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Original Article

Unveiling cryptic diversity of the anemonefish genera *Amphiprion* and *Premnas* (Perciformes: Pomacentridae) in Thailand with mitochondrial DNA barcodes

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ABSTRACT

The genera *Amphiprion* and *Premnas* comprise the common anemonefish that are widely distributed in tropical areas. Species identification of these two genera is difficult due to high, intraspecific, morphological variation. Recently, DNA barcoding has been employed as an efficient tool that uses a short genetic marker in an organism's DNA to enable the identification and recognition of cryptic species. This study applied three regions of mitochondrial DNA—cytochrome c oxidase I (*COI*), cytochrome *b* (*Cytb*) and 16S rRNA—as DNA barcodes for species identification of seven species of *Amphiprion* and one species of *Premnas* in Thailand. Three species-delimitation methods—general mixed Yule-coalescent (GMYC), automatic barcoding gap detection (ABGD) and a Bayesian implementation of the Poisson tree processes model (bPTP)—were also used to estimate the number of species. An overlap was found between the intra- and inter-specific genetic divergence values in *Cytb* and 16S rRNA, but not for the *COI* data. This indicated that *COI* was the most effective for identifying different anemonefish species. A three-gene phylogenetic analysis and species-delimitation methods based on both *COI* and *Cytb* data suggested cryptic diversity in *Amphiprion clarkii*, *A. percula*, *A. ocellaris* and *Premnas biaculeatus*. Different distributions were found also for two cryptic species of *A. clarkia*—one restricted to the Gulf of Thailand and the other to the Andaman Sea. The results confirmed the efficiency of *COI* as a suitable marker for species identification of anemonefish.

Introduction

Anemonefish or clownfish (family Pomacentridae) have a mutualistic symbiotic relationship with the tropical sea anemones (Elliott et al., 1999). Their chance of attack by predators increases when away from their sea anemone host, resulting in only short excursions. Anemonefish are therefore indicators, which can be used to investigate the geographical distribution of species with high ecological speciation in the marine environment (Timm et al., 2008). Thirty species of anemonefish are recognized in the two genera of *Premnas* and *Amphiprion* (Allen, 1975). *Amphiprion* is further divided into four subgenera (Actinicola, Paramphiprion, Phalerebus, and Amphiprion) which are mainly distinguished by their color patterns. However, closely related species exhibit some differences in their color pattern, for example *Amphiprion akallopisos* and *A. sandaracinos* or *A. perideraion* and *A. nigripes* and moreover, *A. frenatus* and *A. melanopus* together with *A. ocellaris* and *A. percula* have highly similar color patterns (Allen, 1975). *A. ocellaris* displays different color patterns according to its geographic distribution. For example, black *A. ocellaris* with white

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bands is found in waters around Northern Australia, Southeast Asia and Japan, while the orange *A. ocellaris* occurs typically in the Eastern Indian Ocean and the Western Pacific Ocean (Allen, 1997). The single species in the genus *Premnas*—the maroon anemonefish (*Premnas biaculeatus*)—has two different patterns with both white and golden yellow striped bands. This striping color variation results from a number of factors including nutrition (Michael, 2008). The maroon clownfish hails from the Indo-West Pacific and is found throughout the Indo-Australian Archipelago including India, Burma, Thailand, Malaysia, Indonesia, Philippines, New Guinea, New Britain, Solomon Islands, Vanuatu and Australia (Froese and Pauly, 2000). The color patterns cannot be used as a diagnostic characteristic for species identification, leading to misidentification and incorrect species boundaries (Allen, 1997).

Modern taxonomy now relies increasingly on molecular tools using DNA sequences of a short standardized region to compare with sequences of a particular species in databases. This is known as 'DNA barcoding' and has been successfully used to determine the biodiversity, ecological applications and forensic wildlife of many organisms (Hebert et al., 2003a,b; Carvalho et al., 2011; Dubey et al., 2011; Feng et al., 2011; Pereira et al., 2011; Gaur et al., 2012; Pramual and Adler, 2014). Barcodes are constructed from reliably identified reference specimens and retained in reference sequence libraries. The degree of nucleotide divergence between individuals is considered for species identification when inter-species variations become greater than intra-species diversity. The gene most commonly used as a marker for the barcode is the mitochondrial cytochrome *c* oxidase I (*COI*) gene (Hebert et al., 2003a, 2004; Chaves et al., 2008; Supikamolseni et al., 2015; Laopichienpong et al., 2016). The 648-bp segment of the 5′ *COI* gene sequences is used as a universal DNA marker for the identification of animals, including fish (Folmer et al., 1994; Ivanova et al., 2007; Steinke et al., 2009a,b). All available sequences and barcodes are submitted to the Consortium for the Barcode of Life (CBOL) which is concerned with DNA barcoding worldwide for global species identification. However, different mitochondrial genes such as cytochrome *b* (*Cytb*) and 16S rRNA have also been used as barcoding markers for several fish species with varying levels of success (Sevilla et al., 2007; Rock et al., 2008; Smith et al., 2008). Mitochondrial *COI* and *Cytb* genes have provided distinctive barcode cut-off scores for anemonefish species identification in India (Dhaneesh et al., 2015). Notably, substitution rates of mitochondrial DNA (mtDNA) vary between and within species, resulting in broad overlaps of intra- and inter-specific genetic distances (Rubinoff et al., 2006; Chao et al., 2014). A large number of samples is therefore required to determine the best candidate gene marker for each species. The assessment of DNA barcodes across broad geographic ranges is also important for effective species identification.

This study constructed a reference database of eight anemonefish, constituting almost 80% of the species found in Thailand (Allen, 1975), using *COI*, *Cytb* and 16S rRNA barcoding. Species identification using DNA barcodes was then assessed using the degree of nucleotide sequence divergence, barcoding gaps and phylogenetic clustering analysis to test both utility and accuracy. Different methods of DNA-based species delimitation were also applied to estimate the number of species.

Materials and methods

Specimen collection and DNA extraction

Forty individual specimens were examined of seven *Amphiprion* spp. (two varieties of *A. ocellaris*—orange and black, *A. clarkii*, *A. polymnus*, *A. akallopisos*, *A. ephippium*, *A. percula*, and *A. frenatus*) and two varieties of *P. biaculeatus*—white stripe and yellow stripe (Table 1). The anemonefish samples were morphologically identified following Allen (1975). Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University, Bangkok, Thailand (approval no. ACKU01257), and conducted according to the Regulations on Animal Experiments in Kasetsart University. Whole-genomic DNA was isolated from fish fins and muscles according to standard salting out protocol following Supikamolseni et al. (2015) and used as a template for polymerase chain reaction (PCR). DNA quality and concentration were determined using 1% agarose gel electrophoresis and spectrophotometric analysis.

