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Molecular phylogeny of the Chinese ranids inferred from nuclear and mitochondrial DNA sequences

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Abstract

The phylogeny of representative species of Chinese ranids was reconstructed using two nuclear (tyrosinase and rhodopsin) and two mitochondrial (12S rRNA, 16S rRNA) DNA fragments. Maximum parsimony, Bayesian, and maximum likelihood analyses were employed. In comparison with the results from nuclear and mitochondrial data, we used nuclear gene data as our preferred phylogenetic hypothesis. We proposed two families (Ranidae, Dicroglossidae) for Chinese ranids, with the exception of genus *Ingerana*. Within Dicroglossidae, four tribes were supported including Dicroglossini, Paini, Limnonectini, and Occidozygini. A broader sampling strategy and evidence from additional molecular markers are required to decisively evaluate the evolutionary history of Chinese ranids.

Keywords: Nuclear genes; Mitochondrial DNA; Ranidae; Molecular phylogeny

1. Introduction

As one of the most diverse amphibian groups, ranids are cosmopolitan and encompass more than 700 species arranged into about 39 genera (Frost, 2004). The taxonomy about Ranidae is extremely problematic, and has received much attention. Recently, a series studies, including several using DNA sequences, have proposed many major revisions.

Dubois (1992) proposed ranids into seven subfamilies and made numerous generic and subgeneric rearrangements. According to Dubois's (1992) proposal, Chinese ranids should be distributed between two subfamilies: Raninae

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Rafinesque-Schmaltz, 1814 and Dicroglossinae Anderson, 1871. However, Fei et al. (1990) classified Chinese ranids into three subfamilies: Raninae, Amolopinae Yang, 1989, and Occidozyginae Fei, Ye *et* Huang, 1990, among which, Amolopinae and Occidozyginae were only identified as genus *Amolops* sensu lato, and genera *Occidozyga*, *Phrynoglossus* to be respectively included in Raninae and Dicroglossinae by Dubois (1992). Recently, Fei et al. (2005) further divided the Chinese ranids among four subfamilies: Raninae, Dicroglossinae, Amolopinae, and Occidozyginae. Meanwhile, Dubois (2005) also amended his previous classification and defined 14 subfamilies within Ranidae. According to this new classification, most Chinese ranids still remain in the two subfamilies Raninae and Dicroglossinae. An exception is the two Chinese species of genus *Ingerana* placed in Dicroglossinae by Dubois (1992), which were split into Ceratobatrachinae Boulenger, 1884 by Dubois (2005). Besides the suprageneric chaos, the supraspecific classification about Chinese ranids is also in a state of flux (Dubois, 1987, 1992, 2005; Fei, 1999; Fei et al., 1990, 2000, 2005), due, in part, to the various divisions of subfamilies within Ranidae. We are not surprised at such taxonomic inconstancy and disagreements, which are mostly due to the facts that these ranks were proposed merely on the basis of overall similarity and with little examination of phylogenies (Inger, 1996; Zhao, 1994). Clearly, morphology alone will not be able to clarify these controversies due to the high morphological homoplasy existing in amphibians, and molecular data could provide additional needed information (Bossuyt et al., 2006; Chen et al., 2005; Emerson et al., 2000a,b; Jiang and Zhou, 2001, 2005; Jiang et al., 2005; Kosuch et al., 2001; Matsui et al., 2005, 2006; Roelants et al., 2004; Scott, 2005; Tanaka et al., 1996; Tanaka-Ueno et al., 1998a,b). More recently, based on combined data of comparative anatomical and molecular evidence, Frost et al. (2006) proposed a new, advanced taxonomy across all living amphibians, and ''Ranidae'' were partitioned into 11 ''family-group'' taxa to avoid paraphyly with regard to Rhacophoridae and Mantellidae. Accordingly, many generic revisions were made to render a monophyletic taxonomy.

Unfortunately, to date, studies of Chinese ranid species have been based on relatively small character sets of partial 12S and 16S rRNA sequences (Jiang and Zhou, 2001, 2005; Jiang et al., 2005). Recently additional sources of phylogenetic characters, e.g., nuclear DNA markers, have begun to receive more attention. In the present study, we used two nuclear genes and two mitochondrial genes to investigate Chinese ranids, with the objective of (1) examining ranid phylogeny based on different gene partitions, with an emphasis on the Chinese taxa, and (2) providing a phylogenetic background to assess and revise the current classification of Chinese ranids, which is based on morphological studies.

