Mitochondrial COI and nuclear RAG1 DNA sequences and analyses of specimens of the three morphologically established species in the genus *Trichopsis* (Perciformes: Osphronemidae) reveal new/cryptic species

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ABSTRACT

Air-breathing fish species of the genus *Trichopsis* have been reported in Cambodia, Lao PDR, Indonesia, Malaysia, Singapore, Thailand and Vietnam. It is only in Thailand that all three recognized species (*Trichopsis vittata*, *Trichopsis schalleri* and *Trichopsis pumila*), as judged by distinct external features, are found. Cambodia and Lao PDR harbor two species each. The present work involves first-time DNA sequencing and analysis based on mitochondrial (COI) and nuclear (RAG1) DNA of numerous specimens of these species and specimens of a controversial Phetchaburi (Thailand) fish population with a mixed outward appearance. In addition to confirming the morphologically clear-cut taxonomic division of the three fish species, our DNA results show that whereas the *T. pumila* populations form one single species, there are cryptic species in the *T. vittata* and *T. schalleri* populations and possibly a new one in the latter. Members of the putative Phetchaburi fish population have been proven to be hybrids between *T. pumila* and *T. vittata*. In addition, a new the phylogenetic tree indicating ancestral relationships is also
presented. This study should generate further research to find new/ cryptic species of the genus *Trichopsis* in all countries harboring the fish. © 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**Introduction**

There are three recognized species in the genus *Trichopsis* (Canestrini, 1860), viz. *Trichopsis vittata* Cuvier and Valenciennes, 1831, *Trichopsis schalleri* Ladiges, 1962, and *Trichopsis pumila* Arnold, 1936. The *T. vittata* is the most widespread and has been reported in the majority of Southeast Asian countries, e.g. Cambodia, Indonesia, Lao PDR, Malaysia, Singapore, Thailand, and Vietnam (Boonyaratpalin, 1971; Cuvier and Valenciennes, 1831; Fowler, 1934; Kottelat, 2001; Rainboth, 1996; Sithtananan, 2010; Smith, 1945; Suvatti, 1950, 1981). In addition to *T. vittata*, Cambodia also has *T. pumila* and Lao PDR has *T. schalleri* (Kottelat, 2001; Rainboth, 1996). Only in Thailand that all three have been found. These anabantoid fishes are similar to the Indo-Malayan bubble-nest building fishes in the genus *Betta* in terms of air-breathing ability (enabled by the labyrinth organ), body shape and fins, albeit less colorful. Members of the two genera (*Trichopsis* and *Betta*) usually share the same habitats of shallow and relatively still fresh waters with covering vegetation.

Like the bubble-nesting bettas, the male *Trichopsis* fish also build bubble nests for the spawned eggs and hatchlings (Liengpornpan et al., 2006, 2007a). Similarities with the bettas’ behavior with respect to aggressiveness, for example, are also exhibited by the *Trichopsis* fish (Liengpornpan et al., 2007b). Although less aggressive than the bettas, the *Trichopsis* males do fight and display courtship in a similar way, albeit less elaborate, than the betta males. The adults’ croaking sound occurs during aggression and courtship display. In the laboratory the sound produced under water can be heard up to 10 m away (Liengpornpan et al., 2006, 2007a). The common name used by the aquarists and hobbyists for these *Trichopsis* fishes is the croaking gourami with *T. schalleri* being called the sparkling croaking gourami and *T. pumila* called the dwarf croaking gourami (Axelrod et al., 2006; Jennings, 2006; Liengpornpan et al., 2007a).

The mature members of these three species differ in size, *T. vittata* being larger than *T. schalleri*, which are larger again than *T. pumila* (renamed by Schaller and Kottelat (1990), in place of *T. vittatus* and *T. pumilus*). The *T. vittata* fish usually has three dark continuous stripes running along the whole of the dull and flat body, the middle one starts from a region below the mouth all the way to the beginning of the caudal fin. Some *T. vittata* show a fourth line on top of the three above but the former is shorter and located just below the upper body hump. When first caught in the wild most *T. vittata* fish exhibit emerald green appearance on the posterior part of the body. The *T. schalleri* has two such dark and more or less continuous stripes along the body, the top one starting behind the operculum and the lower one from the mouth: the body appears shiny, even sparkling. *T. pumila* also has two less dark stripes which are not continuous; in bright light the body is the shiniest of the three species (Arnold, 1936; Herm, 1953; Sithtananan, 2010). Apart from being the largest, mature *T. vittata* fish usually have one or two dark spots arranged longitudinally on the body behind the operculum; some members, though, have none. The popular Dr. Axelrod’s Mini-atlas of Freshwater Aquarium Fishes (Axelrod et al., 2006), Jennings’ 500 Freshwater Aquarium Fish (Jennings, 2006), Kottelat’s Freshwater Fishes of Western Indonesia and Sulawesi (Kottelat et al., 1993), and Horst Linke’s Labyrinth Fish World (Linke, 2014) have good representative photographs of members of the three fishes with brief information provided. Most of these fishes have lanceolate caudal fins in males and females. Others have more rounded tail fins. All have upturn mouths.

