Do Cryptic Species Exist in *Hoplobatrachus rugulosus*? An Examination Using Four Nuclear Genes, the *Cyt b* Gene and the Complete MT Genome

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Abstract

The Chinese tiger frog *Hoplobatrachus rugulosus* is widely distributed in southern China, Malaysia, Myanmar, Thailand, and Vietnam. It is listed in Appendix II of CITES as the only Class II nationally-protected frog in China. The bred tiger frog known as the Thailand tiger frog, is also identified as *H. rugulosus*. Our analysis of the *Cyt b* gene showed high genetic divergence (13.8%) between wild and bred samples of tiger frog. Unexpected genetic divergence of the complete mt genome (14.0%) was also observed between wild and bred samples of tiger frog. Yet, the nuclear genes (*NCX1*, *Rag1*, *Rhod*, *Tyr*) showed little divergence between them. Despite this and their very similar morphology, the features of the mitochondrial genome including genetic divergence of other genes, different three-dimensional structures of ND5 proteins, and gene rearrangements indicate that *H. rugulosus* may be a cryptic species complex. Using Bayesian inference, maximum likelihood, and maximum parsimony analyses, *Hoplobatrachus* was resolved as a sister clade to *Euphlyctis*, and *H. rugulosus* (BT) as a sister clade to *H. rugulosus* (WT). We suggest that we should prevent Thailand tiger frogs (bred type) from escaping into wild environments lest they produce hybrids with Chinese tiger frogs (wild type).

Introduction

Vertebrate mitochondrial DNA (mtDNA) is a closed circular genome which is approximately 16–20 kb long [1]. This genome typically contains 37 genes (2 rRNAs, 22 tRNAs, and 13 protein-coding genes) and a long non-coding region called the control region or D-loop region [1,2]. The mitochondrial (mt) genome has several valuable characteristics, including small size, fast evolutionary rate, relatively conserved gene content and organization, maternal
inheritance, as well as limited recombination [3–6]. Mitochondrial genomes are useful molecular markers in cryptic species identification because of the differences in compositional features, divergence of protein-coding genes, number and size of non-coding regions, and gene arrangement [7–9].

According to the Amphibian Species of the World 5.6, an online reference (19 Mar. 2014) [10], about 6344 species of Order Anura exist worldwide. Among them, 319 species and subspecies can be found in China [11]. The genus Hoplobatrachus with five species is important among the Dicroglossini of Dicroglossinae [12–14]. Only one of these species, known as the Chinese tiger frog occurs naturally in wild environments in China [10,11,15]. This frog has been identified as Hoplobatrachus rugulosus [13], although some Chinese researchers insist that it should be named Hoplobatrachus chinensis, believing this to be a senior synonym of H. rugulosus [15]. The Chinese tiger frog is listed in Appendix II of CITES as the only Class II nationally-protected frog in China. A bred tiger frog introduced to China from Thailand is called the Thailand tiger frog by Chinese, and has also been identified as H. rugulosus according to Frost’s taxonomic methods [10]. Thailand tiger frogs are bred in many farmers for local meat consumption. But more and more Thailand tiger frogs have been captured in the field after escaping from farms, which may affect the diversity of local Chinese tiger frogs. Alam et al. [16] found high divergences in Hoplobatrachus using Cyt b, 12S rRNA, and 16S rRNA genes. They suggested that H. chinensis (= H. rugulosus) may be subdivided into more than one species. Pansook et al. [17] found two distinct clades in H. rugulosus from Thailand using Cyt b gene, and also suggested that two distinct species may be present in H. rugulosus. In this study, we also found a high divergence between Chinese tiger frog and Thailand tiger frog using Cyt b gene. A cryptic species complex is a group of organisms that are typically very closely related yet their precise classification and relationships cannot be easily determined, although some can be separated by DNA sequence analyses [18–20]. Using the complete mt genome, Alam et al. [21] and Yu et al. [22] found duplications of the ND5 gene and the control region in Hoplobatrachus tigerinus and H. rugulosus. The current work aimed to investigate further the differences between Chinese tiger frog and Thailand tiger frog, as well as to determine whether cryptic species are present in H. rugulosus. Accordingly, we determined the complete mt genome sequences of Thailand tiger frog (bred type (BT)), and then compared the differences in gene arrangement, base compositional features, and genetic divergences of mt genes between Chinese tiger frog (wild type (WT)) and Thailand tiger frog (BT). The protein structures of the ND5 genes in Hoplobatrachus were also compared. Additionally, we sequenced nuclear genes (NCX1, Rag1, Rhod, and Tyr) of wild and bred tiger frogs to examine their genetic divergence. We also performed molecular phylogenetic analyses to discuss the relationship between Chinese tiger frog and Thailand tiger frog, and all available dicroglossids including Occidozyga martensii, based on the 11 mt protein-coding genes using five Ranidae species as out-groups. We follow the names proposed by Frost et al. [12] to avoid taxonomic confusion.

Materials and Methods

Ethical statement

Although the Chinese tiger frog is a protected species, the Chinese tiger frog samples (wild tiger frogs) we used were donated by the Committee of Forest Administrative Bureau of Jinhua (CFABJ), People’s Republic of China in 2000 and 2010. The officers of Forest Administrative Bureau of Jinhua have seized these wild frogs, which died during illegal captivity. The Thailand tiger frog samples (bred tiger frogs) were purchased from various farms from Jinhua, Zhejiang province and sacrificed using ether in our laboratory. The husbandry and breeding procedures of the Thailand tiger frogs in the farms were carried out under the Animal Husbandry Law of
the People’s Republic of China. The study protocol was reviewed and approved by the Committee of Animal Research Ethics of Zhejiang Normal University.

**Sample and DNA extraction.** All samples were stored at —70°C in the Institute of Ecology, Zhejiang Normal University. Information for all samples is shown in S1 Table. Whole genomic DNA was extracted from frozen tissue sample of thigh muscle of tiger frogs using a standard proteinase K/SDS digest extraction method followed by phenol–chloroform isolation and ethanol precipitation [23]. A sample of the Thailand tiger frog (No. THW1) was used to amplify the complete mt genome; other samples were used to amplify the partial Cyt b gene.

**Primer design, PCR amplification, and sequencing**

Cyt b gene was amplified by normal PCR using primers described by Pansook et al. [17]. NCX1, Rag1, Rhod, and Tyr genes were amplified by normal PCR using primers described by Che et al. [24] or Freilich et al. [25,26]. We amplified overlapping fragments that covered the entire mt genome of H. rugulosus (BT) by normal PCR and long-and-accurate PCR (LA-PCR) methods according to Yu et al. [22]. Seven DNA fragments were amplified using seven pairs of highly conserved primers (12STY J/N, 16STY J/N, C1 J/N, C2 J/N, CB-1 J/N, TYC2-C3 J/N, and WC3-ND4L J/N) [22,27–30]. Based on acquired sequence information, four pairs of normal PCR primers (W12S-16S J/N, WC1-C2 J/N, WC2-C3 J/N, and W16S-C1 J/N) and four pairs of LA-PCR primers (WND3-CB J/N, WCR-CR J/N, WND5-ND5 J/N, and 2WD J/N) were designed using Primer Premier 5.0 (Primer Biosoft International) (fragments 1–15 in Table 1) [27,30,31].

All PCRs were performed using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). TaKaRa Ex-Taq and LA-Taq Kits (Takara Biomedical, Dalian, China) were used for normal PCR and LA-PCR reactions respectively. The normal PCR was carried out in a 50 μl reaction mixture containing 5 μl of buffer (10× concentration), 4 μl of MgCl2 (25 mM), 4 μl of dNTP (2.5 mM), 2 μl of each primer (20 μM), 0.25 μl of Ex-Taq polymerase, 30.75 μl of sterile distilled water, and 2 μl of template DNA. The PCR reactions consisted of an initial denaturation at 95°C for 4 min; 35 cycles of denaturation at 94°C for 40 s plus annealing at 48°C to 60°C for

