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# Diversity of the snail-eating snakes *Pareas* (Serpentes, Pareatidae) from Taiwan

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Pareatidae are a group of mollusc-eating snakes widely distributed in South-eastern Asia. Due to their dietary specialization, the asymmetric dentition of pareatids has recently become an interesting issue in evolutionary biology. However, phylogenetic relationships and species diversity of pareatids are still poorly studied. A total of three *Pareas* species, *P. formosanus* (Van Denburgh 1909), *P. compressus* (Oshima 1910) and *P. komaii* (Maki 1931), have been reported for Taiwan. However, only *P. formosanus* is currently regarded as a valid species. Using mitochondrial sequence phylogeny, nuclear *c-mos* haplotype network, as well as multivariate morphometrics, we re-evaluated the taxonomic status of *Pareas* from Taiwan, the Ryukyus and adjacent regions. These lines of evidence showed congruent results for the coexistence of three *Pareas* species in Taiwan, with prominent genetic and morphological differentiation and differing level of dentition asymmetry. The currently used name *P. formosanus* should be applied only to the snakes with red iris, comparatively short lower jaw and totally smooth dorsal scales. An examination of the type material indicated that the name *P. compressus* should be regarded as a junior synonym of *P. formosensis sensu stricto*. *Pareas komaii* (Oshima 1910) is confirmed as a valid taxon with yellow iris, elongated lower jaw and strongly keeled dorsals. The third clade is characterized by a yellow iris, elongated lower jaw and weakly keeled dorsals. Despite their sympatric occurrence, every examined individual showed consistent grouping from mitochondrial, nuclear and morphological markers, indicating there is no gene flow among these three clades. Here, we describe the third clade as a new specie, *Pareas atayal* sp. nov.

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## Introduction

Pareatidae are a group of mollusc-eating snakes recently separated from Colubridae (Lawson *et al.* 2005; Vidal *et al.* 2007; Zaher *et al.* 2009; Pyron *et al.* 2011). The members of Pareatidae are small sized arboreal nocturnal snakes characterized by blunt snout, lack of mental groove and the absence of teeth on the anterior part of maxillary (Rao & Yang 1992; Guo & Deng 2009). These snakes are endemic to the Oriental Region with a wide distribution throughout tropical and subtropical Asia (Rao & Yang 1992). Seventeen species of Pareatidae in three genera are currently recognized: one species of *Aplopeltura* (*Ap. boa*), five species of *Asthenodipsas* (*As. laevis*, *As. lasgalenensis*, *As. malaccanus*, *As. tropidonotus* and *As. vertebralis*) and 11 species of *Pareas* (*P. boulengeri*, *P. carinatus*, *P. chinensis*, *P. formosensis*, *P. hamptoni*, *P. iwasakii*, *P. margaritophorus*, *P. monticola*, *P. nigriceps*, *P. nuchalis* and *P. stanleyi*) (Rao & Yang 1992; Ota *et al.* 1997a,b; Huang 2004; Jiang 2004a,b; Guo & Deng 2009).

In Taiwan, the first pareatid snake was described by Van Denburgh (1909) from a single specimen collected in 'Kanshirei' (=now Guanziling, Tainan, south-western Taiwan) as *Amblycephalus formosensis* (Van Denburgh 1909). One year later, Oshima (1910) published *Psammodynastes compressus* based on a single specimen collected from 'Kokwangai, Taihoku' (=now in Xindian, Taipei, northern Taiwan). Both species shared the common character of smooth dorsals. Owing to the morphological similarity, Oshima (1916) himself invalidated this species as a synonym of *A. formosensis*. The third and the final species, *Amblycephalus komaii*, was described by Maki (1931) based on two specimens from 'Arisan' (=now Alishan, Jiayi, south-western Taiwan). According to the original description, *A. komaii* could be distinguished from *A. formosensis* by its keeled dorsals and position of the supralabials in relation to the oculars (Van Denburgh 1909; Maki 1931; Ota *et al.* 1997b). Lue (1989) suggested validity of these two species and noted that the keeled (*A. komaii*) and unkeeled (*A. formosensis*) species were distributed to higher and lower elevations, respectively.

Except for the change of the genus from *Amblycephalus* to *Pareas* (Smith 1943; Taylor 1965; ICZN 1971; Williams & Wallach 1989), the taxonomic status of pareatid snakes in Taiwan has remained stable for several decades. Although both species were listed in the herpetofauna of Taiwan, diagnosis between the two remained problematic for most herpetologists. Having examined 27 specimens from Taiwan in addition to the holotypes of both species, Ota *et al.* (1997b) suggested that the relative position of supralabials and oculars is not a valid character for the subdivision of Taiwanese *Pareas*. Furthermore, the arrangement and degree of development of keels were found to be highly variable, so the authors concluded that *P. komaii* should be regarded as a junior synonym of *P. formosensis*.

Another controversy is the relationship between *P. formosensis* and its congeners in adjacent regions. Jiang (2004b) evaluated morphological characters between *P. formosensis* on Taiwan and *P. chinensis* (Barbour 1912) on mainland Asia based on the literature review. Due to the overlapping number of ventrals and subcaudals, Jiang (2004b) concluded that *P. chinensis* should be a synonym of *P. formosensis*. However, Guo *et al.* (2011) based on prominent divergence in mitochondrial and nuclear sequences and position in phylogeny regarded *P. chinensis* as a valid species.

Due to the specialized feeding habit and foraging behaviour, the evolutionary biology of *Pareas* has received much attention in recent years (Hoso & Hori 2006, 2008; Hoso 2007; Hoso *et al.* 2007, 2010). In the southern Ryukyus, these snakes were found to specialize and coevolve with their molluscan prey (Hoso *et al.* 2010). Considering the fact that most land snails are predominantly dextral (Vermeij 1975), specializations for foraging on dextral snails would be selectively advantageous for snail-eating specialists. Such natural selection forced an ancestor of pareatid snakes in southern Ryukyus to evolve an asymmetric jaw, which could be quantified by a higher mandibular tooth count from their right mandible over the left one. Furthermore, Hoso *et al.* (2007) also predicted that snail-eating *Pareas* spp. would have a more asymmetric mandibular tooth count than slug eaters, who do not need to handle the asymmetric food item.

Variation in prey preference of *Pareas* snakes from Taiwan was first noticed by Lin (2010, personal communication on his master thesis), who noted that the *Pareas* from high- and low-elevation regions in Taiwan show specific preference on slugs and snails, respectively. After a preliminary evaluation on the samples used in his experiments on preying behaviour, we further found that the *Pareas* populations in Taiwan could be preliminarily distinguished by their dorsal keels, colour of iris and the shape of the heads and jaws (Fig. S1). Snakes with a red iris have shorter lower jaws and feed mostly on slugs, whereas snakes with a yellow iris have elongated jaws and might be able to consume a higher variety of prey items. Such variation hinted that these populations of snakes might show a considerable degree of genetic, morphological and behavioural differentiation.