We have been able to observe courting, spawning, bubble-nest tending and aggressive behavior of these fish; they all agree with other reported observations including the croaking sound. Although the bubble-nesting betta and *Trichopsis* fishes usually cohabit, there were places where we found only bettas or *Trichopsis*; the latter case was more prevalent.

In several South-east Asian countries, viz. Cambodia, Indonesia, Lao PDR, Malaysia, Thailand and Vietnam, the *Trichopsis* fishes have previously been given several names which have now settled to the three names above. Earlier Liengpornpan et al. (2007a,b) described the morphology, the behavior and habitats of *T. vittata* caught in parts of Thailand, whereas Sithtananan (2010) in her M.S. thesis from Kasetsart University, Thailand, cited a wide range of catch sites in Thailand and elsewhere together with excellent color photographs of members of all three fish species. She used morphological criteria to see if there were differences...
in specimens of each species from all over Thailand, and those from abroad in terms of fin rays, bones, and body scales, but could not statistically ascertain differences among members of each recognized species caught in various places. Thus she concluded that throughout Southeast Asia, there were only the three species with T. vittata the most widely distributed.

We have carried out Thailand-wide surveys of Trichopsis fishes during 2009–2014. Some fishes were from Lao PDR and Cambodia. In addition to looking at the geographical distribution of these fish species, we have also done the sequences and analyses of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene (the DNA used in barcoding of fish species (Ward et al., 2005, 2009)) and the nuclear recombination activating gene 1 (RAG1) gene. Here we report DNA sequences and analyses of specimens of the Trichopsis fishes and propose the possible existence of new/cryptic species hitherto unreported. We have also derived a new phylogenetic relationship for these fishes.

**Materials and methods**

**Sample collection**

The Trichopsis fishes in this study were wild caught and only adults were used for experimentation. T. vittata were the most widely spread: from 9 provinces in the central plain of Thailand, 5 provinces in the north, 12 in the northeast, 4 in the west, 7 in the east, and 6 in the upper peninsula of Thailand. Bangkok is considered to be at the center of the country (see Table 1 and Fig. 1). In addition, there were T. vittata catch sites in 2 provinces in Cambodia, 1 in Lao PDR and 1 in Vietnam. T. schalleri were from 8 provinces in the northeast of Thailand and 1 province in Lao PDR whereas T. pumila were caught in 2 central, 4 eastern and 1 western provinces of Thailand, plus 1 province in Cambodia.

Preliminary morphology-based species identifications of the specimens were carried out by comparison with published color photographs and descriptions in books, magazines, and journal articles (Axelrod et al., 2006; Jennings, 2006; Kottelat et al., 1993; Liengpornpan et al., 2007a; Linke, 2014; Sitthananan, 2010). The live fish specimens were randomly selected for experimentation. Before muscle tissues were taken for DNA work, the fish were anesthetized in cold water until death. The dead fish were preserved in 95% ethanol for genetic analysis. Fish specimens were deposited at the National Science Museum, Thailand.

Sequence data were submitted to GenBank. All the 162 COI sequences have accession numbers KP200371–532 (www.ncbi.nlm.nih.gov). All the 108 RAG1 sequences have accession numbers KP200533–640 (www.ncbi.nlm.nih.gov).

**Table 1**

Countries and provincial locations of the Trichopsis species/cryptic species based on COI and RAG1 sequences.

| Species/cryptic species | Countries and provinces |
|-------------------------|-------------------------|
| T. sp. (cf. vittata) 1  | Thailand: Amnat Charoen, Bueng Kan, Buri Ram, Kalasin, Maha Sarakham, Nakhon Ratchasima, Nong Khai, Nong Bua Lam Phu, Sakon Nakhon, Sa Kaeo, Si Sa Ket, Surin, Ubon Ratchathani |
|                        | Lao PDR: Vientiane       |
|                        | Cambodia: Banteay Meancheay |
|                        | Thailand: Chon Buri, Prachin Buri, Rayong, Satun |
|                        | Cambodia: Banteay Meancheay, Kampong Thom |
|                        | Viet Nam: Can Tho |
| T. sp. (cf. vittata) 3  | Thailand: Ang Thong, Bangkok, Chachoengsao, Chanthaburi, Chiang Mai, Chiang Rai, Chon Buri, Chumphon, Krabi, Lampang, Lampohon, Nakhon Pathom, Nakhon Si Thammarat, Pathum Thani, Phayao, Phetchaburi, Phuket, Phitsanulok, Prachuap Khiri Khan, Ratchaburi, Samut Prakan, Samut Sakhon, Samut Songkhram, Sukhothai, Surat Thani, Tak, Trat |
| T. sp. (cf. vittata) 4  | Thailand: Nakhon Si Thammarat |
|                        | Indonesia: Buring |
| T. schalleri           | Thailand: Amnat Charoen, Nakhon Ratchasima, Si Sa Ket, Surin, Ubon Ratchathani |
| T. sp. (cf. schalleri) 1| Thailand: Bueng Kan |
|                        | Lao PDR: Vientiane       |
| T. sp. (cf. schalleri) 2| Thailand: Bueng Kan     |
| T. sp. (cf. schalleri) 3| Thailand: Nakhon Phanom, Nong Khai |
| T. pumila               | Thailand: Chanthaburi, Pathum Thani, Prachin Buri, Ratchaburi, Rayong, Sa Kaeo, Samut Songkhram |
|                        | Cambodia: Kampong Thom   |
Fig. 1. The Southeast Asian natural habitats of the fishes in the genus Trichopsis. * in the inset indicates the specimens cited in Siththananan, 2010. Osphronemus striatus and Trichopsis striatus are now renamed Trichopsis vittata; Trichopsis pumilus siamensis and Ctenop pumilus are now renamed Trichopsis pumila.
DNA extraction and amplification reaction

