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A Species Boundary Within the *Tylototriton verrucosus* Group (Urodela: Salamandroidae) Based on Mitochondrial DNA Sequence Evidence

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Abstract: The taxonomic status of red knobby newt (*Tylototriton shanjing*) is under dispute. Molecular phylogenetic tree of *Tylototriton verrucosus* group was reconstructed based on 753 bp of partial mitochondrial *cyt b* gene sequence to determine species boundaries among the species in *T. verrucosus* group. The phylogeny result indicates that four major clades (clade I-IV) can be distinguished within *Tylototriton verrucosus* group. Clade I and IV consist of *T. taliangensis* and *T. kweichowensis*, respectively. Clade II consists of samples of *T. shanjing* derived from Yunnan of China, a form that researchers resurrect from its synonym under *T. verrucosus*. *T. verrucosus* haplotypes from Shan State of Myanmar and pet trade formed clade III. All populations of *T. shanjing* and *T. verrucosus* formed strongly supported (PP = 1.0) reciprocal monophyletic groups. The average uncorrected pairwise genetic distance (p-distance) of cyt b between these four clades ranges from 0.060-0.089 which is obviously higher than within these four major clades (0.001-0.014). Researchers propose that the *T. shanjing* should be a valid species rather than synonym of *T. verrucosus*.

Key words: Species boundary, *Tylototriton verrucosus* group, red knobby newt (*Tylototriton shanjing*), *cyt b* gene, clades

INTRODUCTION

One of the greatest challenges for amphibian biodiversity conservation is the poor understanding of species diversity (Funk *et al*., 2011) in part because of extreme morphological conservation and associated homoplasy (Bossuyt and Milinkovitch, 2000; Wake, 1991). The application of molecular data and associated analytical tools is invaluable which has improved the ability to revealed that amphibian species are often morphologically cryptic as a result leading to a revitalization of amphibian taxonomy or uncovering cryptic species (Hass *et al*., 1995; Chek *et al*., 2001; Stuart *et al*., 2006; Wu *et al*., 2010; Yu *et al*., 2010; Funk *et al*., 2011).

The Asian newt genus *Tylototriton* (Anderson, 1871) contains ten currently recognized species (AmphibiaWeb, 2012; Shen *et al*., 2012) distributed in South, Southeast and East Asia (Fei *et al*., 2010; Stuart *et al*., 2010). These 10 species consist of two species groups, the *T. verrucosus* group (= sub-genus *Tylototriton*, Dubois and Raffaelli, 2009) containing *T. verrucosus* (Anderson, 1871), *T. kweichowensis* (Fang and Chang, 1932), *T. taliangensis* (Liu, 1950) and *T. shanjing* (Nussbaum *et al*., 1995) and the *T. asperrimus* group (= sub-genus *Yaotriton* (Dubois and Raffaelli, 2009)) containing *T. asperrimus*, *T. hainanensis* (Fei *et al*., 1984), *T. wenxianensis* (Fei *et al*., 1984), *T. vietnamensis* (Bohme *et al*., 2005), *T. notialis*, (Stuart *et al*., 2010) and *T. broadoridgus* (Shen *et al*., 2012). However, a number of disagreements and uncertainties about phylogeny and taxonomy of this genus remain (Stuart *et al*., 2010; Yuan *et al*., 2011). The taxonomic uncertainty is especially the case for *T. shanjing*.

Himalayan newt, *T. verrucosus* (Anderson, 1871) occurs in extreme Western Yunnan of China, India, Nepal, Bhutan, Northern Myanmar and red knobby newt (*Tylototriton shanjing*) (Nussbaum *et al*., 1995) is distributed in Western, central and Southern Yunnan province of China (Nussbaum *et al*., 1995). The taxonomic status of *Tylototriton shanjing* has recently been disputed (Weisrock *et al*., 2006; Zhang *et al*., 2007; Yang and Rao, 2008; Fei *et al*., 2010; Stuart *et al*., 2010; Kurabayashi *et al*., 2012). *Tylototriton shanjing* was formerly part of *T. verrucosus* but was diagnosed as a distinct species by its unique orange coloration which distinguishes it from the allopatric brown-colored

