FOLIA PARASITOLOGICA 47: 11-16, 2000

# *Neopolystoma liewi* sp. n. (Monogenea: Polystomatidae) from the eye of the Malayan box turtle (*Cuora amboinensis*)

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Key words: Polystomatidae, *Neopolystoma*, *Cuora amboinensis*

**Abstract.** *Neopolystoma liewi* sp. n. is described from the conjunctival cavity of the Malayan box turtle *Cuora amboinensis* (Daudin, 1802), in Peninsular Malaysia. This is the first record of *Neopolystoma* in Malaysia and the fourth polystomatid species described from *C. amboinensis*. Of the 27 Malayan box turtles examined, 8 were found to be infected. A maximum of 2 parasites per eye and 4 individuals per host was recorded. *N. liewi* sp. n. differs from all other members of the genus by possessing few and short genital spines and small marginal hooks. The oncomiracidium has 64 ciliated cells arranged symmetrically about the sagittal axis.

Polystomatids are mainly parasites of semi-aquatic vertebrates. The majority are parasitic in anurans but they are also found in a Japanese salamander (*Onychodactylus japonicus*), the Australian lungfish (*Neoceratodus forsteri*), aquatic chelonians and the African hippopotamus (*Hippopotamus amphibius*) (see Yamaguti 1963, Reichenbach-Klinke 1966, Stunkard 1924, respectively).

Yamaguti (1963) created the subfamily Polystomoidinae for the polystomatids of chelonians which are represented by three genera namely *Polystomoides* Ward, 1917, *Polystomoidella* Price, 1939 and *Neopolystoma* Price, 1939. In Peninsular Malaysia the Polystomoidinae are represented by six species belonging to two genera: *Polystomoides malayi* Rohde, 1963 and *Polystomoidella mayesi* Richardson et Brooks, 1987 from the urinary bladder of *Cuora amboinensis*; *Polystomoides asiaticus* Rohde, 1965 from the oral cavity of *C. amboinensis*; *Polystomoides siebenrockiella* Rohde, 1965 from the urinary bladder of *Siebenrockiella crassicollis*; *Polystomoides renschi Rohde,* 1965 from the oral cavity of *S. crassicollis*; and *Polystomoides platynotae* Combes et Rohde, 1979 from the oral cavity of *Notochelys platynotae.*

This paper reports the finding of a new species of *Neopolystoma* from the conjunctival cavity of the Malaysian box turtle, *Cuora amboinensis* (Daudin, 1802). It is also the first record of *Neopolystoma* in Malaysia and represents the fourth polystomatid genus to be described from *C. amboinensis.*

## MATERIALS AND METHODS

During April 1998 three Malayan box turtles (*Cuora amboinensis*) were collected in a small pond in the Botanical Garden (Hutan Rimba), on the campus of the University of Malaya in Kuala Lumpur and 9 other specimens from a pet shop in Kuala Lumpur. During May 1999 15 Malayan box turtles were obtained from pet shops in Kuala Lumpur. The chelonians from the pet shops were collected in the Perak area.

The chelonians were placed individually in plastic aquaria (400 mm × 250 mm) with water to a depth of 60 mm. The water in which the chelonians were kept was screened for parasite eggs every second day over a period of 8 days. The water was sieved through a set of three plankton net sieves of mesh sizes 500 µm, 250 µm and 112 µm. The 500µm sieve let all the eggs through but removed all the larger particles from the water. Contents from each sieve were transferred to glass Petri dishes and eggs, if any, were picked out from the debris. Eggs of the new species were distinguished from eggs laid by *Polystomoides* sp. infecting the same host by their elliptical shape (see below). The eggs were transferred to small glass Petri dishes containing dechlorinated tap water and incubated at 30-37°C. Difficulty was experienced with the incubation of the eggs of the new species of *Neopolystoma* and only two oncomiracidia were hatched. Both larvae were stained in silver nitrate (Lynch 1933). Stained preparations were rinsed in distilled water, dehydrated in an ethanol series, cleared in xylene and mounted in Canada balsam. Measurements of larval sclerites are based on these silver-stained preparations.

Infected chelonians were killed with an injection of sodium pentabarbitone (Euthapent) and dissected. The urinary and accessory bladders, cloaca, oral and pharyngeal cavities, nasal cavities and eyes were carefully examined for the presence of polystomatids.

