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# Systematics and molecular phylogenetics of Asian snail-eating snakes (Pareatidae)

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## Abstract

The taxonomy of the Asian snail-eating snakes (Pareatidae) is an ongoing controversy, partly because morphological characters do not yield consistent results across studies. We infer phylogenetic relationships within Pareatidae using ~ 2 kilobases of DNA sequences including two mitochondrial (cyt *b* and ND4) and one nuclear gene (c-mos). Results reveal four major lineages: *Aplopeltura*, *Asthenodipsas*, a clade formed by *Pareas carinatus* and *P. nuchalis,* and a clade comprising all other species of *Pareas* sampled in this study. Our data do not have enough signal to either support or reject a monophyletic *Pareas*. However, large molecular divergence (16.5%) is observed between the two major clades of *Pareas*, a level that is comparable to that between *Pareas* and *Aplopeltura*. Scale characters also suggest that *P. carinatus* and *P. nuchalis* are distinct from congeners, and future morphological and/or molecular studies might assess whether a distinct genus should be recognized. The molecular phylogeny further suggests a distant relationship between *P. chinensis* and *P. formosensis* and supports the validity of the former species.

**Key words:** *Aplopeltura*, *Asthenodipsas*, genetic divergence, mitochondrial genes, nuclear genes, *Pareas*, scale patterns

## Introduction

The Asian snail-eating snakes Pareatidae have long been recognized as a distinct lineage since the early nineteenth century (Boie 1827). They were considered a subfamily (Pareatinae) within Colubridae until recent phylogenetic analyses found strong evidence to support them as a separate family (e.g., Lawson *et al.* 2005; Vidal *et al.* 2007; Wiens *et al.* 2008; Pyron *et al.* 2011). Due to highly conserved morphology, the taxonomy of Asian snail-eating snakes remains contentious and has been frequently revised. Rao and Yang (1992) counted 39 species and subspecies in this family but suggested that most names were synonyms. Grossmann and Tillack (2003) recognized Pareatidae to comprise three genera and 15 species: *Aplopeltura boa* (Boie, 1828); *Asthenodipsas laevis* (Boie, 1827), *Asthenodipsas malaccanus* Peters, 1864, *Asthenodipsas vertebralis* (Boulenger, 1900); *Pareas boulengeri* (Angel, 1920), *P. carinatus* (Boie, 1828), *P. chinensis* (Barbour, 1912), *P. formosensis* (Van Denburgh, 1909), *P. hamptoni* (Boulenger, 1905), *P. iwasakii* (Maki, 1937), *P. macularius* Theobald, 1868, *P. margaritophorus* (Jan, 1866), *P. monticola* (Cantor, 1839), *P. nuchalis* (Boulenger, 1900), *P. stanleyi* (Boulenger, 1914). Jiang (2004) suggested that *P. chinensis and P. formosensis* had no significant difference in coloration and ventral and subcaudal scale pattern so he synonymized the former with the latter. In contrast, Zhao (2006) considered these two species as the *formosensis*-*chinensis* species complex pending evalution of more morphological data. Huang (2004) synonymized *P. macularius* with *P. margaritophorus* based also on morphological characters. Recently, Guo and Deng (2009) described another new species from southwestern China, *P. nigriceps*.

Molecular data are a frequently used and effective tool to help untangle taxonomic controversies when morphological analyses yield inconsistent results. However, molecular phylogenetic research on Pareatidae is limited and studies that included these snakes mainly aimed at questions at and above the family level (Slowinski and Law-

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son 2002; Lawson *et al*. 2005; Vidal *et al*. 2007; Pyron *et al*. 2011). Here we present the first study to address phylogenetic relationships within Pareatidae using mitochondrial and nuclear genes sequences. We consider the results in the light of morphological characteristics and systematic implications. We discuss relationships in the *formosensis*-*chinensis* species complex and evaluate the validity of *P. chinensis*.