| Fragment | Primer | Forward primer sequences (5'→3') | Reverse primer sequences (5'→3') | References |
|----------|--------|----------------------------------|----------------------------------|------------|
| 1        | 12 STY J/N | AAAGGTTGTGGTCTGATGCTT | TACCATGTTGACCTTGCTCTTCTTCTT | [28,30] |
| 2        | 16 STY J/N | AAAGTGGAAGTCATTGGCGATG | ATCTCGGTCTGACTGATCGACTGATG | [29] |
| 3        | C1 J/N | CAACATTTTCATTCATCCTTCT | GTRWANNCCNGARARNG | [27] |
| 4        | C2 J/N | GCACCTCCTCCATATCATCCTT | CCRARATYTCTGARCTGARCTG | [27] |
| 5        | CB-1 J/N | TAYGTYCTNCCNTGGRGNCRATR | ARNAONCCNCNCRARTTNTN | [27] |
| 6        | TYC2-C3 J/N | ARATTTTGGGGCGAAACCACA | GACTGCWGTTATAGGAGGG | [22] |
| 7        | WC3-ND4L J/N | CTATATATATATATATATAT | CCRRTGAKCGGACWTRGCA | [22] |
| 8        | W12S-16S J/N | TTTTTATCCACATATCCCTCA | TGCCCTACCTACTACCTTCC | This study |
| 9        | W16S-C1 J/N | GCCCTTACTGCTCTCTTTCTCCTCAT | TTTAGGCTCGTGAGTAAATGTGAT | This study |
| 10       | WC1-C2 J/N | CTGTTGCCAATTCCTATCTG | GGTAAAGATAGGAGGGG | [22] |
| 11       | WC2-C3 J/N | CTGGCGACCTTCTTTCTTCTT | GATGATGACGACGATTTT | This study |
| 12       | WND3-CB J/N | CCATCTTTACTCTCTCTACGCC | GGGGCATTATGACGCTT | This study |
| 13       | WCR-CR J/N | CACACTAAGGCGACAAACAA | AAAAGGTAGATAGAAGAC | This study |
| 14       | WND5-ND5 J/N | ATAGCTACCTCGTCTTCTT | AAGGTCTACCTTATGATTCTT | This study |
| 15       | 2WD J/N | TTCTACCTCTGCTGACTCATG | CTGGGTTCCTATCGTGCTT | This study |

Notes: Y = C/T, R = A/G, M = A/C, W = A/T, K = G/T, S = G/C, and H = A/T/C.

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30 s to 60 s and extension at 72°C for 1 min to 2 min; and a final extension at 72°C for 10 min. The LA-PCR was carried out in a 50 μl reaction mixture containing 5 μl of buffer (10× concentration), 4 μl of MgCl₂ (25 mM), 8 μl of dNTP (2.5 mM), 2 μl of each primer (20 μM), 0.5 μl of LA-Taq polymerase, 2.65 μl of sterile distilled water, and 2 μl of template DNA. The PCR reactions consisted of an initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s plus annealing at 55°C to 65°C for 1 min and extension at 68°C for 3 min to 5 min; and a final extension at 72°C for 10 min. The resultant PCR fragments were electrophoresed on 1% agarose gels, and all target DNAs were purified from excised pieces of gel using an AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc. SF, CA, USA) for sequencing. The sequence for each fragment was obtained in an automated DNA sequencer (ABI 3730) from both strands. The long fragments were sequenced using specific primer walking of both strands.

Sequence assembly and analysis

Sequences were checked and assembled using SeqMan (Lasergene version 5.0) [32]. The locations of the 13 protein coding genes and 2 rRNA genes were determined by comparing the homologous sequences of other anurans using Clustal W in Mega 5.0 [25,33]. All tRNA genes, except tRNA^Ser (AGY) and tRNA^Cys genes, were identified by their cloverleaf secondary structure in tRNA-scan SE 1.21 [34] using the default parameters, and tRNA^Ser (AGY) and tRNA^Cys genes were determined by comparing the homologous sequences of other anurans. The organization of the *H.rugulosus* (BT) mt genome was formed using GenomeVx (Fig 1)(http://wolfe.gen.tcd.ie/GenomeVx/)[35]. The complete mtDNA sequence of *H. rugulosus* (BT) reported in this article was deposited in GenBank under accession number JX181763. The haplotypes of Cyt b, NCX1, Rag1, Rhod, and Tyr genes of 19 frog samples in this study were deposited in GenBank under accession number AB818459-AB818476, KJ637241-KJ637259, KJ637260-KJ637278, KJ637279-KJ63 7297, KJ637298-KJ637316, respectively. The nucleotide sequences of NCX1, Rag1, Rhod, and Tyr genes of the Chinese tiger frog and Thailand tiger frog were analyzed by Mega5.0 [25,33].

Molecular phylogenetic analyses

A total of 55 sequences of the Cyt b gene, including 19 from this study, in-group species from Pansook et al. [17] and out-group species (S1 Table), were used to evaluate the divergence between the Chinese tiger frog and the Thailand tiger frog. All 55 sequences yielded 565 bp of Cyt b gene fragment, including 231 variable and 197 parsimony-informative sites. Phylogenetic relationships were constructed by neighbor joining (NJ) analysis in Mega 5.0 [33] using Kimura 2-parameter model, gamma distributed, gamma parameter 6, with both transitions and transversions included. Statistical support was evaluated using 1000 bootstrap replicates. Using the data with 20 sequences yielded 1624 bp of Rag1, Rhod, and Tyr genes (excluding NCX1 gene as no sequence of Hoplobatrachus is reported in GenBank), including 36 variable sites. The out-group was combined *H. occipitalis* (Rag1 gene HM163613, Tyr gene AJ564729) and *H. tigerinus* (Rhod gene AB489039). Phylogenetic relationships were also constructed by neighbor joining (NJ) analysis in Mega 5.0 [33] with parameter set as above.

To confirm further the phylogenetic relationships of the Chinese tiger frog and the Thailand tiger frog among dicroglossids, 16 available complete mt genomes of anurans based on the addition of two Hoplobatrachus mt genomes, including five species of Ranidae (*Babina adeno-pleura*, *Pelophylax nigromaculata*, *Pelophylax plancyi*, *Odorrana ishikawae*, and *Odorrana tormotus*) [22,36,37]) as out-groups, were retrieved from GenBank (S2 Table). We constructed the phylogenetic trees based on a concatenated set of 11 mt protein-coding genes excluding
ND5 because of the high sequence divergence of its two copies in *H. rugulosus* BT and ATP8 because of its low number of informative sites in anurans.

The nucleotide sequences for each of the 11 mt protein-coding genes were aligned using Clustal W in Mega 5.0 [25,33]. To select the conserved regions of the sequences, each alignment was analyzed with Gblocks 0.91b [38] using default settings. We concatenated the alignments of the 11 mt protein-coding genes, and recovered an alignment consisting of 2791 amino acid residues. An alignment of 8373 nucleotides was obtained using the amino acid alignment as the backbone reference. A saturation analysis was performed for subsets with first, second, and third codon positions using DAMBE 4.2.13 [39]. The results showed that the third codon positions were saturated. Consequently, they were excluded from the final nucleotide alignment and an alignment of 5582 nucleotides was obtained. Maximum parsimony (MP) analysis with the nucleotide dataset was performed using PAUP*4.0b10 [40]. A total of

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**Fig 1. Mitochondrial map of *H. rugulosus* bred type.** tRNAs are labeled according to the three letter amino acid codes. Gene name inside indicates the direction of transcription from left to right, and gene name outside indicates right to left.

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1000 bootstrap replications were generated, each with 10 replications with random taxon order.

Model selection for the nucleotide dataset was performed with Modeltest version 3.7 [41]. The GTR+I+G model was chosen for the likelihood and Bayesian analyses. Maximum likelihood (ML) analysis of the nucleotide dataset was performed using PAUP* 4.0b10 [40] with 1000 bootstrap replications. Bayesian inference (BI) analysis of the nucleotide dataset was performed with MrBayes 3.1.2 [42]. Eight chains were run in parallel for 10 000 000 generations, with trees sampled every 1000 generations. The burn-in sizes for both nucleotide datasets were determined by checking convergences of −log likelihood (−lnL) values, and the first 50 000 generations were discarded. Bayesian posterior probabilities were calculated according to the remaining set of trees. All Markov chain Monte Carlo runs were repeated twice to confirm consistent estimation of the posterior parameter distributions.

Modeling of ND5 Tertiary Structure
A motif scan of ND5 proteins was performed against databases of motifs (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and by using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) [43,44]. The Automated Mode program of the Swiss-Model server (http://swissmodel.expasy.org/workspace/index) [45] was used to research the optimization model to enable selection of suitable templates for ND5 proteins. Additional assessments of domain structures were performed on ProSA-Web (https://prosa.services.came.sbg.ac.at/prosa.php) [46,47] and Verify3D Structure Evaluation Server (http://nihserver.mbi.ucla.edu/Verify3D/) [48–50]. Modeling of the four ND5 protein tertiary structures was performed using Swiss-PdbViewer (http://www.expasy.ch/spdbv/) [50].

Results
Genome organization and arrangement
The complete mt genome of *H. rugulosus (BT)* was 20 926 bp long and contained 14 protein-coding genes (including the extra copy of the ND5 gene), 2 ribosomal RNAs, 23 transfer RNA genes (including the extra copy of the tRNA^{Met} gene), and 2 non-coding regions (including the extra copy of the control region (D-loop)) (Table 2). In *H. rugulosus (BT)*, the distinctive features included a modified cluster of rearranged tRNA genes (TPF tRNA gene cluster), the tandem duplication of tRNA^{Met} genes (Met1 and Met2), the translocation of tRNA^{Leu(CUN)} and ND5 genes, and the two copies of D-loop-ND5 regions. The tRNA^{Leu(CUN)} gene was located between the two D-loop-ND5 regions, rather than in the typical LTPF tRNA cluster (Fig 1 and Table 2). The first D-loop-ND5 region was located between the Cyt b and tRNA^{Leu(CUN)} genes, and the second D-loop-ND5 region was located between the tRNA^{Leu(CUN)} and tRNA^{Thr} genes (Fig 1 and Table 2). Most of these genes were coded on the H-strand, except for ND6 and eight tRNA genes, as reported in other anurans (Fig 1).

