



Paraphyly of Chinese *Amolops* (Anura, Ranidae) and phylogenetic position of the rare Chinese frog, *Amolops tormotus*

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Abstract

In order to evaluate the five species groups of Chinese *Amolops* based on morphological characteristics, and to clarify the phylogenetic position of the concave-eared torrent frog *Amolops tormotus*, we investigated the phylogeny of *Amolops* by maximum parsimony, Bayesian Inference, and maximum likelihood methods using two mitochondrial DNA fragments (12S rRNA, 16S rRNA). Our results supported a sister group relationship of *Amolops ricketti* and *Amolops hainanensis*. However, the grouping of *Amolops mantzorum* and *Amolops monticola* needs to be resolved with more data. *Amolops tormotus* was nested in genus *Odorrana*. Thus, recognition of the *A. tormotus* group is unwarranted and *A. tormotus* should be referred to genus *Odorrana* as *O. tormota*. This species is the sister group of *O. nasica* plus *O. versabilis*. The new classification implies that the genus *Wurana* is to be considered as junior subjective synonym of *Odorrana*.

Key words: phylogeny, mitochondrial DNA, *Amolops*, *Amolops tormotus*, *Odorrana tormota*

Introduction

Among the Southeast Asian ranids, *Amolops* Cope, 1865 (sensu lato: Dubois, 1992) is characterized by the presence of an abdominal sucker in tadpoles and its peculiar occurrence in torrents. Since the taxonomy of this group was questioned (Inger, 1966), significant advances have been achieved in the past few years.

Yang (1991a) recognized three distinct genera including *Amolops* Cope, 1865, *Huia* Yang, 1991, and *Meristogenys* Yang, 1991. Later, Yang (1991b) united these genera into the subfamily Amolopinae within the Ranidae. However, Dubois (1992) did not accept these taxonomic schemes and merely relegated Yang's three genera as subgenera within his genus *Amolops*, along with the subgenus *Amo*. In China, Jiang et al. (1997) added *Pseudoamolops* to the Amolopinae, with *Rana sauteri* as the type species. However, the monophyly of *Amolops* or Amolopinae was not independently verified using molecular data until very recently (e.g., Matsui et al., 2006; Ngo et al., 2006; Frost et al., 2006).

Most species of *Amolops* sensu stricto, the type genus of Amolopinae (Yang, 1991b) or subgenus of *Amolops* sensu lato (Dubois, 1992), are distributed in China (Yang, 1991a; Frost et al., 2007). They are divided into five groups: *A. tormotus*, *A. monticola*, *A. mantzorum*, *A. marmoratus* and *A. hainanensis* groups (Fei et al., 2005). Matsui et al. (2006) and Ngo et al. (2006) studied some Chinese species of *Amolops*. However, data were lacking for the *A. tormotus* group.

The concave-eared frog *A. tormotus*, initially classified as *Rana tormotus* by Wu (1977), has ultrasonic

communication (Feng et al., 2002, 2006). Fei et al. (1991“1990”) moved it into *Amolops*, and later used the species to form the *A. tormotus* group (Fei, 1999; Fei et al., 2005). Recently, based on the larval characteristics, Li et al. (2006) moved *A. tormotus* into a new monotypic genus, *Wurana*, as *W. tormota* (Family Ranidae; also see Frost et al., 2007).

Because ranid morphology is highly homoplastic (Bossuyt and Milinkovitch, 2000), molecular data are an important form of alternative characters. Herein, we present a nearly comprehensive sampling of Chinese *Amolops* to reassess the five species groups suggested by Fei et al. (2005) and to address the phylogenetic status of *A. tormotus*. We incorporate our own mtDNA sequence data as well as those of Matsui et al. (2006) and Ngo et al. (2006).

Materials and methods

Specimens examined

Our classification followed Frost et al. (2007) and Che et al. (2007). The taxonomic scheme of Fei et al. (2005) was used to discuss the status of *A. tormotus*. The species of *Amolops* surveyed included our own new data (*A. jinjiangensis*, *A. kangtingensis*, *A. liangshanensis*, *A. hainanensis*, *A. torrentis*, and *A. tormotus*) and others from GenBank (Matsui et al., 2006; Ngo et al., 2006). Based on previous molecular studies (Chen et al., 2005; Frost et al. 2006; Ngo et al., 2006), specimens assigned to *A. chapaensis* and *A. nasica* fell in genus *Odorrana*. Therefore, all available representatives of genus *Odorrana* (Matsui et al., 2005; Ngo et al., 2006) were used, including new data for eight Chinese species of *Odorrana*. Representatives of other ranid genera were retrieved from GenBank. The choice of outgroup taxa was based on Matsui et al. (2006) and Ngo et al. (2006). Locality and voucher data are presented in Table 1.

