

KARYOTYPIC ANALYSIS OF THE TERRESTRIAL SNAIL GENUS *PHUPHANIA* (PULMONATA: DYAKIIDAE) WITH DESCRIPTION OF A NEW SPECIES FROM THAILAND

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Abstract The dyakiid genus *Phuphania* is reported for the second time and a new species *Phuphania carinata* is described from a limestone mountain at Phu Kiew National Park, Chaiyaphum Province, Thailand. The first species described, the nominotypic species *Phuphania globosa*, and the current new species described here, *P. carinata*, were karyotyped for the first time and showed distinct inter-specific differentiation in the diploid chromosome number and karyotypic formula (*P. carinata*: $2n = 64$ and $5m + 5sm + 21t + 1a$; *P. globosa*: $2n = 66$ and $7m + 12sm + 14t$). The new species has a semiglobose, pale fulvous, shell, a black ground body colour with cream spots all over the body, remarkable fine radial growth lines and the amatorial organ gland has two lobes forming a cluster of numerous ducts. The comparison of shell, radula and genitalia morphology, plus the karyotype and habitats of *P. carinata* new species with *P. globosa* are discussed.

Key words systematics, *Phuphania*, Dyakiidae, new species, karyotype, Thailand

INTRODUCTION

The terrestrial snail genus *Phuphania* Tumpeesuwan & Panha 2007, was first recognized as a distinct genus on the basis of shell morphology and reproductive characteristics of the type species *P. globosa* (see Tumpeesuwan *et al.*, 2007). *Phuphania globosa* occurs in the sandstone mountains of the Phu Phan mountain range in three provinces of Nakhon Phanom, Mukdaharn and Kalasin in Northeastern Thailand (Tumpeesuwan *et al.*, 2007). The distinguishing characteristics of the genus are a semiglobose to globose shell, short vas deferens and features of the amatorial organ. The amatorial organ is stoutly cylindrical, sac-shaped in which the lobes are fused together as a cap that covers the tip of the amatorial organ and consists of numerous ducts, and a short, large sac shaped gametolytic duct. *Phuphania* belongs to the family Dyakiidae Gude & Woodward 1921, pulmonate snails reported from Sundaland and some parts of Australia and which are endemic to Sundaland (Zilch, 1959; Schileyko, 2003; Wade *et al.*, 2006), and usually live under leaf litter and rotten logs during the day time. The first descriptions of *Phuphania* and related genera were based on morphological characters (Godwin-Austen, 1891, 1906, 1907; Laidlaw,

1931, 1933; Thiele, 1931; Baker, 1941; Zilch, 1959; olem, 1966; Hausdorf, 1995; Schileyko, 2003.

In a recent collection of snails from the foot of a natural forest on limestone hills in the Phu Kiew mountain range in 2010–2012, some samples were found with shells that were thin, semiglobose and pale fulvous throughout and without a band on the periphery. Comparisons of their shells and genitalia with the type species *P. globosa* and with related genera within the family Dyakiidae, such as *Kalamantania*, *Everettia* and *Bertia* by Schileyko (2003) revealed that these new specimens from the Phu Kiew mountain range, though sharing morphological similarities were distinct from *P. globosa*.

Structural changes in chromosomes are considered to often play a crucial role in the establishment and maintenance of reproductive isolation (and so speciation). As a consequence, chromosomal investigations are expected to be one effective method for recognising cryptic species (King, 1993). We therefore conducted a cytogenetic survey of *P. globosa* and the proposed new species from the Phu Kiew mountain range to clarify their status.

MATERIALS AND METHODS

Snails were collected from natural forest areas in the Phu Kiew mountain range during both the