In this study, we aim to solve the long-lasting question on species and genetic diversity of *Pareas* snakes in the island of Taiwan and adjacent regions. For this purpose, we sampled these snakes throughout Taiwan. Specimens from neighbouring regions were also included in the analysis. Based on molecular data, including mitochondrial and nuclear sequences, as well as morphological data, we argue that there are three distinct *Pareas* species coexisting in Taiwan: two of them require taxonomic revision and the third form represents a species new to science.

## Materials and methods

### *Sample collection*

During 2009–2011, we obtained 79 individuals of *Pareas* snakes from 15 localities, covering most of the distribution in Taiwan and southern Ryukyus (Fig. 1; Table S1). Specimens were fixed in 10% formalin and preserved in 70% ethanol, and a sample of their muscle tissues were preserved in 95% ethanol. Types and referred materials are stored in the herpetological collections of the National Museum of Natural Science (NMNS), Taichung, Taiwan and the Zoological Museum of Moscow State University (ZMMU), Moscow, Russia.

We collected four *Pareas* species from adjacent regions and included genetic information on seven species available

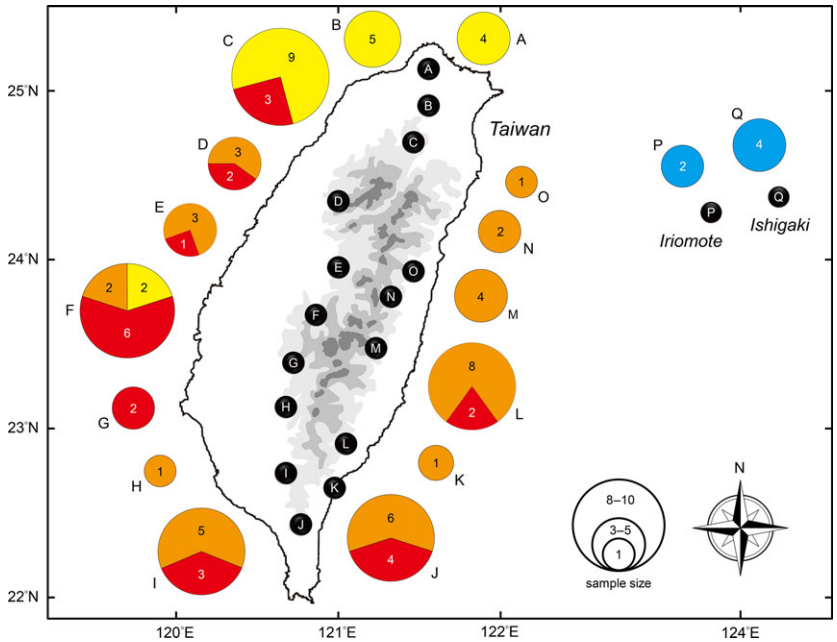


**Fig. 1** Sample localities (A–Q) of *Pareas* samples used in this study. The pie charts represented the ratio and sample size of the red-eyed form (*P. formosensis*, in red), yellow-eyed form 1 (*P. komaii*, in orange), yellow-eyed form 2 (*P. atayal* sp. nov., in yellow) and *P. iwasakii* (blue).

from GenBank to reconstruct the phylogeny of *Pareas* (Table S1). Our collection included five *P. iwasakii* tissues: three from Ishigaki island and two from Iriomote island (locality P and Q in Fig. 1), provided by Dr. Masaki Hoso (Kyoto University, Japan); three *P. hamptoni*: two from Vietnam and one from Guangdong Province, China; three *P. margaritophorus*: two from Vietnam and one from Hong Kong; and two *P. carinatus* from southern Vietnam. Sequences downloaded from GenBank included *P. boulengeri*, *P. chinensis*, *P. macularius*, *P. monticola*, *P. stanleyi* and *P. hamptoni* (identified as *P. tonkinensis*; Guo *et al.* 2011; Ding *et al.* 2011; Fernandes & Malhotra 2003, direct submission sequence; see Table S1). The remaining members of Pareatidae, *Asthenodipsas tropidonotus*, *Asthenodipsas vertebralis* and *Aplopeltura boa*, served as outgroups.

### *Molecular techniques*

Genomic DNA was extracted from ethanol preserved tissues using the EasyPure genomic DNA spin kit (Bioman, Taiwan) according to the manufacturer's instructions and stored at −20 °C until further usage. Complete mitochondrial cytochrome *b* gene and nuclear c-mos gene sequences were amplified by polymerase chain reactions (PCR). The primers used for cytochrome *b* amplification were as follows: L14910: 5′-GAC CTG TGA TMT GAA AAA CCA YCG TTG T-3′ and H16064: 5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′, designed by De Queiroz *et al.* (2002). Primers used for amplification of nuclear c-mos gene were as follows: S77: 5′-CAT GGA CTG GGA TCA GTT ATG-3′and S78: 5′-CCT TGG GTG TGA TTT TCT CAC CT-3′, designed by Slowinski & Lawson (2002). Reactions were conducted in a 20-$\mu$L reaction volume containing 1× PCR buffer [10 mM Tris-HCl, pH 9.0; 50 mM KCl, 0.01% (w/v) gelatine and 0.1% Triton X-100], 0.8 U Taq DNA polymerase (Amersham Biosciences), 0.2 $\mu$M each primer, 0.5 mM dNTP and 50 ng template DNA. The PCR conditions were denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 52 °C for 40 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min using an iCycler Thermal Cycler (Bio-Rad). PCR products were purified with a PCR Product Pre-Sequencing Kit (USB Corporation) and subsequently used as the template for direct DNA sequencing reactions with a DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The same primers used for PCR were used for the sequencing reactions. Sequencing products were run on an ABI 3730 automated DNA sequencer (Amersham Biosciences). The sequences were determined in both directions, and the original signals were proofread using Sequencher 4.7 (Gene Codes Corporation).

For nuclear c-mos gene, with possibly two alleles from this diploid locus, we used the PHASE algorithm (Stephens *et al.* 2001; Stephens & Scheet 2005) performed in software DNASP 5.10.1 (Librado & Rozas 2009) to reconstruct the two haplotypes of each individual from population genotype data. We implemented one million of Monte Carlo Markov Chain (MCMC) iterations while thinning at every 100 steps and discarding the first 1000 samples as burn-in. We excluded haplotypes for which phases were determined with probability <60% (Sotka *et al.* 2004).

***Phylogenetic analyses of mitochondrial sequences***

The obtained sequences were aligned by CLUSTAL W using MEGA 6.0.5 (Tamura *et al.* 2013). The data set after alignment contained 112 OTUs with 1119 bp, including 619 and 510 variable and parsimony-informative sites, respectively. PARTITIONFINDER 1.0.1 (Lanfear *et al.* 2012) was used to determine the best substitution model and partitioning scheme based on Bayesian information criterion (BIC) scores. The results indicated a scheme with three partitions: 1st, 2nd and 3rd positions. The preferred evolutionary model for all three partitions was TrN + I + G (Tamura–Nei's model with a proportion of invariable sites and a gamma distribution shape parameter).

