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ORIGINAL ARTICLE

A new species of the genus *Dugesia* (Tricladida: Dugesiidae) from China

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Abstract A new freshwater turbellarian species, *Dugesia sinensis* Chen & Wang, **sp. nov.**, is described, which was collected from Caoxi rivulet, Shaoguan, Guandong Province, China. The morphology, reproduction, histochemical localization of AChE and phylogenetics based on COI gene of the new species was observed and analysed. The new species differs from its sister species in the developed diagram of the male atrium, the bursal canal to the left of the copulatory apparatus and distance (1 mm) between copulatory bursa and the mouth. In addition, the results indicated that morphological differences of AChE+ nerve structure can be used as a distinguishable character for species identification of genera Dugesiidae.

Key words Flatworm, Tricladida, new species, histochemistry, nerve system, phylogeny.

1 Introduction

The speceis of the genus *Dugesia* are ideal laboratory animals for many studies, especially in the field of regeneration, because they are easy to culture and have strong ability to regenerate. So far, 120 species of the genus *Dugesia* has been recorded all over the world (Tyler *et al*., 2006–2012; Sluys *et al*., 2013; Stocchino *et al*., 2014). The research for the diversity of flatworm in China began in the early twenty century. Until 2014, there are 16 species of the order Tricladida reported from China (Ichikawa & Okugawa, 1958; Liu, 1990, 1994, 1995, 1996, 1997; Ichikawa & Kawakatsu, 1964; Ijima & Kaburaki, 1916; Kenk, 1974; Yu *et al*., 2013).

Chinese mainland stretches over Oriental and Palearctic Realms with various landforms and diverse climatic conditions, and riches in species diversity. In the 20th century, most studies about Order Tricladida were focused on North China, only few on South China. *Dugesia jaonica* is the only species of genus *Dugesia* ever recorded in China, and the source about this genus is also not clear. In recent ten years, 29 planaria species were discovered in South China (Lai *et al*.,2013; Wang & Deng, 2006; Wang & Li, 2005; Wang & Sun, 2011; Wang & Wu, 2005a, 2005b, 2008; Xia *et al*., 2014; Zhang *et al*., 2010; Zhang *et al*., 2014; Wang & Luo, 2004; Wang *et al*., 2004; Wang, 2004, 2005; Zhao *et al*., 2011; Gao *et al*., 2011; Ma *et al*., 2014; Peng *et al*., 2007; Sun & Wang, 2014), indicating the underestimated resource of genus *Dugesia* in this area.

After two years research in South China, a new species of *Dugesia* collected from Caoxi rivulet, Shaoguan, Guangdong Province, China was described and diagnosed as *D. sinensis* Chen & Wang, **sp. nov.** in this study. The morphology, reproduction, histochemical localization of AChE and phylogenetics based on COI gene of the new species was observed and analysed.

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2 Materials and methods

2.1 Materials collecting

To take pebbles from the bottom of the rivulet, flush the faltwoms on the back of the pebbles into a bucket, then transfer the specimens to the laboratory for separation and identification. The faltworms were reared in glass bowls（21 cm × 15 cm × 18 cm）with aquatic plants and a piece of granite, and fed with pork liver slices for 1 hour every day. Change the water every week. Mature individuals were starved at least 5 days, then killed by 1% hydrochloric acid and fixed in Bouin's fluid for 24–36 hours. After that, the fixed samples were embedded in synthetic paraffin and cut into slices with the thickness of 6 μm horizontally and vertically, at last stained by H.E method.

2.2 AChE histochemical localization

The AChE histochemical localization method was similar to Zheng *et al.* (2011), but use a magnetic stirrer to replace the plastic pipette in Zheng *et al.* (2011) to remove paraformaldehyde from faltwoms with acetic acid buffer. Observation was performed with a microscope Olympus BX51 and the photographed by a DP 72 digital dedicated camera. DP 2-BSW software was used to measure the size of the samples. Figures and photographs were edited by Adobe Photoshop® version 7.0. The biological patterns were drawn based on the digital photographs. Type specimens are deposited in IZCAS, Beijing, China.

2.3 DNA extraction, PCR amplification, sequencing and phylogenetic analysis

The specimens used for phylogenic analysis were starved for at least a week. E.Z.N.A.™ Mollusc DNA KIT (Omega, Norcross, GA, USA) was used to extracted genomic DNA from 3 randomly picked starved individuals (marked as *Dugesia sinensis* sp. 1–3). The target COI gene was amplified by polymerase chain reaction (PCR), using a pair of specific primers, which were designed based on the sequence of the COI gene (DCOIF: GCTCATGGTTTARTWATGATTTTYTT; DCOIR: GWGCAACAACATARTAAGTATCAT) (Lázaro *et al.*, 2011; Pongratz *et al.*, 2003). Premix Ex Taq™ Hot Start Version (TaKaRa, Otsu Japan) was used to carry out the PCR. The resulting PCR fragments were cloned into pUCm-T vector (BBI, Toronto, Canada), and then transformed into the competent cells of *Escherichia coli* Top 10. Plasmid inserts were sequenced by Beijing Genomics Institute (BGI, Shenzhen, China). The homologous sequences were obtained from NCBI using NCBI blastn option (Table 1). The phylogenic and molecular evolutionary analyses were conducted using Mega version 6 (Tamura, Stecher, Peterson *et al.*, 2013). All the sequences were trimmed to the shortest length of obtained sequences. *Bipalium adventitium* of Geoplanoidea was chosen as outgroup. Maximum-likelihood (ML) and Neighbor-Joining (NJ) methods with Tamura-Nei model were used to demonstrate the inferred evolutionary relationships among these species. As the homology and recovery rate of COI nucleotide sequences among species of the order Tricladida is low, this study selected 300 bp sequences to root the tree.