A total of 19 specimens was obtained from the eyes of *C. amboinensis*. Twelve of the parasites were fixed flattened in 10% neutral buffered formalin for preparation of whole mounts. Eleven of these were egg-producing. The remaining 7 specimens were fixed for molecular, histological and electron microscope studies which do not form part of the present study. Flattened specimens were stained in alum carmine and mounted permanently in Canada balsam. However, prior to staining, the specimens were cleared in Bouin-glycerin and studied under phase contrast. The sclerotised structures were

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examined and drawn before staining and mounting. Description is based on egg-producing parasites. All measurements (mean with range in parentheses) are given in micrometres (μm).

## RESULTS

### Levels of infection

Two of the three hosts collected in the Botanical Garden on the campus of the University of Malaya were infected (prevalence, 67%; mean intensity, 2.5). Six of the 24 hosts purchased from the pet shops were infected (prevalence, 25%; mean intensity, 2.3). The highest infection in a single host, which was collected on the campus, was 4 parasites and the highest infection per eye was 2 parasites.

### *Neopolystoma liewi* sp. n. Figs. 1-3

T y p e h o s t : *Cuora amboinensis* (Daudin, 1802), the Malayan box turtle.

S i t e : Conjunctival cavity, under the lower eyelid.

T y p e l o c a l i t y : Well vegetated earth-walled dam in the Botanical Garden (Hutan Rimbat) on the campus of the University of Malaya, Kuala Lumpur, Malaysia.

T y p e m a t e r i a l : Eleven mature and egg-producing. Holotype (1999.10.29.1) and three paratypes (1999.10.29.2-4) deposited in the Parasitic Worms Collection, Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom; one paratype (M-355) in the collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic; six paratypes (NMB P222 - NMB P227) in the Parasitic Worm Collection, National Museum, Aliwal Street, Bloemfontein 9301, South Africa.

E t y m o l o g y : The species name *liewi* refers to Mr Liew Kim-Seng George as an acknowledgement of his invaluable assistance and support during this research project.

### Description (based on 11 specimens)

**Mature parasite** (Fig. 1)**.** The general characteristics of mature parasites are typical of *Neopolystoma* Price, 1939 in that they infect turtles and do not have hamuli. Body elongate; total length 3474 (2120-4169); greatest width 1321 (1012-1566); width at vaginae 1246 (1012-1446); haptor length 921 (795-1205); haptor width 1179 (988-1470); haptoral sucker diameter 217 (184-252). Suckers well developed.

Mouth subterminal, ventral. Oral sucker 367 (252-446) wide; pharynx length 349 (296-398); pharynx width 382 (330-417). Intestine bifurcate, caeca not confluent posteriorly, more or less of equal length; extends well beyond posterior margin of testis but not into haptor; caeca without diverticula.

Single testis roughly circular, length 341 (271-446) and width 363 (349-436); seminal vesicle prominent.