## Material and methods

**Data preparation.** We collected 33 specimens representing all three genera and 10 species of pareatids (Table 1). Total DNA was extracted from shed skins, liver tissues or muscle tissues preserved in 95% ethanol. We followed the phenol/chloroform extraction procedure of Sambrook *et al.* (1989). We amplified partial sequences of the mitochondrial ND4 and cytochrome *b* genes and of the nuclear c-mos gene. We used primer pair L14910 (de Queiroz *et al.* 2002) and H16064 (Burbrink *et al.* 2000) to amplify the mitochondrial cyt *b* gene, primer pair ND4L and Leu (Arévalo *et al.* 1994) for ND4, and primer pair S77 and S78 (Lawson *et al.* 2005) for the c-mos fragment. Amplified DNA was purified with the BioStar glassmilk DNA purification kit according to the manufacturer's instructions. Purified DNA was sequenced using dye-labeled dideoxy terminator cycle sequencing on an ABI 3730 capillary sequencer (Applied Biosystems). *Dinodon rufozonatum* (Colubridae) and *Gloydius brevicaudus* (Viperidae) were chosen as outgroups based on the phylogeny of Vidal *et al.* (2007).

**Phylogenetic reconstruction.** Sequences were aligned using ClustalW (Thompson *et al.* 1994) implemented in Bioedit 7.0.9 (Hall 1999) with default parameters and proofread by eye. No premature stop codons or indels were detected. To evaluate potential incongruence among the cyt *b*, ND4 and c-mos sequences, we performed an incongruence length difference (ILD) test (Farris *et al.* 1995) in PAUP v4.0b 10a (Swofford 2003) with 1000 replicates and 10 random addition-sequences.

Phylogenetic relationships were estimated using maximum-likelihood (ML) implemented in Garli (Zwickl 2006), with the best-fitting evolutionary model determined by the Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada and Crandall 1998). The best-fit model for the concatenated data is the unequal-frequency Kimura 3-parameter model with Gamma distribution (G = 0.6636) and proportion of invariable sites (I = 0.4180). The search for the best ML tree was terminated when the likelihood score had not been improved for 100,000 generations. Bootstrap values were calculated for 100 replicates with the termination threshold reduced to 50,000 generations. To address possible saturation, we deleted third codon positions in mitochondrial sequences and re-ran the ML analysis with a newly assessed best-bit model.

We also performed Bayesian analysis on the full data set in MrBayes version 3.1 (Huelsenbeck and Ronquist 2001). Based on previous studies (e.g., Lawson *et al.* 2005; Bryson *et al.* 2007; Wiens *et al.* 2008), the concatenated data were partitioned by gene and codon position, and each partition was assigned an independent GTR+I+G model. Four independent Markov-chain Monte Carlo (MCMC) runs were carried out with random starting trees. Five million generations were sampled and the first 40% trees were discarded as burn in. To assess divergence among major clades recovered by the ML tree and Bayesian tree, we calculated the mean uncorrected *p*-distance among those clades in MEGA 4 (Tamura *et al.* 2007). Only specimens with complete data were included in the *p*-distance calculation.

To evaluate the monophyly of *Pareas* we determined best ML trees under a constraint of *Pareas* monophyly and compared them with the best unconstrained tree using one-tailed KH (Kishino and Hasegawa 1989) and one-tailed SH (Shimodaira and Hasegawa 1999) tests. Both tests were performed with 100 bootstrap replicates under full optimization.

## Results

**Sequence characteristics.** The aligned mitochondrial cyt *b* sequence includes 756 base pairs (bp), of which 329 are variable and parsimony-informative and 92 are uninformative. Within the 642 bp ND4 sequence, 234 variable sites are parsimony-informative and 71 are uninformative. In contrast to the highly variable mitochondrial sequences, the 570 bp of nuclear c-mos sequence contains only 45 variable sites, of which 27 are parsimony-informative. The ILD test detected no significant conflict among those three genes (P = 0.95). The concatenated data

**TABLE 1.** Specimens sampled in this study. Sequences newly generated for this study indicated in bold text. Abbreviations: CAS—California Academy of Sciences, San Francisco, USA; CIB—Chengdu Institute of Biology, the Chinese Academy of Sciences, Chengdu, China; DL—Li Ding collection, Chengdu Institute of Biology, the Chinese Academy of Sciences, Chengdu, China; FK—voucher listed by Kraus and Brown (1998); HC—Cryobanking project, Taipei Zoo, Taipei, China; HM—Mian Hou collection, Sichuan Normal University, Chengdu, China; KIZ—Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming, China; R: Hainan Normal University, Haikou, China; SYN—Shenyang Normal University, Shenyang, China.