The best partitioning scheme was then used for maximum likelihood (ML) and Bayesian phylogeny inferences. The ML analysis was conducted with RAXML 7.3.2 software (Stamatakis 2006). The best ML tree was selected from 200 iterations, each starting with distinct randomized parsimony trees. Clade support was examined by nonparametric bootstrap analyses (1000 replicates) summarized with 50% majority rule consensus trees. Bayesian inference analyses were conducted with MRBAYES 3.2.2 (Ronquist *et al.* 2012). The combined data matrix was partitioned, and models were assigned as suggested by PARTITIONFINDER. Two independent runs of $5 \times 10^7$ generations with eight MCMC chains each were conducted simultaneously, starting from random trees and resampling each tree every 1000 generations. The standard deviation of split frequencies between runs (<0.01) and the effective sample size (ESS) as measured by TRACER 1.5 (Rambaut & Drummond 2009) were monitored to ensure stationarity, convergence and correct mixing of the chains and to determine the correct number of generations to discard as a burn-in for the analyses (first 20%). Converged MRBAYES runs were combined after the exclusion of burn-in, and a majority rule consensus tree was created with nodal confidence assessed by posterior probabilities. Finally, the values of statistical supports from ML bootstraps and BPPs were labelled on corresponding nodes.

***Haplotype network of nuclear c-mos gene***

Owing to the comparatively lower sequence divergence of the c-mos gene, we conducted haplotype network to represent the interrelationship among *Pareas* species from Taiwan and adjacent regions. From 80 individuals, we obtained 160 c-mos partial sequences each of 602 bp in length. The 160 sequences contained 16 variable and 13 parsimony-informative sites, respectively. Individual sequences were transformed to haplotype data set by DNASP 5.10.1 (Librado & Rozas 2009), yielding to 13 haplotypes. Haplotype network was constructed using NETWORK 4.6.1.2 (Fluxus Technology Ltd.), where the size of the circles denoted the relative sample size.

***Morphological examination and multivariate morphometrics***

Morphological data for comparisons are based on examination of original collections by the authors and on information available in the literature and includes data from Pope (1935), Smith (1943), Nakamura & Uéno (1963), Taylor (1965), Rao & Yang (1992), Manthey & Grossmann (1997), Ota *et al.* (1997a,b), Cox *et al.* (1998), Zhao *et al.* (1998), Stuebing *et al.* (1999), Grossmann & Tillack (2003), Jiang (2004a,b), Guo & Deng (2009) and Das (2010).

Morphological description, measurements and scale counts follow Dowling (1951), Guo & Deng (2009) and Vassilieva *et al.* (2013). The terminal scute is excluded from the number of subcaudals. The numbers of dorsal scale rows are counted at one head length behind the head, at midbody and at one head length before the vent. Values for symmetric head characters are given in left/right order. Scale counts and measurements were taken under an Olympus SZ30 or Leica EZ4 microscopes using a digital caliper.

The hemipenal morphology was studied on specimens with hemipenal structures everted before preservation; terminology and description follow Keogh (1999). Cranial osteology of the new species (holotype NMNS 05594 and paratype ZMMU R-14434) was investigated with kind help of Dr. Masaki Hoso (Kyoto University, Japan) using high-resolution X-ray computed tomography (voxel size 10 mm); images were processed using MESHLAB 32 bit v 1.3.3.

The following measurements and counts were taken: snout-vent length (SVL); tail length (TaL); total length (TL); head length (HL, from snout tip to jaw angles); head width (HW); head height (HH); interorbital distance (IO); eye–nostril distance (EN, from anterior edge of orbit to posterior edge of nostril); internarial distance (IN); eye diameter (ED, horizontal); snout length (SnL, from the tip of rostral to the anterior margin of eye); nasal scale (NAS, entire or divided); supralabial scales (SL); infralabial scales (IL); loreal scale(s) (LOR); pre-ocular scales (Pre-OC); postocular scales (Post-OC); temporal scales (Temp); dorsal scale rows (DSR), including number of dorsal scale rows at neck (ASR, about one HL behind head); number of dorsal scales at midbody (MSR, at number of VEN/2); number of dorsal scale rows before vent (PSR); number of keeled dorsal scale rows at neck (KAD); number of keeled dorsal scales at midbody (KMD); number of keeled dorsal scale rows before vent (KPD); ventral scales (VEN); subcaudal scales (SC); and anal scale (entire or divided). Numbers of pattern units (like crossbars or vertebral blotches) are provided as number on body + numbers on tail.

We performed principal component analysis (PCA) and discriminant analysis (DA) to discriminate among the

genetic clades from Taiwan and the Ryukyus. First, we obtained morphological data from 62 intact individuals with clade groupings confirmed by molecular phylogeny. After preliminary tests, we chose nine traits which could provide a best resolution among the clades, including six meristic characters (IL, VEN, SC, KAD, KMD and KPD) and three size-related traits (TaL/TL, HL/TL and SnL/HL). PCA and DA were performed using JMP 7.0 (SAS Institute Inc.)

After PCA and DA have been executed, a series of coefficients was determined which could best discriminate the clades according to linear composition of characters from each individual. We took the same characters from the holotype (NSMT H00529) and the paratype (NSMT H00530) of *Pareas komaii*, and the specimen NSMT H02567 which might represent the type of *Psammodynastes compressus* Oshima 1910 preserved in National Science Museum, Tokyo, Japan. Characters taken from these type specimens were calculated with coefficients by linear composition to obtain their relative position in the scatter plots in PCA and DA.

## Results

### *Genetic differentiation among Taiwanese Pareas*

Phylogeny of cytochrome *b* gene reconstructed by ML and Bayesian approaches yielded to the same tree topology with high statistical supports on each node (Fig. 2A). Therefore, we took the ML tree as the best topology explaining the interrelationship among Taiwanese samples. The species of *Pareas* from East Asian islands (Taiwan and southern Ryukyus) group together with *P. hamptoni* distributed in mainland Asia. We refer to this clade as '*P. hamptoni*