Polymerase chain reaction amplification and sequencing

Partial DNA fragments of the *COI* gene were amplified using primers FF2 and FR1d (Ivanova et al., 2007), and the PCR-product fragment regions were overlapped with those of the universal *COI* primers (Folmer et al., 1994). For *Cytb* and 16S rRNA genes, partial DNA fragments were amplified using AmphicytbF: 5′-ATTGCBACAGCCTTTTCTTC-3′ and AmphicytbR: 5′-GTGGAAGGARATTTTGTCTGC-3′ for the *Cytb* gene and Amphi16SF 5′-AACAACATAAGCTTCTGATTACTC-3′ and AmphicytbR: 5′-GGTTGGTGGTTTCGTCCATG-3′ for the 16S rRNA gene. These were designed based on newly available nucleotide sequence data of teleost fishes. PCR amplification was performed using 15 μL of 1× ThermalPol buffer, containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 μM of primers, 0.5 U of *Taq* polymerase (Vivantis Technologies Sdn Bhd; Selangor Darul Ehsan, Malaysia), and 25 ng of genomic DNA. PCR conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50–60 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 7 min. The PCR products were molecularly cloned using pTG19-T cloning vector (Vivantis Technologies Sdn Bhd; Selangor Darul Ehsan, Malaysia), and the nucleotide sequences of the DNA fragments were determined by the DNA sequencing service of 1st BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor, Malaysia). Nucleotide sequences of three DNA clones were searched for homologies with nucleotide sequences in the National Center for Biotechnology Information (NCBI) database to identify the objective genes using BLASTn and BLASTx programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and then deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/index-e.html) and the Barcode of Life Data Systems (BOLD) (Table 1).

Sequence divergence and phylogenetic clustering analysis

In total, 120 sequences (8 species with 40 individuals) from *COI*, *Cytb*, and 16S rRNA genes were generated, and 13 sequences of 6 *Amphiprion* species (*A. frenatus*, *A. perideraion*, *A. akallopisos*, *A. bicinctus*, *A. percula*, and *A. ocellaris*) and 2 sequences of *Abudefduf vaigiensis* and *Paratilapia polleni* were retrieved from the GenBank database. Multiple sequence alignment was performed using the MEGA6 software with the default parameters (Tamura et al., 2013). All unalignable and gap-containing sites were carefully removed and trimmed from the four datasets (*COI*, *Cytb*, 16S rRNA genes and concatenated sequences), and the level of sequence divergence within and between species was estimated using Kimura's-two-parameter (K2P) model as implemented in MEGA6. Base composition for each nucleotide dataset was measured using PAUP* v4.0b10 (Swofford, 2003). Phylogenetic clustering analyses of the four datasets were then performed using two different methods—Bayesian inference (BI) and maximum likelihood (ML). The best-fit model of DNA substitution was determined for each gene using the program Kakusan4 (Tanabe, 2007). BI analyses were performed using MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). The

Table 1
Classification and accession numbers of samples used in the current study. Bold letters indicate the sequence accession number derived in this study. The remaining sequence accession numbers were taken from the GenBank database (GT, the Gulf of Thailand; ADM, the Andaman Sea).

Species	Collection code	Locality detail	Voucher number	Accession number		
				COI	*Ctyb*	16S rRNA
Amphiprion akallopisos	AAK	Madagascar	NBE1037	JF434726	JF434726	JF434726
	AAK1	Krabi (ADM)	KUGEN2011004	**LC160015**	**LC160016**	**LC160017**
	AAK2	Krabi (ADM)	KUGEN2011005	**LC160018**	**LC160019**	**LC160020**
	AKK3	Krabi (ADM)	KUGEN2011006	**LC160021**	**LC160022**	**LC160023**
A. bicinctus	ABI1	N/A	RSRCmb2010	JQ030887	JQ030887	JQ030887
	ABI2	Saudi Arabia	RS5643	KR023459	DQ343946	DQ343906
	ABI3	Indonesia	GA036	KR023433	KF264300	KF264154
A. clarkia	ACL1	Krabi (ADM)	KUGEN2011013	**LC160042**	**LC160043**	**LC160044**
	ACL2	Krabi (ADM)	KUGEN2011014	**LC160045**	**LC160046**	**LC160047**
	ACL3	Krabi (ADM)	KUGEN2011015	**LC160048**	**LC160049**	**LC160050**
	ACL4	Chonburi (GT)	KUGEN2011016	**LC160051**	**LC160052**	**LC160053**
	ACL5	Chonburi (GT)	KUGEN2011017	**LC160054**	**LC160055**	**LC160056**
	ACL6	Chonburi (GT)	KUGEN2011018	**LC160057**	**LC160058**	**LC160059**
	ACL7	Rayong (GT)	KUGEN2011019	**LC160060**	**LC160061**	**LC160062**
	ACL8	Rayong (GT)	KUGEN2011020	**LC160063**	**LC160064**	**LC160065**
	ACL9	Rayong (GT)	KUGEN2011021	**LC160066**	**LC160067**	**LC160068**
	ACL10	Rayong (GT)	KUGEN2011022	**LC160069**	**LC160070**	**LC160071**
A. ephippium	AEP1	Krabi (ADM)	KUGEN2011001	**LC160006**	**LC160007**	**LC160008**
	AEP2	Krabi (ADM)	KUGEN2011002	**LC160009**	**LC160010**	**LC160011**
	AEP3	Krabi (ADM)	KUGEN2011003	**LC160012**	**LC160013**	**LC160014**
A. frenatus	AFR	China	AF20131222A	KJ833752	KJ833752	KJ833752
	AFR1	Krabi (ADM)	KUGEN2011028	**LC160087**	**LC160088**	**LC160089**
	AFR2	Krabi (ADM)	KUGEN2011029	**LC160090**	**LC160091**	**LC160092**
	AFR3	Rayong (GT)	KUGEN2011030	**LC160093**	**LC160094**	**LC160095**
A. percula	APE	China	AC20130909A	KJ174497	KJ174497	KJ174497
	APE1	Krabi (ADM)	KUGEN2011023	**LC160072**	**LC160073**	**LC160074**
	APE2	Krabi (ADM)	KUGEN2011024	**LC160075**	**LC160076**	**LC160077**
	APE3	Krabi (ADM)	KUGEN2011025	**LC160078**	**LC160079**	**LC160080**
	APE4	Chonburi (GT)	KUGEN2011026	**LC160081**	**LC160082**	**LC160083**
	APE5	Chonburi (GT)	KUGEN2011027	**LC160084**	**LC160085**	**LC160086**
A. perideraion	APR1	China	AP20140328A	KJ833753	KJ833753	KJ833753
	APR2	Indonesia	GA015	FJ582795	KF264300	KF264178
	APR3	Indonesia	GA037	FJ582798	KF264306	KF264184
	APR4	Indonesia	GA020	FJ582797	KF264305	KF264179
A. polymnus	APO1	Krabi (ADM)	KUGEN2011031	**LC160096**	**LC160097**	**LC160098**
	APO2	Krabi (ADM)	KUGEN2011032	**LC160099**	**LC160100**	**LC160101**
	APO3	Rayong (GT)	KUGEN2011033	**LC160102**	**LC160103**	**LC160104**
	APO4	Chonburi (GT)	KUGEN2011034	**LC160105**	**LC160106**	**LC160107**
	APO5	Chonburi (GT)	KUGEN2011035	**LC160108**	**LC160109**	**LC160110**
A. ocellaris	AOC	Japan	N/A	AP006017	AP006017	AP006017
	AOC1	Rayong (GT)	KUGEN2011036	**LC160111**	**LC160112**	**LC160113**
	AOC2	Rayong (GT)	KUGEN2011037	**LC160114**	**LC160115**	**LC160116**
	AOC3	Chonburi (GT)	KUGEN2011038	**LC160117**	**LC160118**	**LC160119**
	AOCB1	Chonburi (GT)	KUGEN2011039	**LC160120**	**LC160121**	**LC160122**
	AOCB2	Chonburi (GT)	KUGEN2011040	**LC160123**	**LC160124**	**LC160125**
Premnas biaculeatus	PBWS1	Krabi (ADM)	KUGEN2011007	**LC160024**	**LC160025**	**LC160026**
	PBWS2	Krabi (ADM)	KUGEN2011008	**LC160027**	**LC160028**	**LC160029**
	PBWS3	Krabi (ADM)	KUGEN2011009	**LC160030**	**LC160031**	**LC160032**
	PBWS4	Petchaburi (GT)	KUGEN2011010	**LC160033**	**LC160034**	**LC160035**
	PBYS1	Chonburi (GT)	KUGEN2011011	**LC160036**	**LC160037**	**LC160038**
	PBYS2	Chonburi (GT)	KUGEN2011012	**LC160039**	**LC160040**	**LC160041**