| Specimen voucher No. | Name in Fig. 1 | Locality | Genbank Accessions | | |
|---|---|---|---|---|---|
| | | | cyt *b* | ND 4 | c-mos |
| ***Aplopeltura*** | | | | | |
| KIZ 011963 | *Aplopeltura boa* | Malaysia | **JF827673** | **JF827650** | **JF827696** |
| ***Asthenodipsas*** | | | | | |
| No voucher | *Asthenodipsas vertebralis1* | N/A (Genbank direct submission) | AY425807 | - | - |
| No voucher | *Asthenodipsas vertebralis2* | N/A (Genbank direct submission) | AY425808 | - | - |
| ***Pareas*** | | | | | |
| KIZ 09965 | *Pareas boulengeri1* | Enshi, Hubei, China | **JF827678** | **JF827655** | **JF827704** |
| KIZ 09966 | *Pareas boulengeri2* | Jiannan, Hubei, China | **JF827679** | **JF827656** | **JF827705** |
| KIZ 09967 | *Pareas boulengeri3* | Jianzhuxi, Hubei, China | **JF827680** | **JF827657** | **JF827706** |
| KIZ 09968 | *Pareas boulengeri4* | Luxi, Hunan, China | **JF827681** | **JF827658** | **JF827707** |
| KIZ 09969 | *Pareas boulengeri5* | Shennongjia, Hubei, China | **JF827682** | **JF827659** | **JF827708** |
| KIZ 09970 | *Pareas boulengeri6* | Luxi, Hunan, China | **JF827683** | **JF827660** | **JF827709** |
| KIZ 09971 | *Pareas boulengeri7* | Shennongjia, Hubei, China | **JF827684** | **JF827661** | **JF827710** |
| CIB 098270 | *Pareas carinatus1* | Menla, Yunnan, China | **JF827676** | **JF827652** | **JF827701** |
| DL 2008-S039 | *Pareas carinatus2* | Malaysia | **JF827677** | **JF827653** | **JF827702** |

continued next page

**TABLE 1.** (continued)

| Specimen voucher No. | Name in Fig. 1 | Locality | Genbank Accessions | | |
|---|---|---|---|---|---|
| | | | cyt *b* | ND 4 | c-mos |
| CIB 010140 | *Pareas chinensis1* | Baoxing, Sichuan, China | **JF827690** | **JF827667** | **JF827716** |
| CIB 098269 | *Pareas chinensis2* | Tianquan, Sichuan, China | **JF827691** | **JF827668** | **JF827717** |
| CIB 010141 | *Pareas chinensis3* | Baoxing, Sichuan, China | **JF827692** | **JF827669** | **JF827718** |
| CIB 010144 | *Pareas chinensis4* | Baoxing, Sichuan, China | **JF827693** | **JF827670** | **JF827719** |
| CIB 098272 | *Pareas chinensis5* | Tianquan, Sichuan, China | **JF827694** | **JF827671** | **JF827720** |
| HC 000618 | *Pareas formosensis1* | Yilan, Taiwan, China | **JF827685** | **JF827662** | **JF827711** |
| HC 000628 | *Pareas formosensis2* | Taoyuan, Taiwan, China | **JF827686** | **JF827663** | **JF827712** |
| HC 000669 | *Pareas formosensis3* | Taidong, Taiwan, China | **JF827687** | **JF827664** | **JF827713** |
| HC 000711 | *Pareas formosensis4* | Taipei, Taiwan, China | **JF827688** | **JF827665** | **JF827714** |
| HM 2007-S001 | *Pareas stanleyi* | Guilin, Guangxi, China | **JN230704** | **JN230705** | **JN230703** |
| R 0721 | *Pareas hamptoni1* | Hainan, China | - | **JF827654** | **JF827703** |
| No voucher | *Pareas hamptoni2* | N/A (Genbank direct submission) | AY425809 | - | - |
| R 0210 | *Pareas margaritophorus1* | Hainan, China | - | - | **JF827698** |
| CIB 098271 | *Pareas margaritophorus2* | Hainan, China | - | - | **JF827699** |
| CIB 098267 | *Pareas margaritophorus3* | Hainan, China | **JF827675** | - | **JF827700** |
| No voucher | *Pareas margaritophorus4* | N/A (Genbank direct submission) | AY425805 | - | - |
| CAS 206620 | *Pareas margaritophorus5* | Bago Division, Myanmar | AF471082 | - | AY471150 |
| SYN U04(Ⅱ)149 | *Pareas monticola* | Motuo, Xizang, China | **JF827689** | **JF827666** | **JF827715** |
| FK 2626 | *Pareas nuchalis* | Belait District, Brunei | - | PNU49311 | - |
| **Outgroups** | | | | | |
| CIB 098274 | *Dinodon rufozonatum* | | **JF827672** | **JF827649** | **JF827695** |
| CIB 088188 | *Gloydius brevicaudus* | | **JF827674** | **JF827651** | **JF827697** |

