

Taxonomic Relationships within the Pan-Oriental Narrow-mouth Toad *Microhyla ornata* as Revealed by mtDNA Analysis (Amphibia, Anura, Microhylidae)

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ABSTRACT—A molecular phylogenetic survey was conducted using mtDNA sequences of 12S and 16S rRNA, and cyt-b genes to examine taxonomic relationships among populations of the Pan-Oriental microhylid, *Microhyla ornata*, from India, Bangladesh, Thailand, Laos, China, Taiwan, and the Ryukyu Archipelago of Japan. Two discrete clades are recognized within this species, one consisting of populations from India and Bangladesh, and the other encompassing the remaining populations. In the latter clade, populations from the Ryukyu Archipelago are clearly split from the rest (populations from Taiwan and the continent) with considerable degrees of genetic differentiations. Each of the three lineages is judged to represent a good species, and the name *Microhyla ornata* is restricted to the South Asian populations. For the populations from Taiwan and a wide region from China to Southeast Asia, the name *Microhyla fissipes* should be applied, whereas the Ryukyu populations are most appropriately referred to as *Microhyla okinavensis*, although further substantial genetic differentiations are recognized among some island group populations within this last species.

Key words: *Microhyla ornata*, *Microhyla fissipes*, *Microhyla okinavensis*, mtDNA sequence, taxonomy

INTRODUCTION

Microhyla ornata (Duméril and Bibron, 1841) is a small-sized, narrow-mouth toad, and is an inhabitant of lowlands and hills where it takes termites and ants among litters (Fei, 1999; Maeda and Matsui, 1999; Hirai and Matsui, 2000; Schleich and Kästle, 2002). The species was originally described from India and is now considered to have a very wide range of distribution from the Ryukyu Archipelago of Japan through Taiwan, southern China, Southeast Asia including northern Malay Peninsula, to Nepal, India, Sri Lanka and northeastern Pakistan (Fig. 1: Frost, 1985; Duellman, 1993; Inger, 1999; Zhao, 1999; Khan, 2000).

Besides *M. ornata*, there were several other Oriental

anurans of different lineages that were also considered as wide-ranging single species. In the last two decades, however, some of them have proved to include several cryptic species: *Polypedates megacephalus* Hallowell, 1861 was resurrected from *P. leucomystax* (Gravenhorst, 1829) (Matsui *et al.*, 1986); *Kalophrynus interlineatus* (Blyth, 1855) was elevated from a subspecific status of *K. pleurostigma* Tschudi, 1838 (Matsui *et al.*, 1996); and *Fejervarya limnocharis* (Gravenhorst, 1829) was shown to include some morphologically similar but reproductively isolated entities (Toda *et al.*, 1998; Veith *et al.*, 2001). These findings were achieved by employing the methods other than the conventional morphological one. In particular, recent arguments on the amphibian taxonomy, like those on many other groups of organisms, tend to have their bases on the results of molecular analyses (e.g., Matsui, 1994; Toda *et al.*, 1998; Veith *et al.*, 2001).