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T. verrucosus (Nussbaum *et al*., 1995). Zhang *et al*. (2007) recommended that *T. shanjing* be considered a synonym of *T. verrucosus* on the basis of similarity in cyt b (average cyt b divergence is 1.2%). This change was followed by Yang and Rao (2008) and Frost (2012). However, only a single sample of Himalayan newt, *T. verrucosus* from type locality (Husa, Longchuan, Yunnan) was analyzed; no samples were included from other parts of the range (India, Nepal, Myanmar, Thailand, Burma, Vietnam and probably Laos and Bhutan). In addition, Ziegler *et al*. (2008) reported that *T. shanjing* breeds true in captivity. Until a more thorough analysis of *T. verrucosus* is undertaken, the systematic decision to remove shanjing must be considered premature (AmphibiaWeb, 2012). Hence, the phylogenetic placement of *T. shanjing* and the validity of it need further examination.

In this study, by analyzing partial mitochondrial Cytochrome b gene sequence (*cyt b*) of *T. verrucosus* group, researchers investigate the phylogenetic relationships within it and re-examine the taxonomy of *T. shanjing*.

MATERIALS AND METHODS

Since, the taxonomic status of *T. shanjing* is inconclusive, the classification of Frost (2012) was followed provisionally for convenience of discussion.

Genomic DNA extraction: Total genomic DNA was extracted from liver tissue from three *T. verrucosus* specimens (CAS 230899, 230933, 230940) fixed in 99% ethanol using the Qiagen Dneasy Tissue kit (Qiagen, Valencia, California, USA). Researchers then checked the DNA quality by running 5 μL of the extraction out on a 1% agarose gel.

Amplification and sequencing of *cyt b* gene: Fragment of mitochondrial DNA *cyt b* gene was amplified and sequenced using primers MVZ15 (5'GAACTAATGG CCCACACWWTACGNAA3') (Moritz *et al*., 1992) and MVZ16(5'AAATAGGAARTATCAYTCTGGTTTRAT3') (Chan *et al*., 2001). PCR amplifications were performed in 50 μL reactions using the following cycling conditions: an initial denaturing step at 94°C for 5 min; 35 cycles of denaturing at 95°C for 25 sec, annealing at 46°C or 30 sec and extending at 72°C for 60 sec and a final extending step of 72°C for 5 min. Negative controls were added to detect contamination. The PCR product was checked on 1% agarose gel and successively purified via ExoSAP-IT (USB, Cleveland, Ohio). DNA sequences of both strands were obtained using the BigDye Terminator v3.1 on an ABI 3130xl Genetic Analyzer following the manufacturer's instructions. Sequences were examined for signal quality and confirmed for complementarity using DNASTAR 6.0 (DNASTAR Inc.). A partial of 753 bp mtDNA cyt b fragment of *Tylototriton verrucosus* of three samples from Shan State of Myanmar was newly sequenced for this research (Table 1).

Molecular data analysis: The mitochondrial dataset was supplemented with homologous sequences retrieved from GenBank (Table 1) which represent 40 individuals of *T. verrucosus* from China (Zhang *et al*., 2007) and two sequences of *T. verrucosus* from pet trade (Veith *et al*., 2004; Kurabayashi *et al*., 2012). The samples span the most known range of *T. verrucosus* from 14 localities in China and three localities in Myanmar (Table 1 and Fig. 1). In addition, mtDNA cyt b sequence of *Tylototriton taliangensis* (EF627455; AF 295684) and *T. kweichowensis* (EF627453; EF627456) were downloaded from GenBank and were used as outgroups for reconstruction the phylogenetic tree based on earlier study (Chan *et al*., 2001; Zhang *et al*., 2007) (Table 1).