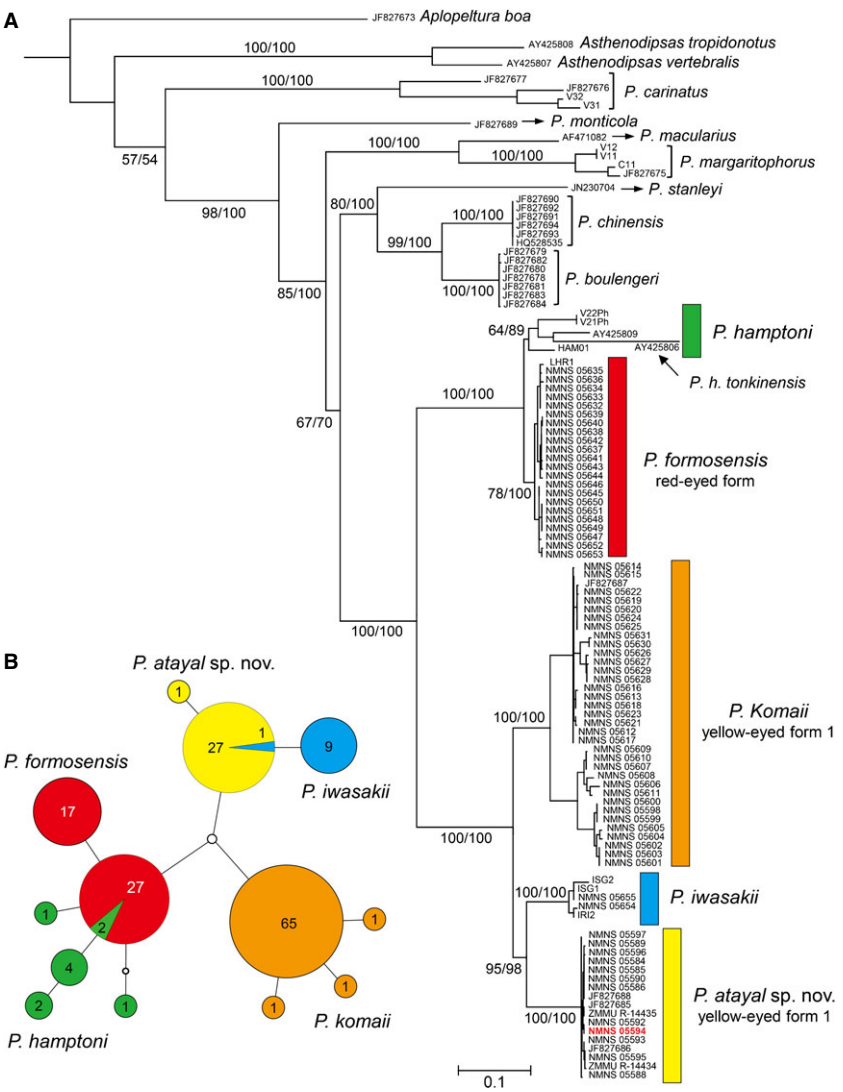


**Fig. 2** (A) Maximum-likelihood (ML) tree of *Pareas* species constructed using mitochondrial cytochrome *b* sequences (1119 bp, full length). Numbers above each node denote the statistic support from 1000 ML replicates and Bayesian posterior probabilities. (B) Haplotype network of nuclear c-mos gene from the 160 haplotypes (80 individuals) of *Pareas* species in Taiwan and southern Ryukyu. The areas of the circles correspond to the samples, while the colours correspond to the clade assignment in the phylogenetic tree. Green: *P. hamptoni*; red: the red-eyed form (*P. formosensis*); orange: the yellow-eyed form 1 (*P. komaii*); blue: *P. iwasakii*; and yellow: the yellow-eyed form 2 (*P. atayal* sp. nov.).

group' in the following discussion. *Pareas* in Taiwan were divided into three distinct clades (noted as red, orange and yellow bars in Fig. 2A) and did not form a monophyly. Specimens with red iris, short lower jaws and smooth dorsals (denoted with the red bar) were closely related to *P. hamptoni* collected from Guagdong (HAM01), from Vietnam (V21Ph and V22Ph) and AY425809 from GenBank (sample location not available). *Pareas tonkinensis* from GenBank is included in the *P. hamptoni* clade. Specimens with yellow iris, elongated lower jaws and keeled dorsals were further assigned into two clades. Snakes with stronger keels and higher keeled dorsal rows formed a unique clade, while those with weaker keels and lower number of keeled dorsals were grouped with *P. iwasakii*. These two clades were defined as 'yellow-eyed form 1' and 'yellow-eyed form 2', respectively. This branching pattern persisted with high statistical support among different analyses.

Sequence divergence among the clades has reached the species level and even exceeded interspecific divergence among some of the other *Pareas* species (Table S2). The two yellow-eyed clades have a divergence of 0.1085, whereas the distance between the red-eyed clade and the two yellow-eyed clades is 0.1810 and 0.1759, respectively. These values exceed genetic distances among *P. boulengeri*, *P. chinensis* and *P. stanleyi* (divergence from 0.1013 to 0.1702; Table S2).

Haplotype network of the 160 nuclear c-mos sequences is given in Fig. 2B, with the area of each haplotype corresponding to its sample size. We report a precise consistency between mitochondrial and nuclear groupings of Taiwanese *Pareas*. Individuals of red-eyed form, yellow-eyed form 1 and yellow-eyed form 2 have unique haplotypes, with at least two mutation steps from each other which are not shared by the other clades. Shared nuclear haplotypes were revealed in two sister pairs: between *P. iwasakii* and the yellow-eyed form 2, where a heterozygous *P. iwasakii* showed one allele identical to the most common one from the latter; and between *P. hamptoni* and the red-eyed form, where two *P. hamptoni* had one allele identical to the latter (Fig. 2B). Thus, although with a large area of sympatric occurrence (Fig. 1), species do not share nuclear haplotypes with the other clades even when they come into contact.

### *Morphological analyses*

All Taiwanese samples could be correctly assigned into morphological groups congruent with their molecular clades (Fig. 3). The PCA ordination plot successfully separated the three groups of snakes, where the first two principal component eigenvectors accounted for 64.72% of variation (Fig. 3A). Similarly, all groups were recovered in DA and each individual could be 100% correctly assigned to its phylogenetic clade (Fig. 3B). All snakes belonging to red-eyed clade shared the characters of red iris, short head, short lower jaw and smooth dorsals; while the yellow-eyed forms have yellow iris, longer head, elongated lower jaw and keeled dorsals (Fig. 4). The yellow-eyed form 2 could be further distinguished from form 1 by its higher numbers of infralabial scales (IL = 8.59 ± 0.59 vs. 7.81 ± 0.65, $P = 0.0013$), ventral scales (VEN = 180.77 ± 4.55 vs. 174.27 ± 4.67, $P = 0.0002$) and subcaudal scales (SC = 75.47 ± 2.23 vs. 69.56 ± 3.84, $P < 0.0001$; Table 1). Yellow-eyed form 1 has significantly higher numbers of keeled dorsal rows in every position of the body (KAD, KMD and KPD) than form 2 (Table 1).

Following the formula of PC1 and PC2 provided by PCA, and dim 1 and dim 2 provided by DA, the specimen NSMT H02567 (which represents the type of *Psammodynastes compressus* Oshima 1910; see Discussion) clearly clusters within the red-eyed form. On the other hand, both the holotype and the paratype of *P. komaii* were both assigned to the yellow-eyed form 1 group (Fig. 3). This indicates