2. Materials and methods

2.1. Taxon sampling

A general consensus regarding ranid taxonomy does not exist, so we mainly followed the classification of Dubois (1992) and Frost (2004) for convenience. Thirty-one species covering most of Chinese genera or subgenera proposed by Dubois (1992) were selected as ingroup taxa, including 12 species new to the present study, and 19 species from Che et al. (in press), along with three species from GenBank (Roelants et al., 2004). Ranidae is paraphyletic with respective to Mantellidae and Rhacophoridae on the basis of molecular evidence (Emerson et al., 2000a; Frost et al., 2006; Roelants et al., 2004), so four species belonging to Mantellidae and Rhacophoridae were also included in this study. Two species from Microhylidae (*Microhyla ornata*) and Hyperoliidae (*Hyperolius* sp.) were chosen as outgroup taxa based on the studies of Frost et al. (2006), Hay et al. (1995), and Roelants et al. (2004). Table 1 lists for all specimens examined in this study.

2.2. Extraction, amplification, and sequencing

Muscle or liver tissue samples were stored in 95% or 100% ethanol. DNA was extracted using the standard 3-step phenol/chloroform extractions. Two mitochondrial and two nuclear DNA fragments (Bossuyt and Milinkovitch, 2000) were PCR-amplified. The mtDNA fragments are as following: ①750 base pair (bp) region covering part of the 12S rRNA gene, the complete $tRNA^{Val}$ gene and part of the 16S rRNA gene; ②550 bp of the 16S rRNA gene. The nuDNA fragments are: ③529–532 bp of exon 1 of the tyrosinase gene; ④316 bp of exon 1 of the rhodopsin gene. Amplification was performed in a 50 μl 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 55 °C, extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 10 min. For rhodopsin and tyrosinase, the same

procedure was used, but with annealing at 51 °C and 49 °C, respectively. Purified PCR products were directly sequenced with an ABI automated DNA sequencer and sequences were then determined in both directions for each species and submitted for BLAST searching in GenBank to ensure that required sequences have been amplified.

2.3. Data analysis

Alignments of the four data sets were first conducted separately using Clustalx 1.81 (Thompson et al., 1997), with default parameters, and were then corrected by eye. After alignments of the rRNA genes (12S and 16S rRNA), hypervariable regions were excluded from further analysis due to the ambiguity of the alignment. Such exclusion increases the reliability of the phylogenetic analysis (Swofford et al., 1996). Pairwise divergence values were estimated by the method of Tamura and Nei (1993) (TN93) for the four genes with MEGA 3.1 (Kumar et al., 2004). Considering that all mtDNA gene sequences are inherited as effective one locus, the two mitochondrial gene segments were concatenated into a single partition for further analyses. An incongruence length difference (ILD) test (Farris et al., 1994, 1995) referred to as a partition homogeneity test in PAUP 4.0b10a (Swofford, 2003) was used to examine possible incongruence between different combinations of genes (tyrosinase gene + rhodopsin gene; mitochondrial DNA + nuclear genes).

Sequence data of different genes were analyzed both separately and in combination. Both parsimony and likelihood criteria were used to infer the best phylogenetic hypotheses. Parsimony analyses were implemented using PAUP 4.0b10a (Swofford, 2003). Heuristic MP searches were executed in 1000 replicates with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. Bootstrapping proportions (BSP; Felsenstein, 1985) with 1000 replicates were used for nodal evaluation. For likelihood-based phylogeny inference, the best fitting models of sequence evolution were determined with Modeltest 3.06 (Posada and Crandall, 1998). The Bayesian analysis (BA) was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). For the combined analysis, the data were separated into two partitions, each following its own model. The Bayesian posterior probabilities (BPP) were estimated with 600 million generations, sampling trees every 100th generation, and calculating a consensus tree after omitting the first 25% trees as burn-in. Two separate runs with four Markov chains were performed. Additionally, we conducted ML analysis in comparison with BA. Heuristic searches were executed in 100 replicates with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. Bootstrapping proportions (BSP; Felsenstein, 1985) with 100 replicates were used for nodal evaluation.

3. Result

3.1. Sequence characteristics

All individuals were successfully amplified and sequenced. The alignment of the rhodopsin exon 1 sequences produced 316 base pairs of sequences, of which 103 sites were variable and 60 sites were parsimony informative. A total of 535 base pair sequences of tyrosinase exon 1 were obtained. The alignment produced one 3-bp gap; *Occidozyga martensii* has a three base pair insertion. Among the sites, 269 were variable and 204 were parsimony informative. After the alignment, a total of 844 base pairs of 12S gene were resolved, and 194 base pairs in total were removed from further analysis due to ambiguous alignment. Among the remaining 650 base pairs, 354 were variable and 294 were parsimony informative. The alignment of 16S gene sequences produced 587 base pairs in length, of which 65 base pairs were removed from further analysis due to ambiguous alignment. Among the remaining 522 base pairs, 245 were variable and 189 were parsimony informative. All sequences are deposited in GenBank and Accession numbers are listed in the Table 1.