Table 1: Samples of *Tylototriton verrucosus* group used in this study

Species	Voucher No.	Localities (Abbreviation in Fig. 1)	Haplotype	GenBank accession
T. verrucosus	JP-218	Jingping, Yunan, China (JP)	E	EF627460[a]
T. verrucosus	JP-219	Jingping, Yunan, China (JP)	E	EF627460[a]
T. verrucosus	JP-220	Jingping, Yunan, China (JP)	D	EF627459[a]
T. verrucosus	LC-228	Lvchun, Yunnan, China (LC)	F	EF627461[a]
T. verrucosus	LC-229	Lvchun, Yunnan, China (LC)	B	EF627457[a]
T. verrucosus	LC-231	Lvchun, Yunnan, China (LC)	B	EF627457[a]
T. verrucosus	JD-113	Jingdong, Yunnan, China (JD)	J	EF627465[a]
T. verrucosus	JD-114	Jingdong, Yunnan, China (JD)	U	EF627476[a]
T. verrucosus	JD-115	Jingdong, Yunnan, China (JD)	U	EF627476[a]
T. verrucosus	YJ-351	Yuanjing, Yunnan, China (YJ)	I	EF627464[a]
T. verrucosus	SB-235	Shuangbai, Yunnan, China (SB)	U	EF627476[a]
T. verrucosus	SB-236	Shuangbai, Yunnan, China (SB)	U	EF627476[a]
T. verrucosus	DY-204	Dayao, Yunnan, China (DY)	M	EF627468[a]
T. verrucosus	DY-205	Dayao, Yunnan, China (DY)	U	EF627476[a]
T. verrucosus	DY-206	Dayao, Yunnan, China (DY)	U	EF627476[a]
T. verrucosus	YD-7	Yongde, Yunnan, China (YD)	K	EF627466[a]
T. verrucosus	YD-8	Yongde, Yunnan, China (YD)	Q	EF627472[a]

Table 1: Continue

Species	Voucher No.	Localities (Abbreviation in Fig. 1)	Haplotype	GenBank accession
T. verrucosus	YD-111	Yongde, Yunnan, China (YD)	K	EF627466[a]
T. verrucosus	YD-112	Yongde, Yunnan, China (YD)	U	EF627476[a]
T. verrucosus	LK-143	Caojian, Yunnan, China (CJ)	S	EF627474[a]
T. verrucosus	LK-144	Caojian, Yunnan, China (CJ)	U	EF627476[a]
T. verrucosus	LLMC256	Mucheng, Yunnan, China (MC)	A	EF627454[a]
T. verrucosus	LLMC257	Mucheng, Yunnan, China (MC)	H	EF627463[a]
T. verrucosus	LL-264	Longling, Yunnan, China (LL)	T	EF627475[a]
T. verrucosus	LL-265	Longling, Yunnan, China (LL)	U	EF627476[a]
T. verrucosus	LL-266	Longling, Yunnan, China (LL)	U	EF627476[a]
T. verrucosus	LL-267	Longling, Yunnan, China (LL)	R	EF627473[a]
T. verrucosus	LL-268	Longling, Yunnan, China (LL)	U	EF627476[a]
T. verrucosus	LL-269	Longling, Yunnan, China (LL)	O	EF627470[a]
T. verrucosus	LL-270	Longling, Yunnan, China (LL)	R	EF627473[a]
T. verrucosus	LL-271	Longling, Yunnan, China (LL)	R	EF627473[a]
T. verrucosus	LL-272	Longling, Yunnan, China (LL)	P	EF627471[a]
T. verrucosus	LL-273	Longling, Yunnan, China (LL)	T	EF627475[a]
T. verrucosus	LL-274	Longling, Yunnan, China (LL)	N	EF627469[a]
T. verrucosus	LL-275	Longling, Yunnan, China (LL)	O	EF627470[a]
T. verrucosus	TC-321	Tengchong, Yunnan, China (TC)	G	EF627462[a]
T. verrucosus	PM-259	Gangfang, Yunnan, China (GF)	C	EF627458[a]
T. verrucosus	PM-260	Gangfang, Yunnan, China (GF)	C	EF627458[a]
T. verrucosus	PM262	Pianma, Yunnan, China (PM)	C	EF627458[a]
T. verrucosus	HS356	Husa, Longchuan, Yunnan, China (HS)	L	EF627467[a]
T. verrucosus	-	pet trade	Vpet1	AY336660[b]
T. verrucosus	-	pet trade	Vpet2	NC017871[c]
T. verrucosus study	CAS 230933	Taunggyi, Shan state, Myanmar	Vver1	JX444703 This
T. verrucosus study	CAS 230899	Taunggyi, Shan state, Myanmar	Vver1	JX444703 This
T. verrucosus study	CAS 230940	Taunggyi, Shan state, Myanmar	Vver1	JX444703 This
T. kweichowensis	ZD192	Zhaotong, Yunnan, China	V	EF627453[a]
T. kweichowensis	ZD195	Zhaotong, Yunnan, China	W	EF627456[a]
T. taliangensis	CAS 195126	Sichuan, China	T1	AF295684[d]
T. taliangensis	SCXC350	Xichang, Sichuan, China	X	EF627455[a]