**Table 1** Measurements and meristic characters of *Pareas formosensis* (the red-eyed form), *P. iwasakii*, *P. komaii* (the yellow-eyed form 1) and *P. atayal* sp. nov. (the yellow-eyed form 2) represented by mean ± SD

| Characters | *P. formosensis* (*n* = 13) | *P. iwasakii* (*n* = 4) | *P. komaii* (*n* = 34) | *P. atayal* (*n* = 17) |
|---|---|---|---|---|
| IL | 6.65 ± 0.59 (6–8) | 9.75 ± 0.87 (9–11) | 7.81 ± 0.65 (6–9) | 8.59 ± 0.59 (7–9) |
| VEN | 174.85 ± 3.08 (170–180) | 192.00 ± 1.41 (190–193) | 174.27 ± 4.67 (162–182) | 180.77 ± 4.55 (174–188) |
| SC | 74.15 ± 3.58 (69–82) | 79.00 ± 3.83 (76–84) | 69.56 ± 3.84 (60–76) | 75.47 ± 2.23 (71–79) |
| KAD | 0 ± 0 (0–0) | 1.5 ± 1.73 (0–3) | 4.82 ± 2.15 (0–9) | 0.47 ± 1.01 (0–3) |
| KMD | 0 ± 0 (0–0) | 6.00 ± 2.00 (3–7) | 10.18 ± 1.91 (7–13) | 5.94 ± 1.60 (3–9) |
| KPD | 0 ± 0 (0–0) | 8.00 ± 1.15 (7–9) | 11.41 ± 1.54 (9–13) | 6.18 ± 1.42 (3–9) |
| TaL/TL | 0.23 ± 0.01 (0.21–0.25) | 0.23 ± 0.01 (0.21–0.24) | 0.22 ± 0.01 (0.20–0.25) | 0.23 ± 0.02 (0.21–0.27) |
| HL/TL | 0.029 ± 0.002 (0.026–0.033) | 0.034 ± 0.003 (0.031–0.039) | 0.036 ± 0.003 (0.031–0.047) | 0.035 ± 0.003 (0.031–0.040) |
| SnL/HL | 0.29 ± 0.02 (0.26–0.32) | 0.20 ± 0.01 (0.19–0.21) | 0.23 ± 0.02 (0.20–0.26) | 0.24 ± 0.02 (0.22–0.28) |

IL, infralabials; VEN, ventrals; SC, subcaudals; KAD, number of keeled dorsal scale rows at neck; KMD, number of keeled dorsal scale rows at midbody; KPD, number of keeled dorsal scale rows before vent; TaL, tail length; TL, total length; HL, head length (from snout tip to jaw angles); SnL, snout length (from the tip of rostral to the anterior margin of eye).

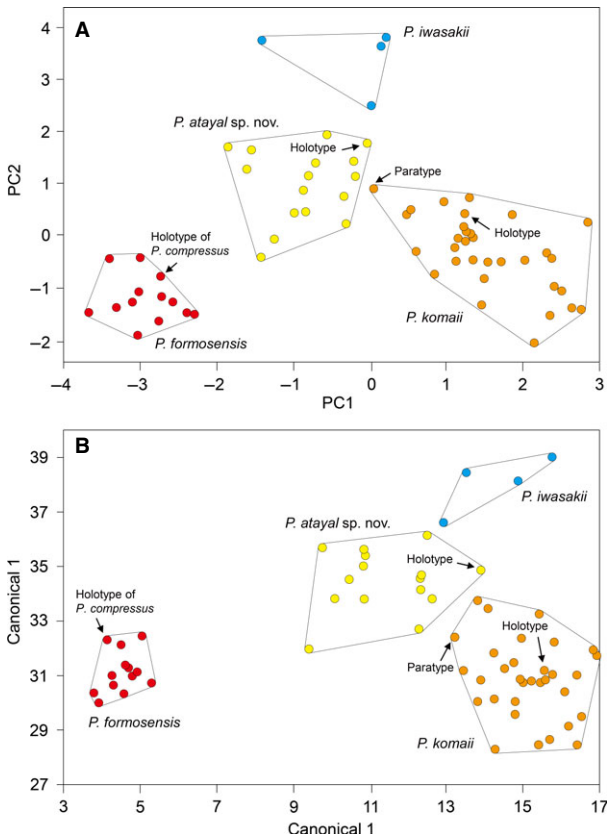


**Fig. 3** Principle component analysis (A) and discriminate analysis (B) from morphology of *Pareas* species in Taiwan and southern Ryukyu. Green: *P. hamptoni*; red: the red-eyed form (*P. formosensis*); orange: the yellow-eyed form 1 (*P. komaii*); blue: *P. iwasakii*; and yellow: the yellow-eyed form 2 (*P. atayal* sp. nov.).

that (i) the specimen Oshima (1910) used in his description of *P. compressus* does not differ from specimens of the red-eyed clade (*P. formosensis*); (ii) the name *P. komaii* should be applied to the yellow-eyed form 1; and (iii) the yellow-eyed form 2 should be treated as a new species.

### *Morphological variation and dentition asymmetry*

Mean and standard errors of the nine diagnostic characters are listed in Table 1, showing that *P. iwasakii* has largest numbers of infralabials (9.75 ± 0.87), ventrals (192.00 ± 1.41) and subcaudals (79.00 ± 3.83). In contrast, *P. formosensis* has lowest numbers of infralabials (6.65 ± 0.59) and ventrals (174.85 ± 3.08), and *P. komaii* has fewest subcaudals (69.56 ± 3.84). The strongly keeled dorsals of *Pareas komaii* are represented by the highest number of keeled dorsal rows (KAD, KMD and KPD), *P. formosensis* is entirely unkeeled. The ratio of tail length to total length (TaL/TL) did not show significant difference among the four species; yet *P. formosensis* has the lowest ratio of head length to tail length (HL/TL = 0.029 ± 0.002), compared to HL/TL > 0.034 in the other species. Differences in head shape were also shown by the differences in the SnL/HL ratio: *P. formosensis* exhibited the highest ratio (indicating the shortest lower jaw; Fig. S1), and *P. iwasakii* represented the lowest (the longest lower jaw; also see Fig. 4 for comparison).

Samples from the three *Pareas* clades in Taiwan also possess differing mandibular teeth counts and asymmetry indices (Table S3). The *P. formosensis* specimen we evaluated has 18 teeth in right mandible and 13 in left, yielding to an index of 16.13, precisely identical to the value proposed by Hoso *et al.* (2007). The two *P. komaii* has 21 teeth in right and 16–18 in left, yielding to 7.69–13.51 asymmetry index, appearing to be one of the most mandibularly symmetric *Pareas* species compared to congeners. *Pareas* sp. nov. has 20 in right, and 11 (holotype, NMNS 05594)—13 (one of the paratypes, ZMMU R-14434) in left (Fig. S2). Asymmetry index of this clade was 21.21 in one of the paratypes and could reach to 29.03 in holotype, ranking as the most asymmetric among all pareatid snakes studied to date.

## Discussion

The highly congruent results from morphology and molecular data indicate the presence of three highly divergent clades which are clearly diagnosable in morphological characters. Such deep differentiation within the Taiwanese *Pareas* contradicts the results of the last review by Ota *et al.* (1997b), who concluded that all Taiwanese populations should be treated as a single species, *P. formosensis* (Van Denburgh 1909). It is worthy to note that two or more *Pareas* clades occur sympatrically at seven of the 15 sites sampled. However, samples collected from these sites never showed evidence of introgression, indicating that these species do not hybridize even when they are in contact in nature. Furthermore, we noticed that all the sampling sites with more than five individuals represent occurrence of at least two different clades, suggesting that coexistence of two or more pareatid species might be common throughout the island.