3.2. Phylogenetic analysis of the nuclear genes

Although initially we intended to analyze each nuclear gene separately, the phylogenetic relationships in general inferred from rhodopsin exon 1 were poorly resolved due to the limited number of informative characters. An alternative combined data set was constructed for phylogenetic inferences because ILD test presented no evidence for phylogenetic conflict between rhodopsin and tyrosinase gene partitions ($P = 0.27$). The 50% majority rule consensus tree

Table 1
Samples and sequences used in this study

Species	Locality	Specimens voucher number	GenBank Accession numbers			
			Exon 1 of rhodopsin gene	Exon 1 of tyrosinase gene	12S rRNA	16S rRNA
Ingroup						
Family Ranidae						
Subfamily Dicroglossinae						
Chaparana unculuana	China: Jingdong, Yunnan	YNUHU2002502601	DQ458262*	DQ458277*	DQ458242*	DQ118490*
Fejervarya cancrivora	China: Hainan	SCUMH021	DQ458259*	DQ458274*	DQ458238*	DQ458252*
Fejervarya limnocharis	China: Hainan	SCUM04H001	DQ458271*	DQ458286*	DQ458239*	DQ458253*
Hoplobatrachus rugulosa H	China: Haikou, Hainan	SCUMH011	DQ458257*	DQ458272*	DQ458236*	DQ458250*
Hoplobatrachus rugulosa X	China: Xishuangbanna, Yunnan	SCUM0437941	DQ458258*	DQ458273*	DQ458237*	DQ458251*
Limnonectes fragilis	China: Mt. Limu, Hainan	SCUMH008	DQ458270*	DQ458285*	DQ458235*	DQ458249*
Limnonectes fujianensis	China: Mt. Wuyi, Fujian	YNUHU20026017	DQ458260*	DQ458275*	DQ458234*	DQ118517*
Limnonectes kuhlii	China: Yunnan	KIZ-RD05DT1	DQ458269*	DQ458284*	DQ458245*	DQ118519*
Nanorana parkeri	China: Nyingchi, Xizang	CIB-XM1096	DQ458261*	DQ458276*	DQ458240*	DQ118498*
Occidozyga martensii H	China: Hainan	SCUMH020	DQ458266*	DQ458281*	DQ458246*	DQ458254*
Occidozyga martensii X1-X2	China: Xishuangbanna, Yunnan	SCUM0437980 SCUM0437983	DQ458267* DQ458268*	DQ458282* DQ458283*	DQ458247* DQ458248*	DQ458255* DQ458256*
Paa boulengeri	China: Mt. Emei, Sichuan	SCUM37989	DQ458264*	DQ458279*	DQ458243*	DQ118477*
Paa robertingeri	China: Hejiang, Sichuan	SCUM0405169	DQ458265*	DQ458280*	DQ458244*	DQ118478*
Paa yunnanensis	China: Huili, Sichuan	SCUM045183CJ	DQ458263*	DQ458278*	DQ458241*	DQ118492*
Subfamily Raninae						
Amolops lifanensis	China: Lixian, Sichuan	SCUM0405177CJ	DQ360034	DQ360065	DQ359981	DQ360003
Amolops loloensis	China: Xichang, Sichuan	SCUM0405178CJ	DQ360012	DQ360043	DQ359959	DQ359990
Amolops mantzorum	China: Maoxian, Sichuan	SCUM030014GP	DQ360023	DQ360054	DQ359970	DQ360000
Amolops ricketti	China: Hejiang, Sichuan	SCUM0405181CJ	DQ360009	DQ360040	DQ359956	DQ359987
Rana adenopleura	China: Mt. Emei, Sichuan	CIB-WU37990	DQ360010	DQ360041	DQ359957	DQ359988
Rana catesbiana	China: Chengdu, Sichuan	SCUM0405176CJ	DQ360013	DQ360044	DQ359960	DQ289127
Rana chensinensis	China: Mt. Qinling, Shannxi	KIZ-RD05SHX001	DQ360030	DQ360061	DQ359977	DQ289119
Rana guentheri	China: Hainan	SCUMH002	DQ360024	DQ360055	DQ359971	DQ360001
Rana kunyuensis	China: Mt. Kunyu, Sichuan	CIB-HUI040001	DQ360033	DQ360064	DQ359973	DQ289111
Rana minima	China: Fuzhou, Fujian	CIB-HUI040003	DQ360021	DQ360052	DQ359968	DQ359998
Rana nigromaculata	China: Hongya, Sichuan	SCUMH0405199CJ	DQ360014	DQ360045	DQ359961	DQ359991
Rana pleuraden	China: Kunming, Yunnan	SCUM0405185CJ	DQ360011	DQ360042	DQ359958	DQ359989
Rana sauteri	China: Gaoxiong, Taiwan	SCUM0405175CJ	DQ360029	DQ360060	DQ359976	DQ289109
Rana schmackeri	China: Mt. Emei, Sichuan	CIB-WU37943	DQ360020	DQ360051	DQ359967	DQ359997