comprises an alignment of 1969 bp. Two different haplotypes are recovered in seven specimens of *P. boulengeri* collected in this study. All five specimens of *P. chinensis* share a single haplotype.

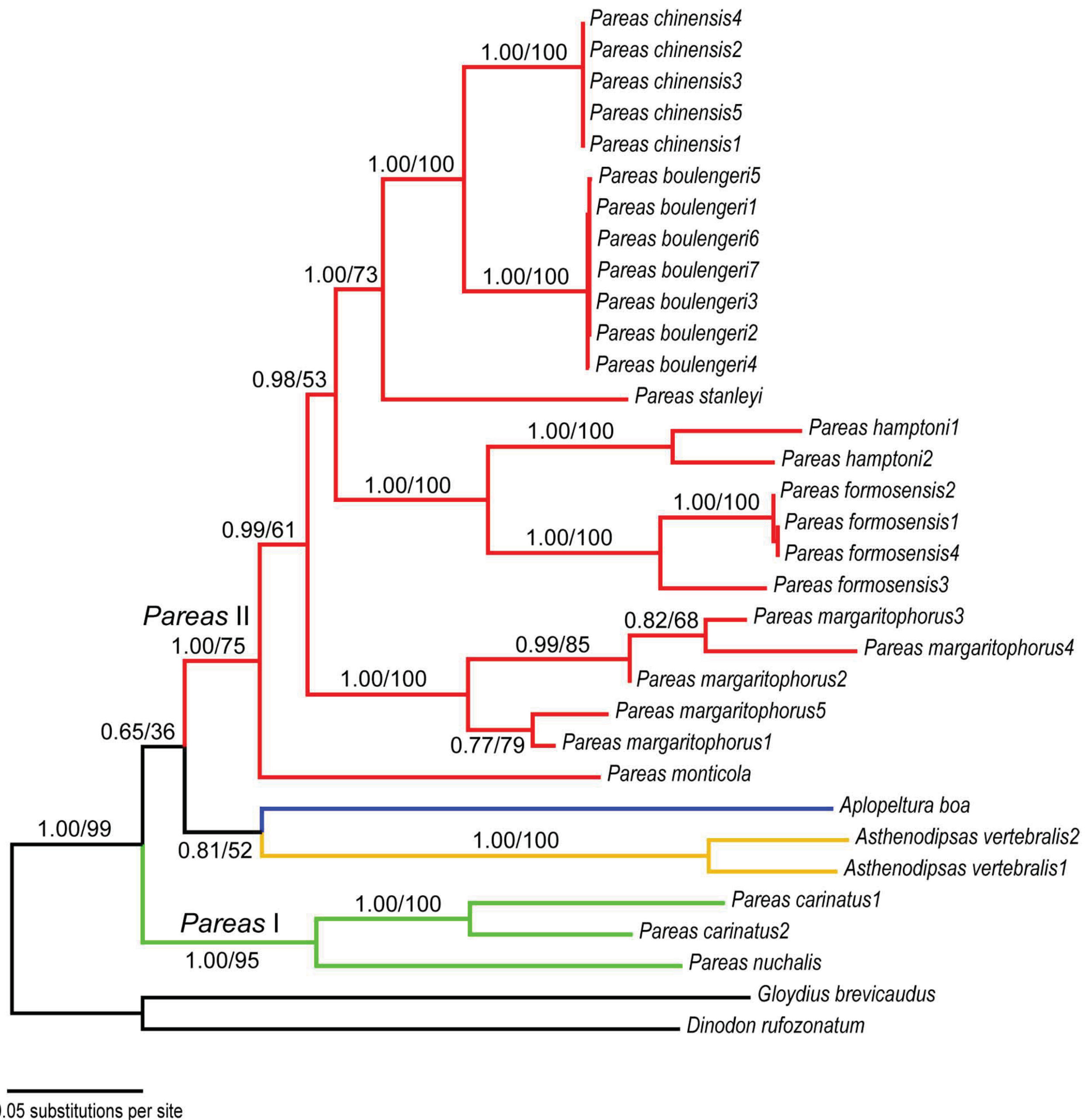


**FIGURE 1.** The maximum-likelihood tree inferred from the concatenated mitochondrial and nuclear sequence data. The four major lineages are coded in different colors: light green (*Pareas* I), red (*Pareas* II), blue (*Aplopeltura*) and light brown (*Asthenodipsas*). Bayesian posterior probability (before slash) and ML bootstrap support (after slash) are denoted above branches.

**Topological results.** The phylogenies estimated from individual genes (not shown) have similar topologies to that derived from the concatenated sequences. Topologies based on ML and Bayesian approaches are also identical. Exclusion of the third codon positions in mitochondrial sequences does not change the topology, which suggests that our results are not unduly affected by saturation. Thus, only the ML tree is shown here (Fig. 1). All species sampled from multiple specimens are monophyletic with strong support values. *Pareas* is not recovered as a monophylum. *Pareas carinatus* and *P. nuchalis* are recovered as strongly supported sister species (*Pareas* I in Fig. 1). All other *Pareas* sampled in our study form a separate clade (*Pareas* II) that is moderately supported. In our best trees, *Pareas* II is more closely related to *Asthenodipsas* and *Aplopeltura* than to *Pareas* I, but