DNA was extracted from fish muscle tissues as previously described by Kowasupat et al. (2014). Two barcoding regions analyzed in this study were the mitochondrial COI gene and nuclear RAG1 gene. Primers and PCR conditions for COI were employed according to protocols summarized in a previous work (Kowasupat et al., 2014). The nuclear RAG1 gene was amplified by a nested PCR protocol established earlier (Li and Orti, 2007) with some minor changes. In brief, the first PCR reaction of 20 μl contained 2 μl of the crude DNA extract mentioned above, 0.5 μl each of 10 μM RAG1-2510F and RAG1-4090R primers (Li and Orti, 2007; López et al., 2004), 0.4 μl of Terra PCR Direct Polymerase Mix (1.25 units/μl, Clontech), 10 μl of 2 × Terra PCR Direct Buffer (with Mg2+ and dNTP), and 6.6 μl of sterile distilled water. The thermal cycling conditions were 98 °C for 2 min followed by 30 cycles of 98 °C for 10 s, annealing at 50 °C for 15 s and extension at 68 °C for 1 min and 40 s. Subsequently, nested amplification was carried out in 20 μl reaction solution containing 2 μl of the first PCR product, 0.5 μl each of 10 μM RAG1-2533F and RAG1-4078R primers (López et al., 2004), 0.25 μl of Taq polymerase (5 units/μl, Invitrogen), 0.5 μl of 10 mM dNTPs, 0.5 μl of 50 mM MgCl2, 2 μl of 10 × reaction buffer, and 13.75 μl of sterile distilled water. The thermal cycling protocol consisted of denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 45 °C for 40 s, and 72 °C for 1 min and 40 s.

DNA cloning and sequencing

After agarose gel electrophoresis, PCR-amplified products of approximately 700 base pairs (bp) for COI and 1500 bp for RAG1 were subjected to gel purification using Gel/PCR Purification Kit (Favorgen Biotech Corp.). DNA cloning was performed using the pPrime cloning vector (5PRIME). After verification of recombinant clones with correct insert size by colony PCR, extracted recombinant plasmids were then sent to 1st BASE Pte Ltd. (Malaysia) for DNA sequencing using vector primers (i.e. T7 and SP6 promoter primers). Two additional sequencing primers, Trichopsis-RAG1-F1 (5′-GAT ACG AYG AGA AGA TGG-3′, this study) and RAG1-3261R (Li and Orti, 2007), were used to read the internal sequence for RAG1-containing plasmids. The next subsection discusses technical details recommended particularly only for readers who are interested in such matters.

Sequence alignment and analysis

The DNA sequencing results were trimmed and assembled using Geneious version 5.5.9 (Biomatters Limited; www.geneious.com). The required regions were then extracted from the assemblies and aligned. The COI sequences of T. vittata from Sriwattanarothai et al. (2010) and those from Trichopsis fish species from Buring, on the southeastern coast town of Sumatra, Indonesia, available in GenBank (accession numbers KM213044–KM213047) were included. The final alignments were inspected and adjusted manually. The COI and RAG1 sequences of the same specimen were also concatenated to form a combined dataset, excluding the sequences of any hybrids. In each dataset, repeat sequences were eliminated so that each variation would appear only once.

According to Rüber et al. (2006), the speciation within the genus Trichopsis happened around 7 million years ago, a very short period of time on the evolutionary scale. Since then the nuclear RAG1 gene apparently has undergone very little variation among the species. The same could be said about the second codon position of the COI gene. Thus, each codon position was thoroughly examined in order to determine whether its informative sites would affect the phylogenetic relationships. As it turned out, only 3 and 4 sites of the first and second positions of the RAG1 codons were informative but they mostly resulted from a few sequences belonging to different species. The same could be said about the only informative site of the second position of the COI codons. So, only the third codon position of the RAG1 gene and the first and second codon positions of the COI gene were used in phylogenetic analyses which were then performed on the three datasets with Betta smaragdina as an outgroup using MrBayes version 3.2.3 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) with B. smaragdina as an outgroup. The analyses followed the steps described in Panijpan et al. (2014) with slight modification. Roughly, for each dataset, a preliminary Bayesian inference with the most extensive parameter set was done in order to provide a basis for model selection. Afterward, Bayesian inferences were carried out for the candidate models and their sums of the means of prior and posterior log likelihoods were compared. If the comparison was not conclusive, the stepping-stone (SS) method (Xie et al., 2011) implemented in MrBayes would then be used to select among the better
candidates. The posterior distribution of the chosen model was then inspected to see whether there could be other candidates. If so, these candidates would be compared to the best candidate so far using the aforementioned procedure. The procedure would be repeated until all the likely candidates were considered.