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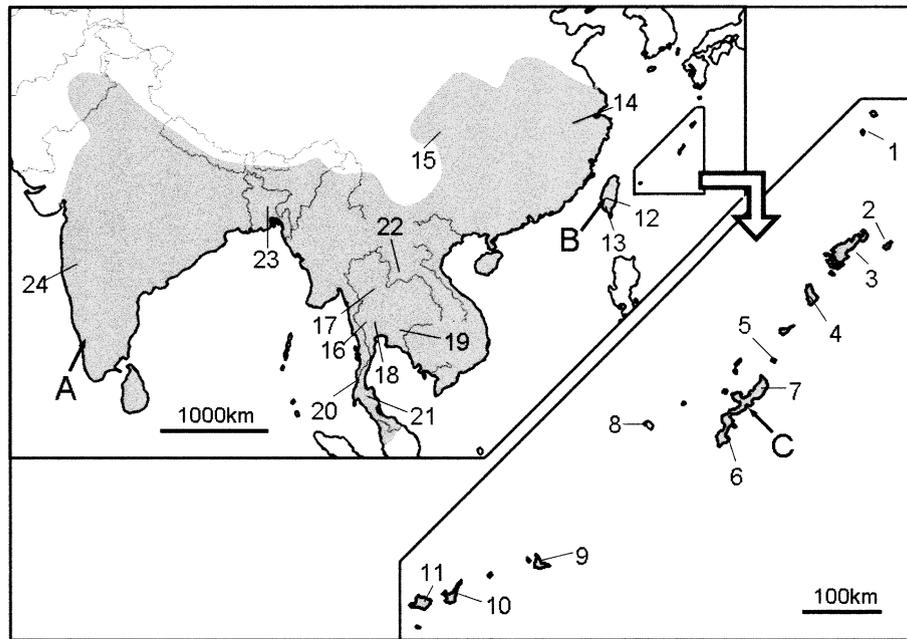


Fig. 1. A map of East to South Asia, showing known range (after Dutta, 1997; Maeda and Matsui, 1999; Fei, 1999; Schleich and Kastle, 2002) and sampling localities of *Microhyla ornata* (sensu lato). For the locality number, refer to Table 1. A, B, and C indicate type localities of *M. ornata*, *M. fissipes*, and *M. okinavensis*, respectively.

Since the description by Duméril and Bibron (1841), several authors proposed different names to different populations of *M. ornata*, but all these are now placed in the synonymy of this nominate species (Frost, 2004). Dubois (1987) recently revived one of such names originally described for a population from Okinawa of the Ryukyu Archipelago. Even if we simply admit his action to recognize distinct specific status of this and adjacent Ryukyu populations, the taxonomic status of populations occurring in the broad area between India and the Ryukyus remains equivocal. Moreover clarification of exact extent of any one species in both taxonomic and geographical senses is indispensable to discuss various issues relevant to biodiversity and biogeography.

Our preliminary analyses of partial sequences of mitochondrial cytochrome b (mt cyt-b) gene revealed great genetic differentiations between populations of *M. ornata* from the Ryukyus, and Taiwan, continental China and Thailand (Matsui *et al.*, unpublished). In this study, we compared *M. ornata* from India with conspecific populations from Southeast and East Asia using mt-DNA sequences. The results, while confirming the distinctness of the Ryukyu populations at the species level, clarified the presence of another distinct species within *M. ornata* (sensu lato) for the first time.

MATERIALS AND METHODS

Sampling strategy

In order to examine gross phylogenetic relationships among *Microhyla*, we first obtained partial sequences of 12S rRNA (ca. 860 bp) and 16S rRNA (ca. 860 bp) for five species of the genus, includ-

ing *M. ornata* (sensu lato) from three disjunct regions (Fig. 1). *Glyphoglossus molossus* Günther, 1869 was also added to the subject, because our preliminary survey among the Oriental microhylids strongly suggested its close affinity with the *Microhyla* species (Matsui *et al.*, unpublished). We selected *Kaloula pulchra* Gray, 1831 as an outgroup (Table 1). We then compared more detailed relationships among populations of *M. ornata*, with *Glyphoglossus molossus* as an outgroup using partial sequences of cytochrome b gene (cyt-b: <629 bp). This domain, known to exhibit more rapid nucleotide substitutions than rRNA genes, is considered to be more informative in examining relationships among conspecific populations or closely related, poorly diverged species (Koike and Matsui, 2003).