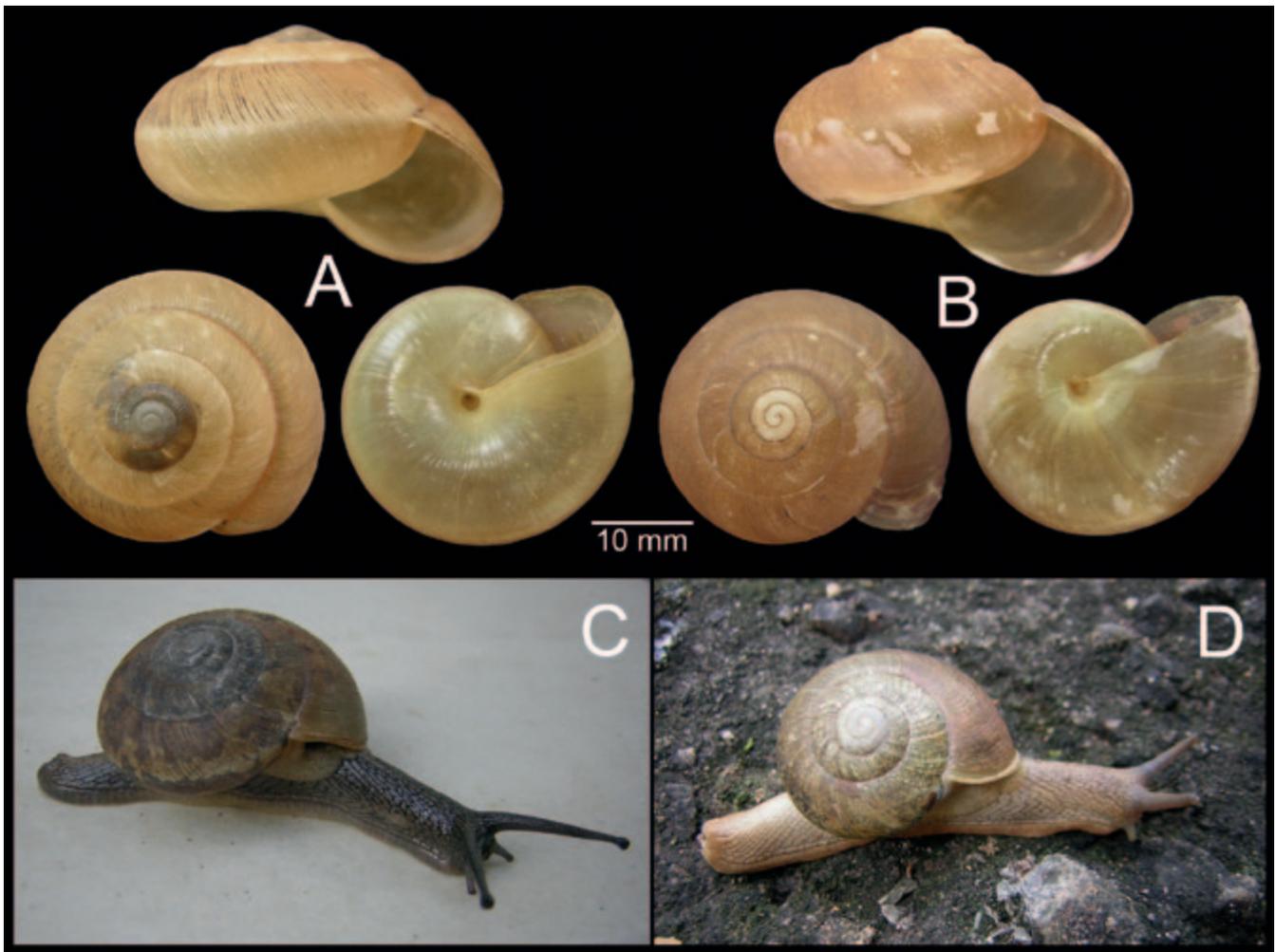


Figure 1 Shell characteristics of: A *Phuphania carinata* sp. nov.; B *P. globosa*. Live adult specimens of: C *P. carinata* sp. nov.; D *P. globosa*.

rainy and dry 2010–2012 seasons in 2010–2012. Live specimens were preserved in 70% (v/v) ethanol for anatomical study. The height and width of adult shells were measured using a digital vernier caliper. Reproductive organs were studied and drawn using a stereo microscope and camera lucida attachment. Radulae were examined under a scanning electron microscope (JEOL, JSM 5410 LV); the formula and shape of the radula teeth were recorded.

