao Phraya to the Mekong River System (Rainboth *et al.*, 2012). In addition, the Miocene Mun and Chi Rivers probably joined the Chao Phraya River System (the Pa Sak and Loei Rivers included) too, connecting the central plain of Thailand with the Khorat Plateau, as the western margin of the plateau had yet to rise (Rainboth *et al.*, 2012). Thus, the nest-builders could easily disperse onto the plateau. The speciation event that separated *B. smaragdina* from *B. splendens* ancestor (~6.8 Ma BP) coincided with the sharp rise in the sea level to about 40 m above the current sea level in the early Messinian age (Woodruff, 2003) which might have submerged not only the central plain of Thailand (Sathiamurthy and Voris, 2006) but also the low-elevation Miocene Khorat Basin, separating those on the Sakon Nakhon Basin from the remaining nest builders.

The first *Trichopsis* to appear was similar to *T. vittata*, some of which probably inhabited the lowland south of the Khorat Plateau around 7 Ma BP. At that time, the uplift of the southern margin of the plateau had yet to happen (Rainboth *et al.*, 2012). Thus *T. vittata* was free to migrate onto the plateau. The aforementioned rise in the sea level might have also separated *T. vittata* into two groups: the original group probably took refuge on the Cardamom Mountains while the migrating group (*T. schalleri* ancestor)

lived on the Sakon Nakhon Basin.

The sea level dropped and then rose sharply in the next 1 Ma. By about 5.5 Ma BP, the sea level was at its highest level, about 100 m above the current sea level (Woodruff, 2003). Then, about 5 Ma BP, the rate of convergence between the North Luzon Arc and the South China block dropped by half, indicating that the two micro-plates had collided (Lee and Lawver, 1995). One or both geological changes might have triggered any of the four speciation events in this study: *B. sp.* (cf. *smaragdina*) 1 from the rest of *B. smaragdina*, *B. ideii* from *B. unimaculata* ancestor, *B. ferox* ancestor from *B. waseri* ancestor, and *T. pumila* from *T. schalleri*. For the last speciation, a group of *T. schalleri* might have dispersed down the Mekong River System to the lowland south of the Khorat Plateau during the drop in the sea level and was later separated from the rest by the following rise in the sea level to finally become *T. pumila*.

The sea level was very high until about 4.2 Ma BP when it dropped again by more than 100 m (~3.5 Ma BP) which might cause the speciation of *B. splendens* ancestor from *B. imbellis* ancestor, *B. pugnax* ancestor from *B. waseri* ancestor, and *B. simplex* from *B. ferox* ancestor. This sharp drop in the late Zanclean age probably provided a higher variety of habitats for the nest-builders and facilitated their dispersal along the Chao Phraya River System. The next sharp drop to about 100 m below the current sea level, exposing the Gulf of Thailand (Voris, 2000), probably enabled the speciation of *B. mahachaiensis* (~3.1 Ma BP), which preferred brackish water.

Interestingly *B. mahachaiensis*, now endemic to a very small area of brackish water, probably migrated along with the movement of the mouth of the Chao Phraya River System for close to 3 Ma during which the sea

level fluctuated periodically according to 4 short (23–400 ka), concurrent glacioeustatic fluctuation cycles (Webb and Bartlein, 1992). Frequent sea level fluctuations probably allowed *B. imbellis* in the east and west of the Gulf of Thailand to contribute to the same gene pool, more so than that between *B. imbellis* and *B. siamorientalis* which were partially separated by the Cardamom Mountains. The admixture of genes from *B. prima* in East Thailand and contiguous Cambodia and *B. pallida* in the southern peninsula of Thailand was also due to these fluctuations. Again, the genes of *B. prima* seemed to be separated from those of nearby *B. sp. (cf. prima)* in Southern Vietnam more than from those of *B. pallida* across the Gulf of Thailand. Another possible cause of the separation between *B. prima* and *B. sp. (cf. prima)* was a big impact event somewhere on the Khorat Basin around 800 ka BP (Rainboth *et al.*, 2012). The wild fluctuations in the sea level were also responsible for a majority of the speciation events in this study as the sea level changes were the results of climate changes.