Table 1 (*continued*)

Species	Locality	Specimens voucher number	GenBank Accession numbers			
			Exon 1 of rhodopsin gene	Exon 1 of tyrosinase gene	12S rRNA	16S rRNA
Rana shuchinae	China: Xichang, Sichuan	CIB-HUI040009	DQ360026	DQ360057	DQ359980	DQ289126
Rana taipehensis	China: Sanya, Hainan	SCUMH019	DQ360036	DQ360067	DQ359983	DQ360005
Rana tientaiensis	China: Huangshan, Anhui	SCUM0405192CJ	DQ360007	DQ360038	DQ359954	DQ359985
Rana versabilis	China: Mt. Limu, Hainan	HNNU-A0019L	DQ360015	DQ360046	DQ359962	DQ359992
Rana weiningensis	China: Kunming, Yunnan	KIZ-RD05KMWN01	DQ360019	DQ360050	DQ359966	DQ359996
Subfamily Micrixalinae						
Micrixalus fuscus	India		AF249120	AF249183	AF249024	AF249056
Subfamily Ranixalinae						
Indirana sp. C.	India		AF249122	AF249185	AF249026	AF249051
Nyctibatrachus aliciae	India		AF249114	AF249177	AF249018	AF249063
Family Mantellidae						
Subfamily Boophiinae						
Boophis tephraeomystax	Madagascar		AF249105	AF249168	AF249009	AF249039
Subfamily Laliostominae						
Laliostoma labrosa	Madagascar	Genbank	AF249106	AF249169	AF249010	AF249037
Subfamily Mantellinae						
Mantella madagascariensis	Madagascar		AF249101	AF249164	AF249005	AF249049
Family Rhacophorinae						
Subfamily Rhacophorinae						
Polypedates cruciger	Sri Lanka		AF249124	AF249187	AF249028	AF249045
Outgroup						
Family Hyperoliidae						
Hyperolius sp.	Kenya		AF249098	AF249161	AF249002	AF249033
Family Microhylidae						
Microhyla ornata	India		AF249099	AF249162	AF249003	AF249060

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

inferred from the Bayesian analysis produces completely identical topology with ML analysis (Fig. 1A). Parsimony analysis using equal weights result in 142 MPTs (length, 1065; CI = 0.469, RI = 0.646). The strict consensus tree is shown in Fig. 1B, which essentially is compatible with the result of the Bayesian and ML analyses.

Our phylogeny supported the monophyly of Ranidae, Mantellidae, and Rhacophoridae with respect to Hyperoliidae and Microhylidae. The Chinese ranids examined clearly formed two major groups (I, II). *Occidozyga* was nested in group I, and *Amolops* within group II. In group I, there were four well-supported clades (Fig. 1: ①②③④), nevertheless, the relationships among them remain unresolved. Within clade ①, the sister group relationship of *Hoplobatrachus* and *Fejervarya* was strongly supported (100% BPP and 100% BSP). *Paa*, *Chaparana*, and *Nanorana* constituted the clade ②, among which, *Paa yunnanensis* and *Chaparana unculuana* were closely related to each other (99% BPP, 90% and 78% BSP). *Paa robertingeri* and *Paa boulengeri* constituted another sister taxon pair (100% BPP and 100%