Markov chain Monte Carlo (MCMC) process was set to run four chains simultaneously for one million generations. After the log-likelihood value plateaued, a sampling procedure was performed every 100 generations to obtain 10,000 trees, and subsequently to provide a majority-rule consensus tree with average branch lengths. All sample points prior to reaching convergence were discarded as burn-in, and the Bayesian posterior probability in the sampled tree population was obtained in percentage terms. ML analyses for all datasets were performed using Treefinder software (Jobb et al., 2004) within the tool package Phylogear v2.0 (Tanabe, 2008), followed by bootstrap analysis conducted with 1000 replications.

Species delimitation

DNA-based species delimitations were tested using separate datasets of *COI* and *Cytb* by three different methods—general mixed Yule-coalescent (GMYC) (Pons et al., 2006), automatic barcoding gap detection (ABGD) (Puillandre et al., 2012) and a Bayesian implementation of the Poisson tree processes model (bPTP) (Zhang et al., 2013). For the GMYC delimitation method, an ultrametric tree was constructed in BEAST v2.0.2 (Drummond et al., 2012), relying on the uncorrelated lognormal relaxed clock GTR + I + G, and a coalescent tree prior. MCMC was run for 20 million generations, and trees and parameters were sampled every 1000 generations. Log files were visualized in Tracers v1.6 (Rambaut et al., 2015) to assess the stationary state of parameters by the value of their estimated effective sample size (ESS). After removing 25% of the trees as burn-in, the remainders were used to generate a single summarized tree in TreeAnnotator v2.0.2 (part of the BEAST v2.0.2 package) as an input file for GMYC analyses. The GMYC analyses with a single threshold model were performed in R (R Development Core Team, http://www.R-project.org) under the 'splits' package

using the 'gmyc' function (R-Forge, http://r-forge.r-project.org/projects/splits/). The ABGD method was tested via a web interface (ABGD web, http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Before analysis, model criteria were set as follows: intraspecific variability (*P*) between 0.001 (P_{min}) and 0.1 (P_{max}), minimum gap width (*X*) of 0.1, K2P and 50 screening steps. In addition, bPTP analyses of *COI* and *Cytb* datasets were performed on the web (http://species.h-its.org/ptp/) with a Bayesian tree obtained in MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001).

Results

Barcode construction

The nucleotide sequences of *COI*, *Cytb* and 16S rRNA barcodes were successfully retrieved from 40 samples (eight species). All the 16S rRNA, *COI* and *Cytb* datasets showed no insertions, deletions or stop codons in the sequences of *COI* and *Cytb* datasets. All nucleotide sequences were trimmed, and the datasets of aligned *COI*, *Cytb* and 16S rRNA fragments showed 589, 392 and 376 nucleotides, comprising 202, 126 and 191 variable sites with 133, 99 and 103 parsimony informative sites, respectively. The average nucleotide frequencies for the entire datasets were A = 24.19%, T = 30.02%, C = 28.60% and G = 17.19% for the *COI* dataset, A = 25.93%, T = 30.94%, C = 26.48% and G = 16.65% for the *Cytb* dataset, and A = 32.34%, T = 22.81%, C = 23.36% and G = 21.49% for the 16S rRNA dataset. The eventual aligned dataset of the concatenated sequences of *COI*, *Cytb* and 16S rRNA provided 1346 characters for 51 samples (10 species) with two outgroup species excluded.

Specific nucleotide mutation analysis

The occurrence of species-specific nucleotide sequences was examined by evaluating the same single nucleotide among members of a species. All the *COI*, *Cytb* and 16S rRNA sequences obtained in this study and those previously deposited in the database were investigated for species-specific nucleotide polymorphisms (Table 2). The specific nucleotides found in the *COI*, *Cytb* and 16S rRNA sequences were respectively 42 (9 species), 21 (7 species) and 17 (6 species) specific nucleotides, which could feasibly be used to discriminate between species.