Abbreviations used in the text and figures:

PLA. Platyhelminthes;
IZCAS. Institute of Zoology, Chinese Academy of Sciences;
$AChE^{+}$. Acetylcholinesterase positive reaction;
ChAT. choline acetyltransferase;
bc. bursa canal;
ca. common atrium;
cb. copulatory bursa;
cgg. cerebral ganglion;
cg. cement glands;
cm. circular muscle;
cnr. circular nerve ring;
d. diaphragm;
e. eye;
ed. ejaculatory duct;
go. gonopore;

lm. longitudinal muscle;
lnc. lateral nerve cord;
lod. left oviduct;
m. mouth;
ma. male atrium;
md. male diaphragm;
o. ovary;
pb. penis bulb;
pg. penis glands;
ph. pharynx;
pp. penis papilla;
rod. right oviduct;
sg. shell glands;
sv. seminal vesicle;
tn. transverse nerves;
vd. vas deferens;
vnc. ventral nerve cord.

Table 1. Nucleotide sequences selected for phylogenetic analysis.

Family	Species	GeneBank acc. no.	Reference
Geoplanoidea	*Bipalium adventitium*	AF178306	Huh & Jo, 2010
	Schmidtea mediterranea	JF837062	Lazaro *et al*., 2011
	Schmidtea polychroa	AF287133	Pongratz *et al*., 2003
	Dugesia japonica	AB618487	Sakai & Sakaizumi, 2012
	Dugesia ryukyuensis	AB618488	Sakai & Sakaizumi, 2012
	Dugesia sicula	KF308799	Sluys *et al*., 2013
	Duegsia naiadis	KF308757	Sluys *et al*., 2013
	Dugesia sagitta	KF308818	Sluys *et al*., 2013
	Dugesia arcadia	KF308795	Sluys *et al*., 2013
	Duegsia improvisa	KF308774	Sluys *et al*., 2013
	Dugesia gonocephala	DQ666033	Lazaro *et al*., 2006
	Girardia tigrina	AF178316	Riutort *et al*., 2001
	Girardia schubarti	DQ666041	Alvarez *et al*., 2006
	Dugesia sinensis sp.1	KP401590	This study
	Dugesia sinensis sp.2	KP401591	This study
	Dugesia sinensis sp.3	KP401592	This study

3 Systematic account

Tricladida Lang, 1884
Dugesiidae Ball, 1974
***Dugesia* Girard, 1850**

***Dugesia sinensis* Chen & Wang, sp. nov.**

Locality. Specimens were collected from the ventral side of the pebbles in the rivulet (0.4 m in maximum depth, 25 m in width) beside the Caoxi spring resort, Shaoguan, Guandong Province, China (24°39′42″N, 113°36′50″E; elev. 75 m). There are plenty of pebbles in the bottom of the rivulet. Wheel animalcule and different insect's larvae can be observed in the water samples.

Material examined. Holotype PLA-0090-1–21, serial section vertically, rivulet (0.4 m in maximum depth, 25 m in width) beside Caoxi Spring Resort, Shaoguan, Guandong Province, China (24°39′42″N, 113°36′50″E; elev. 75 m). Paratypes: PLA

-0091-1–9, PLA-0092-1–5, PLA-0093-1–4, serial section horizontally, same data as holotype. Other materials: PLA-0094–0098, the whole body is fixed in 10% formaldehyde, same data as holotype. All specimens are deposited in IZCAS, Beijing, China.