The overall base composition of the H-strand was as follows: A (26.8%), T (26.0%), G (15.7%), C (31.5%), and total A+T content (52.8%) which was lower than those of other anurans (from 53.1% to 65.3%) (S2 Table). The nucleotide divergence of the complete mt genome was 15.8% between *H. rugulosus (WT)* and *H. tigerinus*, 18.0% between *H. rugulosus (BT)* and *H. tigerinus*, and 14.0% between *H. rugulosus (BT)* and *H. rugulosus (WT)*.

Protein-coding genes
There were six reading frame overlaps in the mt genome of *H. rugulosus (BT)*: COI and tRNA^{Ser (UCN)} shared nine nucleotides; ATP8 and ATP6 shared seven nucleotides; ND4L
and ND4 shared seven nucleotides; ND2 and tRNA\textsuperscript{Trp} shared two nucleotides; COIII and tRNA\textsuperscript{Gly} shared one nucleotide; and ND3 and tRNA\textsuperscript{Arg} shared two nucleotides. Other overlaps are shown in Table 2. Two ND5 genes (with 84.1% similarity) were found in \textit{H. rugulosus} (BT) as well as \textit{H. tigerinus} (two identical ND5 genes) and \textit{H. rugulosus} (WT) (two identical

| Gene/region          | Start position | Stop position | Spacer (+) | Overlap (-) | Length (bp) | Start codon | Stop codon | Strand |
|----------------------|----------------|---------------|------------|-------------|-------------|-------------|------------|--------|
| D-loop (CR2)         | 1              | 1815          |            |             | 1815        | ATG         | TAG        | H      |
| ND5-2                | 1816           | 3663          | +9         |             | 1848        | ATG         | TAG        | H      |
| tRNA\textsuperscript{Thr} | 3673          | 3741          | -1         |             | 69          |             |            | H      |
| tRNA\textsuperscript{Pro} | 3741          | 3809          | -1         |             | 69          |             |            | L      |
| tRNA\textsuperscript{Phe} | 3809          | 3876          |            |             | 68          |             |            | H      |
| 12S rRNA             | 3877           | 4816          |            |             | 940         |             |            | H      |
| tRNA\textsuperscript{Val} | 4817          | 4885          |            |             | 69          |             |            | H      |
| 16S rRNA             | 4886           | 6472          |            |             | 1587        |             |            | H      |
| tRNA\textsuperscript{Leu (UUR)} | 6473          | 6545          |            |             | 73          |             |            | H      |
| ND1                  | 6546           | 7506          |            |             | 961         | ATG         | T          | H      |
| tRNA\textsuperscript{Leu (AUN)} | 7507          | 7577          |            |             | 71          |             |            | H      |
| tRNA\textsuperscript{Gln} | 7578          | 7648          | -1         |             | 71          |             |            | L      |
| tRNA\textsuperscript{Met (AUN)} | 7648          | 7719          | +3         |             | 72          |             |            | H      |
| tRNA\textsuperscript{Met (AUN)} | 7723          | 7791          |            |             | 69          |             |            | H      |
| ND2                  | 7792           | 8826          | -2         |             | 1035        | ATG         | TAG        | H      |
| tRNA\textsuperscript{Trp} | 8825          | 8896          |            |             | 72          |             |            | H      |
| tRNA\textsuperscript{Ala} | 8897          | 8965          | +2         |             | 69          |             |            | L      |
| tRNA\textsuperscript{Asn} | 8868          | 9040          |            |             | 73          |             |            | L      |
| OL                   | 9041           | 9070          | -3         |             | 30          |             |            | L      |
| tRNA\textsuperscript{Cys} | 9068          | 9132          |            |             | 65          |             |            | L      |
| tRNA\textsuperscript{Tyr} | 9133          | 9199          | +4         |             | 67          |             |            | L      |
| COI                  | 9204           | 10 754        | -9         |             | 1551        | ATA         | AGG        | H      |
| tRNA\textsuperscript{Ser (UCN)} | 10 746        | 10 816        |            |             | 71          |             |            | L      |
| tRNA\textsuperscript{Asp} | 10 817        | 10 884        | +1         |             | 68          |             |            | H      |
| COII                 | 10 886         | 11 567        |            |             | 682         | ATG         | T          | H      |
| tRNA\textsuperscript{Lys} | 11 568        | 11 636        | +1         |             | 69          |             |            | H      |
| ATP8                 | 11 638         | 11 799        | -7         |             | 162         | ATG         | TAA        | H      |
| ATP6                 | 11 793         | 12 474        |            |             | 682         | ATG         | T          | H      |
| COIII                | 12 475         | 13 260        | -1         |             | 786         | ATG         | TAA        | H      |
| tRNA\textsuperscript{Gly} | 13 260        | 13 328        | -3         |             | 69          |             |            | H      |
| ND3                  | 13 326         | 13 670        | -2         |             | 345         | ATG         | TAA        | H      |
| tRNA\textsuperscript{Arg} | 13 669        | 13 737        |            |             | 69          |             |            | H      |
| ND4L                 | 13 738         | 14 019        | -7         |             | 282         | ATG         | TAA        | H      |
| ND4                  | 14 013         | 15 369        |            |             | 1357        | ATG         | T          | H      |
| tRNA\textsuperscript{His} | 15 370        | 15 438        |            |             | 69          |             |            | H      |
| tRNA\textsuperscript{Ser (AGY)} | 15 439        | 15 506        | +23        |             | 68          |             |            | H      |
| ND6                  | 15 530         | 16 015        | +1         |             | 486         | ATG         | AGG        | L      |
| tRNA\textsuperscript{Glu} | 16 017        | 16 085        | +9         |             | 69          |             |            | L      |
| Cyt b                | 16 095         | 17 240        |            |             | 1146        | ATG         | TAA        | H      |
| D-loop (CR1)         | 17 241         | 19 012        |            |             | 1772        |             |            | H      |
| ND5-1                | 19 013         | 20 854        | +2         |             | 1842        | ATG         | TAA        | H      |
| tRNA\textsuperscript{Leu (CUN)} | 20 855        | 20 926        |            |             | 72          |             |            | H      |

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The first ND5 gene (ND5-1) was located between the D-loop and tRNA^{Leu (CUN)}\textsuperscript{1}, and the second ND5 gene (ND5-2) was located between the D-loop and tRNA^{Thr} (Fig 1 and Table 2). The dataset comparing the two ND5 genes in \textit{H. rugulosus} (BT) included 288 variable sites in a total of 1848 aligned nucleotide sites and 131 variable sites in amino acid sequence over a total of 616 alignment amino acid sites. The codon frequency of the two ND5 genes in \textit{H. rugulosus} (BT) is shown in Table 3.

Protein-coding genes in \textit{H. rugulosus} (BT) begin with ATG as the start codon, except COI with ATA and the ND6 gene, for which \textit{H. rugulosus} (WT) used ACA—\textit{H. rugulosus} (BT) used ATG. ND5-1, ATP8, COIII, ND3, ND4L, and Cyt b genes are terminated with TAA as the stop codon, COI and ND6 end with AGG, ND2 and ND5-2 end with TAG, and the other four protein-coding genes end with an incomplete stop codon (a single stop nucleotide T) (Table 2).