Sources of sequences retrieved from GenBank; [a]Zhang *et al.* (2007); [b]Veith *et al.* (2004); [c]Kurabayashi *et al.* (2012) and [d]Chan *et al.* (2001)

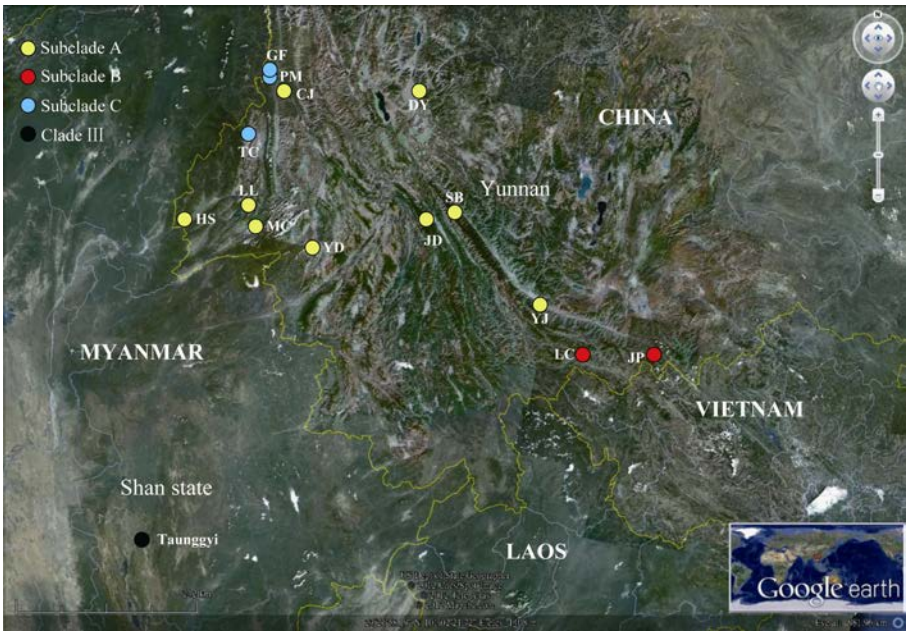

Fig. 1: Map showing the sampling localities of *T. shanjing* (red, yellow and blue dots) and *T. verrucosus* (black dot) in this study. A single black dot represents three adjacent localities; population name correspond to those (in parentheses) in Table 1

DNA sequences were aligned with Clustal X1.83 (Thompson *et al*., 1997) with default parameters. Researchers estimated the numbers of haplotypes using DNASP 5.1 (Librado and Rozas, 2009). The average uncorrected pairwise genetic distances (p-distance) among and within taxa was calculated in MEGA 5.0 (Tamura *et al*., 2011). MODELTEST 3.7 (Posada and Crandall, 1998) and PAUP×4.0b10 (Swofford, 2000) were used to find the best fit model of nucleotide evolution under the Akaike Information Criterion (AIC) for the sequence data. Researchers apply Bayesian Inference (BI) to reconstruct phylogenetic relationships based on GTR+G Model by using MRBAYES Version 3.1.2 (Ronquist and Huelsenbeck, 2003). Four independent Markov chain Monte Carlo analyses were run for 5×10^6 generations simultaneously, sampling every 100 generations and with the first 25% discarded as burn-in after the average standard deviation of split frequencies below 0.01.