DNA extraction, amplification, and sequencing

DNA was extracted from muscle or liver tissue samples stored in 95% or 100% ethanol using a standard 3-step phenol/chloroform extraction procedure. The primers used for PCR amplification were as stated in Matsui et al. (2006). Amplification was performed in a 50 μ l volume reaction with the following procedures for both 12S and 16S fragments: initial denaturation step with 4 min at 94°C, 35 cycles of denaturation 1 min at 94°C, annealing for 1 min at 46°C for 12S primers and 50°C for 16S primers, extension for 1 min at 72°C. Final extension at 72°C was conducted for 10 min.

Purified PCR products were sequenced from both directions with an ABI automated DNA sequencer (ABI 3700). Acquired sequences were blasted against the GenBank database to verify that required sequences had been amplified.

Phylogenetic analyses

Alignments were first conducted using ClustalX 1.81 (Thompson et al., 1997) with default parameters, and subsequently verified manually. Considering all mtDNA gene sequences are effectively inherited as one locus, the two mitochondrial gene segments were concatenated into a single partition for the analyses.

The sequence data were analyzed using maximum parsimony (MP) and maximum likelihood (ML) implemented in PAUP* 4.0b10 (Swofford, 2003). Heuristic MP Searches were executed in 1000 random addition replicates with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch-swapping. Bootstrap branch proportions (BBP) were calculated with 1000 MP replicates. Under the Akaike Information Criterion (AIC), the GTR+I+G model was selected for maximum likelihood (ML) analyses by Modeltest 3.7 (Posada et al., 2004). Heuristic Searches were executed in 10 replicates with the GTR+I+G model, and using tree bisection reconnection (TBR) branch swapping. BBP values were calculated with 10 ML replicates.

TABLE 1. Samples and sequences used in this study.

Species	Voucher	Locality	GeneBank No. (12S;16S)
<i>Amolops bellulus</i>	KIZ9810021	Yunnan, China	DQ204429; DQ204473
<i>Amolops chunganensis</i>	KUHE27699	Sichuan, China	AB211454; AB211477
<i>Amolops cremnobatus</i>	N24538	Ha Tinh, Vietnam	AB211460; AB211483
<i>Amolops daiyunensis</i>	C93075	Fujian, China	AB211451; AB211474
<i>Amolops granulosus</i>	C93161	Sichuan, China	AB211458; AB211481
<i>Amolops hainanensis</i>	SCUM050245YJ	Hainan, China	*EF453725; EF453740
<i>Amolops hongkongensis</i>	KUZ30210	Hong kong, China	AB211450; AB211473
<i>Amolops jinjiangensis</i>	SCUM050435CHX	Deqing, Yunnan, China	*EF453726; EF453741
<i>Amolops kangtingensis</i>	SCUM045822HX	Kangding, Sichuan, China	*EF453727; EF453742
<i>Amolops larutensis</i>	KUHE15488	Perak, Malaysia	AB211461; AB211484
<i>Amolops liangshanensis</i>	SCUM045807HX	Xichang, Sichuan, China	*EF453728; EF453743
<i>Amolops lifanensis</i>	C9350	Sichuan, China	AB211459; AB211482
<i>Amolops loloensis</i>	C18	Sichuan, China	AB211455; AB211478
<i>Amolops mantzorum</i>	C62	Sichuan, China	AB211456; AB211479
<i>Amolops marmoratus</i>	KUHE19089	Chieng Mai, Thailand	AB211463; AB211486
<i>Amolops ricketti</i>	C-F93066	Fujian, China	AB211452; AB211475
<i>Amolops</i> sp.	KUHE20133	Phetchaburi, Thailand	AB211465; AB211488
<i>Amolops spinapectoralis</i>	ROM27424	Kon Tum Prov., Vietnam	DQ204444; DQ204488
<i>Amolops torrentis</i>	SCUM050253YJ	Hainan, China	*EF453729; EF453744
<i>Amolops tormotus</i>	SCUM052068	Huangshan, Anhui, China	*EF453738; EF453753
<i>Amolops tormotus</i>	SCUM052069	Huangshan, Anhui, China	*EF453739; EF453754
<i>Amolops viridimaculatus</i>	C-green 05	Yunnan, China	AB211457; AB211480
<i>Amolops wuyiensis</i>	C-F93066	Fujian, China	AB211453; AB211476
<i>Babina adenopleura</i>	UMMZ189963	Taiwan, China	DQ283117; DQ283117
<i>Fejervarya limnocharis</i>	GeneBank sequence	China	AY158705; AY158705
<i>Huia cavitympanum</i>	BOR uncatalogued	Sabah, Malaysia	AB211466; AB211489
<i>Huia</i> sp.	N6468	Sumatra, Indonesia	AB211468; AB211491
<i>Huia sumatrana</i>	N6474	Sumatra, Indonesia	AB211467; AB211490
<i>Hydrophylax chalconota</i>	KUHE23936	Ranong, Thailand	AB200932; AB200956
<i>Hylarana erythraea</i>	ROM7296	Gia Lai Prov., Vietnam	AF206094; AF206475
<i>Lithobates catesbeianus</i>	BLC	New Mexico, USA	DQ283257; DQ283257
<i>Meristogenys jerboa</i>	KUHE12028	Sarawak, Malaysia	AB211470; AB211493
<i>Meristogenys kinabaluensis</i>	BOR uncatalogued	Sabah, Malaysia	AB211469; AB211492
<i>Meristogenys orphnocnemis</i>	BOR22352	Sabah, Malaysia	AB211471; AB211494
<i>Odorrana amamiensis</i>	KUHE24635	Ryukyu, Japan	AB200923; AB200947
<i>Odorrana andersonii</i>	KIZ-RD02YNJD01	Jingdong, Yunnan, China	*EF453730; EF453745
<i>Odorrana banaorum</i>	ROM7472	Gia Lai Prov., Vietnam	AF206106; AF206487
<i>Odorrana chapaensis</i>	AMNH-A161439	Lai Chau Prov, Vietnam	DQ283372; DQ283372
<i>Odorrana chloronota</i>	KUZ30216	Hong kong, China	AB200930; AB200954
<i>Odorrana daorum</i>	ROM19053	Lao Cai Prov., Vietnam	AF206101; AF206482
<i>Odorrana grahami</i>	SCUM0405186CJ	Xichang, Sichuan, China	*EF453731; EF453746
<i>Odorrana hejiangensis</i>	SCUM0405180CJ	Hejiang, Sichuan, China	*EF453732; EF453747