### Table 3. The RSCU in two different ND5 genes of \textit{H. rugulosus} (BT).

| Codon | Count | RSCU | Codon | Count | RSCU | Codon | Count | RSCU | Codon | Count | RSCU |
|-------|-------|------|-------|-------|------|-------|-------|------|-------|-------|------|
| UUU(F) | 16 | 0.64 | UCU(S) | 11 | 1.22 | UAU(Y) | 5 | 0.71 | UGU(C) | 3 | 0.86 |
| UUU(F) | 11 | 0.5 | UCU(S) | 14 | 1.47 | UAU(Y) | 5 | 0.67 | UGU(C) | 2 | 0.57 |
| UUC(F) | 34 | 1.36 | UCC(S) | 17 | 1.89 | UAC(Y) | 9 | 1.29 | UGC(C) | 4 | 1.14 |
| UUC(F) | 33 | 1.5 | UCC(S) | 20 | 2.11 | UAC(Y) | 10 | 1.33 | UGC(C) | 5 | 1.43 |
| UUA(L) | 8 | 0.5 | UCA(S) | 13 | 1.44 | UAA(*) | 0 | 0 | UGA(W) | 12 | 1.5 |
| UUA(L) | 11 | 0.67 | UCA(S) | 9 | 0.95 | UAA(*) | 1 | 4 | UGA(W) | 15 | 1.76 |
| UUG(L) | 0 | 0 | UCG(S) | 5 | 0.56 | UAG(*) | 0 | 0 | UGG(W) | 4 | 0.5 |
| UUG(L) | 1 | 0.06 | UCG(S) | 6 | 0.63 | UAG(*) | 1 | 4 | UGG(W) | 2 | 0.24 |
| CUU(L) | 25 | 1.56 | CCU(P) | 5 | 0.65 | CAU(H) | 3 | 0.26 | CGU(R) | 0 | 0 |
| CUU(L) | 21 | 1.27 | CCU(P) | 5 | 0.54 | CAU(H) | 3 | 0.29 | CGU(R) | 1 | 0.31 |
| CUC(L) | 40 | 2.5 | CCC(P) | 11 | 1.42 | CAC(H) | 20 | 1.74 | CGC(R) | 6 | 1.6 |
| CUC(L) | 39 | 2.36 | CCC(P) | 15 | 1.62 | CAC(H) | 18 | 1.71 | CGC(R) | 7 | 2.15 |
| CUA(L) | 14 | 0.88 | CCA(P) | 14 | 1.81 | CAA(Q) | 9 | 1.8 | CGA(R) | 9 | 2.4 |
| CUA(L) | 19 | 1.15 | CCA(P) | 17 | 1.84 | CAA(Q) | 11 | 1.57 | CGA(R) | 5 | 1.54 |
| CUG(L) | 9 | 0.56 | CGG(P) | 1 | 0.13 | CAG(Q) | 1 | 0.2 | CGG(R) | 0 | 0 |
| CUG(L) | 8 | 0.48 | CGG(P) | 0 | 0 | CAG(Q) | 3 | 0.43 | CGG(R) | 0 | 0 |
| AUG(I) | 15 | 0.73 | ACA(T) | 9 | 0.8 | AAU(N) | 7 | 0.88 | AGU(S) | 0 | 0 |
| AUG(I) | 21 | 0.93 | ACA(T) | 8 | 0.74 | AAU(N) | 5 | 0.48 | AGU(S) | 1 | 0.11 |
| AUC(I) | 26 | 1.27 | ACC(T) | 23 | 2.04 | AAC(N) | 9 | 1.13 | AGC(S) | 8 | 0.89 |
| AUC(I) | 24 | 1.07 | ACC(T) | 20 | 1.86 | AAC(N) | 16 | 1.52 | AGC(S) | 7 | 0.74 |
| AUA(M) | 13 | 1.04 | ACA(T) | 12 | 1.07 | AAA(K) | 13 | 1.37 | AGA(*) | 0 | 0 |
| AUA(M) | 13 | 1.24 | ACA(T) | 12 | 1.12 | AAA(K) | 14 | 1.33 | AGA(*) | 0 | 0 |
| AUG(M) | 12 | 0.96 | ACG(T) | 1 | 0.09 | AAG(K) | 6 | 0.63 | AGG(*) | 0 | 0 |
| AUG(M) | 8 | 0.76 | ACG(T) | 3 | 0.28 | AAG(K) | 7 | 0.67 | AGG(*) | 0 | 0 |
| GUU(V) | 10 | 1.18 | GCU(A) | 12 | 0.77 | GAU(D) | 4 | 0.5 | GGU(G) | 1 | 0.15 |
| GUU(V) | 9 | 1.03 | GCU(A) | 14 | 1.08 | GAU(D) | 5 | 0.91 | GGU(G) | 1 | 0.16 |
| GUC(V) | 16 | 1.88 | GCC(A) | 29 | 1.87 | GAC(D) | 12 | 1.5 | GGC(G) | 11 | 1.69 |
| GUC(V) | 16 | 1.83 | GCC(A) | 21 | 1.62 | GAC(D) | 6 | 1.09 | GGC(G) | 10 | 1.6 |
| GUA(V) | 6 | 0.71 | GCA(A) | 17 | 1.1 | GAA(E) | 10 | 1.33 | GGA(G) | 9 | 1.38 |
| GUA(V) | 9 | 1.03 | GCA(A) | 16 | 1.23 | GAA(E) | 11 | 1.47 | GGA(G) | 6 | 0.96 |
| GUG(V) | 2 | 0.24 | GCG(A) | 4 | 0.26 | GAG(E) | 5 | 0.67 | GGG(G) | 5 | 0.77 |
| GUG(V) | 1 | 0.11 | GCG(A) | 1 | 0.08 | GAG(E) | 4 | 0.53 | GGG(G) | 8 | 1.28 |

All frequencies are averages over two ND5 genes in \textit{H. rugulosus} (BT), and relative synonymous codon usage is given in the column following the codon count column.

All frequencies are averages over two ND5 genes in \textit{H. rugulosus} (BT), and relative synonymous codon usage is given in the column following the codon count column.

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the ND5-2 gene, *H. rugulosus* (WT) used TAA as the stop codon, whereas *H. rugulosus* (BT) used TAG (Table 2).

Three protein-coding genes surprisingly showed different lengths between *H. rugulosus* WT and BT: ND3 differed by 3 bp (one amino acid), ND5-1 differed by 6 bp (two amino acids), and ND5-2 differed by 12 bp (four amino acids).

The divergence of nucleotides and amino acids in protein-coding genes using the uncorrected p-distance model between *H. rugulosus* (WT) and *H. rugulosus* (BT) ranged from 11.2% (COIII) to 24.0% (ND5-2) and from 2.1% (COIII) to 28.3% (ATP8), respectively (Table 4).

The divergence of nucleotides and amino acids in protein-coding genes among *Hoplobatrachus* ranged from 11.2% (COIII) to 28.2% (ND5-2) and from 1% (COI) to 30.5% (ND6), respectively (Table 4). The nucleotide divergence of the two pairs of ND5 genes in *H. rugulosus* (WT) and *H. rugulosus* (BT) was 18.8% and 24.0%, respectively, and the divergences in amino acid sequence were 14.2% and 26.8%.

**Ribosomal and transfer RNA genes**

In *H. rugulosus* (WT), the 12S rRNA gene (940 bp long) was located between tRNA^Phe^ and tRNA^Val^ genes, and the 16S rRNA gene (1587 bp long) was located between tRNA^Val^ and tRNA^Leu^ (UUR) genes. The mt genome of *H. rugulosus* (BT) contained 23 tRNA genes (including the extra copy of tRNA^Met^ gene) (Fig 2), that were interspersed in the genome and ranged in size from 65 bp to 73 bp. As reported in anurans, all tRNA genes can be folded into typical cloverleaf secondary structures with the known exception of the tRNA^Ser^ (AGY) gene (Fig 2).

The divergence of 12S rRNA and 16S rRNA genes between *H. rugulosus* (WT) and *H. rugulosus* (BT) was 7.6% and 6.6%, respectively, using the uncorrected p-distance model. Table 4 shows the divergences (uncorrected p-distances) of the 23 tRNA genes, 12S rRNA genes, and 16S rRNA genes among *H. tigerinus* and *H. rugulosus* (WT and BT).

**Noncoding regions**

The non-coding regions of *H. rugulosus* (BT) included the duplicated control regions (D-loop) and a few intergenic spacers. In *H. rugulosus* (BT), the first control region (CR1) was located between the *Cyt b* and ND5 genes, whereas in *H. tigerinus* and *H. rugulosus* (WT), the second control region (CR2) was located between the tRNA^Leu^ (UUR) and ND5 genes (Fig 1 and Table 2). The two control regions had almost identical nucleotide sequences, except the extra A (or C) AATATGCT5 in CR2. The length of control regions was similar to those of other anurans (from 851 bp to 4704 bp). However, the A+T content of the control regions was 57.0% or 56.6% (A, 29.0% or 28.8%; C, 28.7% or 29.0%; G, 14.3% or 14.4%; T, 28.0% or 27.8% in CR1 and CR2, respectively), which was between 54.9% and 73.3% in other anurans (S2 Table). The two control regions contained TASs (5’-ACATTAATCTGCTG-3’), CSBs (CSB-1, 5’-AGCCCTATTAAGGTGATGGACATAG-3’; CSB-2, 5’-GACCCCCCCCTAGGGATGCT-3’), as well as 7 and 12 tandem repeat units of 5’-TCAATATGCT-3’ in CR1 and CR2, respectively. Some short non-coding sequences also occurred in *H. rugulosus* (BT), and the longest intergenic space (about 23 bp) was between tRNA^Ser^ (AGY) and ND6 gene (Table 2).