RESULTS AND DISCUSSION

In the aligned 753 bp partial mtDNA cyt b sequences, researchers identified 28 haplotypes among the 49 *Tylototriton verrucosus* group individuals which including 24 haplotypes from 45 individuals of *T. verrucosus* (Table 1). Three new haplotypes were identified in this study which contained one haplotype among the three *T. verrucosus* individuals from Shan State of Myanmar and the other two from pet trade by Veith *et al*. (2004) and Kurabayashi *et al*. (2012), respectively (Table 1). No premature stop codons, insertions or deletions were observed and cyt b exhibited a compositional bias against guanines (15.1%). In the total 753 base pairs, there were 610 constant sites, 143 variable sites, 109 parsimony informative and 34 singleton sites in the *Tylototriton verrucosus* group. Among the 143 variable sites, there are 33 (23.1%) in the 1st, 10 (7%) in the 2nd and 100 (69.9%) in the 3rd codon position. Twenty eight of 251 (11.2%) amino acid sites were variable. The new sequence of cyt b haplotype was deposited in GenBank under accession numbers JX444703 (Table 1).

Researchers used 28 unique sequences of *T. verrucosus* group in the phylogenetic analysis. The phylogenetic tree of Bayesian Inference using the cyt b sequence data showed that *T. verrucosus* group consists of four clades (I-IV) with strong support (Fig. 2), clade I containing *T. taliangensis*, clade II containing *T. verrucosus* from Yunnan province of China, clade III containing *T. verrucosus* from Shan State of Myanmar and pet trade and clade IV containing *T. kweichowensis*.

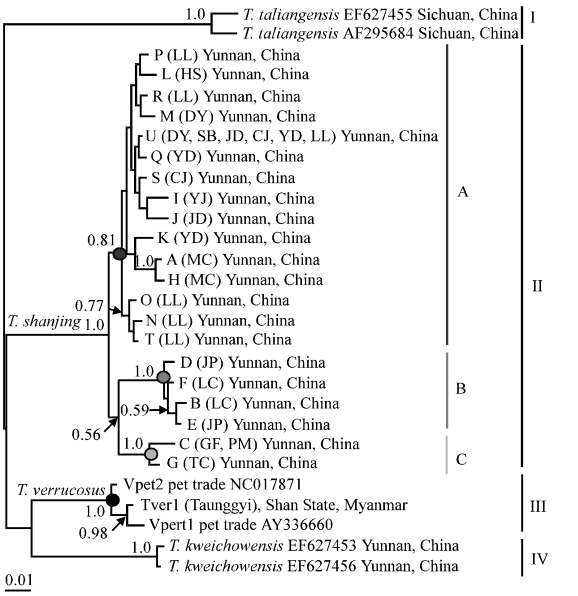

Fig. 2: Bayesian consensus tree of the *Tylototriton verrucosus* group based on 753 bp of *cyt b* gene with *T. taliangensis* as outgroup. Bayesian posterior probabilities are given only for those nodes which obtained values >0.60 with this method. The abbreviations in parentheses refer to localities in Table 1 and Fig. 1

Table 2: The average uncorrected pairwise distance of the mtDNA sequences used in this study within (diagonal in italic) and between (below diagonal) members of the *Tylototriton verrucosus* group

	Clade			
Groups	II	III	IV	I
Clade II (*T. shanjing*)	0.014	-	-	-
Clade III (*T. verrucosus*)	0.062	0.005	-	-
Clade IV (*T. kweichowensis*)	0.065	0.060	0.001	-
Clade I (*T. taliangensis*)	0.089	0.078	0.089	0.014