Chromosome preparations were made from gonad tissue by an air-drying method as reported previously (Kongim *et al.*, 2010). To increase the proportion of cells in metaphase, gonad cells were subject to microtubule polymerization arrest (and so G₁ and G₂ cell cycle arrest) by injection with 0.1% (w/v) colchicine solution (Sigma D-89552) and left for 3 h. Thereafter the gonads were cut into small pieces in 0.07% (w/v) hypotonic KCl solution. Separated cells were collected by

centrifugation at 1,000 rpm for 10 min and fixed in fresh Carnoy's fixative (3:1 (v/v) ratio of absolute ethanol: glacial acetic acid). The supernatant was replaced with fresh fixative for each of the two centrifugations at 1,000 rpm for 10 min. Cell suspensions were then dropped onto clean glass slides pre-heated to 60 °C and allowed to air-dry before staining in 4% (w/v) Giemsa solution for 15 min. Photomicrographs of ten well-spread metaphase cells were measured for their relative length and centromeric index. Mitotic karyotypes were arranged and numbered for chromosome pairs in order of the decreasing mean relative length. The nomenclature of morphological chromosome types follows that of Levan *et al.* (1964).

Anatomical abbreviations ag, albumin gland; am, amatorial organ; amg, amatorial organ gland; amr, amatorial organ retractor; at, atrium; e, epiphallus; f, flagellum; fo, free oviduct; gs,