### *The name P. formosensis* should be applied only to the red-eyed form

Both the original description of Van Denburgh (1909) and the re-evaluation by Ota *et al.* (1997b) of the holotype of *P. formosensis* indicated that this snake is characterized by its smooth dorsal scales. Although we did not have a chance to check the holotype of *P. formosensis* preserved in California Academy of Sciences (CAS 18006), the photograph provided by Ota *et al.* (1997b) clearly shows that this specimen is a smooth-scaled snake with short head and jaw. According to our study, these characters are found only in the red-eyed clade of Taiwanese *Pareas*. Based on these lines of evidence,

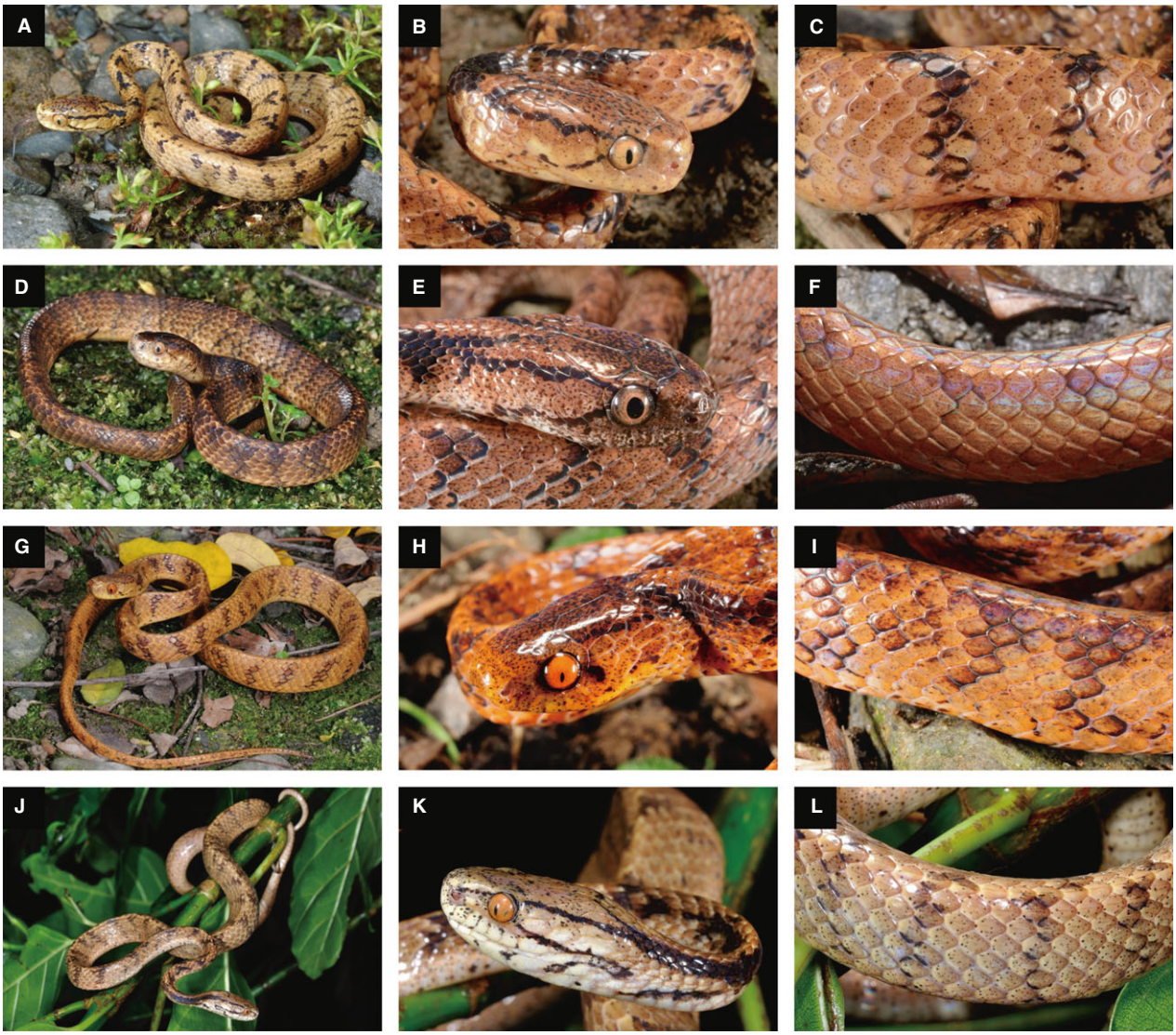


**Fig. 4** A comparison of body coloration, iris coloration and scales among *Pareas atayal* sp. nov., *P. komaii*, *P. formosensis* and *P. iwasakii*. *Pareas atayal* sp. nov. is characterized by its yellow iris and slightly keeled dorsals (A, B, and C); *P. komaii* is characterized by its yellow iris, strongly keeled dorsals and usually darker coloration (D, E and F). *Pareas formosensis* is especially distinguishable from the formers by its red iris and totally smooth dorsals (G, H and I), and *P. iwasakii* is distinguishable by its extra-elongated head and jaw (J, K and L). Photographed by C.-W. You.

we conclude that the name *P. formosensis* (Van Denburgh 1909) should be applied only to the red-eyed taxon.

However, the taxonomic status of *P. hamptoni* in different regions remains a controversial issue. *Pareas hamptoni* has wide distribution in South-east Asia from Myanmar to Thailand, Indochina and southern China (Yunnan, Hainan, Guangdong and Guangxi provinces) in the east (Nguyen *et al.* 2009). The mean divergence of cytochrome *b* sequences within this species is 0.0513, comparable to the level of differentiation between *P. formosensis* and *P. hamptoni* (0.0571). Such high divergence indicates that *P. hamptoni* might represent a complicated species complex with pronounced geographic structure. Furthermore, although the name *P. tonkinensis* has been treated as a synonym of *P. hamptoni* in some recent literature (Nguyen *et al.* 2009) or as a subspecies *P. h. tonkinensis* (Orlov *et al.* 2000), the long branch of *P. h. tonkinensis* in the phylogenetic tree makes their taxonomy more problematic. Despite this potential problem, none of the recent studies (including this one) included the specimens or tissues from the *P. hamptoni* type locality, Mogok (Myanmar) or provided a detailed description of the holotype of this species. The

original description of *P. hamptoni* mentioned that dorsals are 'feebly keeled' (Boulenger 1905), yet this character did not show up in Chinese nor Vietnamese samples in our disposal (all specimens have completely smooth dorsals). The samples collected across Vietnam, China and Taiwan should not represent typical morphology of *P. hamptoni*.

Considering the fact that *P. formosensis* represents a reciprocally monophyletic group in respect to *P. hamptoni* samples in our study (Fig. 2A), we can offer two probable taxonomic treatments: (i) If *P. hamptoni* is defined as a widely distributed species with huge geographic differentiation in morphology, the Taiwanese population could be treated as a local subspecies, that is *P. hamptoni formosensis*. (ii) If topotypic *P. hamptoni* from Myanmar prove to be greatly distinct from populations in the eastern part of species range, the widely distributed red-eyed *Pareas* with smooth dorsals from Taiwan, China and South-east Asia can be regarded as full species *P. formosensis*, while the extent of distribution of this species will need a more thorough review. Unfortunately, we failed to obtain the topotype material from Myanmar and new samples from this country are unlikely to be available in the near future.