Results

Phylogenetic relationship

The phylogenetic trees reconstructed from the COI and COI + RAG1 datasets are shown in Fig. 2. The most distinct common feature in all the trees was the presence of the three clades found previously (Rüber et al., 2006), namely T. pumila, T. schalleri, and T. vittata. This commonality was about the only feature the RAG1 tree had in common with the other two trees (results not shown). In fact, the posterior probability for the T. schalleri clade in the RAG1 tree was extremely low (0.35) and the clade seemed to separate from the other two clades while it formed a sister group with T. pumila with very high posterior probability (0.97) in the COI tree. The latter grouping agrees with that in Rüber et al. (2006). Thus, unless explicitly stated, all further results and discussion will be based on the COI and COI + RAG1 trees.

There were several potential cryptic species in both the T. schalleri and T. vittata clades. The former might contain up to 3 cryptic species, namely T. spp. (cf. schalleri) 1–3 and all the subclades, including the type-locality one, seemed to be monophyletic. For ease of discussion, T. spp. (cf. schalleri) 1–3 will be abbreviated to S1–S3. The posterior probabilities for all the subclades were at least 0.99 in both trees. The average intraspecific differences among the COI sequences in the subclades ranged from 0.41% (S1) to 0.56% (S3) (see Table 2) while the average interspecific differences ranged from 2.34% (T. schalleri and S3) to 7.24% (T. schalleri and S1) (see Table 3).

On the other hand, the phylogenetic relationships among T. vittata’s subclades, namely T. spp. (cf. vittata) 1–4 (so named without the T. vittata subclade because a type-locality specimen could not be acquired), were not the same in both trees and some of the posterior probabilities were not as high. From now on, T. spp. (cf. vittata) 1–4 will be called V1–V4. V1 was a monophyletic subgroup of V2 with the posterior probability of 1 in both trees while V3 was sister to V4 with the posterior probability of 1 in the COI + RAG1 tree and 0.89 in the COI tree. The posterior probability for V3 was at least 0.99 in both trees while that for V4 was 0.94 in the COI tree. However, the relationship of the two subclades (V1 + V2 and V3 + V4) was not as clear; they seemed to be separated in the COI tree with the posterior probabilities of 1 and 0.89 respectively while the latter was another monophyletic subgroup of V2 in the COI + RAG1 tree with the posterior probability of 1. The average intraspecific differences among the COI sequences in the subclades ranged from 0.36% (V1) to 1.17% (V4) (see Table 2) while the average interspecific differences ranged from 1.62% (V1 and V2) to 5.23% (V1 and V3) (see Table 3).

However, for T. pumila, our genetic analyses by COI, RAG1 and COI + RAG1 yielded only one genetic species with the posterior probability of 1 in all three trees.

Fish biodiversity and the habitats

T. vittata are the most widespread throughout Thailand and other Southeast Asian countries. Contrary to the findings of others prior to ours, especially those of Sithitananan (2010), our genetic results and analyses indicated that there were at least two distinct subdivisions of the T. vittata species (V1 + V2 and V3 + V4, see Fig. 2) which were distributed in quite distinct geographical locations in Thailand. One group (V1 + V2) predominated in the east and northeast of the country and Lao PDR and parts of Cambodia bordering Thailand. There might even be two distinct species (V1 and V2) within this group. The other group (V3 + V4) predominated in the northern, central, west, south and some eastern regions of Thailand.

Our genetic analyses also indicated that in addition to the type-locality fish, T. schalleri might also be split into S1, S2 and S3 (see Fig. 2); S1 being from Bueng Khong Long in Bueng Kan province and northern Lao PDR and S2 fish being also from Bueng Kan. S3 is predominantly in Nong Khai province. The type-locality T. schalleri were more widespread in places along the Mekong River and Thailand boundaries with Lao PDR and Cambodia. Thus there were places that at least 2 commonly recognized species cohabited. Again the northeast of Thailand having geographical features of dividing mountain ranges, high plateaus and river systems seemed to promote speciation of Trichopsis as well as Betta fishes (Kowasupat et al., 2014).
**Fig. 2.** The phylogenetic trees of the fishes in the genus *Trichopsis* reconstructed from the COI sequences (a) and the COI + RAG1 sequences (b) using partitioned Bayesian analyses. Only the first and third positions of COI codons and the third position of RAG1 codons were used. The posterior probability supporting each clade is shown next to the node. *Betta smaragdina* was used as the outgroup. Each isosceles triangle represents the most extreme difference (horizontal length from apex to base) and the number of variations (length of vertical base) within each clade. Each terminating branch represents one variation.
T. pumila were found in eastern provinces plus a town in Cambodia and two Cambodian western provinces. We also found T. pumila in provinces west of Bangkok.

Among the aquarists and hobbyists, there has been a question of whether some Phetchaburi or Hua Hin Trichopsis fish with the size similar to that of T. schalleri should have a new species status (Linke, 2014). Our DNA analyses confirmed that some wild-caught fish from these places were hybrids as a result of crossing between female T. vittata and male T. pumila: the fish inherited the COI gene from maternal T. vittata and the RAG1 gene from paternal T. pumila. Thus, we are able to spot hybrids when they exist in nature. However, so far, we have not found other natural hybrids; this fact may just indicate that the undetected hybrid populations are so small or so rare that we have not found any specimens.