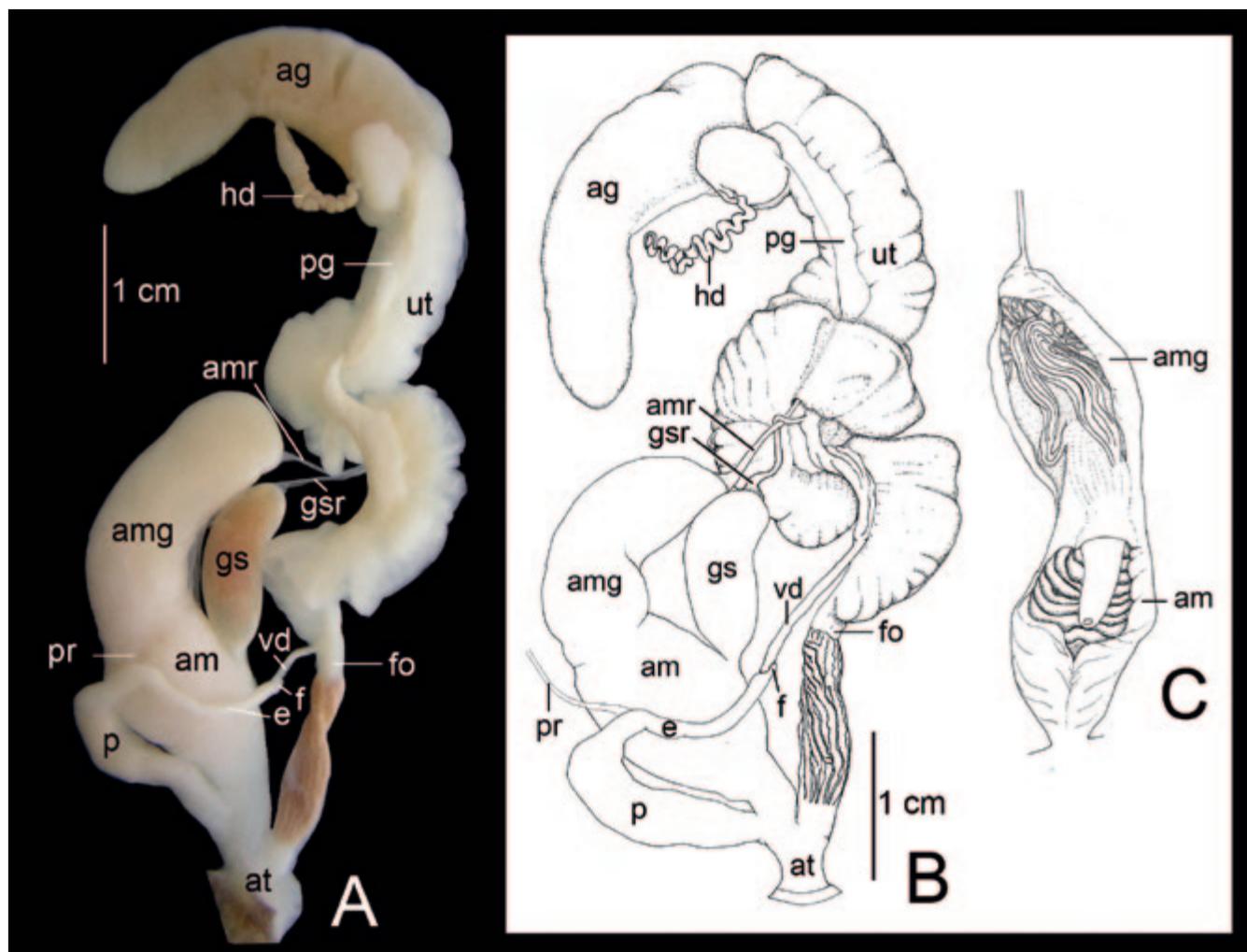


Figure 2 Genitalia of *Phuphania carinata* sp. nov.: A genitalia photograph; B illustration of genitalia; C inner structures of amatorial organ (am) and amatorial organ gland (amg).

gametolytic sac; gsr, gametolytic retractor; hd, hermaphroditic duct; p, penis; pg, prostate gland; pr, penial retractor; ut, uterus; vd, vas deferens.

Institutional abbreviations ZMMSU, Zoological Museum of Mahasarakham University, Thailand.

SYSTEMATICS

Family Dyakiidae Gude & Woodward 1921

Subfamily Dyakiinae Gude & Woodward 1921

Genus *Phuphania* Tumpeesuwan, Naggs & Panha 2007

Phuphania carinata sp. nov.
(Figs 1A, 1C, 2, 3, 4C & 4D)

Holotype ZMMSU 00250

Type locality Phu Kiew National Park (16°9.7'N 101°39.6'E), Chaiyaphum Province, west of northeastern Thailand.

Paratypes 10 paratypes: ZMMSU 00251. Collected throughout the Phu Kiew mountain range covering the three provinces of Chaiyaphum, Khon Kaen and Loie.

Etymology The specific name "*carinata*" referred to the distinctive subangulate shell character.

Diagnosis Shell medium-sized, semiglobose, pale fulvous, thin, dextral, with six convex whorls, smooth surface, with very fine radial growth lines. Aperture, ovate and oblique, lip not expanded or reflected. Umbilicus, narrow. Body with head and tentacles and whole body dark with cream colour on the mantle lobes.