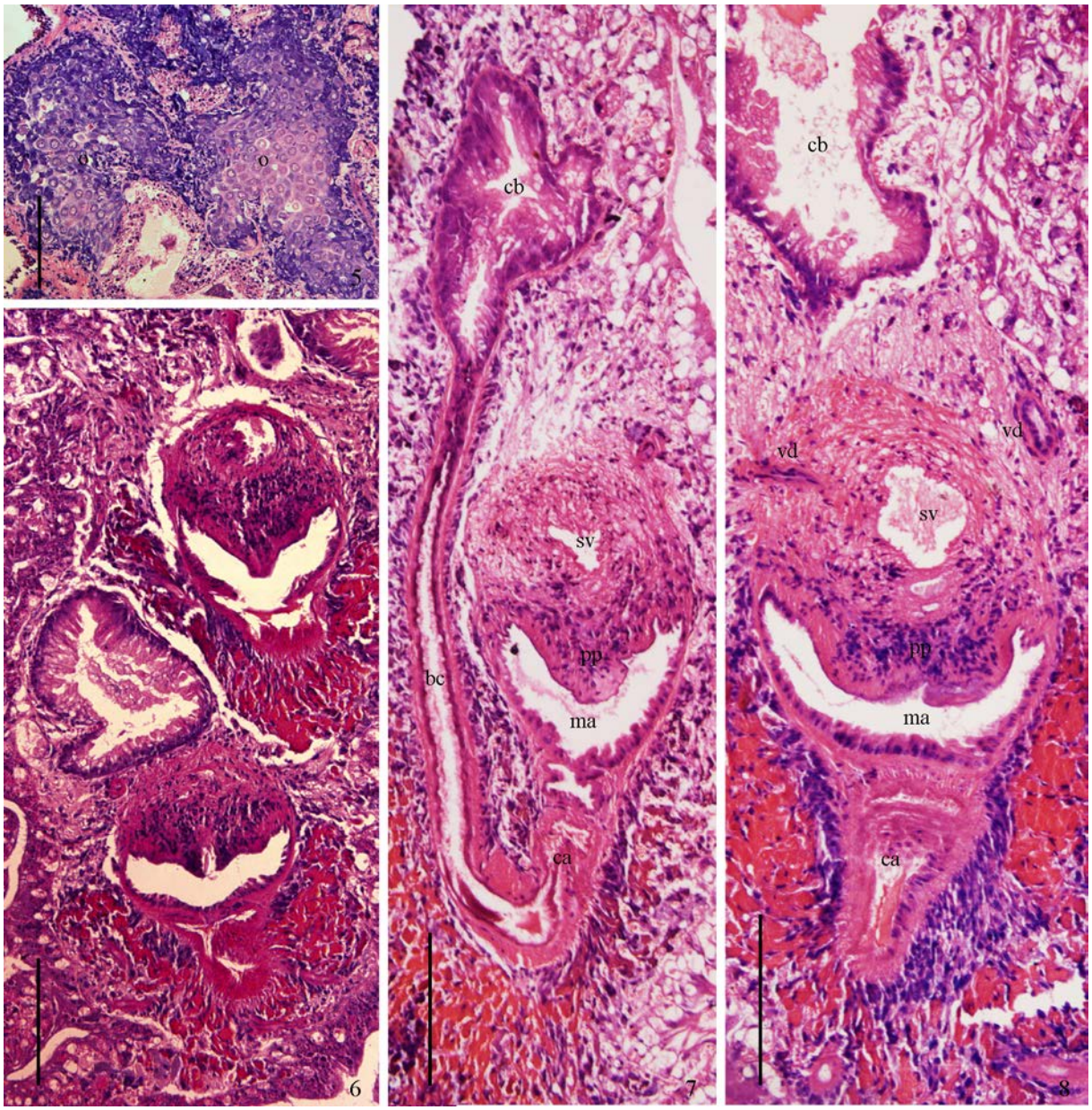
Etymology. The species is referring to its type locality, China.

Figs 1–4. *Dugesia sinensis* Chen & Wang, **sp. nov.**, habitus of living specimens. 1. Mature individual, dorsal view. 2. Mature individual, ventral view. 3. Egg. 4. Immature individual, dorsal view. Scale bars: 1–2 = 2 mm, 3–4 = 500 μm.

Diagnosis. The new species is characterized by the following features: triangle-shaped head, two hyperplastic ovaries, asymmetric opening of oviducts, bursal canal to the left of the copulatory apparatus, a distance between copulatory bursa and the mouth, developed diagram in the male atrium.

Description. Body size of the living mature individuals ranges from 12.0–15.0 mm in length and 2.48 mm in width. Dorsal side of body uniformly light brown while ventral side pale. Margin of body and areas of copulatory apparatus appear gray in color. Mobilizable triangle head has two blunt-pointed auricles. Bar-shape unpigmented auricular grooves locate posteriorly to two kidney-shaped eyes. Fan-shaped unpigmented area placed just at the lateral part of eyes (Figs 1–2). Mouth opens at 1/3 rear part of body and distance between mouth and gonopore 3.0–3.3 mm in living individuals (1.6–2.0 in fixed samples).

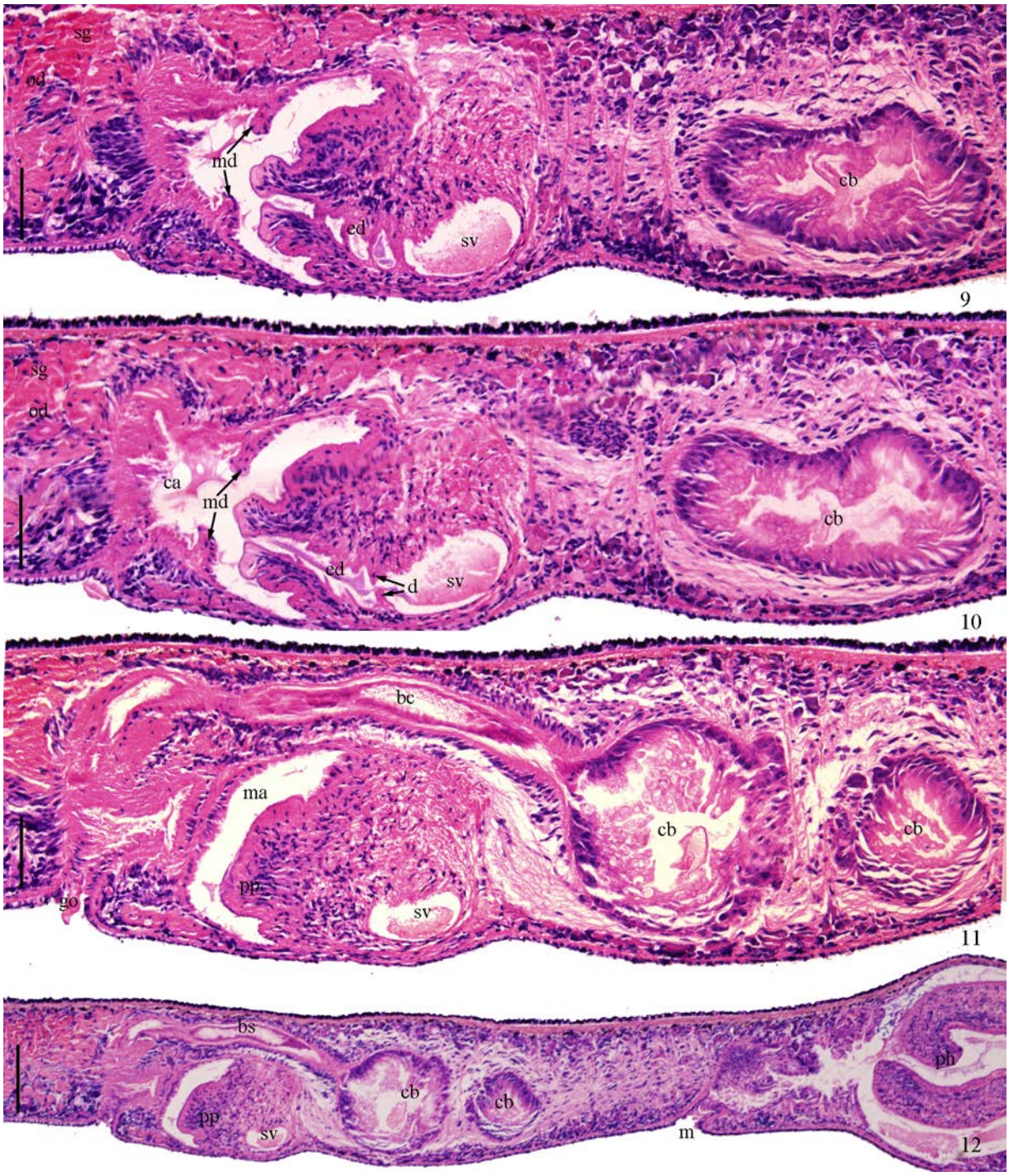
Habits. Newly collected individuals cannot adapt to the environment in the laboratory and refuse to eat either the pork liver slices or cooked egg yolk, resulting in disintegration. After using the mineral water to rear, the animals start to ingest the