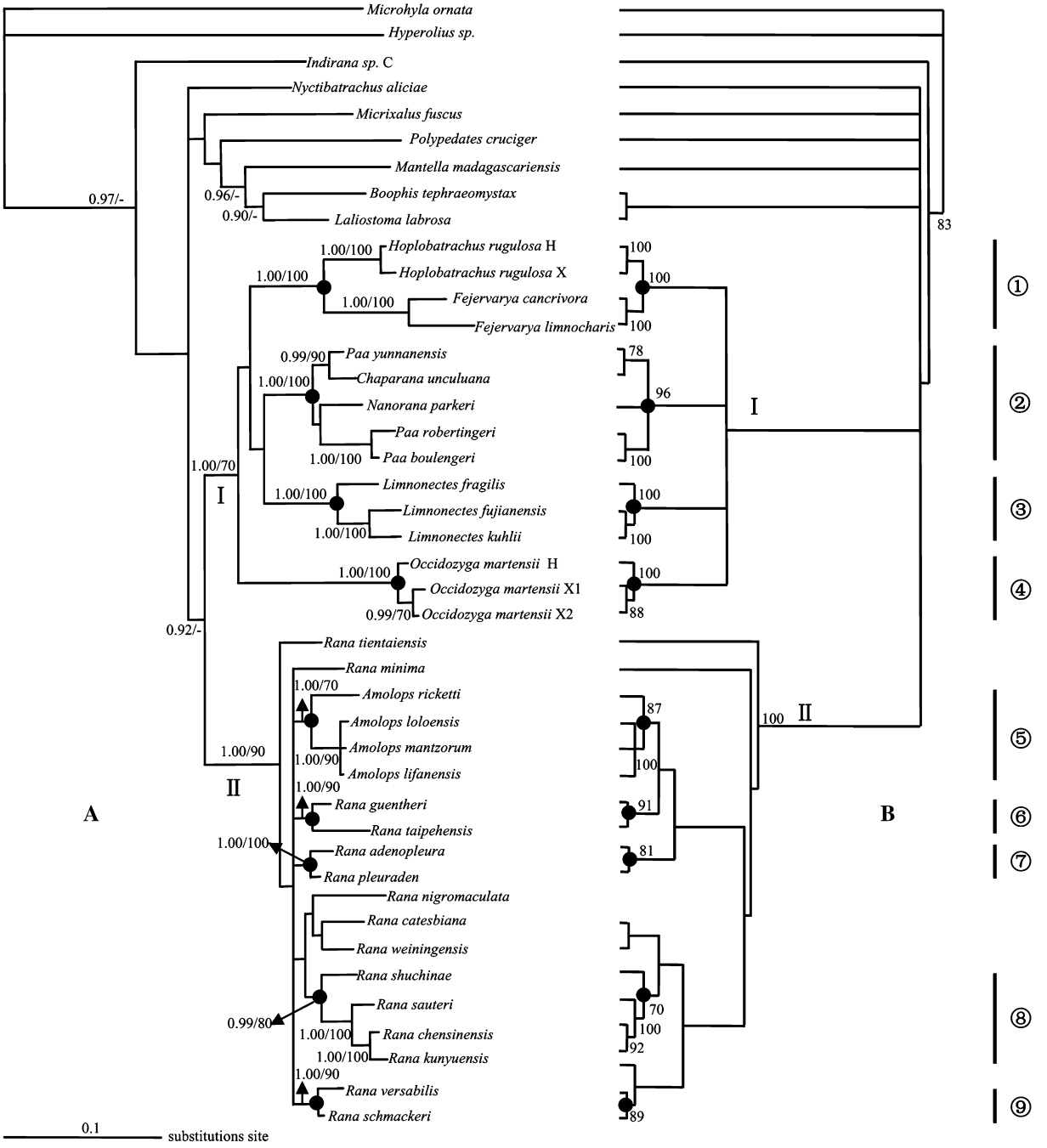

Fig. 1. The phylogenetic hypothesis derived from partial DNA sequences of the nuclear gene (exon 1 from tyrosinase gene and rhodopsin gene). *Microhyla ornata* and *Hyperolius* sp. are outgroups. (A) The 50% majority rule consensus from the Bayesian analysis. Numbers near branches represent Bayesian posterior probability (≥90% retained)/bootstrap support for ML (100 replicates) inference (≥70% retained). (B) The strict consensus tree from the parsimony analysis. Numbers near branches are bootstrap proportions calculated with 1000 replicates (≥70% retained). ①②③④⑤⑥⑦⑧⑨ indicate the clade assignment, which correspond to the black circles as in Fig. 2.

BSP). The status of *Nanorana parkeri* is uncertain. Within clade ③, *Limnonectes fujianensis* and *Limnonectes kuhlii* were identified as sister taxa, then followed by *Limnonectes fragilis* with high nodal support (100% BPP and 100% BSP). *O. martensii* from Hainan, Xishuangbannan of China was placed as clade ④, which was nested in group I with robust BPP (100%), but low BSP in ML (70%) and MP (52%, not shown).

Among group II, five clades (⑤⑥⑦⑧⑨) received high nodal support. The group of *Amolops loloensis*, *Amolops mantzorum*, and *Amolops lifanensis* was sister to *Amolops ricketti*, and they constituted the clade ⑤ (100% BPP, 70% and 87% BSP). Clade ⑥ consisted of *Rana guentheri* and *Rana taipehensis* (100% BPP, 90% and 91% BSP), and clade ⑦ included *Rana adenopleura* and *Rana pleuraden* (100% BPP, 100% and 81% BSP). Within clade ⑧, *Rana chensinensis* and *Rana kunyuensis* were first clustered as sister taxa, and then followed by *Rana sauteri*. The close association of *Rana shuchinae* with the three brown frogs was supported with 99% BPP, 80% and 70% BSP. *Rana versabilis* and *Rana schmackeri* constituted the clade ⑨ (100% BPP, 90% and 89% BSP). The position of other species, e.g., *Rana tientaiensis*, *Rana minima*, *Rana catesbiana*, *Rana weiningensis*, and *Rana nigromaculata* was not resolved.