Our results about fish species and their phylogenetic relationship were made possible by the following results about DNA sequence alignments and analyses. We recommend that only the readers who wish to delve into such details should read the next subsection.

Sequencing results and their alignment

There were 177 COI sequences and 108 RAG1 sequences of the specimens in the genus Trichopsis. The length of a complete COI sequence was 652 base pairs (bp) while that of a complete RAG1 sequence was 1544 bp. All the 11 COI sequences from Sriwattanarothai et al. (2010) were identical and also identical with the most prevalent COI sequence of T. vittata in this study. The four COI sequences of Indonesian T. vittata (Buring town) from GenBank were identical but their length was only 515 bp. Several RAG1 sequences were considerably shorter than 1544 bp (1108–1288 bp) due to the incompleteness of the sequencing results.

The COI alignment (excluding the outgroup) contained 202 variable sites, 147 of which were informative, while the RAG1 alignment contained 252 variable sites but only 50 of them were informative. After eliminating the three codon positions, the resulting alignments contained 84 unique COI sequences (435 bp), 84 unique RAG1 sequences (515 bp), and 87 non-hybrid, unique concatenated sequences (950 bp) upon which phylogenetic analyses were performed.

There were 35 informative sites of the COI alignment that clearly exhibited the grouping of the three clades, namely T. schalleri, T. pumila, and T. vittata, as shown in Table 4. One can see that T. pumila could be sister to either of the other two clades. The phylogenetic results were also inconclusive in this regard, with

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**Table 2**

Average intraspecific differences for the cytochrome c oxidase subunit 1 (COI) gene.

| Species            | Differences (%) | No. of varieties |
|--------------------|-----------------|------------------|
| T. pumila          | 0.92            | 10               |
| T. schalleri (S)   | 0.54            | 11               |
| T. sp. (cf. schalleri) 1 (S1) | 0.41 | 4                |
| T. sp. (cf. schalleri) 2 (S2) | 0.46 | 2                |
| T. sp. (cf. schalleri) 3 (S3) | 0.56 | 4                |
| T. sp. (cf. vittata) 1 (V1) | 0.36 | 17               |
| T. sp. (cf. vittata) 2 (V2) | 0.63 | 12               |
| T. sp. (cf. vittata) 3 (V3) | 0.44 | 22               |
| T. sp. (cf. vittata) 4 (V4) | 1.17 | 2                |

**Table 3**

Average pair-wise interspecific differences (%) for the cytochrome c oxidase subunit 1 (COI) gene.

| Species            | T. pumila | S  | S1  | S2  | S3  | V1  | V2  | V3  |
|--------------------|-----------|----|-----|-----|-----|-----|-----|-----|
| T. schalleri (S)   | 11.22     |    |     |     |     |     |     |     |
| T. sp. (cf. schalleri) 1 (S1) | 11.51 | 7.24 |     |     |     |     |     |     |
| T. sp. (cf. schalleri) 2 (S2) | 11.87 | 6.34 | 3.53 |     |     |     |     |     |
| T. sp. (cf. schalleri) 3 (S3) | 11.19 | 2.34 | 6.67 | 5.75 |     |     |     |     |
| T. sp. (cf. vittata) 1 (V1) | 12.33 | 10.67 | 12.70 | 12.34 | 10.76 |     |     |     |
| T. sp. (cf. vittata) 2 (V2) | 12.00 | 10.45 | 12.24 | 11.82 | 10.56 | 1.62 |     |     |
| T. sp. (cf. vittata) 3 (V3) | 13.04 | 11.10 | 12.44 | 11.52 | 10.86 | 5.23 | 4.72 |     |
| T. sp. (cf. vittata) 4 (V4) | 12.49 | 10.80 | 12.79 | 11.61 | 10.90 | 5.13 | 4.61 | 2.25 |
Table 4
COI informative sites exhibiting the grouping of the three clades. The numbers (read vertically from the top of the header of each column) indicate the positions of the sites in the COI alignment (43 to 604). A period (.) represents the same nucleotide as that in the first row. The vertical boxes highlight the differences within the clades; the solid ones are informative while the dotted ones are not.

| Site no. | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T. sp. (cf. schalleri) 1 | A | T | T | T | A | A | T | C | A | T | G | T | C | C | G | A | C | T | C | T | T | T | T | T | C | C |
| T. sp. (cf. schalleri) 2 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| T. sp. (cf. schalleri) 3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| T. schalleri | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| T. pumila | T | C | . | G | . | A | T | . | C | . | T | T | T | . | A | G | A | C | G | . | . | C | C | C | . | T | . | C | C | T |
| T. sp. (cf. vittata) 1 | T | C | C | C | G | C | A | T | C | C | A | C | T | T | T | T | A | G | C | G | T | T | A | C | C | C | G | G | A | C | A | A | T | T |
| T. sp. (cf. vittata) 2 | T | C | C | C | G | C | A | T | T | C | C | A | C | T | T | T | T | A | G | C | G | T | T | A | C | C | C | G | A | A | C | A | G | T | T |
| T. sp. (cf. vittata) 3 | T | C | C | C | G | C | A | T | T | C | C | A | C | T | T | T | T | A | G | C | A | T | T | A | C | C | C | G | A | A | C | A | A | T | T |
| T. sp. (cf. vittata) 4 | T | C | C | C | G | G | A | T | T | C | C | A | C | T | T | T | T | A | G | C | A | T | T | A | C | C | C | G | A | A | C | A | A | T | T |
the COI tree supporting the \textit{T. pumila–} \textit{T. schalleri} clade with the posterior probability of 0.97 (Fig. 2a) while the RAG1 tree pointing to the \textit{T. pumila–} \textit{T. vittata} grouping with a posterior probability of 0.98 (result not shown). The combined evidence favored the former albeit with a relatively low posterior probability of 0.77 (Fig. 2b). The sequences of more than one gene are thus needed to resolve this relationship. It was also evident from the table that the phylogenetic relationship within the \textit{T. schalleri} clade was more resolved than that within the \textit{T. vittata} clade, with three informative sites (solid vertical boxes) for the former versus one for the latter. Again, this was reflected by the posterior probabilities in the COI tree (Fig. 2a).