support for this resolution is not high. The best ML tree is not a statistically better fit to the data than the best tree enforcing a

monophyletic *Pareas* (KH and SH tests: P = 0.18). The mean uncorrected *p*-distance between *Pareas* I (represented by *P. carinatus*) and II is 16.5%, which is nearly as great as that between *P. carinatus* and *Aplopeltura boa* (17.7%), and between *Pareas* II and *Aplopeltura boa* (17.3%). *Pareas nuchalis* and species of *Asthenodipsas* have a missing gene fragment and thus were excluded from these calculation. Thus, we identify four major lineages within Pareatidae: *Aplopeltura*, *Asthenodipsas*, "*Pareas* I" and "*Pareas* II".

In contrast to the observed morphological similarity of *Pareas formosensis* and *P. chinensis* (Jiang 2004; Zhao 2006), molecular data reveal a large divergence between the two species. Our results do not support the monophyly of the *formosensis-chinensis* species complex. Instead *P. formosensis* is more closely related to *P. hamptoni*, and *P. chinensis* is the sister species of *P. boulengeri*. This result receives strong bootstrap support and high posterior probabilities (Fig. 1).

## Discussion

Pareatidae is a family of small snakes with a high degree of phenotypic similarity (such as coloration and the number of dorsal scales), thus causing difficulties in diagnoses and taxonomy. Molecular sequences in conjunction with morphological characters provide the opportunity to reexamine the phylogenetic relationships within Pareatidae.