Figs 5–8. *Dugesia sinensis* Chen & Wang, **sp. nov.**, horizontal sections of copulatory apparatus. 5. Ovary. 6. Duplications of reproductive apparatus. 7–8. Copulatory apparatus. Scale bars = 200 μm.

pork livers. In the daytime, they like to flock under the ventral side of the granite while moving slowly along the wall of the glass bowl during the night. When preying, they stretch out the pharynx and swallow the pork liver tissue, then the intestine turns into dark red in the next few hours. The whole length of the body in stationary state is 2/3 of the length in the state of movement.

Reproduction. The animals were collected in August 2011. They started the state of sexual reproduction in June 2013, and appeared fissiparous states in November 2013. Until May 2014, the individuals presented the sexual reproduction mode



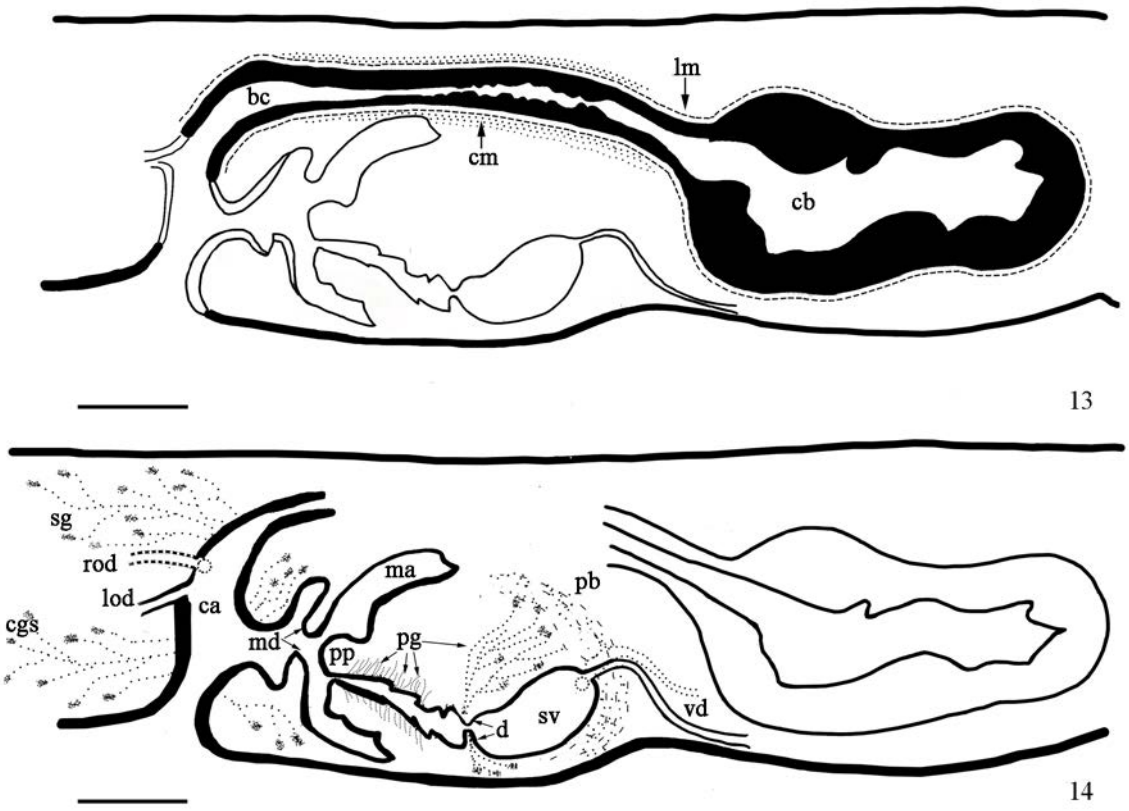
Figs 9–12. *Dugesia sinensis* Chen & Wang, **sp. nov.**, longitudinal sections of copulatory apparatus. 9–10. Male copulatory appararus. 11. Female copulattory apparatus. 12. Location of copulattory apparatus. Scale bars: 9–11 = 100 μm, 12 = 200 μm.