<i>Odorrana hmongorum</i>	ROM19112	Lao Cai Prov., Vietnam	AF206102; AF206483
<i>Odorrana hosii</i>	KUHE34491	Malay Peninsula	AB200933; AB200957
<i>Odorrana ishikawae</i>	KUHE10069	Ryukyu, Japan	AB200921; AB200945
<i>Odorrana livida</i>	SCUM050518CHX	Hekou, Yunnan, China	*EF453733; EF453748
<i>Odorrana margaretae</i>	SCUM045830HX	Anxian, Sichuan, China	*EF453734; EF453749
<i>Odorrana megalotympanum</i>	ROM13046	Nghe An Prov., Vietnam	AF206100; AF206481
<i>Odorrana morafkai</i>	ROM7446	Gia Lai Prov., Vietnam	AF206103; AF206484
<i>Odorrana narina</i>	KUNHE12788	Ryukyu, Japan	AB200924; AB200948
<i>Odorrana nasica</i>	AMNH-A161169	Ha Tinh Prov, Vietnam	DQ283345; DQ283345
<i>Odorrana schmackeri</i>	CIB-WU37990	Omei, Sichuan, China	*EF453735; EF453750
<i>Odorrana supranarina</i>	KUHE24141	Ryukyu, Japan	AB200925; AB200949
<i>Odorrana swinhoana</i>	KUHE34731	Taiwan, China	AB200929; AB200953
<i>Odorrana tiannanensis</i>	SCUM050510CHX	Hekou, Yunnan, China	*EF453736; EF453751
<i>Odorrana utsunomiyaorum</i>	KUHE24144	Ryukyu, Japan	AB200927; AB200951
<i>Odorrana versabilis</i>	HNNU-A0019L	Mt. Limu, Hainan, China	*EF453737; EF453752
<i>Nanorana quadranus</i>	C91	Fujian, China	AB200943; AB200967
<i>Pelophylax nigromaculatus</i>	FMNH232879	Sichuan, China	DQ283137; DQ283137
<i>Rana johnsi</i>	AMNHA161191	Nghe An Prov, Vietnam,	DQ283214; DQ283214
<i>Rana sauteri</i>	KUHE18098	Taiwan, China	AB211472; AB211495
<i>Staurois latopalmaris</i>	BOR8098	Sabah, Malaysia	AB200942; AB200966

*Sequences new to this study. CIB, Chengdu Institute of Biology, the Chinese Academy of Sciences; HNNU, Hainan Normal University; KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; SCUM, Sichuan University Museum.