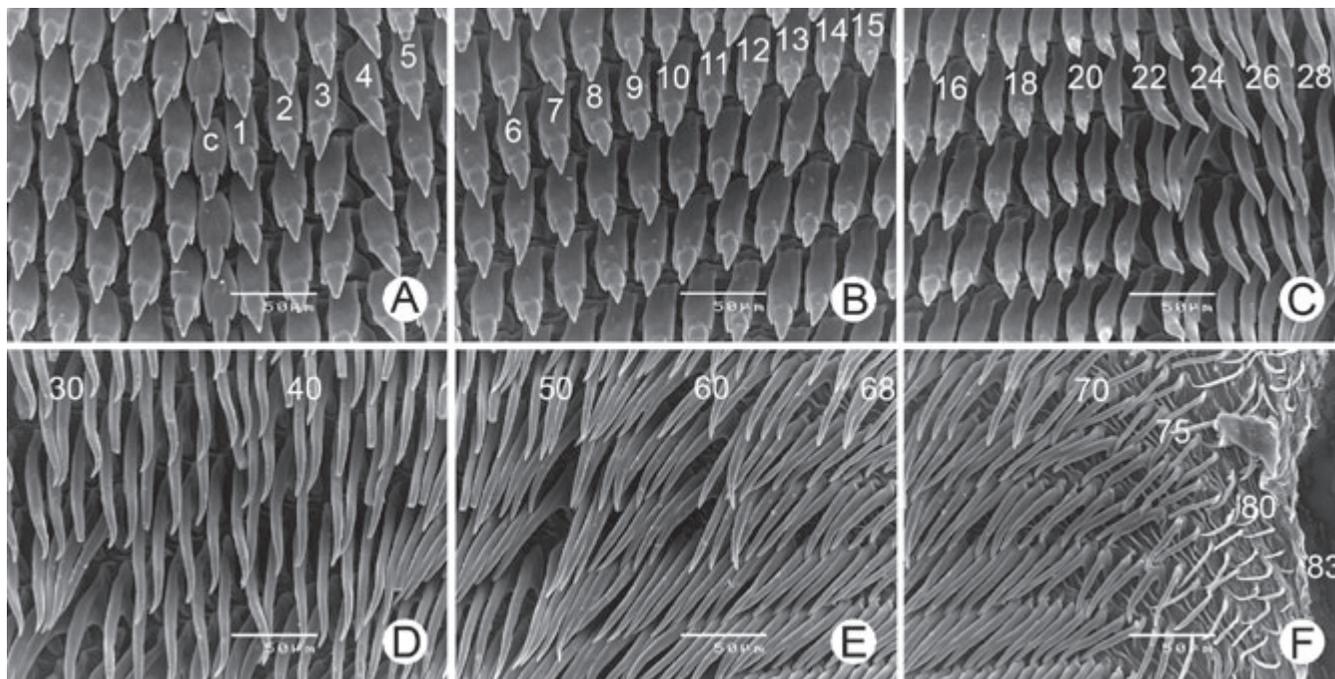


Figure 3 Radula characters of *Phuphania carinata* sp. nov. (Paratype ZMMSU 00253): A central tooth with the first to fifth lateral teeth; B lateral teeth with three cusps; C lateral teeth and premarginal teeth; D–F marginal teeth without lateral cusps. The numbers indicate the order of the teeth and the letter ‘C’ in the images indicates the central tooth.

Description of holotype Shell, height from apex to aperture 22.5 mm, diameter 33.5 mm, dextral, semiglobose, thin, pale fulvous, upper surface finely striate, lower surface polished. Whorls 6, regularly increasing in size, slightly convex, body whorl large consisting of almost $\frac{3}{4}$ of the shell height, last whorl periphery slightly sub-angulate, embryonic whorls smooth. Aperture: distinctly oblique, broadly lunar. Peristome: discontinuous, thin, lip not expanded or reflected. Umbilicus: narrow (Fig. 1A). Body: dark colour. Foot fringe distinct, about 5 mm wide, foot sole undivided, caudal gland without overhanging lobe (Fig. 1C).

In descriptions of the genitalia, we use ‘proximal’ to refer to the region closest to the genital opening and ‘distal’ to refer to the region furthest away from the genital opening.

Genitalia Penis (p) cylindrical sac, longer and wider than epiphallus (e) and vas deferens (vd), distally curved, narrows proximally (Fig. 2A & B). Penial retractor muscle (pr) inserts between the penis and epiphallus. Penis entering to vas deferens, distal end portion joining to a very short flagellum. Vas deferens runs directly to prostate gland (pg), and links proximally to the

long epiphallus. Amatorial organ (am) stoutly tumid sac, inner circular fold. Amatorial organ gland (amg) long and inflated sausage liked structure, inner portion consists of two lobes forming a cluster of numerous ducts (Fig. 2C), distal end attached to amatorial organ retractor muscle (amr). Amatorial organ retractor muscle long, thin and attached to the prostate gland. Gametolytic sac (gs) long, inflated, translucent. The gametolytic sac connects directly to the amatorial organ through a short, narrow constriction, internally orange in fresh specimens, distal end attached to gametolytic retractor muscle (gsr). Thin gametolytic retractor muscle small passes under and then over the amatorial organ retractor muscle before attached to prostate gland. Hermaphroditic duct (hd) small, convoluted entering to a small pouch before opening to uterus (ut).

Radula Teeth are arranged in v-shaped rows. Each row contains about 167 (83-1-83) teeth, central tooth symmetrical tricuspid, mesocone large with pointed cusp, ectocones small and located in middle of tooth narrow and elongate (Fig. 4A). Lateral teeth elongate, asymmetric bicuspid with tiny ectocone towards central tooth (Fig. 4B & C).