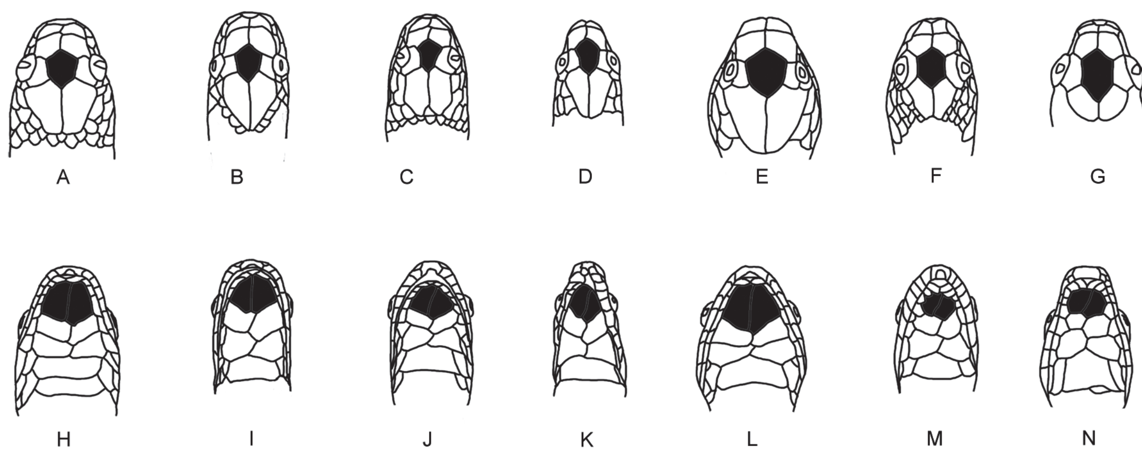


**FIGURE 2.** Variation in the frontal scale (top row) and the anterior pair of the chin shields (lower row) in *Pareas*. Both characters are shaded black. A & H: *P. hamptoni* (A–C, H–J all modified from Pope 1935); B & I: *P. stanleyi*; C & J: *P. boulengeri*; D & K: *P. iwasakii* (modified from Ota *et al.* 1997); E & L: *P. nigriceps* (modified from Guo and Deng 2009); F & M: *P. carinatus* (modified from Rao and Yang 1992); G & N: *P. nuchalis* (modified from Boulenger 1900).

The combined mitochondrial and nuclear sequence data indicate that Pareatidae is composed of four major lineages. Although phylogenetic signal is weak and does not provide compelling resolution of the (non-)monophyly of *Pareas*, the large uncorrected *p*-distance between *Pareas* I and II indicates that *P. carinatus* and *P. nuchalis* are genetically quite divergent from their congeners. This result is consistent with the phylogeny of Pyron *et al.* (2011), which sampled six species of pareatids. *Pareas carinatus* and *P. nuchalis* also differ from other *Pareas* in cephalic scalation and distribution pattern. *Pareas carinatus* and *P. nuchalis* share three anterior temporals in contrast to the one or two (rarely three) anterior temporals in other *Pareas* species. The frontal scale of the former two species is hexagonal with the lateral sides parallel to the body axis; this scale in other *Pareas* is almost diamond-shaped or shield-shaped with the lateral sides converging posteriorly (Fig. 2). A third distinction between the two groups can be found in the shape of the anterior pair of chin shields (Fig. 2). These two scales are broader than long in *P. carinatus* and *P. nuchalis* but longer than broad in congeners (Boulenger 1900; Rao and Yang 1992). Although we were unable to acquire tissue samples from *P. iwasakii* and the recently described *P. nigriceps* at this time, their cephalic scale characters match those of *Pareas* II species (Ota *et al.* 1997; Guo and Deng 2009). A study of scale micro-ornamentation of colubroid snakes revealed that *P. carinatus* has a distinct micro-ornamentation from *P. chinensis* and *P. boulengeri,* with the latter two species sharing similar patterns (He 2009). Scale micro-ornamentation has been shown to be somewhat congruent with phylogenetic relationships in other snakes (e.g., Price 1982; Gower

2003). Geographically, *P. carinatus* and *P. nuchalis* occur mainly throughout the Indochinese Peninsular and the Sunda Islands. Most species in the *Pareas* II clade occur in central and southern China and the northern Indochinese Peninsular, and only *P. margaritophorus* and *P. hamptoni* are found in the southern Indochinese Peninsular. Future studies extending character (morphological and molecular) and taxonomic sampling should consider whether the two major groups of *Pareas* identified here should be recognized as distinct genera.

*Pareas formosensis* and *P. chinensis* share very similar coloration and scale patterns (Jiang 2004). However, our molecular phylogenetic results indicate that the two species are not especially closely related within *Pareas*. *Pareas formosensis* is the sister species to *P. hamptoni*, and *P. chinensis* the sister of *P. boulengeri*. Close examination of maxillary teeth is consistent with this result. Both *P. formosensis* and *P. hamptoni* have 7–8 maxillary teeth, while *P. chinensis* and *P. boulengeri* have only 4–5 maxillary teeth (Rao and Yang, 1992). Thus, *P. chinensis* is a valid species and does not form a complex with *P. formosensis*. Our molecular data also finds large divergences among the sampled specimens of *P. margaritophorus*, which includes *Pareas macularius* as a junior synonym (Huang 2004). Future studies should also pay attention to variation within *P. margaritophorus*.

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