### *Psammodynastes compressus* Oshima 1910 is synonym of *P. formosensis*

In 1910, Masamitsu Oshima provided a brief description of a new species *Psammodynastes compressus*, known from a single specimen collected in 'Kokwangai, Taihoku'. Later, Oshima (1916) himself invalidated *P. compressus* as a junior synonym of *Amblycephalus formosensis*. Our study showed that two forms of *Pareas* can be found on the territory of the former Taihoku Prefecture: the red-eyed form inhabiting the southernmost part and the yellow-eyed form 2 distributed more widely within the range of Taipei and New Taipei City. Thus, for evaluation of taxonomic status of Taiwanese *Pareas*, more detailed information on taxonomic assignation of *Psammodynastes compressus* Oshima 1910 is needed.

The single specimen of pareatid snake, which was collected by Masamitsu Oshima from 'Taihoku, Taiwan', is now tagged as *Pareas formosensis* (NSMT H02567) preserved in National Museum of Nature and Science, Tsukuba, Japan. A careful examination of this specimen indicated remarkable similarities with counts, measurements and descriptions provided by Oshima in his original paper (1910). The NSMT H02567 specimen shows the following morphological character states (data from Oshima 1910 given in brackets): IL 7 [5]; VEN 182 [182]; SC 75 [75]; TL 60.50 [60.80] cm; TaL 11.40 [13.50] cm; HL 16.39 [N/A] mm; SnL 4.27 [N/A] mm. Slight differences in measurements could be explained by the long preservation time of the specimen in alcohol. Difference in numbers of infralabials is likely caused by alternative methods of calculation: Oshima gives no details on counting method he used and no figure of head scalation of the new species; however, the IL count he reports as 5 is never observed in the genus *Pareas* (Guo & Deng 2009); obviously Oshima did not include two posterior elongated infralabials in his count. However, precise match of ventral and subcaudal counts, as well as temporal region scalation, indicates that Oshima likely made his 1910 description based on this specimen. NSMT H02567 has entirely smooth dorsal scales; according to the results of our multivariate analyses, it undoubtedly belongs in the red-eyed clade of Taiwanese *Pareas* (Fig. 3). We redefine the geographic locality 'Kokwangai' as environs of Ankeng, Xindian District (Taihoku Administrative Office 1919), where both red-eyed and yellow-eyed forms of *Pareas* can be found today. Therefore, we conclude that specimen NSMT H02567 is the holotype of *Psammodynastes compressus* Oshima 1910; the name appears to be a junior synonym of *P. formosensis* (Van Denburgh 1909) *sensu stricto*.

### *The yellowed-eyed form of Pareas* contains two valid species

In their review of East Asian *Pareas*, Ota *et al.* (1997b) examined material collected from Taiwan and Japanese islands of Iriomote and Ishigaki (Okinawa, Yaeyama group) and concluded that *P. komaii* (Maki 1931) should be regarded as a junior synonym of *P. formosensis* (Van Denburgh 1909). This taxonomic revision was based on their examination of preserved specimens, including holotypes of *P. komaii* (Maki 1931) and *P. formosensis* (Van Denburgh 1909). However, they did not examine living specimens for characterization of iris coloration. As snakes with smooth and keeled dorsals occur in some areas and are often found sympatrically, they interpreted that snakes with keeled or smooth scales represent intraspecific variation within *P. formosensis* (Van Denburgh 1909). Our PCA and DA demonstrated that both the holotype and the paratype of *P. komaii* (Maki 1931) were assigned to the yellow-eyed form 1 (Fig. 3). In contrary to Ota *et al.* (1997b), our morphological and molecular data suggest that *P. komaii* should be restored as a valid name. This species can be easily distinguished from *P. formosensis* by its yellow iris and strongly keeled dorsals.

The third clade in Taiwan represents snakes with yellow iris and weakly keeled dorsals (yellow-eyed form 2). A small proportion of *P. iwasakii* represent shared c-mos haplotype that was found in these snakes, indicating the comparatively shorter separation history between these two closely related species. However, owing to a relatively large mitochondrial sequence divergence, as well as diagnostic morphological

characters (a more elongated head and significantly higher numbers of infralabials, ventrals and subcaudals in *P. iwasakii*; see Table 1), we suggest the yellow-eyed form 2 should be evaluated as a new species which we describe below.

### *Species diversity of Pareas* remains to be uncovered in the future

Based on the literature data, Jiang (2004b) proposed that *P. chinensis* (Barbour 1912) was a synonym of *P. formosensis* (Van Denburgh 1909). However, this decision was tentatively made without referring to any certain specimens. To clarify this problem, Guo *et al.* (2011) used four *Pareas* tissues from our collection in Taiwan and rejected Jiang's rearrangement based on molecular phylogenetic data. However, the taxonomy of Guo *et al.* (2011) is incomplete, as their collection lacks the real *P. formosensis sensu stricto* (the red-eyed form). According to our phylogenetic tree (Fig. 2A), the four '*P. formosensis*' individuals they used were assigned to either *P. komaii* (JF827687) or the new *Pareas* species (JF827685, JF827686, JF827688). The validity of *P. chinensis* is still supported in our study; while the additional material from Taiwan hinted the under-estimation of pareatid diversity in East Asia.

Our study also uncovered the distribution pattern of the three Taiwanese pareatids. *Pareas formosensis* is distributed throughout the whole mountain regions except for the north-eastern tip of the island. Across this area, one other species, either the yellow-eyed form 1 (*P. komaii*) or the yellow-eyed form 2 (*Pareas* sp. nov.), coexists with *P. formosensis* (Fig. 1). *Pareas komaii* and the nominal new species occupy central-southern and northern Taiwan, respectively. The ranges of the three clades overlap in central Taiwan; however, we have not found any evidence of hybridization.

The speciation of *Pareas* in Taiwan might be attributed to two probable factors: the complicated geological history of the island and the evolution of their asymmetric dentition. The repetitive connection and separation among island groups between Taiwan, Ryukyu and mainland Asia has contributed to species or genetic differentiation in this region, which has been widely discussed in the literature (Lin *et al.* 2002; Tseng *et al.* 2014). In our samples, *P. komaii* (the yellow-eyed form 1) has shown significant genetic differentiation between eastern and western populations, congruent to the patterns discovered in vipers (Creer *et al.* 2001), cobras (Lin *et al.* 2008) and tree frogs (Lin *et al.* 2012). On the other hand, studies on the asymmetric mandibular teeth of *Pareas* snakes and their mollusc prey have highlighted a novel aspect on the evolution of these snakes. Although the sample size is limited, preliminary evaluation on dentition asymmetric index showed substantial variation among the specimens which we evaluated in this study (Table S3). Further studies of correlation between dentition asymmetry and their prey preference with higher sample size will give insight into dietary resource partitioning and niche differentiation between these species.