again. The area copulatory apparatus on the dorsal side of the ex-fissiparous individuals is clearly unpigmented (Fig. 1). The sphere-shape eggs are dark red in color and the egg stalk is twisted (Fig. 3). The eggs scattered in the ventral side of the granite or the back of aquatic plants' leaves. The larva is 1.9 mm in length and 0.6 mm in width without evident pigmentation throughout the dorsal side of the body, and the intestine is clear under the microscope (Fig. 4).

Digestive system. Digestive system is comprised of mouth, pharynx and intestine. The cylinder-shape pharynx is located in the central part of body, extending longitudinally to the rear. The surface of pharynx is generally pale without visible pigment. The inner and outer pharynxgeal musculature is bilayered. The base of pharynx was connected to 3 intestinal branches. While the middle one reaches to the anterior part of eyes, the other two extend to the end of the body (Fig. 4).

Reproductive system. Female reproductive system consists of ovaries, bursa canal, copulatory bursa and common atrium. A pair of hyperplasic ovaries are closely next to each other, showing an irregular shape (Fig. 5). The distance between ovaries and the brain is 760 μm. Each ovary has an oviduct elongating ventrally along the outer side of the ventral nerve cord and opening asymmetrically into the common atrium. While the right oviduct opens at the ventral side of the common atrium, the left oviduct opens more dorsally. There is a significant distance (1.0 mm) from the anterior wall of copulatory bursa to the mouth (Fig. 12). The irregular copulatory bursa is lined by glandular epithelium, surrounded by a layer of longitudinal muscle (Figs 7–11). Copulatory bursa connects the common atrium by bursal canal. Pipe-shaped bursal canal lies dorsally to the left of the penis (Figs 7, 13–14). The area around the opening of oviducts is covered by abundant shell glands and the gonopore receives the a few cement glands (Figs 7–11). A few samples present 2–3 duplicated copulatory bursas (Fig. 6).

Male reproductive system is made up of tests, vas deferens, penis, male atrium, common atrium. The vas deferentia extend ventrally to the base of the penis bulb, and then go up anterior-dorsally before penetrating the penis bulb, and finally open at the proximal anterior part of the seminal vesicle (Fig. 14). The penis consists of penis bulb, seminal vesicle and penis papilla. The penis bulb is comprised of interwoven circular and longitudinal muscle fibers with an oval-shape seminal



Figs 13–14. *Dugesia sinensis* Chen & Wang, **sp. nov.**, holotype PLA-0090, sagittal reconstructions of the copulatory apparatus. 13. Female copulatory apparatus. 14. Male copulatory apparatus. Scale bars = 200 μm.

vesicle inside (Figs 9–10, 14). A small diaphragm, receiving abundant penis glands, separates the seminal vesicle and ejaculatory duct (Fig. 14). The ejaculatory duct runs ventrally and opens at the blunt tip of the penis papilla, making penis papilla in an asymmetric shape. The irregular lumen in the ejaculatory duct receives numerous penis glands. The conical penis papilla has a rather blunt tip and the margin of the papilla is folded, without distinguishing penis folds. There is a developed male diaphragm separating the male atrium and common atrium. The male diaphragm is lined by nucleated epithelium which is underlain by thick circular muscle fibers (Figs 9–11, 14). The specimen with 3 duplicated penises has one ejaculatory duct opening opposite to the mouth and the others are arranged like the normal individuals. All size of the duplicated penis is relatively small than that in normal individuals (Fig. 6).

AChE$^+$ nerve structure. After histochemical localization, the AChE$^+$ nerve structure appears copper−red ladder structure (Figs 15–16). The AChE$^+$ nerve structure is composed of cerebral ganglion, ventral nerve cord, lateral nerve cord, circular nerve ring and transverse nerves. The control specimens present a negative reaction.

The reverse "U" shape cerebral ganglions are filled with numerous nerve cells. Each side of the cerebral ganglions extends backward, narrows gradually and forms the ventral nerve cord (Fig. 16). The sites where the ventral nerve cord sent out the transverse nerves always bulge and the transverse nerves end at the lateral nerve cord (Fig. 17). The lateral nerve cords originate from the cerebral ganglions, terminate at the end of the tail and are linked by a crescent-shaped transverse nerve (Figs 15–16). Transverse nerves rarely send out branch nerves. A thick circular nerve ring locates near the mouth and connects the pharynx nerve by pharyngal nerve net (Fig. 17). The boundary between cerebral ganglion and ventral nerve cord is hard to define.

Molecular phylogenetic analysis. The NJ and ML trees based on COI sequences are closely similar (Figs 18–19). Three individuals of *Duegsia sinensis* Chen & Wang, **sp. nov.** comprise a sister group to the other *Duegsia* group with 100% bootstrap in both trees. All the COI sequences of genus *Duegsia* used in analysis form a clade with 100% bootstrap in both trees, suggesting the genus *Dugesia* is likely to be a sister group of the genus *Bipalium*, *Schmidtea*, *Girardia* from the family Geoplanoidea.