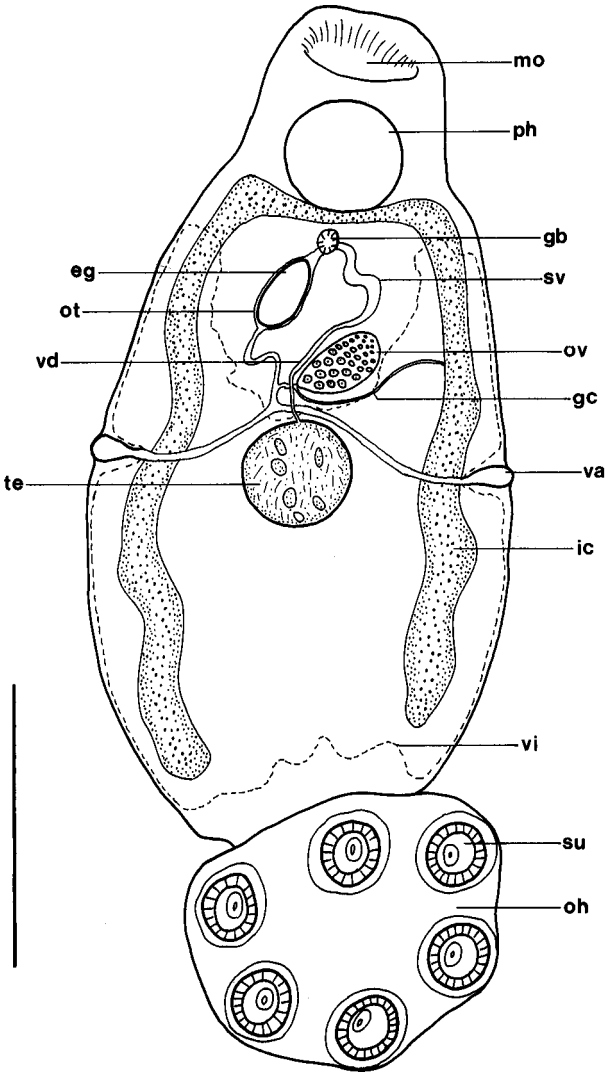


**Fig. 1.** *Neopolystoma liewi* sp. n. Ventral view of holotype. eg – egg; gb – genital bulb; gc – genitointestinal canal; ic – intestinal caecum; mo – mouth; oh – opisthaptor; ot – ootype; ov – ovary; ph – pharynx; su – sucker; sv – seminal vesicle; te – testis; va – vagina; vd – vas deferens; vi – vitelline distribution. Scale bar = 1 mm.

Vaginae 2, lateral. Vitellaria follicular cover most of prehaptoral body except the anterior third of body.

Ovary sinistral, submedian, 287 (213-339) × 136 (116-155); ootype well developed containing a single egg at a time. Genito-intestinal canal on same side as ovary, joins left intestinal caecum at mid-ovarian position. Uterus absent. Common genital atrium median, ventral. Genital bulb posterior to intestinal bifurcation, length 78 (63-87) and width 72 (53-92), 8-11 genital spines 11.8 (10.8-13.2), sharply hooked with straight shaft and fan-shaped root.

Two types of eggs (spherical and ellipsoid) were obtained from the 250μm and the 112μm sieves respectively. Eggs collected were compared with intra-uterine eggs of parasites removed. Round eggs were

found to be eggs of *Polystomoides*, while the ellipsoid eggs were those of *Neopolystoma liewi*. Egg of *N. liewi* length and width 283 (265-294) × 120 (109-126). Egg production measured over 30 days at 28°C was 1.5 (0-3) eggs per parasite per 24 h.

**Oncomiracidium.** The ciliated oncomiracidium resembles a typical polystomatid larva, having a narrow cylindrical body with a circular cup-shaped haptor. The haptor bears 16 marginal hooklets. Marginal hooklet 1 (postero-medial), 12.6 (12.3-12.8) in length. The ratio of total length (*a* in Fig. 2C) to handle length (*b* in Fig. 2C), 2.1 (see Murith 1981). All 8 pairs of marginal hooks are retained in adult specimens and remain the same length (Fig. 2B,C). Hooks 1 and 2 (postero-medial) are located in between the first sucker pair, hooks 3, 4 and 5 associated with suckers 1, 2 and 3, respectively; hooks 6, 7 and 8 located around anterior marginal region of haptor. There are no hamuli primordia present. Staining with silver nitrate revealed bands of ciliated cells on the surface of the larvae and surface sensilla. Arrangement of ciliary bands as shown in Fig. 3. There are 64 ciliated cells arranged symmetrically about the sagittal axis of the oncomiracidium in 5 cell groups recognised by Tinsley (1981) for polystomatid oncomiracidia. The number of ciliated cells in each of the cell groups is as follows: Apical group: 2 × 1 cell, anterior; cephalic group: 2 × 14 cells, dorsal, lateral and ventral; medioanterior group: 2 × 3 cells, ventral; medioposterior group: 2 × 6 cells, dorsal, lateral and ventral; haptoral group: 2 × 8 cells, dorsal, lateral and ventral. The same number of cells were found in both larval specimens studied.

**Differential diagnosis**

*Neopolystoma liewi* differs from all other members of the genus by possessing few and short genital spines and small marginal hooks. Its genital spines vary in number from 8 to 11. Of the 11 specimens of *N. liewi* studied, two had 8 genital spines, two had 9, five had 10, and two had 11. This character separates it from all other members of the genus except *Neopolystoma cribbi* Pichelin, 1995, which has 6-10, and *Neopolystoma macleayi* Rohde, 1984, which has 11-13 (Table 1). *N. liewi* has very short genital spines (11.8), which separates this species from the other known species for which this measurement is known including *N. cribbi* (17.8) and *N. macleayi* (23.6). Genital spines in a single circle compared to the two circles of larger and smaller spines found in *Neopolystoma palpebrae* Strelkov, 1950. The marginal hooks 1 for *N. liewi* have a length of 12.6 (12.2-12.7), which separates it from all species for which this character is known, except *N. palpebrae*, for which the marginal hooklet length varies between 11 and 14 (Table 1).