The putative origin of the L-strand replication (O1) [51] was located in the WANCY tRNA gene cluster between the tRNA^Asn^ and tRNA^Cys^ genes (Fig 1). This region was 30 bp long and had the potential to fold into a stem-loop secondary structure with a stem formed by 9 paired nucleotides and a loop of 12 nucleotides. In this study, we analyzed 12 sequences of O1 in Dicroglossidae, and found that the sequence of *H. rugulosus* (BT) was similar to those of *E. hexadactylyus*, *H. tigerinus*, *H. rugulosus* (WT), *Nanorana pleskei*, and *Quasipaa spinosa* (Fig 3).
Table 4. Statistics describing the divergence (\(p\)-distance) in mitochondrial genes between *H. rugulosus* wild type and *H. tigerinus*, between *H. rugulosus* wild type and bred type, as well as between *H. rugulosus* bred type and *H. tigerinus*. W: *H. rugulosus* wild type; B: *H. rugulosus* bred type; T: *H. Tigerinus*.

| Name of Gene or D-loop | No. of nucleotide differences | Pairwise distances of nucleotide | No. of amino acid differences | Pairwise distances divergence of amino acid |
|------------------------|-------------------------------|---------------------------------|------------------------------|------------------------------------------|
|                        | W-T                          | W-B                            | B-T                          | W-T                          | W-B                            | B-T                          |
| D-loop-2               | 232                          | 225                            | 350                          | 0.156                        | 0.151                          | 0.222                        |
| ND5-2                  | 375                          | 347                            | 423                          | 0.205                        | 0.188                          | 0.232                        |
| tRNA{Thr}              | 8                            | 8                              | 10                           | 0.114                        | 0.114                          | 0.145                        |
| tRNA{Pro}              | 6                            | 11                             | 12                           | 0.087                        | 0.159                          | 0.174                        |
| tRNA{Phe}              | 2                            | 4                              | 4                            | 0.029                        | 0.059                          | 0.059                        |
| 12S rRNA               | 61                           | 71                             | 80                           | 0.065                        | 0.076                          | 0.086                        |
| tRNA{Val}              | 8                            | 10                             | 12                           | 0.116                        | 0.145                          | 0.174                        |
| 16S rRNA               | 128                          | 105                            | 139                          | 0.081                        | 0.066                          | 0.088                        |
| tRNA{Lau} (UUR)        | 5                            | 0                              | 5                            | 0.068                        | 0                              | 0.068                        |
| ND1                    | 154                          | 142                            | 187                          | 0.161                        | 0.148                          | 0.195                        |
| tRNA{Ser}              | 4                            | 3                              | 3                            | 0.056                        | 0.042                          | 0.042                        |
| tRNA{Gln}              | 6                            | 10                             | 8                            | 0.085                        | 0.141                          | 0.113                        |
| tRNA{Met} (AUN)        | 6                            | 6                              | 9                            | 0.083                        | 0.083                          | 0.125                        |
| tRNA{Met} (AUN)        | 2                            | 1                              | 3                            | 0.029                        | 0.014                          | 0.043                        |
| ND2                    | 208                          | 182                            | 208                          | 0.201                        | 0.176                          | 0.201                        |
| tRNA{Trp}              | 2                            | 4                              | 1                            | 0.029                        | 0.052                          | 0.014                        |
| tRNA{Asp}              | 6                            | 2                              | 5                            | 0.087                        | 0.029                          | 0.072                        |
| tRNA{Ash}              | 3                            | 2                              | 3                            | 0.041                        | 0.027                          | 0.041                        |
| tRNA{Cys}              | 1                            | 7                              | 6                            | 0.015                        | 0.108                          | 0.092                        |
| tRNA{Ty}               | 3                            | 1                              | 4                            | 0.045                        | 0.015                          | 0.060                        |
| COI                    | 236                          | 198                            | 248                          | 0.152                        | 0.128                          | 0.160                        |
| tRNA{Ser} (UCN)        | 1                            | 1                              | 2                            | 0.014                        | 0.014                          | 0.029                        |
| tRNA{Asp}              | 3                            | 9                              | 8                            | 0.044                        | 0.132                          | 0.118                        |
| COII                   | 104                          | 89                             | 120                          | 0.152                        | 0.130                          | 0.176                        |
| tRNA{Lys}              | 2                            | 1                              | 1                            | 0.029                        | 0.014                          | 0.014                        |
| ATP8                   | 40                           | 29                             | 42                            | 0.247                        | 0.179                          | 0.259                        |
| ATP6                   | 123                          | 123                            | 138                           | 0.180                        | 0.180                          | 0.202                        |
| COIII                  | 116                          | 88                             | 123                           | 0.148                        | 0.112                          | 0.156                        |
| tRNA{Gly}              | 3                            | 6                              | 7                            | 0.043                        | 0.087                          | 0.101                        |
| ND3                    | 66                           | 52                             | 67                            | 0.194                        | 0.152                          | 0.196                        |
| tRNA{Arg}              | 8                            | 2                              | 8                            | 0.116                        | 0.029                          | 0.116                        |
| ND4L                   | 54                           | 44                             | 50                            | 0.191                        | 0.156                          | 0.177                        |
| ND4                    | 277                          | 211                            | 292                           | 0.204                        | 0.155                          | 0.215                        |
| tRNA{His}              | 10                           | 10                             | 14                            | 0.145                        | 0.145                          | 0.203                        |
| tRNA{Ser} (AGY)        | 3                            | 4                              | 1                            | 0.044                        | 0.059                          | 0.015                        |
| ND6                    | 85                           | 56                             | 103                           | 0.175                        | 0.177                          | 0.212                        |
| tRNA{Glu}              | 3                            | 3                              | 4                            | 0.043                        | 0.043                          | 0.058                        |
| Cyt b                  | 179                          | 171                            | 209                           | 0.156                        | 0.149                          | 0.182                        |
| D-loop-1               | 232                          | 548                            | 454                           | 0.156                        | 0.362                          | 0.257                        |
| ND5-1                  | 374                          | 440                            | 520                           | 0.205                        | 0.240                          | 0.282                        |
| tRNA{Lau} (UUR)        | 5                            | 0                              | 5                            | 0.069                        | 0.069                          | 0                            |

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Fig 2. Predicted secondary structures for the 22 tRNA genes of \textit{H. rugulosus} bred type. Dashes (\textendash) indicate Watson–Crick base pairing and plus (+) indicate G+U base pairing. Arms of tRNAs (clockwise from the top) are the amino acid acceptor (AA) arm, the TyC (T) arm, the anticodon (AC) arm, and the dihydrouridine (DHU) arm.

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Phylogenetic analyses in *Hoplobatrachus*

Fig 4 shows the NJ phylogenetic analysis among *Hoplobatrachus* based on Cyt b gene. *H. rugulosus* was divided into two distinct clades. Chinese tiger frog (*H. rugulosus* WT) was clustered with the clade of *H. rugulosus* from northern, northeastern, and eastern Thailand. Thailand tiger frog (*H. rugulosus* BT) was clustered with the clade of *H. rugulosus* from western, central, eastern, and southern Thailand. The divergence between two clades of *H. rugulosus* was 11.5% using the uncorrected p-distance model.

BI, ML, and MP phylogenetic analyses based on the nucleotide dataset of 11 protein-coding genes had a similar topology (Fig 5), which is consistent with Zhang et al. [52]. In this study, we recovered topological relationships among dicroglossid clades with high bootstrap and posterior probability (Fig 5). *O. martensii* (Occidozyginae: Occidozygini) occupied the basal phylogenetic position among the dicroglossid frogs (posterior probabilities 1.00 in BI, bootstrap value 100% in ML and MP). The nucleotide dataset also favored a topology that placed *Hoplobatrachus* as a sister clade to *Euphlyctis* (posterior probabilities 1.00 in BI, bootstrap value 100% in ML and MP), and *H. rugulosus* (BT) was a sister clade to *H. rugulosus* (WT) (posterior probabilities 0.93 in BI, bootstrap value 54% in ML and 83% in MP).

Analyses in *Hoplobatrachus* using the nuclear genes. A total data of 19 sequences of the NCX gene with 7 variable sites of 892 nucleotides, 21 sequences of Rag1 gene including *H. rugulosus* (HM163612) and *H. occipitalis* (HM163613) from GenBank with 20 variable sites of 783 nucleotides, 21 sequences of Rhod gene including *H. rugulosus* (AJ564731) and *H. tigerinus* (AB489039) from GenBank with 4 variable sites of 313 nucleotides, and 21 sequences of Tyr
Fig 4. Phylogenetic relationships among *Hoplobatrachus*. Phylogenetic analysis was carried out for the 41 tiger frogs using the Cyt b gene. The tree was rooted with two out-groups (*E. cyanophlyctis* and *E. hexadactylus*). Numbers at the nodes are NJ bootstrap values.
gene including *H. tigerinus* (AB277358) and *H. occipitalis* (AJ564729) from GenBank with 14 variable sites of 532 nucleotides, were used to evaluate the divergence between the Chinese tiger frog and the Thailand tiger frog, respectively. S3–S6 Tables show the nucleotide genetic divergences among the samples with NCX1, Rag1, Rhod, and Tyr genes, respectively. The nucleotide mean genetic divergences between *H. rugulosus* (BT) and *H. rugulosus* (WT) using NCX1, Rag1, Rhod, and Tyr genes were 0.7%, 0.3%, 0.1%, 0.1%, respectively. We found fixed different nucleotide sites in the Rag1 gene: C and G in *H. rugulosus* (BT) but T and A in *H. rugulosus* (WT) on 386th and 695th site, respectively. Using NJ analysis based on the data of Rag1, Rhod, and Tyr genes, we also found two clades (clade 1 and 2) in *Hoplobatrachus* (S1 Fig).