4 Discussion

4.1 Classification

As reported, 17 of 120 nominal species of genus *Dugesia* recorded in the world are distributed in Oriental realm (Sakai & Sakaizumi, 2012; Alvarez-Presas *et al.*, 2008; Kenk, 1974; Kawakatsu *et al.*, 1980; Kawakatsu, 1972,1973, 1980; Kawakatsu & Basil, 1975; Kawakatsu *et al.*, 1980; Ball, 1970; Kawakatsu *et al.*, 1991; Kawakatsu *et al.*, 1989; Sivickis, 1928; De Beauchamp, 1959), including 5 species in India (*D. andamanensis* Kaburaki, 1925; *D. bengalensis* Kawakatsu, 1983; *D. siamana* Kawakatsu, 1980; *D. indica* Kawakatsu, 1969; *D. krishnaswamyi* Kawakatsu, 1975; *D. tamilensis* Kawakatsu, 1980), 1 species in Sri Lanka (*D. nannophallus* Ball, 1970), 2 species in Indonesia (*D. indonesiana* Kawakatsu, 1973; *D. leclerci* Kawakatsu & Mitchel, 1995), 2 species in Malaysia (*D. batuensis* Ball, 1970; *D. borneana* Kawakatsu, 1972), 1 species in Myanmar (*D. burmaensis* Kaburaki, 1918), 2 species in Thailand (*D. deharvengi* Kawakatsu & Mitchell, 1989), 1 species in Philippine (*D. hymanae* Sivickis, 1928), 2 species in Pakistan (*D. lindbergi* de Beauchamp, 1959; *D. bactriana* de Beauchamp, 1959). Before the work, *Dugesia japonica* is the only species of genus *Dugesia* reported in China, distributed mainly in Chinese mainland, Taiwan, Hong Kong, Japan, South Korea and Russia (Kawakatsu *et al*., 1995).

Dugesia sinensis Chen & Wang, **sp. nov.** is similar to *D. gonocehpala* Duges,1830, *D. ryukyuensis* Kawakatsu, 1976 and *D. japonica* Ichikawa & Kawakatsu, 1964, according to the morphological characteristics of copulatory apparatus (Ichikawa & Kawakatsu, 1964).

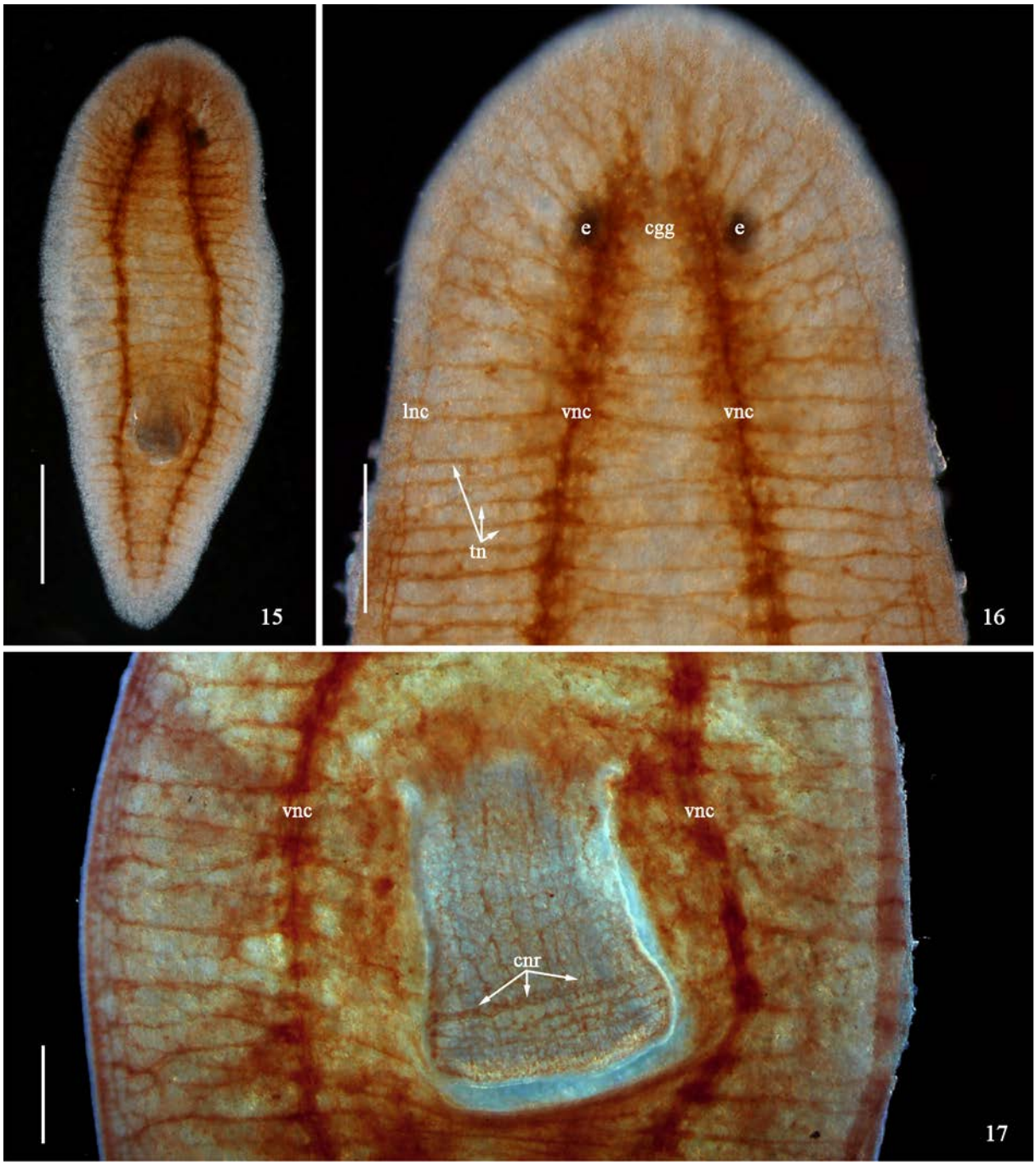
The new species is similar to *D. gonocehpala* by the oval−shape seminal vesicle and penis papilla tip-opening ejaculatory duct. However, *D. sinensis* Chen & Wang, **sp. nov.** has the inner and outer pharynxgeal musculature bilayered, while *D. gonocehpala* has the third outer longitudinal muscle layer. In addition *D. sinensis* has a developed male diaphragm which is absent in *D.gonocehpala*, separating the male atrium and common atrium.