Sequence divergence

Multiple samples in each species were used to search for intraspecific sequence divergence. The average *p*-distance and K2P divergences, respectively, were 0.99 ± 0.01% and 4.15 ± 0.52% for *COI*, 1.35 ± 0.04% and 4.32 ± 0.34% for *Cytb*, and 0.85 ± 0.02% and 2.14 ± 0.29% for 16S rRNA genes (Table 3). The degree of *p*-distance and K2P divergences, respectively, of *COI*, *Cytb* and 16S rRNA fragments obtained in this study, and those previously deposited in the database for each species were 0–1.19% and 0–1.20% for *COI*, 0–3.29% and 0–3.32% for *Cytb*, and 0–4.01% and 0–4.03% for 16S rRNA. The average interspecific K2P divergences of *COI*, *Cytb* and 16S rRNA were 7 ± 0.77%, 9 ± 0.54% and 6.50 ± 0.31%, respectively (Table 3). The dataset of interspecific sequence divergence is shown in Table 4.

The maximum intraspecific K2P divergences were 2.30% in *A. frenatus* for *COI*, 4.20% in *A. bicinctus* for *Cytb*, and 1.9% in *A. clarkii* for 16S rRNA genes, respectively. By contrast, the minimum interspecific K2P divergences were 2.2% between *A. akallopisos* and *A. perideraion* for *COI*, 1% between *A. frenatus* and *A. ephippium* for *Cytb* and 0% between *A. frenatus* and *A. ephippium* for 16S rRNA, respectively. These divergence values between the minimum interspecific and maximum intraspecific divergences were taken to be the threshold level of species identification and thereby considered as a barcoding gap. Within the *COI* dataset, gaps were found in *A. akallopisos*, *A. bicinctus*, *A. clarkiil*, *A. ephippium*, *A. frenatus*, *A. percula*, *A. perideraion*, *A. polymnus*, *A. ocellaris* and *P. biaculeatus*. Within *Cytb* dataset, the gaps were found in *A. akallopisos*, *A. clarkii*, *A. ephippium*, *A. frenatus*, *A. ocellaris*, *A. percula*, *A. polymnus* and *P. biaculeatus*. Within the 16S rRNA dataset, gaps were found in *A. akallopisos*, *A. bicinctus*, *A. clarkii*, *A. polymnus*, *A. perideraion* and *P. biaculeatus*. However, there was a significant overlap without gaps in the *Cytb* and 16S rRNA datasets. Using a two-marker combination of *COI* and *Cytb* genes, gaps were found in all species.

Because of the high intraspecific and low interspecific values of 16S rRNA, their 16S rRNA values were not examined for concatenated sequences. The *COI* + *Cytb* concatenated maximum intraspecific K2P divergence was 3.30% in *A. percula* and the minimum interspecific K2P divergence was 1.2% between *A. frenatus* and *A. ephippium*. The results showed that marker combinations can differentiate all anemonefish species.

Table 2
Specific nucleotide mutations in partial mitochondrial gene sequences for anemonefish species.

Species	Nucleotide position
COI (589 bp)	
Amphiprion akallopisos	T(369), A(392), T(440)
A. bicinctus	G(143), G(263), C(353)
A. clarkia	G(107), G(224), G(344), A(518), T(578)
A. frenatus	T(527)
A. ocellaris	C(140), T(305), T(392), A(512)
A. polymnus	C(170), A(281), A(371), C(395)
A. percula	C(23), T(309), T(326), T(584)
A. perideraion	C(348)
Premnas biaculeatus	G(32), G(53), C(86), G(182), A(185), C(233), C(245), C(278), G(350), T(365), C(377), G(458), A(461), G(473), C(488), C(533), A(548)
Cytb (392 bp)	
A. akallopisos	G(114), T(201), A(279)
A. clarkia	C(84), T(145), C(198)
A. ephippium	G(21), T(390)
A. percula	C(132), T(294)
A. polymnus	T(4), T(327)
A. ocellaris	T(24), G(84), T(132)
P. biaculeatus	T(258), C(288), A(321), C(333), A(339), C(361)
16S rRNA (376 bp)	
A. akallopisos	A(300), A(352)
A. bicinctus	A(124), T(267), T(289), G(296)
A. clarkia	G(161), A(367)
A. frenatus	A(187)
A. polymnus	T(184)
P. biaculeatus	T(53), A(211), C(223), T(280), G(303), C(325), C(347)

Table 3
Comparison of average intraspecific sequence divergence (%) obtained in this study.

Species	*COI*		*Cytb*		16S rRNA	
	p-distance	K2P	*p*-distance	K2P	*p*-distance	K2P
Amphiprion akallopisos	1.19	1.20	0.40	0.40	0.11	0.12
A. bicinctus	0.30	0.33	2.58	2.59	0.20	0.21
A. clarkia	0.68	0.69	0.77	0.80	0.90	0.92
A. ephippium	0.00	0.00	0.00	0.00	0.00	0.00
A. frenatus	1.10	1.11	1.16	1.18	0.14	0.16
A. percula	1.10	1.12	3.29	3.32	4.01	4.03
A. perideraion	0.00	0.00	0.47	0.49	0.71	0.73
A. polymnus	0.00	0.00	0.37	0.38	0.20	0.22
A. ocellaris	0.67	0.69	1.31	1.32	2.59	2.60
Premnas biaculeatus	0.90	0.92	0.55	0.56	0.73	0.76

Table 4
Comparison of average interspecific sequence divergence (%) obtained in this study.