However, the sister relationship between clade II and III was not supported. In addition, Fig. 2 showed that within clade II, all 40 samples from Yunnan of China were grouped into three subclades (A, B and C); this is concordant with Zhang *et al*. (2007). At the same time, the relationships between the three subclades within clade II were not resolved by this analysis (Fig. 2). Table 2 showed the p-distance between the four clades (I-IV) of the *T. verrucosus* group (0.060-0.089) is obviously higher than within these four major clades (0.001-0.014) indicating differentiation at species level. In addition, differentiation of the three subclades within clade II (sub-clades A, B and C) is less pronounced (0.019-0.026) and may be regarded as subspecific.

The observed genetic differences (Table 2) between clade II and III (0.062) together with their phylogenetic relationships (Fig. 2) above indicate that the genetic

differentiation between the clade II and III has possibly reached a species level differentiation. Furthermore, the results indicate that clade II includes the samples from Jingdong, Yunnan of China (Table 1 and Fig. 1) which is near the type locality of *T. shanjing* (Nussbaum *et al*., 1995). Consequently, the analysis cyt b data corresponded with the conclusions that *T. shanjing* (clade II) should be recognized a valid species (Nussbaum *et al*., 1995; Weisrock *et al*., 2006; Fei *et al*., 2010; Stuart *et al*., 2010, Kurabayashi *et al*., 2012) and the clade III represents *T. verrucosus*. It is notable that only one sample from Husa, Longchuan, Yunnan (type locality of *T. verrucosus*) clustered within *T. shanjing* (clade II), indicating it probably does not belong to *T. verrucosus*. Further, examination need including more samples from this region (Husa, Longchuang, Yunnan) and nuclear gene to determine precisely the geographic ranges of *Tylototriton verrucosus* and *T. shanjing*.

Tylototriton verrucosus and *T. shanjing* are regularly caught and dried for traditional medicinal usage but also increasingly threatened by the habitat destruction inflicted by growing human populations (Zhao, 1998). At the same time because of its beautiful coloration patterns, it is commonly sold in the pet trade industry. The newts *Tylototriton verrucosus* and *T. shanjing* are categorized as globally Least Concern and Near Threatened on the IUCN Red List of endangered species (IUCN, 2012), respectively. A strong and well-funded science base in taxonomy and systematics could have profound implications for biodiversity assessment and effective conservation planning (Bickford *et al*., 2007; Fouquet *et al*., 2007; Funk *et al*., 2011). The result shows that *Tylototriton shanjing* should be a valid species and harbors three distinct divergent geographic sub-clades (A, B and C) indicating strong population genetic structure. Therefore, future conservation policy of this species should not only strengthen law to limit human exploitation and habitat destruction but also protection the three distinct regional subclades which represent important components in the evolutionary and adaptive structure of this species.

CONCLUSION

Molecular phylogenetic relationships and genetic distance analysis within the *Tylototriton verrucosus* group indicate that clade II and III actually represent two different species and clade II (subclade A, B and C) corresponds to the nominotypical *T. shanjing*. Detailed field research in Eastern Myanmar and Western Yunnan are needed to determine precisely the geographic ranges of *Tylototriton verrucosus* and *T. shanjing*. Recently, two new species within genus *Tylototriton* were found and reported (Stuart *et al*., 2010; Shen *et al*., 2012), this suggests that species-level diversity within Tylototriton may remain underestimated, especially in poorly surveyed areas of South, Southeast and East Asian. At the same time, it is possible of that more than one species of *T. verrucosus* and more than one species of *T. shanjing* exist which has been suggested by Nussbaum *et al*. (1995). Therefore, further decision should be based on denser sampling with accurate locality data in other parts of these two species range (India, Nepal, Myanmar, Thailand, Burma, Viet Nam and probably Laos and Bhutan) and additional nuclear genes.

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