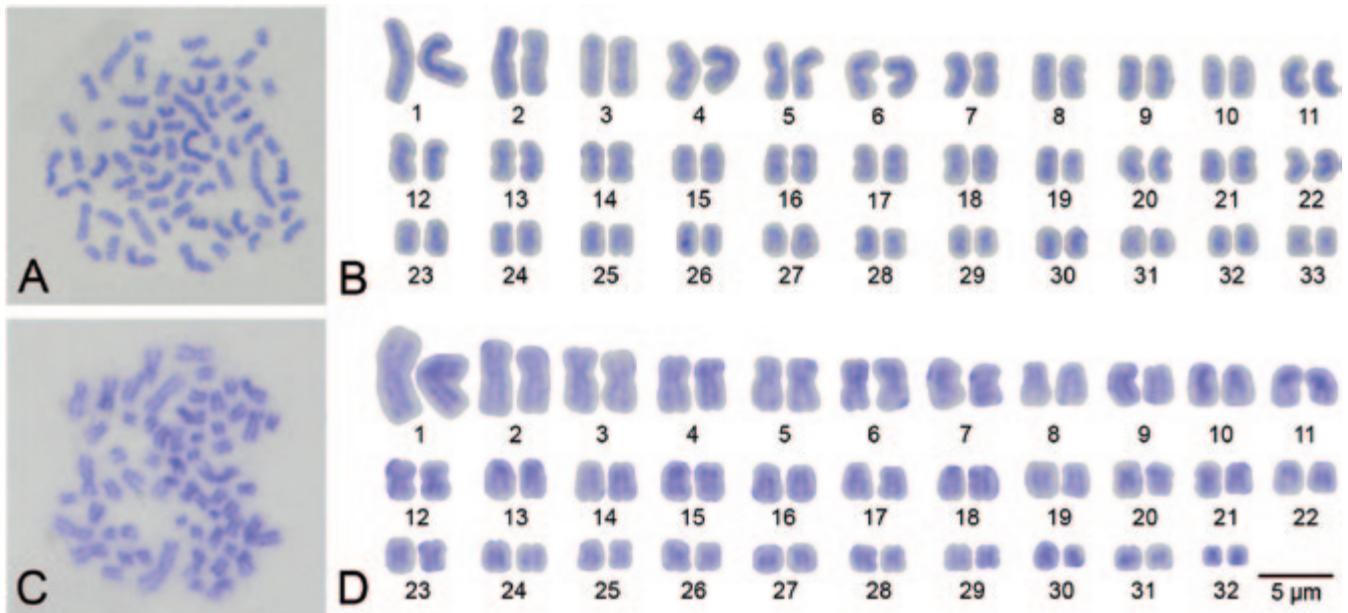


Figure 4 Mitotic chromosomes and karyotypes: A–B *Phuphania globosa* with chromosome number of $2n = 66$, karyotype formula of $7m + 12sm + 14t$; C–D *P. carinata* sp. nov. with diploid number of $2n = 64$, karyotype formula of $5m + 5sm + 21t + 1a$. Abbreviations: m metacentric; sm submetacentric; t telocentric; a acrocentric.

Marginal teeth narrow, elongate, without lateral cusps, outer marginals progressively shorter and smaller (Fig. 4D, E & F).

Remark The new species can be distinguished from the type species *P. globosa* in having a semiglobose shell, dark body colour and a distinctive amatorial organ. The new species occurs 300 kilometers west of the known habitat of *P. globosa*.

Karyotypes Karyotypes of the two species showed distinct differences with the diploid chromosome number being $2n = 66$ in *P. globosa* but $2n = 64$ in *P. carinata*. The karyotypes of *P. carinata* sp. nov. from the Phu Kiew mountain range contain five metacentric (m) chromosome pairs (7, 12, 15, 21 and 26), five submetacentric (sm) chromosome pairs (1, 2, 3, 5 and 6), 21 telocentric (t) chromosome pairs (4, 8, 9, 10, 13, 14, 16–20, 22–25 and 27–32) and one acrocentric (a) chromosome pair (11), with a karyotype formula of $5m + 5sm + 21t + 1a$. The karyotype of *P. globosa* from the Phu Phan mountain range, however, contains seven metacentric chromosome pairs (13, 14, 16, 17, 29, 31 and 33), 12 submetacentric chromosome pairs (1, 2, 4–7, 10, 11, 22, 25 and 28) and 14 telocentric chromosome pairs (3, 8, 9, 12, 15, 18, 19, 20, 21, 24, 26, 27, 30 and 32), with a karyotype formula of $7m + 12sm + 14t$ (Fig. 4). In addition,

the new species shows a distinct dark short arm on chromosome pair 11.