Species1	Species2	*COI*		*Cytb*		16S rRNA	
		p-distance	K2P	*p*-distance	K2P	*p*-distance	K2P
A. ephippium	*A. akallopisos*	3.10	3.19	6.57	6.97	2.57	2.63
A. ephippium	*P. biaculeatus*	9.28	10.06	10.14	11.11	6.99	7.47
A. akallopisos	*P. biaculeatus*	9.45	10.29	10.24	11.25	7.62	8.17
A. ephippium	*A. clarkii*	4.43	4.61	7.40	7.88	4.47	4.65
A. akallopisos	*A. clarkii*	4.75	4.95	6.79	7.19	4.82	5.02
P. biaculeatus	*A. clarkii*	9.87	10.77	10.20	11.15	7.11	7.59
A. ephippium	*A. percula*	8.90	9.62	12.11	13.61	6.57	7.08
A. akallopisos	*A. percula*	9.22	10.00	12.20	13.71	7.19	7.77
P. biaculeatus	*A. percula*	10.03	10.94	9.99	10.93	7.41	8.08
A. clarkii	*A. percula*	9.87	10.77	11.90	13.30	7.81	8.48
A. ephippium	*A. frenatus*	0.79	0.79	2.00	2.04	0.21	0.21
A. akallopisos	*A. frenatus*	3.27	3.38	5.93	6.24	2.36	2.41
P. biaculeatus	*A. frenatus*	9.35	10.14	10.24	11.25	6.78	7.23
A. clarkii	*A. frenatus*	5.13	5.37	6.16	6.48	4.26	4.42
A. percula	*A. frenatus*	8.89	9.61	11.60	12.95	6.47	6.97
A. ephippium	*A. polymnus*	3.32	3.41	5.15	5.42	2.33	2.39
A. akallopisos	*A. polymnus*	4.71	4.91	6.73	7.16	3.24	3.34
P. biaculeatus	*A. polymnus*	8.99	9.68	10.45	11.52	6.44	6.84
A. clarkii	*A. polymnus*	6.07	6.40	7.48	7.99	5.03	5.26
A. percula	*A. polymnus*	9.08	9.81	11.67	13.05	6.53	7.03
A. frenatus	*A. polymnus*	3.75	3.87	4.91	5.15	2.13	2.17
A. ephippium	*A. ocellaris*	8.96	9.69	12.89	14.54	9.35	10.68
A. akallopisos	*A. ocellaris*	8.80	9.50	12.67	14.24	10.16	11.67
P. biaculeatus	*A. ocellaris*	10.49	11.49	10.25	11.23	10.48	12.06
A. clarkii	*A. ocellaris*	9.15	9.93	12.13	13.49	10.62	12.19
A. percula	*A. ocellaris*	3.69	3.81	4.80	5.03	9.79	11.20
A. frenatus	*A. ocellaris*	8.73	9.42	12.41	13.92	9.21	10.53
A. polymnus	*A. ocellaris*	8.76	9.43	11.41	12.67	9.57	11.01
A. ephippium	*A. perideraion*	2.44	2.50	5.22	5.49	1.18	1.19
A. akallopisos	*A. perideraion*	1.66	1.68	2.51	2.56	1.53	1.55
P. biaculeatus	*A. perideraion*	9.45	10.27	10.20	11.23	6.50	6.90
A. clarkii	*A. perideraion*	4.66	4.85	6.29	6.64	3.99	4.12
A. percula	*A. perideraion*	8.87	9.61	12.41	14.01	6.60	7.10
A. frenatus	*A. perideraion*	2.62	2.68	4.51	4.71	0.97	0.98
A. polymnus	*A. perideraion*	4.54	4.72	5.10	5.27	1.85	1.88
A. ocellaris	*A. perideraion*	8.61	9.29	12.65	14.23	9.51	10.93
A. ephippium	*A. bicinctus*	3.03	3.11	4.81	5.02	2.59	2.65
A. akallopisos	*A. bicinctus*	4.33	4.52	4.25	4.41	3.22	3.30
P. biaculeatus	*A. bicinctus*	8.70	9.39	10.74	11.86	7.36	7.86
A. clarkii	*A. bicinctus*	5.47	5.76	6.16	6.48	5.12	5.34
A. percula	*A. bicinctus*	9.31	10.13	12.11	13.63	7.31	7.90
A. frenatus	*A. bicinctus*	3.42	3.53	4.23	4.39	2.38	2.43
A. polymnus	*A. bicinctus*	4.42	4.59	5.38	5.66	2.70	2.77
A. ocellaris	*A. bicinctus*	8.58	9.26	12.60	14.20	10.19	11.70
A. perideraion	*A. bicinctus*	3.66	3.79	2.68	2.76	2.11	2.14

Phylogenetic reconstruction

The ML and Bayesian methods yielded very similar tree topologies for the three individual markers and the combined dataset. The results were presented using the combined dataset-based Bayesian topology with the support value from both analyses indicated at each node (Fig. 1). Within the *Amphiprion* clade, two well-supported clades were recognized, being clade 2A consisting of *A. percula* and *A. ocellaris* (ML = 100%, BI = 1.00), corresponding to their morphology-based species assignment in the clownfish complex, and clade 1A consisting of the remaining species (ML = 95%, BI = 1.00). In clade 2A containing *A. percula* and *A. ocellaris*, well-supported clades were recognized (Fig. 1, ML > 85%, BI > 95%).

In clade 1A, *A. clarkii* was recovered as a sister to the remaining species. *A. akallopisos*, *A. polymnus*, *A. frenatus* and *A. ephippium* formed a monophyletic clade with strong support (ML > 85%, BI = 1.00). *A. perideration* and *A. akallopisos* formed a well-supported clade (ML = 100%, BI = 1.00), corresponding to the morphological features of the skunk complex. Clades of *A. ephippium* and *A. frenatus* were grouped together (ML = 100%, BI = 1.00), representing the morphology of the ephippium complex. The sequence of *A. frenatus* (KJ833753) retrieved from GenBank was placed with the samples of *A. ephippium*. The *A. ephippium*-*A. frenatus* clade was positioned close to *A. polymnus* (saddleback complex).

The clade of *P. biaculeatus* was composed of samples from both coasts of Thailand. This species was morphologically assigned to the maroon complex as the samples clearly showed two different body bar colors of white (PBWS1–4) and yellow (PBYS1–2). Several well-supported clades were recognized (ML > 85%, BI = 100).