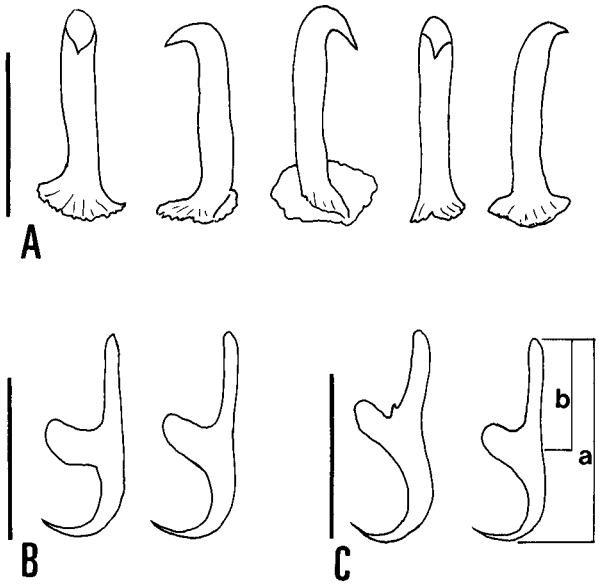


**Fig. 2.** *Neopolystoma liewi* sp. n. **A** – genital spines drawn from holotype; **B** – marginal hooklets 1 from holotype; **C** – marginal hooklets 1 from oncomiracidia that hatched from eggs laid by holotype and paratypes. a – total length of marginal hooklet 1; b – handle length of marginal hooklet 1 measured from tip to centre of guard base (see Murith 1981). Scale bars: A-C = 10 μm.

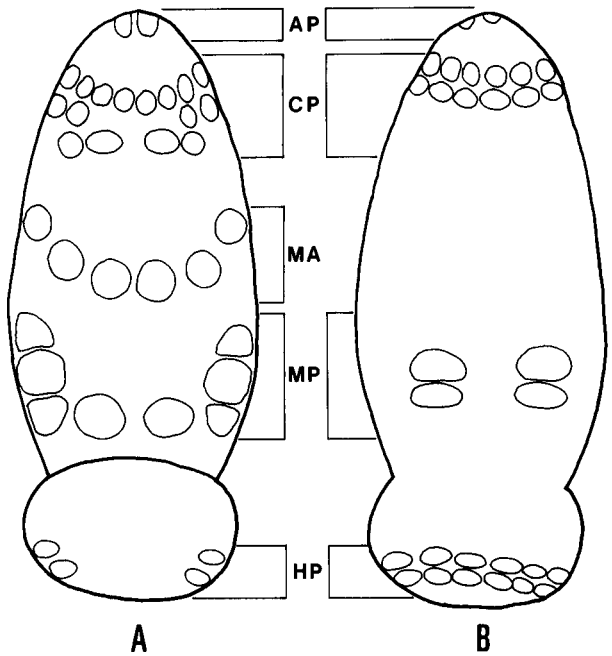


**Fig. 3.** *Neopolystoma liewi* sp. n., oncomiracidium. Ventral (**A**) and dorsal (**B**) aspects showing the distribution of ciliated cells. AP – apical group; CP – cephalic group; MA – medioanterior group; MP – medioposterior group; HP – haptoral group.

**Table 1.** List of known species of *Neopolystoma* with information on the number and length of genital spines as well as the length of the marginal hooklets.