**Structure analyses on ND5 gene in *Hoplobatrachus***

SMART program analysis revealed the presence in ND5-1 of *H. rugulosus* (BT) of two transmembrane domains (residues 7 to 29 in the amino acid sequence and residues 44 to 66), an Oxidored_q1_N domain (residues 73 to 134), an Oxidored_q1 domain (residues 146 to 408), and a NADH5_C domain (residues 431 to 611) (Fig 6A). Three transmembrane domains (residues 15 to 34 in the amino acid sequence, residues 51 to 73, and residues 93 to 115), an Oxidored_q1 domain (residues 143 to 406), and a NADH5_C domain (residues 429 to 609) existed in the ND5-2 of *H. rugulosus* (BT) (Fig 6B). A signal peptide (residues 1 to 34), a transmembrane domain (residues 41 to 63), an Oxidored_q1_N domain (residues 69 to 130), an Oxidored_q1 domain (residues 141 to 404), and a NADH5_C domain (residues 427 to 607) were present in ND5-1 and ND5-2 of *H. rugulosus* (WT) (Fig 6C). A signal peptide (residues 1 to 20), a transmembrane domain (residues 38 to 60), an Oxidored_q1_N domain (residues 66 to 127), an Oxidored_q1 domain (residues 138 to 401), and a NADH5_C domain (residues 424 to 604) were present in ND5-1 and ND5-2 of *H. tigerinus* (Fig 6D).
Do Cryptic Species Exist in H. rugulosus?
The three-dimensional structure analyses of ND5 proteins revealed that ND5-1 of *H. rugulosus* (BT) contained 2 β-sheet and 22 α-helices; ND5-2 of *H. rugulosus* (BT) contained 2 β-sheet and 23 α-helices; ND5-1 and ND5-2 of *H. rugulosus* (WT) contained 2 β-sheet and 23 α-helices; and ND5-1 and ND5-2 of *H. tigerinus* contained 2 β-sheet and 21 α-helices (Fig 7). β-Sheet and α-helices were attached to one another by relatively flexible, highly charged loops (Fig 7). The overall model quality Z-scores were —5.82 for ND5-1 of *H. rugulosus* (BT); —4.77
for ND5-2 of *H. rugulosus* (BT); −5.61 for ND5-1 and ND5-2 of *H. rugulosus* (WT); and −6.27 for ND5-1 and ND5-2 of *H. tigerinus*. Local model quality values were generally below zero for all ND5 proteins. The 3D–1D averaged score was generally distributed from −0.55 to 0.91 for ND5-1 of *H. rugulosus* (BT); from −0.46 to 0.86 for ND5-2 of *H. rugulosus* (BT); from −0.6 to 0.92 for ND5-1 and ND5-2 of *H. rugulosus* (WT); and from −0.51 to 0.79 for ND5-1 and ND5-2 of *H. tigerinus*.

**Discussion**

**Evidence of cryptic species**

The divergences of partial *Cyt b* gene and mt genome in *H. rugulosus*. The taxonomic problem of *H. rugulosus* has been debated by a number of researchers [10,13,16,53]. Alam et al. [16] and Pansook et al. [17] found high sequence divergences in *H. rugulosus*. In our study, we found high divergence (13.8%) between *H. rugulosus* WT and BT using the *Cyt b* gene, and high divergence (11.5%) between two different clades (clade 1 and 2) of the phylogenetic relationship in *H. rugulosus* (Fig 4). Based on the *Cyt b* gene divergence we support the suggestion [17] that *H. rugulosus* is a cryptic species complex.

Nucleotide divergence within the complete mt genome was 14.0% between *H. rugulosus* WT and BT, which is very high but not beyond the genetic divergence found in cryptic species of diverse other taxa such as *Ciona intestinalis*, *Friesea grisea*, and *Taenia taeniaeformis* [7–9]. Deep nucleotide divergence in protein-coding genes (11.2–24%) was also found using the uncorrected *p*-distance model between *H. rugulosus* (WT) and *H. rugulosus* (BT), which is similar to the values for cryptic species in some frogs [18,20].

The two ND5 genes in *H. rugulosus* (WT) and *H. tigerinus* were identical within each species whereas the two ND5 genes in *H. rugulosus* (BT) had only 84.1% similarity sequence. Although the transmembrane domains and three-dimensional structure analyses of ND5 proteins had differences among *H. tigerinus*, *H. rugulosus* (BT), and *H. rugulosus* (WT), the two ND5 genes in *H. rugulosus* (WT) had an identical tertiary structure (Fig 7). The predicted results (Fig 7) showed that ND5-1 and ND5-2 of *H. rugulosus* (BT) had a similar structural plan, which suggested a common origin in the two ND5 genes. A comparison of all four types of ND5 genes in *Hoplobatrachus*, the protein structure of the two ND5 genes in *H. rugulosus* (BT) differed from those in *H. rugulosus* (WT) and *H. tigerinus*, which suggested that *H. rugulosus* (BT) diverged from *H. rugulosus* (WT) in evolution. According to the different nucleotide divergences, the transmembrane domains, three-dimensional structure, and gene arrangement of the ND5 gene between *H. rugulosus* (WT) and *H. rugulosus* (BT), *H. rugulosus* (WT) and *H. rugulosus* (BT), two different duplication of ND5 genes may have been independently formed in phylogenetic evolution.

Based on the mt genome divergence of the total nucleotide and protein-coding genes (Table 4), as well as the character of ND5 gene in *H. rugulosus* (BT), we can also conclude that *H. rugulosus* is a cryptic species complex. Yet, we failed to find the high genetic divergence between *H. rugulosus* (BT) and *H. rugulosus* (WT) using NCX1, Rag1, Rhod, and Tyr genes.

**mt genome rearrangement in *H. rugulosus* (BT).** In all published dicroglossid sequences, tRNA^Leu^ (CUN), tRNA^Thr^, tRNA^Pro^, and tRNA^Phe^ genes were translocated from their original position of Archaeobatrachia and formed a LTPF tRNA gene cluster the upstream of the 12S rRNA gene. However, the members and arrangement of this tRNA gene cluster are slightly modified in some taxa [21,54]. In our study, the tRNA^Leu^ (CUN) gene was found between the duplicated D-loop–ND5 regions as previously observed in *H. tigerinus* [21] and *H. rugulosus* (WT) [22]. A TPF tRNA gene cluster at the upstream of 12S rRNA gene was formed, which was consistent with *H. tigerinus* [21] and *H. rugulosus* (WT) [22]. We analyzed the four
samples of Thailand tiger frogs by amplifying sequences from ND5 to 12S RNA genes, and the TPF tRNA gene cluster was also found. Thus, the TPF tRNA gene cluster can be regarded as a synapomorphy of Hoplobatrachus, which was consistent with the finding of Alam et al. [21]. Zhou et al. [5], Alam et al. [21], and Chen et al. [55] suggested that the tandem duplication of tRNA\textsuperscript{Met} gene can be regarded as a synapomorphy of Dicroglossinae. In our results, tandem tRNA\textsuperscript{Met} genes were found to exist in \textit{H. rugulosus} (BT).

Identical tandem duplication of ND5-D-loop was observed in \textit{H. tigerinus} and \textit{H. rugulosus} (WT), but only a similar tandem duplication of ND5-D-loop was observed in \textit{H. rugulosus} (BT). The possible rearrangement pathway of the two different ND5 genes in \textit{H. rugulosus} (BT) can be explained by the random duplication model as well as \textit{H. tigerinus} [21]. Yet, the random loss has not happened in two ND5-D-loop regions. The mutation in two ND5 genes of \textit{H. rugulosus} (BT) maybe happened dependently by some unknown selection pressures during the evolution history. The different arrangement pathways of the ND5 gene and the three-dimensional structure of ND5 proteins in \textit{H. rugulosus} (BT) also suggested that \textit{H. rugulosus} represents a cryptic species complex comprising at least two well supported lineages (BT and WT).