D. sinensis Chen & Wang, **sp. nov.** is similar to D. *ryukyuensis* by the arrangement of the pharynxgeal musculature, but the latter species with the ejaculatory duct opens ventrally, and the upper section of the penis papilla much longer. The penis folds does not present in the male atrium of *D. sinensis* Chen & Wang, **sp. nov.** but in *D. ryukyuensis*. The margin of the penis papilla in *D. sinensis* Chen & Wang, **sp. nov.** is clearly folded, while it is smooth in the *D. ryukyuensis.* Futhermore,

there is no visible male diaphragm in *D. ryukyuensis*

D. sinensis Chen & Wang, **sp. nov.** has bilayered pharynxgeal musculature, hyperplasic ovaries and asymmetric opening of the oviducts as *D. japonica*, but differs from the latter by: a). different opening of the ejaculatory duct (ventral side in *D. japonica*, tip of the papilla in *D. sinensis* Chen & Wang, **sp. nov.**); b). a male diaphragm in *D. sinensis* Chen & Wang, **sp. nov.**; c). different shapes of seminal vesicle (oval shape in *D. sinensis* Chen & Wang, **sp. nov.**, retort-shape in *D. japonica*); d). different courses of bursal canal (to the right of penis in *D. japonica* and left in *D. sinensis* Chen & Wang, **sp. nov.**).

A few samples of *D. sinensis* Chen & Wang, **sp. nov.** are present the duplicated copulatory apparatus. These copulatory



Figs 15–17. *Dugesia sinensis* Chen & Wang, **sp. nov.**, AChE$^+$ nerve structure. 15. Ventral view. 16. Head. 17. Pharynx nerve structure. Scale bars: 15 = 400 μm, 16–17 = 200 μm.

apparatus are smaller than that in normal specimens. The significant distance (1 mm) between the mouth and copulatory bursa is rare found in the genus *Dugesia*. Because the individuals are fed with pork liver slice which may contains large amount of hormones, the situation is natural or not needs further study in futher.

4.2 AChE$^+$ nerve structure characteristics

The simple ladder nerve structure is considered as the classic structure of the central nerve system of genus *Dugesia*. According to the study of Nishimura (Nishimura *et al*., 2010), each side of the cerebral ganglion in *D. japoncia* send out 9 branches, using anti-*Dugesia japonica* ChAT antibody, and no evident branch nerves from ventral nerve cords are observed. In 2011, Zheng *et al.* revealed the lateral cords and dorsal and ventral nerve net in *D. japoncia*, and change the recognition towards the nerve structure of *D. japonica*. However the morphology of specimens for Zheng's study is greatly different with *Dugesia japonica*. Those specimens were collected in Wenshan Lake, Shenzhen, Guangdong Province and its classification status remains to be further identified.

Through careful comparison, the differences of nerve structure between *D. sinensis* Chen & Wang, **sp. nov.** and specimens used in Zheng's study are following: a). bulged central nerve cord in Zheng's specimens; b). dorsal and ventral nerve net formed by numerous nerve branches in Zheng's specimens, while the lack of branch nerves emitted from central nerve cord in *D. sinensis* Chen & Wang, **sp. nov.**; c). a thick circular nerve ring present in *D. sinensis* Chen & Wang, **sp. nov.**

With respect to the number of the branch nerve originating from cerebral ganglion, *D. sinensis* Chen & Wang, **sp. nov.** has 9–10 sents from each side, whereras10–12 in Zheng's specimens and 8 in *Schmidtea mediterranea* Benazzi, Baguna, Ballester & del Papa, 1975 (Salo, 2006).

Based on the comparison above, it is suggested that the morphological differences of AChE+ nerve structure can be used as a distinguishable character for identification among the genus of Dugesiidae.