3.3. Phylogenetic analysis of the mitochondrial genes

The 50% majority consensus tree from the Bayesian analysis (Fig. 2A) revealed an almost identical topology to the ML analysis. Maximum parsimony analysis resulted in five most parsimonious trees of 2943 steps (CI = 0.327, RI = 0.528). Fig. 2B shows the strict consensus MP tree, which essentially is consistent with Fig. 2A. Notably, all topological differences were weakly supported (BPP < 90%, BSP < 50%).

The results of the analysis of the mitochondrial genes (Fig. 2) differed from those based on the nuclear genes (Fig. 1). First, group I from nuclear data was not recovered by mtDNA data, and *O. martensii* constituted another separate evolutionary clade ④. Second, the well-supported monophyly of *Amolops* (Fig. 1: ⑤) was not resolved by mtDNA data, as shown in Fig. 2. Third, the monophyly of *R. tientaiensis* and *R. minima* inferred from mtDNA data sets (Fig. 2) was not recovered in the nuclear analysis (Fig. 1).

4. Discussion

4.1. The comparison of different gene partitions

We used nuclear and mitochondrial data sets for comparison due to the significant incongruence between them ($P = 0.001$). A simultaneous combined analyses (mtDNA + nuDNA: not shown) were conducted merely for discussion here. Apparently, there are some consistent aspects. First, all analyses supported the monophyly comprising Ranidae, Mantellidae, and Rhacophoridae, but with unresolved relationships among them. Second, monophyly of group II was recovered with strong nodal support by all analyses. In addition, all analyses recovered the monophyly of each clade (①③④⑥⑦⑧⑨), with high support values. Monophyly of clade ② is also identical, but with low BPP (52%, not shown) and moderate BSP (80%) based on mitochondrial data (Fig. 2).

The most distinct difference between mtDNA and nuclear data is the status of *Occidozyga*. Our mitochondrial data (Fig. 2) indicated that *Occidozyga* was not within group I, as suggested by Delorme et al. (2004) and Marmayou et al. (2000) based on mtDNA evidence. However, our nuclear data (Fig. 1) suggested that *Occidozyga* is nested within that group, which was supported strongly by Bayesian analysis (100%), but weakly by ML (70%) and MP (<50%, not shown). The nuclear gene analyses are congruent with the results of Bossuyt et al. (2006), Frost et al. (2006), Roelants et al. (2004), and Van der Meijden et al. (2005) based on combined data (mtDNA + nuDNA). Combining different data sets is controversial (Bull et al., 1993; De Queiroz et al., 1995). Usually, combined analysis increased character numbers and could improve the resolve ability. However, the topology of combined data of our own (mtDNA + nuDNA) recovered group I only by Bayesian and ML analyses, with relatively weak nodal values (BPP = 76%, BSP < 50%, not shown), which was possibly ascribed to the heterogeneity between mtDNA and nuclear data ($P = 0.001$).

In addition, regardless of the tree reconstruction methods employed, all nuclear analyses showed consistently that representatives of *Amolops* confined to subgenus *Amolops* (clade ⑤) proposed by Dubois (1992) formed a clade (Fig. 1: 100% BPP, 70% and 87% BSP). The diagnostic morphological character of this group is the presence of an abdominal sucker in tadpoles, presumably as an adaptation to a torrential life. Our nuclear data, along with the combined data (mtDNA + nuDNA) clearly supported the group; however, clade ⑤ remained unresolved on the basis of mtDNA data (Fig. 2). Furthermore, *R. tientaiensis* and *R. minima* formed a clade with high BPP (100%) and low BSP (72%, 77%) based on mtDNA data (Fig. 2), as already suggested by Jiang and Zhou (2005). However, the clade *R. tientaiensis* + *R. minima* was not recovered by nuclear data (Fig. 1). In this regard, the close relationships between the two species were also supported by our combined data (100% BPP and 83% BSP, not shown).