Even though the India Plate had been colliding into the Eurasia Plate for about 55 Ma (Lee and Lawver, 1995), the elevation of the western rim of the Khorat Plateau was still very low (Rainboth *et al.*, 2012). The rapid uplift of the western margin of the plateau began about 3 Ma BP, tilting the plateau eastward and reversing the flow of the rivers on the plateau in the process. As a result, the Mekong, Mun, and Chi Rivers significantly changed their courses. In the early stages of the uplift, the flow of rivers along the western and southern margins of the plateau gradually slowed down. By about 1.5 Ma BP, there was no longer any water flowing through the gaps along the two margins (Rainboth *et al.*, 2012), effectively separating the nest-builders on the plateau from those on the central plain. The rapid

and constant changes in the environment provided ample opportunities for the fish to evolve and occupy new niches.

As mentioned earlier, the ancestors of *B. smaragdina* species group and *T. schalleri* probably took refuge on the Sakon Nakhon Basin about 5 Ma BP. Interestingly, the spatial patterns of speciations of both groups of fishes were very similar, starting from inhabiting the Sakon Nakhon Basin south (*B. sp. (cf. smaragdina)* 4 and *T. sp. (cf. schalleri)* 3) and north (*B. sp. (cf. smaragdina)* 1 and *T. sp. (cf. schalleri)* 2) of the Songkhram River respectively, followed by dispersals into the Nam Ngum River Basin (*B. sp. (cf. smaragdina)* 3 and *T. sp. (cf. schalleri)* 1) and then into the Khorat Basin (*B. smaragdina* and *T. schalleri*). The newest member of *B. smaragdina* species group was *B. stiktos*, a variation of *B. smaragdina* on the lower Khorat Basin which migrated down the Mekong River very recently (~0.3 Ma BP).

Finally, from our observation, the females of both the bubble-nesters and the brooders can also help gather newly fertilized eggs. In fact, female brooders have been observed to pass eggs from their mouths into the water to be snapped up by the mouths of male brooders. Male bubble-nesters, like male brooders, can snap up young fry by their mouths when sensing danger. Thus both groups can use their mouths to take care of the young; only their paternal care preferences differ. Male brooders can also blow bubbles on occasions but not as a raft, as in the case of male bubble-nesters. Thus, it should not take much adaptation for the fish of one parental-care type to switch to the other type while adapting to a more stagnant water body as against a flowing one. Besides, the courtship behavior of the two types shows close similarity.

CONCLUSIONS

In order to determine the geographical pattern of evolution of different clades of fishes in the genera *Betta* and *Trichopsis*, we reconstructed a clock phylogenetic tree, inferred ancestral COI sequences in the tree, and estimated the likelihoods of the extant COI sequences given the inferred ancestral sequences. Correlating the tree and the sequence likelihoods with the geographical history, we inferred that the first *Betta* species to appear was a mouth brooder, probably somewhat similar to a *B. waseri* inhabiting the present-day Peninsular Malaysia. The first speciation in the genus involved *B. macrostoma* occurring at about 22 Ma BP while the first bubble-nester, probably similar to a *B. imbellis*, emerged at about 16.5 Ma BP. The mouth brooders on Borneo do not form a monophyletic group as *B. macrostoma* seemed to be the first to have migrated there but did not give rise to any other species in this study. Other Borneo mouth brooders were more closely related, stemming from their common ancestor that appeared at about 14.5 Ma BP. Around the same time, *B. bellica* separated from *B. splendens* ancestor. Adapting bubble-nest building behavior seemed to have happened along with the adaptation to stagnant water as another clade of bubble-nesters emerged again in *B. persephone* ancestor, which appeared at about 10 Ma BP. The northeastern variety of bubble-nesters in Thailand appeared at around 6.8 Ma BP, probably after having migrated to the area via the western rim of the Khorat Plateau which was still quite low at that time. The first *Trichopsis*, probably similar to a *T. vittata*, appeared around this period somewhere on the lowland south of the plateau. Whereas the *Betta* in Thailand spread from the south across the present-day Gulf of Thailand to the central plain of Thailand and to the plain south of the Khorat Plateau, *T. vittata* ancestor seemed to have

spread in the opposite direction. *T. schalleri* ancestor speciated from *T. vittata* ancestor at about 6.3 Ma BP while *Trichopsis pumila* separated from the former at about 5.1 Ma BP. Interestingly, the spatial orders of the speciation of *B. smaragdina* and *T. schalleri*, both of which originated on the Sakon Nakhon Basin, were similar.

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