4.3 Phylogenetic analysis

The results show the three individual used in constructing NJ and ML trees comprise a sister group to the other *Duegsia*

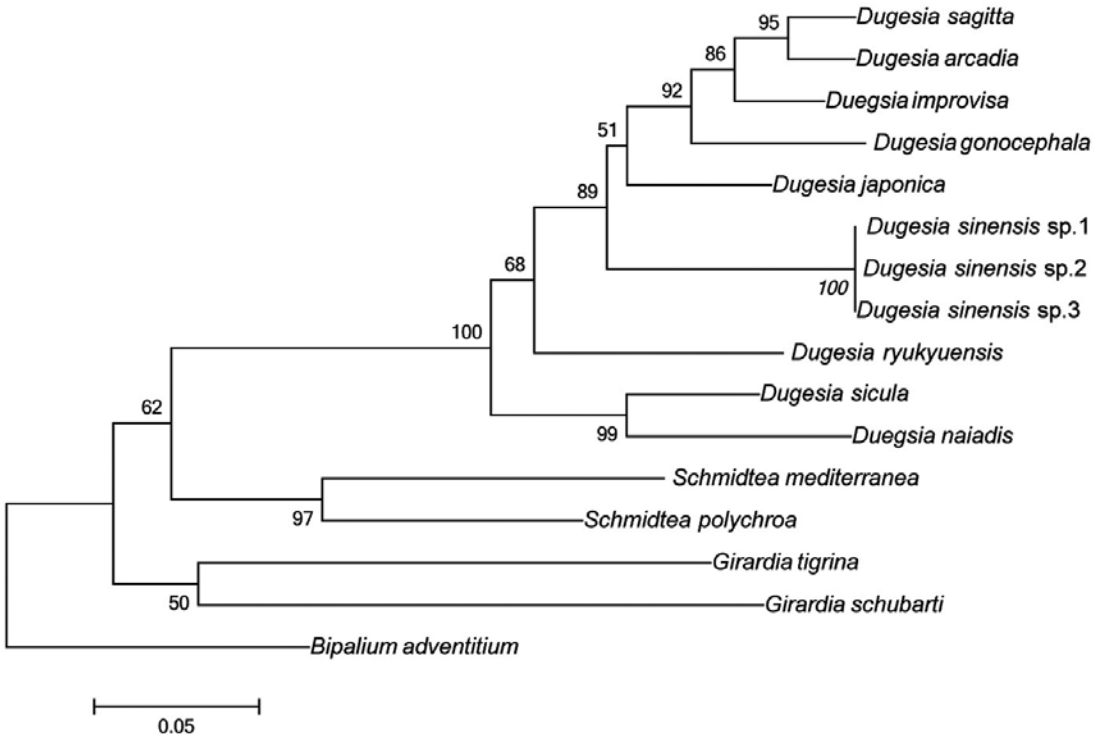


Fig. 18. Neighbor-Joining tree of COI gene, the scale indicates the branch length in substitutions per site.

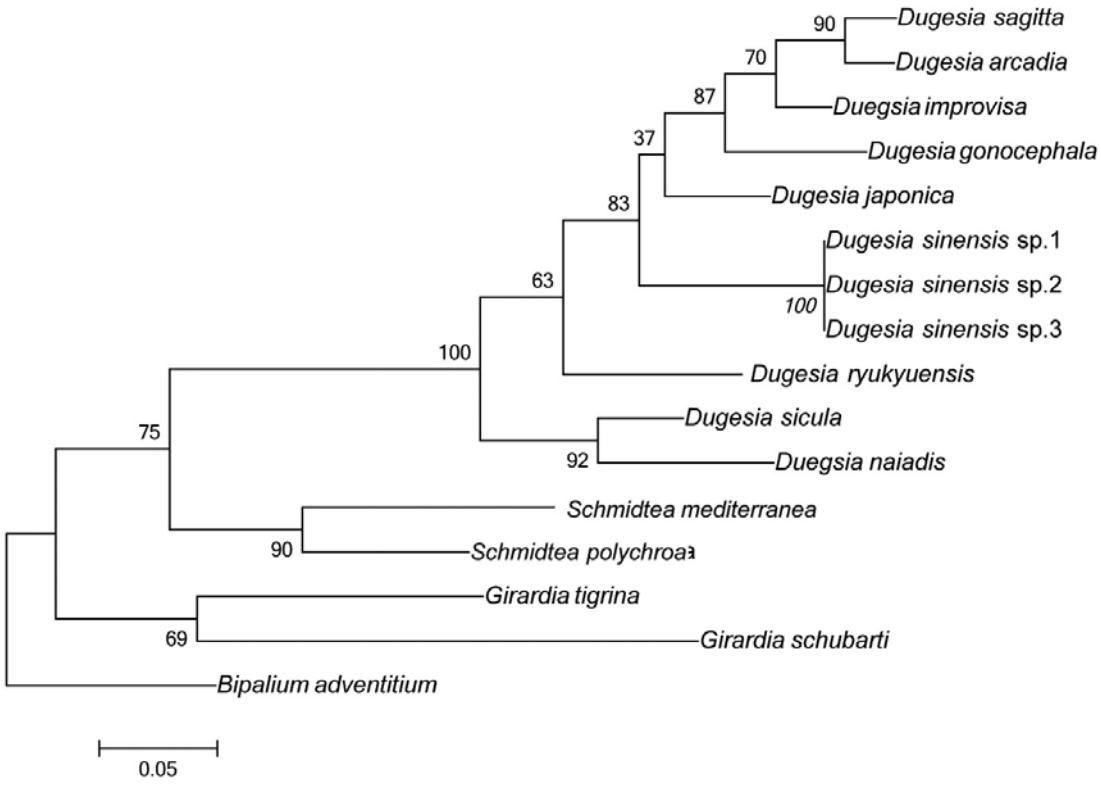


Fig. 19. Maximum-Likelihood tree of COI gene, the scale indicates the branch length in substitutions per site.

group with 100% bootstrap. *D. sinensis* is closely related to the *D. ryukyuensis* and *D. japonica*, which matches the mophorlogical description, thus providing the firmed molecular phylogenetics evidence that *D. sinensis* is distinct species belong to the genus *Dugesia.*

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