**Discussion**

\textit{Trichopsis} species diversity and ancestral relationship

The phylogenetic tree by COI + RAG1 gene analysis is the first ever proposed. This phylogenetic tree indicating ancestral relationship between the various species/subspecies was well supported by DNA sequence analyses described in the \textit{Sequence alignment and analysis} subsection in the \textit{Materials and methods} section and the \textit{Sequencing results and their alignment} subsection in the \textit{Results} section.

Before our findings it has been generally accepted that there were only three species of \textit{Trichopsis} fish in Thailand and only one or two species in other Southeast Asian countries. As stated earlier, despite the fact that members of the \textit{T. vittata} may exhibit zero, one or two dark spots (behind the operculum) among other variations, only one species of \textit{T. vittata} has been assumed to exist and also only one species of \textit{T. schalleri} and one species of \textit{T. pumila}.

In \textit{T. vittata}, we found the number of post-opercular spots not to correlate in any way with the DNA sequence results nor with the locations of catch sites. In fact, Sithitananan’s (2010) detailed descriptions and comparative statistical meristic and morphometric analyses of numbers of the three established species, inter-specifically as well as intra-specifically, indicated no significant differences among numbers of each species collected by her and others all over the Southeast Asian countries mentioned above.

Our genetic sequencing of mitochondrial COI (proposed for DNA barcoding of fish species (Ward et al., 2005, 2009)) and nuclear RAG1, and analysis of the two genes in combination led to the discovery of genetically significant differences in members of \textit{T. vittata} and \textit{T. schalleri} whereas in \textit{T. pumila} we observed no such significant differences. We were able to build a new phylogenetic tree showing relationships between the three established species as well as the subdivision of each “species” into cryptic and new species as shown in Fig. 2. The results above came from the concordance or near concordance between COI and the combined COI + RAG1 analyses yielding a phylogenetic tree with good statistical supports.

The \textit{T. schalleri} clade should contain one new species and another cryptic species. More conservatively S3 might be the same species as the type-locality \textit{T. schalleri} while S1 and S2 together might be the same new species. More likely is the possibility that S1 and S2 are two distinct subspecies of the aforementioned new species while S3 is a cryptic species of \textit{T. schalleri} in addition to the type-locality one as depicted in the COI and COI + RAG1 trees (Fig. 2). The new species is supported by the posterior probabilities of 1 in both trees and relatively high (more than 6%) genetic distance from \textit{T. schalleri} (Table 3). Genetic distance of 5% or higher is considered a good cutoff for a new species (Paniypan et al., 2014). The evidence for subspecies and cryptic species is their posterior probabilities being 1 in both trees and the average COI interspecific differences of at least 4 times the corresponding intraspecific ones among all the subclades (Tables 2 and 3).

The \textit{T. vittata} clade should also contain four cryptic species: one of which (V4), however, might be the same as the type-locality one. V1 inhabit the northeastern region of Thailand and the neighboring Laos PDR and Cambodia. Among the four potential cryptic species, V1 live farthest from the habitat of the type-locality fish. V1’s position on the two trees reveals that it might be a subgroup of V2. This is strongly supported by the posterior probability of 1 in both trees even though the average difference between the COI sequences of the two clades is a mediocre 1.62% (Table 3). V3 are the most widely spread: from the north all the way to the south of Thailand. The cryptic species status of V3 is equally well supported with the posterior probability of at least 0.99 in both trees. Moreover, their COI sequences differed from those of the other clades more substantially (about 5% from V1 and V2 and 2% from V4). The COI sequence of one specimen from Nakhon Si Thammarat province in the south formed a group (V4) with the sequences from Buring, Indonesia, a place relatively close to \textit{T. vittata}’s type locality, even though Nakhon Si Thammarat is rather far from Buring. Thus, V4 might be the same as the type-locality \textit{T. vittata} which inhabit the whole Malay Peninsula and
Sumatra. The remaining question is whether V3 + V4 should be another subgroup of V2 as indicated by the COI + RAG1 tree or separated from the other two clades as suggested by the COI tree. Again, additional genes are required to answer this question.