**mt genome rearrangement and phylogeny of Dicroglossidae**

The phylogenetic relationships among dicroglossids were mostly similar to the previous molecular phylogeny [5,52,56]. Although the phylogenetic topology is the similar to Zhang et al [52], the bootstrap values of MP, ML and NJ and the posterior probabilities of BI are well supported (Fig 5). In the complete mtDNA of dicroglossids retrieved from GenBank, ND5 gene translocation was observed in \textit{O. martenstii}, \textit{Fejervarya limnocharis}, \textit{Fejervarya cancrivora}, \textit{E. hexadactylus}, \textit{H. rugulosus} (WT), and \textit{H. tigerinus}. In this study, the location of two ND5 genes of \textit{H. rugulosus} (BT) was consistent with \textit{H. tigerinus} and \textit{H. rugulosus} (WT) [21,22]. We identified all different mt genomes of anurans in GenBank. ND5 gene translocation occurred in three distinct lineages of focusing on families closely related to Dicroglossidae: the lineage to \textit{O. martenstii} (Occidozyginae), the lineages to Mantellidae and Rhacophoridae, as well as the lineages to \textit{Fejervarya}, \textit{Euphlyctis}, and \textit{Hoplobatrachus} (Dicroglossinae).

Generally, mt genome arrangements are believed to reflect phylogenetic relationships [57–59]. Using such information, three major lineages can be separated in Dicroglossidae: translocation of ND5, LTPF tRNA gene cluster, and tandem duplication of tRNA\textsuperscript{Met}, which were observed in \textit{O. martenstii} (Occidozyginae). The LTPF tRNA gene cluster and the tandem duplication of tRNA\textsuperscript{Met} were observed in \textit{Quasipaa}, \textit{Nanorana}, and \textit{Limnonectes}. The translocation of ND5, the modified LTPF tRNA gene cluster, and the tandem duplication of tRNA\textsuperscript{Met} were observed in \textit{Fejervarya}, \textit{Hoplobatrachus}, and \textit{Euphlyctis}. The evolutionary relationships of dicroglossid taxa indicated in the phylogenetic trees, which was based on the concatenated sequences of the 11 protein coding genes (Fig 4), were similar to the traditional classification [14,54,60–63]. Based on the results of genome rearrangement and phylogenetic relationship in dicroglossids, we found that the genome rearrangement of Dicroglossidae was consistent with the results of phylogenetic analyses (Fig 4).

Although NJ analysis based on the data of \textit{Rag1}, \textit{Rhod}, and \textit{Tyr} genes, two clades (clade 1 and 2) in \textit{Hoplobatrachus} (S1 Fig) were supported, the genetic divergences of those genes is low. Our findings highlight the need for further nuclear gene and crossbreeding studies of \textit{H. rugulosus} (WT) and \textit{H. rugulosus} (BT). Whether cryptic species exist or not in \textit{H. rugulosus}, Thailand tiger frogs (\textit{H. rugulosus} (BT)) should be prevented from escaping into wild environments to protect Chinese tiger frogs. We strongly advocate that Thailand tiger frogs in the farms should be strictly managed and that release of these frogs into the wild should be prohibited lest they produce hybrids with Chinese tiger frogs.
Conclusion

Based on the Cyt b gene divergence (13.8%) between *H. rugulosus* WT and BT, the mt genome divergence of the total nucleotide (14.0%) between *H. rugulosus* WT and BT, different three-dimensional structure of ND5 proteins and different rearrangement pathways of the ND5 gene between *H. rugulosus* WT and BT, all suggest that *H. rugulosus* is a cryptic species complex. Although using the nuclear gene (NCX1, Rag1, Rhod, and Tyr genes), we failed to find the high genetic divergence between *H. rugulosus* WT and BT, we found two clades (clade 1 and 2) in *Hoplobatrachus* (S1 Fig) using NJ analysis based on the data of Rag1, Rhod, and Tyr genes. Because of the genetic difference between *H. rugulosus* WT and BT, we suggest that we should prevent Thailand tiger frogs from escaping into wild environments lest they produce hybrids with Chinese tiger frogs.

Supporting Information

S1 Fig. Phylogenetic relationships among *Hoplobatrachus* using nuclear genes. Phylogenetic analysis was carried out for the 20 tiger frogs using Rag1, Rhod and Tyr gene. The tree was rooted with a chimeric sequence combining data from two species (*H. tigerinus* with Rhod and Tyr, *H. occipitalis* with Rag1). Numbers at the nodes are NJ bootstrap values. (EPS)

S1 Table. Information about the Cyt b gene in species with GenBank accession numbers used in this study. (DOC)

S2 Table. List of species used in this study, along with GenBank accession numbers and A+T content of total mitochondrial genome and control region (D-loop). (DOC)

S3 Table. List of the nucleotide genetic divergences among 19 samples of NCX gene in species used in this study. The meaning of symbols is shown in S2 Table. (XLS)

S4 Table. List of the nucleotide genetic divergences among 21 samples of Rag1 gene in species used in this study including *H. rugulosus* (HM163612) and *H. occipitalis* (HM163613). The meaning of symbols is shown in S2 Table. (XLS)

S5 Table. List of the nucleotide genetic divergences among 21 samples of Rhod gene in species used in this study including *H. rugulosus* (AJ564731) and *H. tigerinus* (AB489039). The meaning of symbols is shown in S2 Table. (XLS)

S6 Table. List of the nucleotide genetic divergences among 21 samples of Tyr gene in species used in this study including *H. tigerinus* (AB277358) and *H. occipitalis* (AJ564729). The meaning of symbols is shown in S2 Table. (XLS)

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Author Contributions

Conceived and designed the experiments: JZ DY. Performed the experiments: JZ DY. Analyzed the data: JZ PL DY CS. Contributed reagents/materials/analysis tools: JZ PL. Wrote the paper: DY JZ PL RZ CS.

References

1. Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. In: Wolstenholme DR, Jeon KW, editors. Mitochondrial genomes. New York: Academic Press. pp. 173–216.
2. Sbisa E, Tanzariello F, Reyes A, Pesole G, Saccone C (1997) Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene 205: 125–140. PMID: 9461386
3. Brown WM (1983) Evolution of animal mitochondrial DNA. In: Nei M, Koehn RK, editors. Evolution of genes and proteins. Sinauer, Sunderland, MA: Sinauer Assotition Inc Press. pp. 62–88.
4. Avise JC (1994) Molecular markers, natural history and evolution. New York: Champman and Hall.
5. Zhou Y, Zhang JY, Zheng RQ, Yu BG, Yang G (2009) Complete nucleotide sequence and gene organization of the mitochondrial genome of Paao spinosa (Anura: Ranoidae). Gene 447: 86–96. doi: 10.1016/j.gene.2009.07.009 PMID: 19631263
6. Zhang JY, Zhou CF, Gai YH, Song DX, Zhou KY (2008) The complete mitochondrial genome of Parafurronus youi (Insecta: Ephemeroptera) and phylogenetic position of the Ephemeroptera. Gene 424: 18–24. doi: 10.1016/j.gene.2008.07.037 PMID: 18725275
7. Jannelli F, Pesole G, Sordino P, Gissi C (2007) Mitogenomics reveals two cryptic species in Ciona intestinalis. Trends Genet 23: 419–422. PMID: 17640763
8. Torricelli G, Carapelli A, Convey P, Nardi F, Boore JL, Frati F. (2010) High divergence across the whole mitochondrial genome in the “pan-Antarctic” springtail Friesea grisea: Evidence for cryptic species? Gene 449: 30–40. doi: 10.1016/j.gene.2009.09.006 PMID: 19782734
9. Jia W, Yan H, Lou Z, Ni X, Dyachenko V, Li H, et al. (2012) Mitochondrial genes and genomes support a cryptic species of tapeworm within Taenia taeniaeformis. Acta Trop 123: 154–163. doi: 10.1016/j.actatropica.2012.04.006 PMID: 22569565
10. Frost DR (2011) Amphibian Species of the World: an Online Reference. Version 5.5 (31 January, 2014). Electronic Database Available: http://research.amnh.org/vz/herpetology/amphibia/American Museum of Natural History, New York, USA. Version 6.0 ed.
11. Fei L, Ye C, Jiang J (2010) Colored atlas of Chinese amphibians. Chengdu: Sichuan Publishing House of Science and Technology. pp. 1–519.
12. Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad C, et al. (2006) The amphibian tree of life. Bull Am Mus Nat Hist. 1–291.
13. Kosuch J, Vences M, Dubois A, Ohler A, Böhme W (2001) Out of Asia: mitochondrial DNA evidence for an oriental origin of tiger frogs, genus Hoplobatrachus. Mol Phylogenet Evol 21: 398–407. PMID: 11741382
14. Dubois A (2005) Amphibia Mundi. 1.1. An ergotaxonomy of recent amphibians. Alytes 23: 1–24.
15. Fei L, Hu S, Ye C, Huang Y (2009) Fauna Sinica. Amphibia. Vol. 3. Anura Ranidae. Beijing: Chinese Academy of Science. Science Press.
16. Alom MS, Igawa T, Khan MMR, Islama MM, Kuramoto M, Matsui M, et al. (2008) Genetic divergence and evolutionary relationships in six species of genera Hoplobatrachus and Euphlyctis (Amphibia: Anura) from Bangladesh and other Asian countries revealed by mitochondrial gene sequences. Mol Phylogenet Evol 48: 515–527. doi: 10.1016/j.mpev.2008.04.020 PMID: 18513995
17. Pansook A, Khonsue W, Piyapattanakom S, Pariyanonth P (2012) Phylogenetic Relationships among Hoplobatrachus rugulosus in Thailand as inferred from mitochondrial DNA sequences of the cytochrome-b gene (Amphibia, Anura, Dicroglossidae). Zool Sci 29: 54–58. doi: 10.2108/zsj.29.54 PMID: 22234487
18. Elmer KR, Dávila JA, Lougheed SC (2007) Cryptic diversity and deep divergence in an upper Amazonian leaflitter frog, Eleutherodactylus ockendeni. BMC Evol Biol 7: 247. PMID: 18154647
19. Funk WC, Caminer M, Ron SR (2012) High levels of cryptic species diversity uncovered in Amazonian frogs. P Roy Soc B-Biol Sci 279: 1806–1814. doi: 10.1098/rspb.2011.1653 PMID: 22130600