DISCUSSION

Karyotypes of the two *Phuphania* species showed distinct differences in both their diploid chromosome number ($2n = 66$ and 64 in *P. globosa* and *P. carinata* sp. nov., respectively) and their karyotypes ($7m + 12sm + 14t$ and $5m + 5sm + 21t + 1a$ in *P. globosa* and *P. carinata* sp. nov., respectively). In addition, *P. carinata* n. sp. exhibits distinct thick short arms of chromosome 11 and very small chromosome pairs 31 and 32As with other pulmonate species, such as *Cantareus aspersus* and *C. mazzullii* reported in Vitturi *et al.* (2005), in this study no heteromorphic sex chromosomes were detected. Moreover, Thiriou-Quie'vreux (2003) reported that there were no sex chromosomes in the Opisthobranchia and Pulmonata. This is different to the land operculate snails where the variations in the chromosome number of the subclass Streptoneura (Caenogastropoda) range from $2n = 14$ to 120 (Burch, 1967; Patterson & Burch, 1978). It is clearly different within the superfamily Cyclophoroidea which contains a conservative constant chromosome numbers in many taxa, such as $2n = 28$ in Cyclophoridae (Kongim *et al.*, 2006), $2n = 26$ in Pupinidae (Kongim *et al.*, 2009),

2010), and $2n = 26$ in Diplommatinidae (Ieyama & Ogaito, 2000).

In addition to the chromosomal differences between *P. carinata* sp. nov. and the type species *P. globosa*, they were also found to differ morphologically in the shell shape, shell surface, structure of the genital organs and radula. The shell of *P. carinata* sp. nov. is broader than high and the upper side of the whorls is rather coarsely striated or finely ribbed according to the lines of growth. Anatomically, the genital system of *P. carinata* sp. nov. differs from *P. globosa* in containing two amatorial organ gland lobes, compared to more than four in *P. globosa* (Tumpeesuwan *et al.*, 2007). The duct of the gametolytic sac is connected to the middle of the amatorial organ in *P. carinata* sp. nov., but to the base in *P. globosa*, whilst *P. carinata* sp. nov. has a distinct small pouch in the hermaphroditic duct before opening to the uterus. The body ground colour is black in *P. carinata* sp. nov. but grey in *P. globosa*. Moreover, the two species show a clear difference in their geographical distribution. *P. globosa* occurs in sandstone of the Phu Phan mountain range at an altitude of about 400 m amsl in dry dipterocarp forest, whereas *P. carinata* sp. nov. occurs on limestone in the Phu Kiew mountain range at about 680 m amsl in deciduous forest. The distinguishing characters between *P. carinata* sp. nov. and *P. globosa* are summarized in Table 1.

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Table 1 Comparisons of shell, body colour, genitalia, habitat and chromosome studies of the new species and type species of *Phuphania*.

Characters	<i>P. globosa</i>	<i>P. carinata</i> sp. nov.
Shell	Semiglobose-globose	Semiglobose
Shell surface	Fine growth line not clear	Distinct fine growth line
Body ground colour	Grey	Black
Colour shell	Fulvous	Pale fulvous
Penis	As long as the epiphallus and vas deferens	Longer than the epiphallus and vas deferens
Amatorial organ	Cylindrical sac	Tumid sac
Amatorial organ gland	Consists of numerous ducts fused together to form at least four lobes	Consists of numerous ducts fused together to form two lobes
Gametolytic duct entrance	Base of amatorial organ	Middle of amatorial organ
Habitat	Sandstone; ~400 m amsl	Limestone; ~680 m amsl
Chromosome number ($2n$)	66	64
Karyotype formula	7 m + 12sm + 14t	5 m + 5sm + 21t + 1a

Abbreviation: m, metacentric; sm, submetacentric; t, telocentric; a, acrocentric

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