One might argue that the aforementioned new/cryptic species of both T. schalleri and T. vittata were in fact variations of each of the species. This might be the case if the catch sites were few and far between. However, in our opinion, sufficient efforts had been spent in achieving sufficient numbers of habitats and specimens and the latter’s DNAs analyzed to ensure that there should not be any Trichopsis fishes forming gradual genetic linkages between these new/cryptic species. The cases of V3 and V4 are still doubtful because more fish habitats in Malay Peninsula and Indonesian islands are required to ascertain their cryptic-species status. V4 caught in Buring, a town on Sumatra island facing the Java Island nearby, could be closely related to the type-locality fish caught on the latter island.

**Geographical distribution**

As a result of the above we found the geographical distribution of the Trichopsis to agree generally with previous findings of Sithtananan (2010). She also recorded the geographical distribution of these fish species in Thailand which agreed with our results generally. Also she stated that she had access to some museum specimens which have not been available to us. However, now we can propose finer subdivisions among these previously established morphology-based species.

Morphological criteria showed the Phetchaburi (Hua Hin) fish to be different from T. schalleri. For example, the fish has features in the front part very similar to those of T. vittata. We were able to obtain some wild Phetchaburi fish specimens from which we did try to breed offspring. These fish were very difficult to breed. Also the gender imbalance in the grown progeny (ca. 95% females, ca. 5% males) is also worth noticing. The combination of COI and RAG1 helped in establishing that the Phetchaburi fish is actually a hybrid of mixed parentage (maternal T. vittata and paternal T. pumila) and definitely not related to T. schalleri. The closest habitat of T. schalleri to the province of Phetchaburi is 500 km!

The eastern T. pumila COI sequences were not found in southern fish specimens at all and only one specimen of T. vittata (V2) was from the south. Exclusively T. vittata (V3 and V4) COI sequences were regularly found in specimens from the south. Earlier, it was found that eastern bettas (be it nest-builder or mouth-brooder) resembled the southern bettas, e.g. the nesters Betta siamorientalis to Betta imbellis, and the brooders Betta prima to Betta pallida (Kowasupat et al., 2012; Panijpan et al., 2014). But such resemblances do not occur among the Trichopsis fishes from the two regions as in the case of bettas. One possibility is that T. pumila from the eastern region did not reach the south via the land bridge during the last ice age (~20,000 years) (Panijpan et al., 2014). V2 also might not have crossed this land bridge during that time or some fish evolved independently since the ice age.

The existence of V3 and V4 near Thailand–Malaysia border should lead to more research on T. vittata south of the Thai peninsula all the way down to Indonesian islands. Since our results point to more than one population of T. vittata and more than one population of T. schalleri in Thailand, we are quite optimistic that other Southeast Asian countries may harbor the non-Thailand Trichopsis populations as well.

Although we did not have the type-locality fishes from Indonesia (T. vittata), our results were important by themselves because of their wider implication. Anyway, even if we had had some Trichopsis fish (T. vittata) sent from the locations as reported much earlier (Cuvier and Valenciennes, 1831), we might not have obtained the type-locality ones. Thus new research should be conducted to identify the fishes in these localities.

**Conclusion**

We have found greater biodiversity in Indochina T. vittata and T. schalleri by DNA analysis. As a result of our work, the previous beliefs in the species uniformity of T. vittata and T. schalleri throughout Southeast Asia should be reinvestigated morphologically and genetically.

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References

Arnold, J.P., 1936. *Ctenops pumilus* Arnold, 1936. Wochenschr. f. Aquar. Terr 33(11).

Axelrod, G.S., Burgess, W.E., Scott, B.M., Emmens, C.W., Pronek, N., Axelrod, H.R., 2006. Dr. Axelrod's Mini-atlas of Freshwater Aquarium Fishes. T.F.H. Publications, Inc., U.S.A.

Boonyaratpam, S., 1971. Fishes of the genus *Trichopsis* Kner found in Thailand. Proceedings of the 10th National Conference on Agricultural and Biological Science: Animal Science. Kasetsart University, Bangkok, Thailand, pp. 41–59.

Canestrini, G., 1860. Zur systematik und charakteristik der Anabatinen. Verh. K.-K. Zool.-Bot. Ges. Wien. 10 p. 708.

Cuvier, G., Valenciennes, A., 1831. Histoire naturelle des poissons. Tome septième. Livre septième. Des Squamipennes. Livre huitième. Des poissons à pharyngiens labyrinthiformes. Hist. Nat. Poisson. 7 pp. 170–208.

Fowler, H.W., 1934. Zoological results of the third de Schauensee Siamese expedition, part V: additional fishes. Proc. Acad. Nat. Sci. Phila. 86, 348–350.

Herm, F., 1953. *Fisch im Gartenteich*. Aquar. Terr. Z. 6, 276–280.

Huelsenbeck, J.P., Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinform 17, 754–755. http://dx.doi.org/10.1093/bioinformatics/17.8.754

Jennings, G., 2006. 500 Freshwater Aquarium Fish: A Visual Reference to the Most Popular Species. Firefly Books Ltd., U.S.A.

Kottelat, M., 2001. Fishes of Laos. WHT Publications (Pte) Ltd., Sri Lanka.

Kottelat, M., Whitten, A.J., Kartikasari, S.N., Wirjoatmodjo, S., 1993. Freshwater Fishes of Western Indonesia and Sulawesi. Periplus ed. Elsevier, Jakarta, Indonesia.