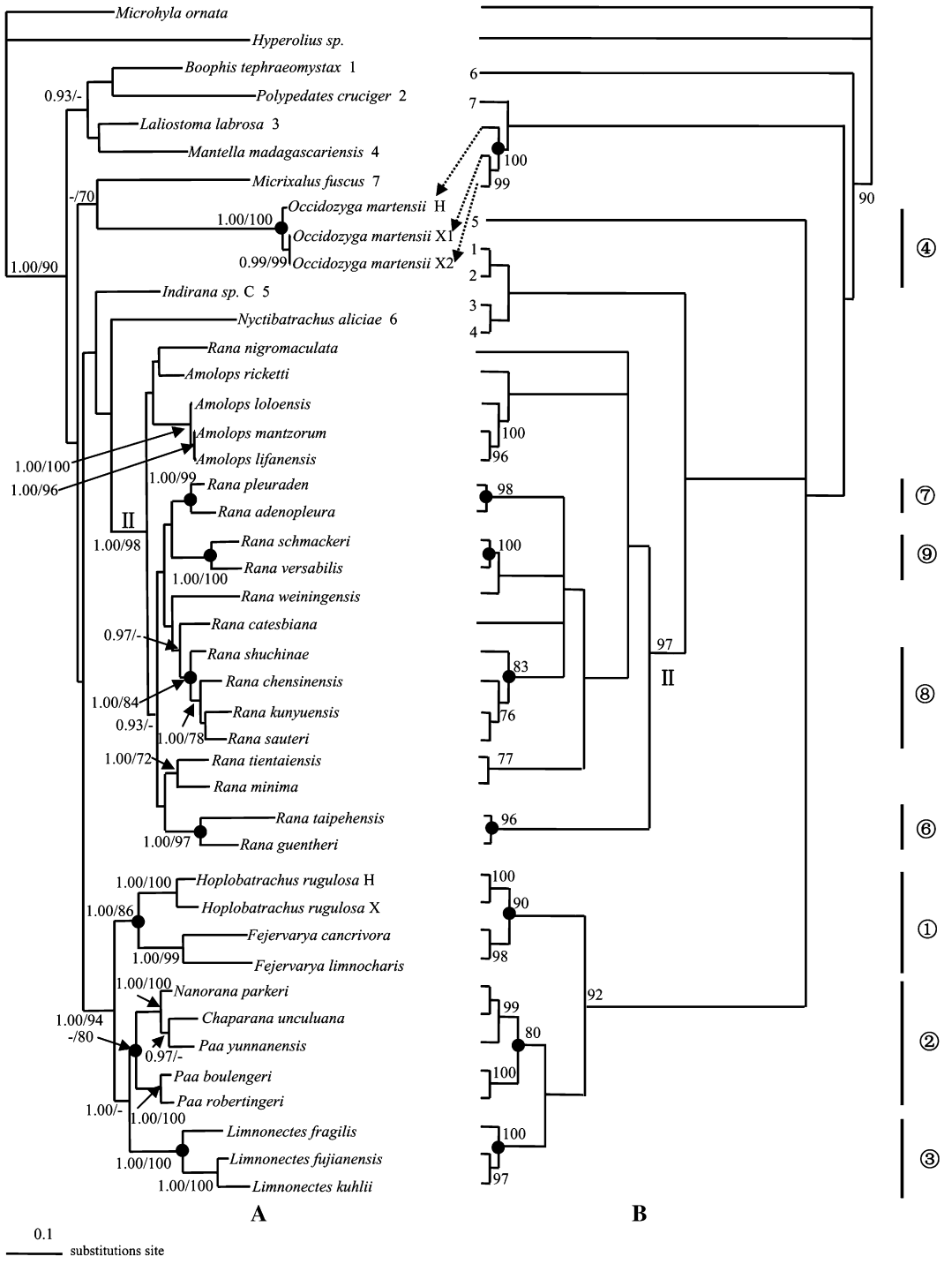

Fig. 2. The phylogenetic hypothesis derived from partial DNA sequences of the mitochondrial gene 12S and 16S. *Microhyla ornata* and *Hyperolius* sp. are outgroups. (A) The 50% majority rule consensus from the Bayesian analysis. Numbers near branches represent Bayesian posterior probability (≥90% retained)/bootstrap support for ML (100 replicates) inference (≥70% retained). (B) The strict consensus tree from the parsimony analysis. Numbers near branches are bootstrap proportions calculated with 1000 replicates (≥70% retained). 1–7 represent seven species taxa (1, *Boophis tephraeomystax*; 2, *Polypedates cruciger*; 3, *Laliostoma labrosa*; 4, *Mantella madagascariensis*; 5, *Indirana* sp. C.; 6, *Nyctibatrachus aliciae*; 7, *Micrixalus fuscus*).

Although some discrepancy was present between the topologies of mitochondrial and nuclear data, most tip nodes are congruent. More data sets and detailed sampling could resolve this problem, because we only have a small data set presently. Recent studies have shown that mitochondrial genome, which is inherited as a single, haploid linkage unit, has a limitation on phylogenetic reconstruction, i.e., incomplete lineage sorting of retained ancestral polymorphisms, introgression, and sex-biased life histories (Avise, 1994, 2000; Harrison, 1991; Moore, 1995; Niegel and Avise, 1986). Furthermore, the nuclear phylogenetic analysis showed CI = 0.469 and RI = 0.646. These values are higher than those obtained in mtDNA analysis (CI = 0.327 and RI = 0.528). However, this kind of inference is speculative, and further studies are needed, including more nuclear and mitochondrial gene characters. Based on present data, we choose the nuclear data as our preferred phylogeny for taxonomic deduction as below.