## Species description

Pareas atayal *sp. nov*.

*Holotype*. NMNS 05594 (Fig. S3), adult male collected by Chung-Wei You on August 27th, 2009 from TAIWAN, Taoyuan County, Fuxing Township, Sileng (24.653570 N, 121.409266 E) with elevation *ca.* 925 m.

*Paratypes*. NMNS 05584, 05585, 05586, 05587, 05588, 05589, 05591, 05593, 05595, 05596; ZMMU R-14434, R-14435 and R-14436. All the paratypes collected during 2009–2013 by Chung-Wei You, Jia-Wei Lin and Ren-Jay Wang in northern Taiwan.

*Other material*. NMNS 05590, 05592, 05597 collected during 2009–2011 by Jia-Wei Lin and Chung-Wei You in northern Taiwan.

*Etymology*. The new species is named with reference to its distribution which is similar to the native Taiwan aboriginal people, the Atayal, inhabiting mountain regions of northern Taiwan. Common name in English: '*Atayal Slug-eating Snake*'.

*Diagnosis*. *Pareas atayal* sp. nov. is a small (about 50 cm) slender yellow-brown snake, a member of *Pareas hamptoni* group on the basis of the following combination of morphological attributes: (1) nasal simple, not divided; (2) loreal not contacting the eye; (3) prefrontal contacting the eye; (4) one pre-ocular; (5) subocular single, crescent-shaped; (6) two anterior temporals, three to four posterior temporals (2 + 4); (7) slightly enlarged median vertebral and two adjacent rows of scales. *Pareas atayal* sp. nov. differs from all other members of the *Pareas* by the combination of the following morphological characters: (8) tail comprising 22% of the TL; (9) 7–9 infralabial scales; (10) 15 dorsal scale rows slightly keeled in seven rows at mid-body; dorsal scales smooth or one row slightly keeled in the anterior 1/4 of TL; (11) head notably elongated with HW/HL ratio 28%; (12) 174–188 ventrals without lateral keels; (13) 71–77 divided subcaudals; (14) iris colour bright yellow to light orange; (15) 50 slightly billowing vertical dark bars on the trunk (the bars about 1–2 scales wide); (16) two very clear thin black postorbital stripes beginning from lower and upper edges of each postorbital scale, with lower postorbital stripe reaching the anterior part of SL7, not continuing to the lower jaw and chin; the left and right upper postorbital stripes forming a bifurcation at the base

of the head forming an M-shaped figure (about 4–6 scale-length) and often connecting together behind head; (17) 6–7 maxillary teeth; 11–13 functional teeth on the left mandible and 19–20 on the right.

*Description of holotype*. The holotype (Fig. S3) is an adult female with body slender and notably flattened laterally (425.5 mm SVL; 132.0 mm TaL; 560.0 mm TL); head comparatively small (17.7 mm HL; 11.4 mm HW; 6.9 mm HH; HL/SVL ratio 4.2%), narrowly elongated, distinctly compressed laterally and oval in dorsal view (HW/HL ratio 27%), clearly distinct from neck; vertebral ridge poorly developed; snout blunt, widely spatulate (4.0 mm SnL; 2.6 mm EN); eye very large (2.9 mm ED; 5.2 mm IO), about 0.17 times the head length, rounded in shape and notably protuberant; pupil vertical; tail slightly laterally compressed at the basis, oval-shaped in cross section in the middle of length and round in the posterior 1/3, ending in an acuminated tail cap (TaL/TL ratio 24%).

*Dentition*. Maxillary teeth: six functional teeth on the left side and seven on the right side. Mandibular teeth: 11 functional teeth on the left lower jaw and 20 on the right (Fig. S2).

*Head scalation*. In dorsal view (Fig. S4B): one rostral, large, slightly wider than high; two internasals, much wider than long; two prefrontals, large, pentagonal, almost as long as wide; two supraoculars, pentagonal, elongated; one frontal, large, subhexagonal; and two parietals, irregularly trapezoid in shape, about twice as large as frontal; no enlarged nuchal scales present. In lateral view (Fig. S4A): nasal 1/1, pierced by a large crescent-shape nostril; squarish loreal scale 1/1, not contacting eye; pre-oculars 1/1, in contact with eye; no presubocular; 7/7 supralabials increasing in size from front to back, separated from eye by crescent-shaped subocular scale; postocular distinct, not fused with subocular, in contact with eye; distance between eye and snout tip (snout length) 1.4 times eye diameter; pupil vertical and slightly elliptical; temporals 2 + 4/2 + 4. In ventral view (Fig. S4C): mental triangular, fully separating first pair of infralabials; infralabials 9/9 (in ventral view only seven pairs apparent); three pairs of chin shields interlaced, no mental groove under chin and throat.

*Body scalation*. Dorsal scales in 15 rows along whole body, DSR formula: 15–15–15; on neck (approximately one HL from the head basis) median three rows (vertebral scale row and two lateral rows) feebly keeled, at middle of body length (VEN92–VEN93) seven dorsal scale rows notably keeled; at posterior third of body length (approximately one HL to the vent) nine dorsal scale rows notably keeled; vertebral scales slightly enlarged (one medial row); ventrals VEN 185, with rounded outer margins, without lateral keels; anal plate entire, crescent in shape; subcaudals SC 76 (excluding terminal spine), all divided/paired; terminal caudal scale forming acuminated tail cap.

*Natural history notes*. *Pareas atayal* inhabits various types of forests at altitudes of *ca.* 100–2000 m above sea level. The new species feeds on a variety of land snails and slugs; strictly nocturnal, mostly active after twilight around 20:00.

*Distribution*. The new species is endemic to Taiwan. To date, *Pareas atayal* is confined to Taipei and New Taipei City, Yilan, Taoyuan, Hsinchu and Nantou counties of Taiwan.

*Comparisons*. For comparisons of the new species with other congeners, see Table 1 and Table S4. For the full species description, see Appendix S1.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Species description of *Pareas atayal* sp. nov. in full details.

**Table S1.** Sample IDs, original and revised identification of species, sample locality, GenBank accession number, and source of specimens used in this study.

**Table S2.** Between-species divergence and within-species polymorphism among *Pareas* spp. from mitochondrial cytochrome *b* sequences.

**Table S3.** Mandibular teeth count and asymmetry index for *Pareas atayal* sp. nov., *P. formosensis* and *P. komaii*.

**Table S4.** Diagnostic features of scalation and color pattern of currently recognized species of *Pareas*.

**Fig. S1.** Lateral and dorsal views of a yellow-eyed *Pareas* and a red-eyed *Pareas* with identical body length.

**Fig. S2.** Volume reconstruction of high-resolution X-ray computed tomography data showing cranium and mandibles of the holotype of *Pareas atayal* sp. nov.

**Fig. S3.** Dorsal and ventral view of the holotype of *Pareas atayal* sp. nov.

**Fig. S4.** Head scalation of the holotype of *Pareas atayal* sp. nov.

**Fig. S5.** Hemipenial sructures of the paratype of *Pareas atayal* sp. nov.