Kowasupat, C., Panijpan, B., Ruenwongsa, P., Jeenthong, T., 2012. *Betta siamomartialis*, a new species of bubble-nest building fishing fish (Teleostei: Osphronemidae) from eastern Thailand. Veretbr. Zool. 62, 387–397.

Kowasupat, C., Panijpan, B., Laosinchai, P., Ruenwongsa, P., Phongdara, A., Wanna, W., Senapin, S., Phiszaiya, K., 2014. Biodiversity of the *Betta maragadina* (Teleostei: Perciformes) in the northeastern region of Thailand as determined by mitochondrial COI and nuclear ITS1 gene sequences. Meta Gene 2, 83–95. http://dx.doi.org/10.1016/j.mgene.2013.12.004.

Ladies, W., 1962. *Trichopsis schalleri* spec. nov., ein neuer Gurami aus Thailand. Aquar. Terr. Z. 15, 101–103.

Li, C.H., Orti, G., 2007. Molecular phylogeny of Clupeiformes (Actinopterygii) inferred from nuclear and mitochondrial DNA sequences. Mol. Phylogenetics Evol. 44, 386–398. http://dx.doi.org/10.1016/j.ympev.2006.10.030.

Liengpornpan, S., Jaroensutasinee, M., Jaroenutsasitine, K., 2006. Mating habits and nesting habitats of the croaking gourami *Trichopsis vittata*. Acta Zool. Sinica 52, 846–853.

Liengpornpan, S., Jaroenutsasitine, M., Jaroenutsasitine, K., 2007a. Biology of croaking gourami *Trichopsis vittata*: the fish that croaks! Thaksin University J. 10, 72–83.

Liengpornpan, S., Jaroenutsasitine, M., Jaroenutsasitine, K., 2007b. Male body size, female preference and male–male competition in croaking gourami. Acta Zool. Sinica 53, 233–240.

Linke, H., 2014. Labyrinth Fish World. Best View Fish Magazine Taiwan, Taiwan.

López, J.A., Chen, W.-J., Ortí, G., 2004. Esociform phylogeny. Copeia 2004, 449–464.

Panijpan, B., Kowasupat, C., Laosinchai, P., Ruenwongsa, P., Phongdara, A., Wanna, W., Senapin, N., Phiszaiya, K., Kühne, J., Fasquel, F., 2014. Southeast Asian mouth-brooding *Betta* fighting fish (Teleostei: Perciformes) species and their phylogenetic relationships based on mitochondrial COI and nuclear ITS1 DNA sequences and analyses. Meta Gene 2, 862–879. http://dx.doi.org/10.1016/j.mgene.2014.10.007.

Rainboth, W.J., 1996. FAO Species Identification Field Guide for Fishery Purposes. Fishes of the Cambodian Mekong. FAO, Rome, Italy.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinform 19, 1572–1574. http://dx.doi.org/10.1093/bioinformatics/19.8.754

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. http://dx.doi.org/10.1093/sysbio/sys029.

Rüberger, L., Britz, R., Zardoya, R., 2006. Molecular phylogenetics and evolutionary diversification of labyrinth fishes (Perciformes: Anabantoidae). Syst. Biol. 55, 374–384. http://dx.doi.org/10.1080/10635150500541664.

Schaller, D., Kottelat, M., 1990. *Betta striolepis* sp. n., ein neuer Kampfisch aus Südborneo (*Osphronemidae: Belontiidae*). Aquar. Terr. Z. 43, 31–37.

Sithtananan, P., 2010. Taxonomic Review of the Anabantoid Fish Genus Trichopsis Canestrini, 1860 from Indochina (Perciformes: Osphronemidae). (Master's thesis) Retrieved from. http://research.rdi.ku.ac.th.

Smith, H.M., 1945. The Fresh-water Fishes of Siam, or Thailand. United States Government Printing Office, Washington, D.C.

Sriwattanarothis, N., Steinké, D., Ruenwongsa, P., Hannner, R., Panijpan, B., 2010. Molecular and morphological evidence supports the species status of the Mahachai fighter *Betta* sp. Mahachai and reveals new species of *Betta* from Thailand. J. Fish Biol. 77, 414–424. http://dx.doi.org/10.1111/j.1095-8649.2010.02715.x.

Suwatti, C., 1981. *Fishes of Thailand*. Royal Institute of Thailand, Bangkok, Thailand.

Suwatti, C., 1990. *Fauna of Thailand. Department of Fisheries, Bangkok, Thailand.*

Suwatti, C., 1950. *The Freshwater Fishes of Siam, or Thailand*. United States Government Printing Office, Washington, D.C.

Talee, D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N., 2005. DNA barcoding Australia's fish species. Philos. Trans. Royal Soc B 360, 1847–1857. http://dx.doi.org/10.1098/rstb.2005.1716.

Talee, D., Hanner, R., Hebert, P.D.N., 2009. The campaign to DNA barcode all fishes, FISH-BOL. J. Fish Biol. 74, 329–356. http://dx.doi.org/10.1111/j.1095-8649.2008.02080.x.

Xie, W., Lewis, P.O., Fan, Y., Kuo, L., Chen, M.H., 2011. Improving marginal likelihood estimation for Bayesian phylogenetic model selection. Syst. Biol. 60, 150–160. http://dx.doi.org/10.1093/sysbio/syr085.