Preparation of DNA, PCR, and DNA sequencing

DNA was extracted from small amounts of frozen or ethanol preserved tissues using standard Phenol-Chloroform extraction procedures (Hillis *et al.*, 1996). Amplification were done by the polymerase chain reaction (PCR), using the primers 12Sh (Cannatella *et al.*, 1998) and H1548: 5'-TAC CAT GTT ACG ACT TTC CTC TTC T-3' made in the present study for 12S, the primers 16L2 (Hedges *et al.*, 1993) and 16SH1 (Hedges, 1994) for 16S, and the primers L14883: 5'-TCT GCT TAA TTG CTC AAA TCG C-3' and H15548: 5'-AAT AGG AAG TAC CAC TCT GGT TTA AT3' made in the present study, and H15502 (Tanaka-Ueno *et al.*, 1998) for cyt-b. The numbering system followed the human sequence (Anderson *et al.*, 1981). The amplified fragments were sequenced in an automated DNA sequencer (ABI PRISM 3100) using the PCR primers and following the manufacturers instructions. Newly obtained sequences were deposited in GenBank (Table 1).

Phylogenetic analysis

Sequences were aligned using the default parameters of ClustalX 1.83 (Thompson *et al.*, 1997). For nucleotide sequences, the 12S and 16S were combined into a single data set of ca. 1720 bp. Maximum-parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) phylogenies were calculated using PAUP*4.0b10 (Swofford, 2002). Pairwise comparisons of corrected sequence divergences [Kimura-2 parameter (K2p) distances (Kimura, 1980)]

Table 1. Samples used in this study and GenBank accession numbers.

Species	Locality*	Voucher**	Accession number		
			12S,	16S,	cyt-b
<i>Microhyla ornata</i>	Ryukyu, Tokara, Suwanosejima (1)	KUHE11965	-	-	AB201195
<i>Microhyla ornata</i>	Ryukyu, Amami, Kikaijima (2)	KUHE33968	-	-	AB201196
<i>Microhyla ornata</i>	Ryukyu, Amami, Amamioshima (3a)	KUHE12840	AB201173,	AB201184,	AB201197
<i>Microhyla ornata</i>	Ryukyu, Amami, Amamioshima (3b)	KUHE32563	-	-	AB201198
<i>Microhyla ornata</i>	Ryukyu, Amami, Tokunoshima (4)	KUHE24172	-	-	AB201199
<i>Microhyla ornata</i>	Ryukyu, Amami, Yoronjima (5a)	KUHE34982	-	-	AB201200
<i>Microhyla ornata</i>	Ryukyu, Amami, Yoronjima (5b)	KUHE34984	-	-	AB201201
<i>Microhyla ornata</i>	Ryukyu, Okinawa, Okinawajima (6)	KUHE24489	-	-	AB201202
<i>Microhyla ornata</i>	Ryukyu, Okinawa, Okinawajima (7a)	KUHE22554	-	-	AB201203
<i>Microhyla ornata</i>	Ryukyu, Okinawa, Okinawajima (7b)	KUHE33444	-	-	AB201204
<i>Microhyla ornata</i>	Ryukyu, Okinawa, Kumejima (8)	KUHE12708	-	-	AB201205
<i>Microhyla ornata</i>	Ryukyu, Miyako, Miyakojima (9a)	KUHE32473	-	-	AB201206
<i>Microhyla ornata</i>	Ryukyu, Miyako, Miyakojima (9b)	KUHE33966	-	-	AB201207
<i>Microhyla ornata</i>	Ryukyu, Yaeyama, Ishigakijima (10)	KUHE29773	-	-	AB201208
<i>Microhyla ornata</i>	Ryukyu, Yaeyama, Iriomotejima (11a)	KUHE24920	-	-	AB201209
<i>Microhyla ornata</i>	Ryukyu, Yaeyama, Iriomotejima (11b)	KUHE22403	-	-	AB201210
<i>Microhyla ornata</i>	Taiwan, Jiayi (12)	KUHE12962	-	-	AB201211
<i>Microhyla ornata</i>	Taiwan, Gaoxiong (13)	KUHE32759	-	-	AB201212
<i>Microhyla ornata</i>	China, Anhui, Huangshan (14)	KUHE32943	AB201174,	AB201185,	AB201213
<i>Microhyla ornata</i>	China, Sichuan, Chengdu (15)	KUHE27705	-	-	AB201214
<i>Microhyla ornata</i>	Thailand, Kanchanaburi, Thong Pha Phum (16)	KUHE35165	AB201175,	AB201186,	AB201215
<i>Microhyla ornata</i>	Thailand, Phrae (17)	KUHE21982	-	-	AB201216
<i>Microhyla ornata</i>	Thailand, Bangkok (18)	KUHE22064	-	-	AB201217
<i>Microhyla ornata</i>	Thailand, Chanthaburi, Namtok Phliu (19)	KUHE34130	-	-	AB201218
<i>Microhyla ornata</i>	Thailand, Ranong (20)	KUHE23891	-	-	AB201219
<i>Microhyla ornata</i>	Thailand, Khaosok (21)	KUHE19687	-	-	AB201220
<i>Microhyla ornata</i>	Laos, Vientiane (22)	KUHE34324	-	-	AB201221
<i>Microhyla ornata</i>	Bangladesh, Dinajpur, Parbatipur (23)	DB-Hi-FROG12005	AB201176,	AB201187,	AB201222
<i>Microhyla ornata</i>	India, Karnataka, Dharwad (24)	ZSIK-A9119	AB201177,	AB201188,	AB201223
<i>Microhyla butleri</i>	Thailand, Bangkok	KUHE33557	AB201178,	AB201189,	-
<i>Microhyla heymonsi</i>	Thailand, Kanchanaburi, Thong Pha Phum	KUHEK1845	AB201179,	AB201190,	-
<i>Microhyla pulchra</i>	Thailand, Kanchanaburi, Thong Pha Phum	KUHE35119	AB201180,	AB201191,	-
<i>Microhyla rubra</i>	India, Karnataka, Dharwad	released	AB201181,	AB201192,	-
<i>Glyphoglossus Molossus</i>	Thailand, Tak, Barrntak	KUHE35182	AB201182,	AB201193,	-
<i>Glyphoglossus Molossus</i>	Thailand, Tak, Barrntak	KUHE35182	-	-	AB201225
<i>Kaloula pulchra</i>	Thailand, Kanchanaburi, Thong Pha Phum	KUHE35171	AB201183,	AB201194,	-