Species delimitation

Three different methods for species delimitation (GMYC, ABGD and bPTP) based on the separate *COI* and *Cytb* datasets supported a single species of *A. ephippium* and *A. frenatus*, but indicated multiple cryptic species in other *Amphiprion* species and *P. biaculeatus* (Fig. 1). Singletons were also observed from sequences retrieved from GenBank (*A. akallopisos* JF434726, *A. frenatus* KJ833753 and *A. percula* KJ174497). Species delimitation based on the *COI* dataset

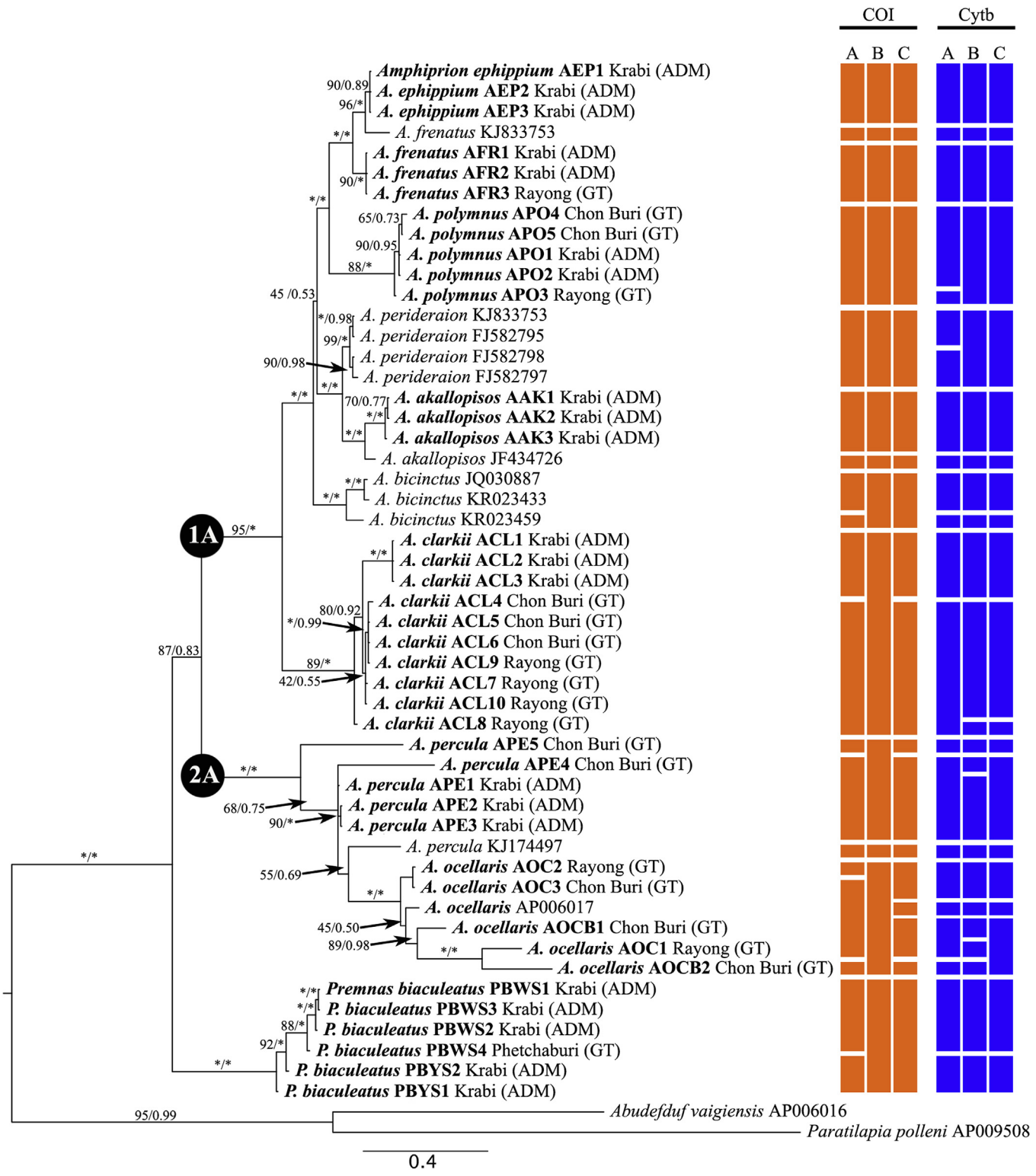

Fig. 1. Phylogenetic tree inferred from BI analyses of the combined dataset of *COI*, *Cytb* and 16S rRNA for *Amphiprion* and *Premnas* species. Support values at each node arebootstrap values from ML (left) and Bayesian posterior probability (right). Asterisk (*) indicates full support (100%, 1.0) in both analyses and hyphen (–) indicates no support. The results of three species delimitation methods: GMYC model (column A), ABGD (column B) and bPTP (column C) based on *COI* (orange color) and *Cytb* (blue color) are indicated at the right edge of the tree (GT, the Gulf of Thailand; ADM, the Andaman Sea).

appeared slightly more conservative than that based on the *Cytb* alignment.

Delimiting *Amphiprion* and *Premnas* species using the *COI* dataset yielded different numbers of putative species depending on the methods. The GMYC method ($\ln L_{GMYC} = 232.146 > \ln L_0 = 214.212$, $P = 0.001$) recognized 19 species, followed by the ABGD method with 18 species ($p = 0.001$) and the bPTP method recovered 13 species. All the species delimitation methods

indicated a single species of *A. ephippium*, *A. frenatus*, *A. polymnus* and *A. perideration*, and multiple species of *A. bicinctus* (2 putative species), *A. clarkii* (2 putative species), *A. percula* (2 putative species), *A. ocellaris* (3 or 4 putative species) and *P. biaculeatus* (2 putative species).

For *Cytb*, three different methods provided incongruent numbers of putative species as 22 for GMYC, 20 for ABGD and 23 for bPTP. Three species (*A. ephippium*, *A. frenatus* and *A. akallopisos*) were recognized as a single cluster across the three different methods, while several single morphospecies were genetically identified as multiple cryptic species (for example, *A. bicinctus*, *A. clarkii*, *A. ocellaris*, *A. percula and P. biaculeatus*).

Discussion

Species identification using DNA barcoding

This was the first study to assess the resolution of DNA barcoding for identifying anemonefish species in Thailand. Species identification through DNA barcoding is constructed where interspecific divergences are sufficiently greater than intraspecific divergences (Hebert et al., 2003a,b). Among the three markers, *COI* and *Cytb* were effective for species identification of Thai anemonefish, while 16S rRNA showed a low level of genetic divergence that occasionally failed to identify fish species. These findings were congruent with several previous studies, indicating that the 16S rRNA gene was a powerful but not perfect barcode for fish species (Zhang and Hanner, 2012) and other organisms (Xia et al., 2012; Lv et al., 2014; Sanchez et al., 2016).