4.2. Taxonomic implication

All present representatives of Chinese ranids were divided into two major groups (group I, II) based on the nuclear data (Fig. 1). Although group I is not strongly supported by statistical tests, the conclusion is concordant with Bossuyt et al. (2006), Frost et al. (2006), Roelants et al. (2004), and Van der Meijden et al. (2005). Based on our phylogeny, we propose to place *Occidozyga* in group I provisionally. Two groups (I, II) did not reject two subfamilies division among Ranidae as proposed by Dubois (2005), however, we prefer to accept the new taxonomic schemes as proposed by Bossuyt et al. (2006), Frost et al. (2006), because we believe that taxonomy should reflect the evolutionary history. Obviously, Mantellidae and Rhacophoridae were well nested in Ranidae sensu Dubois. To avoid the paraphyly of Ranidae, it is sound that present representatives of Chinese ranids should be rearranged into two families, including Dicroglossidae (group I) and Ranidae (group II), as proposed by Frost et al. (2006).

In family Dicroglossidae (group I), four clades ①②③④ well supported four tribes: Dicroglossini, Paini, Limnonectini, and Occidozygini as suggested by Dubois (2005), however, the intercladal relationships were unresolved based on either separate or concatenated data. So we did not conduct further subfamilies division as proposed by Frost et al. (2006). Clearly, more gene markers should be added to elucidate their relationships. Accordingly, four genera could be supported, such as *Hoplobatrachus*, *Fejervarya*, *Limnonectes*, and *Occidozyga*. Consistent with the result of Jiang et al. (2005) and Roelants et al. (2004), all analyses here supported a monophyletic Paini (clade ②: *Paa*, *Chaparana*, and *Nanorana*), which was incompatible with that of Frost et al. (2006) in which Paini proved to be paraphyletic. One possible explanation is the difference of samplings between ours and Frost et al.'s (2006). Another possibility is that the incomplete sequences of several taxa in Frost et al. (2006) might cause the incongruence. Our mtDNA data (Fig. 2) and combined data (mtDNA + nuDNA, not shown) both supported two subclades in Paini, as recognized by Jiang et al. (2005). However, due to the unresolved status of *N. parkeri* based on nuclear data, we did not conduct further generic reassignments among Paini as Frost et al. (2006) and Jiang et al. (2005). Notably, the interesting association of *P. yunnanensis* and *C. unculuana*, in which both have an increased number of chromosomes (Li and Hu, 1994, 1996; Liu and Jiu, 1984; Tan and Wu, 1987; Wu and Zhao, 1984), was found based on nuclear data. The karyotype of *P. yunnanensis* and *C. unculuana*, displaying 64T or 40 chromosomes, deviates from the standard ranid karyological formula, which is considered a derived condition from the $2n = 26$ biarmed chromosome state (Li and Hu, 1999). However, relationships between the two species were not resolved on the basis of our mtDNA data or combined data (mtDNA + nuDNA), as suggested by the mtDNA evidence of Jiang et al. (2005). Presently, more than 40 species are recognized in the tribe Paini, so it is clear that more other species and evidences from more additional molecular markers are required to elucidate these interesting evolutionary problems.

In China, only three species belong to fanged frogs (*Limnonectes*). The name of the group originates from the fact that both males and females have protruding fangs in the lower jaw. Comprehensive studies about this group have been done (Emerson et al., 2000b); nevertheless, data were previously lacking for *L. fragilis*, which is endemic to Hainan of China. Our analyses support its sister relationship with the group of *L. kuhlii* and *L. fujianensis*.

For group II, our nuclear data (Fig. 1) did not support the monophyly of *Amolops* and *Pseudoamolops* (*R. sauteri*), which indicated the invalidation of distinct subfamily status of Amolopinae, as suggested by our mtDNA data (Fig. 2) and Matsui et al. (2006). We refrain from further discussion about the supraspecific problem because the data from Che et al. (in press) recovered a different topology based on greater species sampling among group II. The influence of sampling size for phylogenetic reconstruction attracts much attention (Hillis, 1998; Hillis et al., 2003; Rosenberg and Kumar, 2003). A further investigation about species sampling among this group is required.

The present phylogenetic hypothesis from either separate or combined data is not fully resolved, especially for the basal branches. Meanwhile, the lack of Chinese representatives from genus *Ingerana*, which only distributed in Xizang prevents our overall understanding of the relationships among Chinese ranids owing to the difficulty of sampling. Clearly, additional further work is necessary.

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