*Locality numbers correspond to those of Fig. 1; **KUHE=Graduate School of Human and Environmental Studies, Kyoto University; DB-Hi-FROG=Laboratory for Amphibian Biology, Hiroshima University; ZSIK=Zoological Survey of India, Kolkata.

were also calculated with PAUP. The best-fitting model of sequence evolution for ML analysis was obtained by Modeltest 3.06 (Posada and Crandall, 1998). NJ trees were based on TrN+I+G distance (Tamura and Nei, 1993). Heuristic searches were performed using 10 replicates of a stepwise addition and tree bisection-reconnection (TBR) branch swapping. For MP, all characters were weighted equally, and gaps were treated as missing data. The bootstrap technique was used to test the reliabilities of the MP, NJ, and ML

trees (2000, 2000, 100 replicates, respectively). Bayesian inference was conducted with MRBAYES 3.0 (Huelsenbeck and Ronquist, 2001) using GTR substitution model with 1,000,000 generations, sampling trees every 100th generation, and calculating a consensus tree after omitting the first 2000 trees.

composed of the Yaeyama populations (100, 99, 99, and 100%) and the other of the remaining Ryukyu populations (96, 96, 94, and 100%). In the latter subclade, northern populations from the Tokara and most of the Amami Groups (100, 93, 95, and 100%) and southern populations from part of the Amami, the Okinawa, and the Miyako Groups (100, 100, 100, and 100%) were clearly separated.

Sequence divergences of the South Asian clade from the China-Thailand clade and the Ryukyu clade were great, being 19.1–24.0% and 18.4–21.1%, respectively. The latter two clades also showed great sequence divergences (16.1–20.6%), whereas the divergence within the South Asian clade was also large (15.4%). In the China-Thailand clade, the two subclades showed the divergence of 8.2–11.0%, which is smaller than that observed between the two subclades of the Ryukyu clade (13.3–16.7%). In the second subclade of the Ryukyu clade, the northern and the southern populations were also fairly divergent (11.3–13.8%).