20. Angulo A, Icochea J (2010) Cryptic species complexes, widespread species and conservation: lessons from Amazonian frogs of the Leptodactylus marmoratus group (Anura: Leptodactylidae). Syst Biodivers 8: 357–370.

21. Alam MS, Kurabayashi A, Hayashi Y, Sano N, Khan MMR, Fujii T, et al. (2010) Complete mitochondrial genomes and novel gene rearrangements in two dicroglossid frogs, Hoplobatrachus tigrinus and Euphlyctis hexadactylus, from Bangladesh. Genes Genet Syst 85: 219–232. PMID: 21041980

22. Yu DN, Zhang JY, Zheng RQ, Shao C (2012) The complete mitochondrial genome of Hoplobatrachus rugulosus (Anura: Dicroglossidae). Mitochondr DNA 23: 336–337.

23. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. New York: Cold spring harbor laboratory.

24. Che J, Zhou WW, Hu JS, Yan F, Papenfuss TJ, Wake DB, et al. (2010) Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia. P Natl Acad Sci 107: 13765–13770. doi: 10.1073/pnas.1008415107 PMID: 20643945

25. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680. PMID: 7984417

26. Freileich X, Tollis M, Boissinot S (2014) Hiding in the highlands: Evolution of a frog species complex of the genu Ptychadena in the Ethiopian highlands. Mol Phylogenet Evol 71: 157–169. doi: 10.1016/j.ympev.2013.11.015 PMID: 24315867

27. Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87: 651–701.

28. Cannatella DC, Hillis DM, Chippindale PT, Weigt L, Rand AS, Ryan MJ (1998) Phylogeny of frogs of the Physalaemus pustulosus species group, with an examination of data incongruence. Syst Biol 47: 311–335. PMID: 12064230

29. Hedges SB (1994) Molecular evidence for the origin of birds. P Natl Acad Sci USA 91: 2621–2624. PMID: 8146164

30. Matsui M, Shimada T, Ota H, Tanaka-Ueno T (2005) Multiple invasions of the Ryukyu Archipelago by Oriental frogs of the subgenus Odorrana with phylogenetic reassessment of the related subgenera of the genus Rana. Mol Phylogenet Evol 37: 733–742. PMID: 15964212

31. Su X, Wu XB, Yan P, Cao SY, Hu YL (2007) Rearrangement of a mitochondrial tRNA gene of the cave-eared torrent frog, Amolops tormotus Gene 394: 25–34. PMID: 17368759

32. Burland TG (2000) DNASTAR's Lasergene sequence analysis software. Methods Mol Biol 132: 71–91. PMID: 10547932

33. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGAS: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353

34. Lowe TM, Eddy SR (1997) IRNsascan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25: 955–964. PMID: 9023104

35. Conant GC, Wolfe KH (2008) GenomeVx: simple web-based creation of editable circular chromosome maps. Bioinformatics 24: 861–862. doi: 10.1093/bioinformatics/btm986 PMID: 18227121

36. Sumida M, Kanamori Y, Kaneda H, Kato Y, Nishioka M, Haseqawa M, et al. (2001) Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog Rana nigromaculata. Genes Genet Syst 76: 311–325. PMID: 11817647

37. Kurabayashi A, Yoshikawa N, Sato N, Hayashi Y, Oumi S, Fujii T, et al. (2010) Complete mitochondrial DNA sequence of the endangered frog Odorrana ishikawae (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Mol Phylogenet Evol 56: 543–553. doi: 10.1016/j.ympev.2010.01.022 PMID: 20102742

38. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540–552. PMID: 10742046

39. Xia X, Xie Z (2001) DAMBE: software package for data analysis in molecular biology and evolution. J Hered 92: 371–373. PMID: 11535656

40. Swofford DL (2003) PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Sunderland: Sinauer Associates.

41. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818. PMID: 9918953
42. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. PMID: 11524383

43. Hoegg S, Vences M, Brinkmann H, Meyer A (2004) Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. Mol Biol Evol 21: 1188–1200. PMID: 14963093

44. Letunic I, Doerks T, Bork P (2012) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res 40: D302–D305. doi: 10.1093/nar/gkr931 PMID: 22053084

45. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195–201. PMID: 16301204

46. Sippl MJ (1993) Recognition of errors in three-dimensional structures of proteins. Proteins: Structure, Function, and Bioinformatics 17: 355–362. PMID: 8108378

47. Wiederstein M, Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 35: W407–W410. PMID: 17517781

48. Bowie JU, Lüthy R, Eisenberg D (1991) A method to identify protein sequences that fold into a known three-dimensional structure. Science 253: 164–170. PMID: 1853201

49. Lüthy R, Bowie JU, Eisenberg D (1992) Assessment of protein models with three-dimensional profiles. Nature 356: 83–85. PMID: 1538787

50. Benkert P, Biasini M, Schwede T (2011) Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 27: 343–350. doi: 10.1093/bioinformatics/btq662 PMID: 21134891

51. Kumazawa Y, Nishida M (1999) Complete mitochondrial DNA sequences of the green turtle and blue-tailed mole skink: statistical evidence for archosaurian affinity of turtles. Mol Biol Evol 16: 784. PMID: 10368956

52. Zhang P, Liang D, Mao R-L, Hillis DM, Wake DB, et al. (2013) Efficient sequencing of anuran mtDNAs and a mitogenomic exploration of the phylogeny and evolution of frogs. Mol Biol Evol 30: 1899–1915. doi: 10.1093/molbev/mst091 PMID: 23666244

53. Dubois A (1992) Notes sur la classification des Ranidae (Amphibiens Anoures). B Mens Soc Linn Lyon 61: 305–352.

54. Ren Z, Zhu B, Ma E, Wen J, Tu T, Cao Y, et al. (2009) Complete nucleotide sequence and gene arrangement of the mitochondrial genome of the crab-eating frog Fejervarya cancrivora and evolutionary implications. Gene 441: 148–155. doi: 10.1016/j.gene.2008.09.010 PMID: 19368956

55. Chen G, Wang B, Liu J, Xie F, Jiang JP (2011) Complete mitochondrial genome of Nanorana pleskei (Amphibia: Anura: Dicroglossidae) and evolutionary characteristics of the amphibian mitochondrial genomes. Curr Zool 57: 785–805.

56. Xia Y, Zheng Y, Miura I, Wong PB, Murphy RW, Zeng X (2014) The evolution of mitochondrial genomes in modern frogs (Neobatrachia): nonadaptive evolution of mitochondrial genome reorganization. BMC Genomics 15: 691. doi: 10.1186/1471-2164-15-691 PMID: 25138662

57. Boore J, Brown W (1994) Mitochondrial genomes and the phylogeny of mollusks. Nautilus-Greenville 108: 61–78.

58. Boore JL, Brown WM (1998) Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev 8: 668–674. PMID: 9914213

59. Macey JR, Schulte JA, Larson A (2000) Evolution and phylogenetic information content of mitochondrial genomic structural features illustrated with acrodont lizards. Syst Biol 49: 257–277. PMID: 12118408

60. Hay JM, Ruvinsky I, Hedges SB, Maxson LR (1995) Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. Mol Biol Evol 12: 928. PMID: 7476139

61. Channing A (1999) Historical overview of amphibian systematics in southern Africa. Trans Roy Soc South Africa 54: 121–135.

62. Chen L, Murphy RW, Lathrop A, Ngo A, Orlov NL, Ho CT, et al. (2005) Taxonomic chaos in Asian ranid frogs: an initial phylogenetic resolution. Herp J 15: 231–243.

63. Che J, Pang J, Zhao H, Wu GF, Zhao EM, Zhang YP (2007) Phylogeny of Raninae (Anura: Ranidae) inferred from mitochondrial and nuclear sequences. Mol Phylogenet Evol 43: 1–13. PMID: 17300963