The *COI* and *Cytb* genes have often provided distinctive, barcoded, cut-off scores which have been widely used as genetic markers for identification of fish species (Ivanova et al., 2007; Nicolas et al., 2012). The current results indicated that all anemonefish species were readily distinguishable by *COI* barcodes, while *Cytb* could identify some species. The overlap of inter- and intraspecific genetic divergence was also observed in *Cytb*. Additionally, many diagnostic nucleotide sequences that differentiated each species were found in the *COI* dataset, but not for *Cytb*. Furthermore, the analysis of intra- and inter-specific distances using two different algorithms (*p*-distance and K2P) found that the K2P value was higher than the *p*-distance divergences for all markers. Therefore, K2P divergences were applied to evaluate the reliability and usefulness of a pure distance-based approach in each marker, using a 10× threshold of average K2P divergence for congeneric species as suggested by Hebert et al. (2004). Sequence divergence among the average congeneric species was determined at 11.29 times higher than the average divergence within species for the *COI* barcode. By contrast, the average interspecific sequence divergences of the *Cytb* and 16S rRNA genes within each genus were 8.1 and 4.7 times higher than the intraspecific distance. Therefore, it was proposed that the *COI* gene is a suitable DNA barcode marker for anemonefish species identification.

Phylogeny and species delimitation

The molecular data indicated a monophyletic group of the genera *Amphiprion* and *Premnas* (Allen, 1975). Within the genus *Amphiprion*, two major clades (1A and 2A) were recognized, and the separation of these two clades was congruent with the differences in morphology and ecological behavior of the members of each major clade (Allen, 1975, 1997). For example, the body shape (body depth and total length) of *A. percula* and *A. ocellaris* (clade 2A) are relatively distinct from other species in the clade 1A. However, the morphological features and ecological behavior of our anemonefish samples were not thoroughly observed; hence, it is not possible to clarify the relationship between genetic data and their morphology and ecology. Further study on morphometrics, geographic distribution and ecological behavior is required to verify the taxonomic status and true diversity of these anemonefish species in Thailand and adjacent waters.

The current phylogenetic results strongly indicated the occurrence of multiple well-supported clades within the genera *Amphiprion* and *Premnas*. In clade 2A, the relationship between *A. percula* and *A. ocellaris* was incompletely resolved. However, both the three-gene phylogenetic analyses and the three different species delimitations indicated that *A. percula* had at least two genetic lineages and *A. ocellaris* had at least three genetic lineages. This suggests the possibility of cryptic diversity in these two species. Interestingly, the sequence of *A. percula* from China (KJ174497) was positioned with the *A. ocellaris* group, and the congruent result of all species delimitation algorithms suggested putative species of the Chinese samples. This could either be due to the morphological misidentification of the Chinese samples, or the recognition of undiscovered species of the genus *Amphiprion*. Increased sampling with more variable genetic markers could help to clarify the relationship and taxonomic status of these anemonefish species.

Within clade 1A, the sequences of *A. ephippium*, *A. frenatus*, *A. polymnus* and *A. akallopisos* indicated a monophyletic group for each species. Samples of *A. ephippium* were clustered with *A. frenatus* from China (KJ833753), and this ambiguous grouping could be due to the misidentification of the Chinese species. In addition, the molecular data in the current study suggested the cryptic species of *A. akallopisos*, with the recognition of two genetic lineages—one from Thailand and the other from Madagascar (JF434726). This implies that the occurrence of cryptic diversity of this species was likely caused by geographic disjunction. Future study on the global phylogeographic pattern of *A. akallopisos* will contribute to knowledge of the speciation process of cryptic species and the connectivity of marine populations.

Amphiprion clarkii has two genetic lineages across the Gulf of Thailand and the Andaman Sea. One lineage (*A. clarkii* ACL 1–3) is confined to the Andaman Sea, while the other (*A. clarkii* ACL 4–10) is only found in the Gulf of Thailand. Current data indicates genetic differentiation between the two Thai coastal areas, suggesting a lack of connectivity between the two coasts. A genetic break between the Gulf of Thailand and the Andaman Sea populations was also observed in other marine organisms, for example, the mangrove *Rhizophora apiculata* Blume (Ng et al., 2015), the Asian moon scallop *Amusium pleuronectes* Linnaeus (Mahidol et al., 2007), and the marine red algae *Bostrychia* (Saengkaew et al., 2016).

Molecular data indicated two cryptic species of *P. biaculeatus* which correlated with the different color patterns on their bodies as either a white stripe or a yellow stripe. While the current data revealed the possibility of cryptic species of *P. biaculeatus* in Thailand, more intensive sampling and morphometric analyses are needed to verify their true taxonomic position.

Conflicts of interest

There is no conflict of interest.

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References

Allen, G.R., 1975. The Anemonefishes: Their Classification and Biology, second ed. Tropical Fish Hobbyist Publications, Neptune, NJ, USA.

Allen, G.R., 1997. Marine Fishes of Tropical Australia and South-East Asia. Western Australian Museum, Perth, WA, Australia.

Carvalho, D.C., Neto, D.A.P., Brasil, B.S.A.F., Oliveira, D.A.A., 2011. DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil. Mitochondrial DNA 22, 97–105.

Chao, Z., Liao, J., Liang, Z., Huang, S., Zhang, L., Li, J., 2014. Cytochrome C oxidase subunit I barcodes provide an efficient tool for Jinqian Baihua She (*Bungarus parvus*) authentication. Pharmacogn. Mag. 10, 449–457.

Chaves, A.V., Clozato, C.L., Lacerda, D.R., Sari, E.H.R., Santos, F.R., 2008. Molecular taxonomy of Brazilian tyrant-flycatchers (Passeriformes: Tyrannidae). Mol. Ecol. Resour. 8, 1169–1177.

Dhaneesh, K.V., Kumar, T.T.A., Kumar, A.B., 2015. Barcoding, phylogeography and species boundaries in clownfishes of the Indian Ocean. DNA Barcodes 3, 5–16.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.

Dubey, B., Meganathan, P.R., Haque, I., 2011. DNA mini-barcoding: an approach for forensic identification of some endangered Indian snake species. Forensic Sci. Int. Genet. 5, 181–184.

Elliott, J.K., Lougheed, S.C., Bateman, B., McPhee, L.K., Boag, P.T., 1999. Molecular phylogenetic evidence for the evolution of specialization in anemonefishes. Proc. R. Soc. Lond. B Biol. Sci. 266, 677–685.

Feng, Y., Li, Q.I., Kong, L., Zheng, X., 2011. COI-based DNA barcoding of Arcoida species (Bivalvia: Pteriomorphia) along the coast of China. Mol. Ecol. Resour. 11, 435–441.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Froese, R., Pauly, D. (Eds.), 2000. FishBase 2000: Concepts, Design and Data Sources. ICLARM, Los Baños, Laguna, the Philippines.