DISCUSSION

The results of analyses of the mitochondrial 12S and 16S rRNA data strongly suggest that *M. ornata* (sensu lato) is a composite of at least two discrete lineages, because the clade consisting of populations from South Asia was shown to be sister to *M. rubra* rather than to the other “conspecific” clade encompassing populations from Southeast and East Asia. Likewise, the latter clade constituted a monophyletic group together with *M. pulchra* and *M. heymonsi* in the phylogenetic trees.

The locality where the type specimen of *M. ornata*

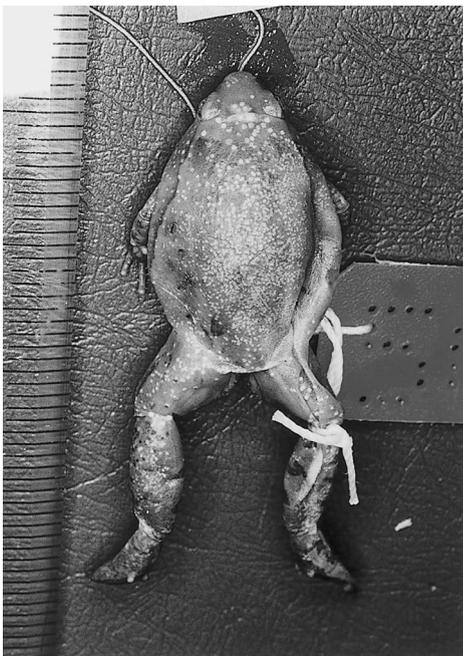


Fig. 4. The holotype of *Microhyla ornata* from India (Muséum National d'Histoire Naturelle, Paris 5035, Snout-vent length=22.7 mm).

(MNHNP 5035: Fig. 4) was obtained is reported to be “côte Malabar”, India (Duméril and Bibron, 1841), and our Indian sample was obtained from a locality not much far from the type locality. The population from Bangladesh was sister to the Indian population, but, as shown by great divergence value, fairly differed genetically from the latter. This may suggest the presence of more than one species within the South Asian populations of *M. ornata*.

Populations from Southeast and East Asia formed a monophylum, which is not sister to the one including *M. ornata* (sensu stricto), in our phylogenetic trees (see above), and it is thus obvious that those populations do not belong to *M. ornata*. Furthermore, of the Southeast and East Asian populations, the Ryukyu populations are suspected to be split from the remainder at the species rank, since the level of genetic divergence between them largely overlapped those between each of them and of *M. ornata* (sensu stricto), and was close to the values obtained for combinations of other distinct species such as *M. pulchra* and *M. heymonsi*. Lai *et al.* (1996) studied phylogenetic relationships among populations of four microhylid species from Taiwan and the Ryukyu Islands (Iriomotejima and Kumejima) through analyses of allozyme variations. In their results, Taiwan populations of *M. ornata* formed a distinct group and split from a group including three other species and Ryukyu populations of *M. ornata*. In the latter group, *M. ornata* from Kumejima was sister to the clade encompassing populations of *M. heymonsi* and *M. ornata* from Iriomotejima. These curious relationships are completely different from those obtained by us (see results) and might have resulted from misinterpretations of allozyme data. The different inheritance mechanisms of mitochondrial and nuclear markers (e.g., Avise, 2000; Kim *et al.*, 2004) cannot be ruled out, but, even so, their results do not contradict, but strengthen, our taxonomic idea to split populations from Taiwan and the Ryukyu Islands.