Odorrana livida = *O. chloronota* (See Bain et al. 2003).

Bayesian inference (BI) was carried out using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The Bayesian posterior probabilities (BPP) used models estimated with Modeltest 3.7 (Posada et al., 2004) under the Akaike Information Criterion (AIC). Two separate runs were performed with four Markov chains. Each run was conducted with 1,000,000 generations and sampled every 100 generations. When the log-likelihood scores were found to stabilize, a consensus tree was calculated after omitting the first 25% trees as burn-in.

Results

Sequence variation

The combined dataset included 974 variable sites and 778 potentially parsimony-informative sites over a total of 1793 alignment nucleotide sites. Newly obtained sequences were submitted to GenBank (accession numbers EF453725-EF453754).

Phylogenetic analyses

MP analysis produced ten most parsimonious trees (TL = 5630, CI = 0.296, RI = 0.560, RC = 0.166), which were virtually identical to the trees recovered in ML (-lnL = 25945.8457) and BI analyses (Fig. 1). The analyses found that Chinese *Amolops* were not a monophyletic group. *Amolops tormotus* fell out of the clade containing *Amolops* and nested in the genus *Odorrana*. The monophyly of *Odorrana* was strongly supported (100, 98, and 100% support for MP, ML bootstrap proportions and Bayesian posterior probability, respectively). Within *Odorrana*, *A. tormotus* was close to the sister groups of *O. versabilis* and *O. nasica* with medium support values (75, 58 and 100% support).

Discussion

Phylogeny of Chinese *Amolops*

Fei et al. (2005) recognized five species groups including *A. tormotus*, *A. monticola*, *A. mantzorum*, *A. marmoratus* and *A. hainanensis* within Chinese *Amolops* (sensu stricto) based on morphology. Matsui et al. (2006) confirmed the monophyly of *A. marmoratus* group and suggested retaining the name *A. ricketti* group (Fei, 1999). They also indicated unresolved relationships for the *A. mantzorum* and *A. monticola* groups. Monophyly of the *A. hainanensis* group was supported by Ngo et al. (2006). Unfortunately, the *A. tormotus* group was not included in previous studies.

Regarding the relationship of Asian *Amolops*, our data findings were essentially identical to those of Matsui et al. (2006) and Ngo et al. (2006). However, the inclusion of the *A. tormotus* group revealed some new, interesting information.

The *A. tormotus* group did not cluster with other species of *Amolops* but rather fell into the monophyletic genus *Odorrana*. Consequently, *Amolops* (sensu stricto), as envisioned by Fei et al. (2005), is not monophyletic and recognition of the *A. tormotus* group is not justified.

Phylogenetic status of *Amolops tormotus*

Amolops tormotus was originally named as *Rana tormotus* by Wu (1977). The distinctive characters of this species were described as follows: distinct dorsolateral folds which were thick and wide; tympanum deeply sunken, forming an external auditory canal especially in males; the males possess a pair of external sub-gular vocal sacs. Its phylogenetic status is controversial (Fei et al., 1991“1990”, 2005; Fei, 1999; Zhao Adler, 1993; Li et al., 2006; Su et al., 2007). Because *A. tormotus* is similar to *A. (H.) cavitympanum* in having a deeply sunken tympanum, Fei et al. (1991“1990”) suggested to transfer *tormotus* from the genus *Rana* into the genus *Amolops*. This transfer assumes that the tadpole of *A. tormotus* is similar to those of other *Amolops*. However, Li et al. (2006) reported that the tadpole of *A. tormotus* has neither an abdominal disk nor poison glands that characterize *Amolops*. In addition, adults of *A. tormotus* did not have drastically enlarged digital pads. Therefore, Li et al. (2006) suggested to establish a new genus, *Wurana*, for *A. tormotus*.

A solid taxonomy is fundamental to biology. Phylogenies provide a logical foundation for establishing taxonomy. Our study strongly supports the transfer of *A. tormotus* into the genus *Odorrana*. *Amolops tormotus* is the sister group of *O. nasica* plus *O. versabilis* (Fig. 1). Although we agree with Li et al. (2006) in removing *A. tormotus* from *Amolops*, in order to avoid paraphyly in *Odorrana*, we suggest that *A. tormotus* be transferred to the genus *Odorrana* as *O. tormota*. In this scheme, the genus *Wurana* (Li et al., 2006) becomes a junior subjective synonym of *Odorrana* (Fei et al., 1991“1990”).

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