Gaur, A., Singh, C.S., Sreenivas, A., Singh, L., 2012. DNA-based identification of a snake in a wine bottle using universal primers: a case of mistaken identity. Forensic Sci. Int. 214, 51–53.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. 270, 313–321.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H., Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. U. S. A. 101, 14812–14817.

Hebert, P.D.N., Ratnasingham, S., deWaard, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. Sci. 270, 96–99.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes 7, 544–548.

Jobb, G., von Haeseler, A., Strimmer, K., 2004. RETRACTED ARTICLE: TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 1–9.

Laopichienpong, N., Muangmai, N., Supikamolseni, A., Twilprawat, P., Chanhome, L., Suntrarachun, S., Peyachoknagul, S., Srikulnath, K., 2016. Assessment of snake DNA barcodes based on mitochondrial *COI* and *Cytb* genes revealed multiple putative cryptic species in Thailand. Gene 594, 238–247.

Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., et al., 2014. Assessment of four DNA fragments (*COI*, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasit. Vectors 7, 93.

Mahidol, C., Na-Nakorn, U., Sukmanomon, S., Taniguchi, N., Nguyen, T.T., 2007. Mitochondrial DNA diversity of the Asian moon scallop, *Amusium pleuronextes* (Pectinidae), in Thailand. Mar. Biotechnol. 9, 352–359.

Michael, S.W., 2008. Damselfishes & Anemonefishes: the Complete Illustrated Guide to Their Identification, Behaviors, and Captive Care. TFH Publications, Neptune, NJ, USA.

Ng, W.L., Onishi, Y., Inomuta, N., Teshima, K.M., Chan, T.H., Baba, S., Changtragoon, S., Siregar, I.S., Szmidt, A.E., 2015. Closely related and sympatric but not all the same: genetic variation of Indo-West Pacific Rhizophora mangroves across the Malay Peninsula. Conserv. Genet. 16, 137–150.

Nicolas, V., Schaeffer, B., Missoup, A.D., Kennis, J., Colyn, M., Denys, C., Tatard, C., Cruaud, C., Laredo, C., 2012. Assessment of three mitochondrial genes (*16S*, *Cytb*, *COI*) for identifying species in the Praomyini tribe (Rodentia: Muridae). PLoS One 7, e36586.

Pramual, P., Adler, P.H., 2014. DNA barcoding of tropical black flies (Diptera: Simuliidae) of Thailand. Mol. Ecol. Resour. 14, 262–271.

Pereira, L.H.G., Pazian, M.F., Hanner, R., Foresti, F., Oliveira, C., 2011. DNA barcoding reveals hidden diversity in the Neotropical freshwater fish *Piabina argentea* (Characiformes: Characidae) from the Upper Paraná Basin of Brazil. Mitochondrial DNA 22, 87–96.

Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D., Vogler, A.P., 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol. 55, 595–609.

Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, automatic barcode gap discovery for primary species delimitation. Mol. Ecol. 21, 1864–1877.

Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2015. Tracer v1.6, 1 February 2017. http://beast.bio.ed.ac.uk/Tracer.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F., Carvalho, G.R., 2008. DNA barcodes of fish of the Scotia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. Antarct. Sci. 20, 253–262.

Rubinoff, D., Cameron, S., Will, K., 2006. A genomic perspective on the shortcomings of mitochondrial DNA for "Barcoding" identification. J. Hered. 97, 581–594.

Saengkaew, J., Muangmai, N., Zuccarello, G.C., 2016. Cryptic diversity of the mangrove-associated alga *Bostrychia* (Rhodomelaceae, Rhodophyta) from Thailand. Bot. Mar. 59, 363–371.

Sanchez, G., Tomano, S., Umino, T., Wakabayashi, T., Sakui, M., 2016. Evaluation of the 5′ end of the 16S rRNA gene as a DNA barcode marker for the Cephalopoda. Fish. Sci. 82, 279.

Sevilla, R.G., Diez, A., Norén, M., Mouchel, O., Jérôme, M., Verrez-Bagnis, V., van Pelt, H., Favre-Krey, L., et al., 2007. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome *b* and nuclear rhodopsin genes. Mol. Ecol. Notes 7, 730–734.

Smith, P.J., McVeagh, S.M., Steinke, D., 2008. DNA barcoding for the identification of smoked fish products. J. Fish. Biol. 72, 464–471.

Steinke, D., Zemlak, T.S., Boutillier, J.A., Hebert, P.D.N., 2009a. DNA barcoding of Pacific Canada's fishes. Mar. Biol. 156, 2641–2647.

Steinke, D., Zemlak, T.S., Hebert, P.D.N., 2009b. Barcoding Nemo: DNA-based identifications for the Ornamental Fish Trade. PLoS One 4, e6300.

Supikamolseni, A., Ngaoburanawit, N., Sumontha, M., Chanhome, L., Suntrarachun, S., Peyachoknagul, S., Srikulnath, K., 2015. Molecular barcoding of venomous snakes and species-specific multiplex PCR assay to identify snake groups for which antivenom is available in Thailand. Genet. Mol. Res. 14, 13981–13997.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates., Sunderland, MA, USA, Version 4.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis. Mol. Biol. Evol. 30, 2725–2729, version 6.0.

Tanabe, A.S., 2007. Kakusan: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. Mol. Ecol. Notes 7, 962–964.

Tanabe, A.S., 2008. Phylogears, 1 February 2017, v2.0. http://www.fifthdimension.jp/.

Timm, J., Figiel, M., Kochzius, M., 2008. Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity. Mol. Phylogenet. Evol. 49, 268–276.

Xia, Y., Gu, H.F., Peng, R., Chen, Q., Zheng, Y.C., Murphy, R.W., Zeng, X.M., 2012. COI is better than 16S rRNA for DNA barcoding Asiatic salamanders (Amphibia: Caudata: Hynobiidae). Mol. Ecol. Resour. 12, 48–56.

Zhang, J., Hanner, R., 2012. Molecular approach to the identification of fish in the South China Sea. PLoS One 7, e30621.

Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29, 2869–2876.

Further reading

Hubert, N., Hanner, R., Holm, E., Mandrak, N.E., Taylor, E., Burridge, M., Watkinson, D., Dumont, P., et al., 2008. Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3, e2490.