Our analyses, although showing certain degrees of genetic divergences among populations from Taiwan, China, Thailand, and Laos, yielded no convincing support to the further specific split of the Southeast and East Asian assemblages exclusive of the Ryukyu populations. Very few studies have been made to estimate variation within this assemblage. Acoustic characteristics are reported to be similar between Taiwan and Thailand populations (Kuratomo, 1987). Our preliminary analyses of calls recorded in Anhui, China, and many localities in Thailand also failed to detect any notable difference (Matsui, unpublished data). Flower (1899) stated that there are some morphological differences in both adults and tadpoles of *M. ornata* between the Malay Peninsula and Indochina. Liu (1950) described the cloacal tail piece in tadpoles of *M. ornata* from western continental China as dextral, although this organ is medial in tadpoles from Taiwan (Chou and Lin, 1997), as well as in those from the Ryukyu Archipelago (Shimizu and Ota, 2003). Validity and taxonomic significance of such variations in the continental-Taiwanese assemblage require future ver-

ification and considerations.

The oldest available name applicable to the frogs in this region is *M. fissipes* Boulenger, 1884 [type locality=Tainan (=Tainan), S. Formosa (Taiwan)]. *Microhyla fissipes* is reported to be morphologically very similar to *M. ornata* (sensu stricto) by Parker (1934), who synonymized it with the latter. Khan (2000), however, argued that tadpoles of *M. ornata* from northeastern Pakistan, South Asia, are differentiated from those from Thailand by several characteristics, such as longer infralabial papillae, more filter rows, and the presence of postnarial membraneous ridge. Future study is needed to examine the validity of these differences in discriminating *M. ornata* (sensu stricto) from *M. fissipes* as a whole. It is also strongly desired to find out reliable diagnostic characteristics for adults of these species.

The populations from the Ryukyu Archipelago were originally described as a distinct species *M. okinavensis* by Stejneger (1901) [type locality=Okinawa Shima, Riu Kiu (=Ryukyu) Archipelago, Japan], but later relegated to the synonymy of *M. ornata* (Inger, 1947). In establishing subgenera in *Microhyla*, Dubois (1987) simply resurrected this old name, but without any comment for this change. Duellman (1993) "automatically" adopted this treatment, and Zhao (1999) listed both *M. ornata* and *M. okinavensis* in the fauna of the Ryukyus. From our results, it is now obvious that the population from the Ryukyu Archipelago should be separated not only from *M. ornata* from India, but also from *M. fissipes*, as a distinct species *M. okinavensis*. From *M. ornata* (sensu stricto) and *M. fissipes*, *M. okinavensis* can be distinguished by the combination of morphological characteristics (shape of the loreal region, relationships of interorbital and upper eyelid widths, shape of toe tips, presence or absence of a median cleft on surfaces of digits, presence or absence of lateral fringes on toes, and position of tibio-tarsal articulation when hindlimb is bent forwards along body: Parker, 1934). In the embryonic development, *M. okinavensis* may differ from *M. fissipes* in the timing of a few important changes, such as darkening of the stomodium area (the neural fold stage vs. neural tube stage: Shimizu and Ota, 2003). The egg diameter is reportedly larger in *M. okinavensis* than in *M. fissipes* (mean=0.97–1.20 mm vs. 0.90: Matsui and Ota, 1984; Liu *et al.*, 1996; Shimizu and Ota, 2003). Acoustically, *M. okinavensis* and *M. fissipes* are slightly different as already reported by Kuramoto (1987).

From our results, lesser, but yet substantial degrees of genetic differentiations are demonstrated for a few combinations of populations of *M. okinavensis*, such as those from Yaeyama and from the other islands of the Ryukyus, and from southern and northern islands of the Miyako to Tokara Groups. The egg diameter of the Okinawajima population (0.93–1.04 mm, mean=0.97, SD=0.04) is substantially smaller than that of the Yaeyama populations (1.00–1.30 mm, mean=1.20, SD=0.03). Furthermore, Kuramoto (1976) has suggested presence of differences in acoustic characteristics between populations of *M. okinavensis* (as *M. ornata*), but detailed studies are still lacking. It is necessary

to morphologically and acoustically reassess relationships among populations of *M. okinavensis* from its whole range of distribution in the Ryukyu Archipelago.

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ERRATA

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page 495, Received date

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