| Species | Reference | Number of genital spines | Length of genital spines | Length of marginal hooklet 1 |
|---|---|---|---|---|
| *N. chelodinae* (MacCallum, 1919) | Pichelin 1995 | 14 (12-16) | 23.8 (20.8-27.2) | 23.8 (21.6-25.6) |
| *N. cyclovitellum* Caballero, Zerecero et Grocott, 1956 | Caballero et al. 1956 | 16 | (19-23) | Not available |
| *N. cribbi* Pichelin, 1995 | Pichelin 1995 | 8 (6-10) | 17.8 (11.2-22.4) | 16.5 (15.2-17.6) |
| *N. domitilae* (Caballero, 1938) | Lamothe-Argumedo 1972 | (20-21) | (55-71) | Not available |
| *N. euzeti* Combes et Ktari, 1976 | Combes and Ktari 1976 | 34 (33-36) | (48-57) | Not available |
| *N. exhaumatum* Ozaki, 1935 | Ozaki 1935 | (16-18) | Not available | Not available |
| *N. kreffti* Rohde, 1984 | Pichelin 1995 | 24 (20-26) | 27.5 (24-32) | 26.1 (22.4-27.2) |
| *N. macleayi* Rohde, 1984 | Pichelin 1995 | 12 (11-13) | 23.6 (20.8-27.4) | 26.4 (25.6-28.8) |
| *N. novaeguineae* Fairfax, 1990 | Fairfax 1990 | 32 (32-33) | 26 (23-29) | 23 (18-26) |
| *N. orbiculare* (Stunkard, 1916) | Baruš and Moravec 1967 | 16 (15-17) | (45-51) | 21 |
| *N. palpebrae* Strelkov, 1950 | Strelkov 1950 | 16 | (14-16) | (11-14) |
| *N. queenslandensis* Pichelin, 1995 | Pichelin 1995 | (22-28) | 21.6 (19.2-25.6) | 17.8 (16.8-19.2) |
| *N. rugosa* (MacCallum, 1919) | Price 1939 | 14 | 9 | Not available |
| *N. spratti* Pichelin, 1995 | Pichelin 1995 | 23 (20-26) | 23.9 (19.2-27.2) | 17.3 (14.4-19.2) |
| *N. terrapensis* (Harwood, 1932) | Harwood 1932 | 16 | Not available | Not available |
| *N. tinsleyi* Pichelin, 1995 | Pichelin 1995 | 23 (21-27) | 16.8 (14.4-20.8) | 17.1 (14.4-19.2) |

## DISCUSSION

The polystomatid genus *Neopolystoma* Price, 1939 is represented currently by 16 species. *Neopolystoma palpebrae* was for four and a half decades the only *Neopolystoma* known to infect the conjunctival cavity of a chelonian. Pichelin (1995) described four more species from the eyes of Australian chelonians: *Neopolystoma cribbi* was described from *Emydura signata, E. macquarii, Elseya latisternum* and *Chelodina expansa*; *Neopolystoma spratti* Pichelin, 1995 from *Chelodina longicollis*; *Neopolystoma tinsleyi* Pichelin, 1995 from *C. expansa*; and *Neopolystoma queenslandensis* Pichelin, 1995 from *Emydura signata* and *E. macquarii*.

Besides *Neopolystoma, Cuora amboinensis* also serves as host to *Polystomoides* and *Polystomoidella* (see Rohde 1963, 1965, Richardson and Brooks 1987). *Polystomoides malayi* was described from the urinary and accessory bladders, *P. asiaticus* from the oral cavity and *Polystomoidella mayesi* from the urinary bladder of *C. amboinensis* (see Rohde 1963). The polystomatid genus *Polystomoidella* is known mainly from the Nearctic region where it is represented by five species, but Richardson and Brooks (1987) described *Polystomoidella mayesi* from *C. amboinensis* in Malaysia. The presence of *Polystomoidella* in the Sunda region raises questions of possible misidentifications or a possible parasite transfer. The possibility of transfer from an introduced American turtle can only be confirmed or refuted if and when *P. mayesi* is found in this chelonian.

Polystomatids of chelonians are known to be site-specific and in a molecular study Littlewood et al. (1997) found that congeneric species infecting the same site in different hosts are more closely related than congeneric species infecting different sites in the same host. *Neopolystoma* is found in the cloaca, urinary and accessory bladders, oral, pharyngeal and nasal cavities, as well as in the conjunctival cavities of freshwater chelonians (Pichelin 1995). The high degree of site-specificity allows for speciation and could explain the three different polystomatids from *C. amboinensis.*

World-wide, chelonians have been very poorly studied for monogeneans. Africa, for example, has no less than 24 species of freshwater chelonians (Branch 1988) but only three species of polystomatids belonging to two genera are known from the Afrotropical region: *Polystomoides chabaudi* Euzet et Combes, 1965 from *Pelomedusa subrufa* in Madagascar (Euzet and Combes 1965) and Uganda (Tinsley 1973), *Polystomoides bourgati* Combes et Kulu, 1978 from *Pelusios adansonii* in Togo (Combes and Kulu 1978) and Senegal (Combes and Justine 1982) and *Neopolystoma euzeti* Combes et Ktari, 1976 from *Clemys caspica* in Tunisia (Combes and Ktari 1976). Although there are 17 species of freshwater turtles in Malaysia (Gregory and Sharma 1997), only 3 species (*Cuora amboinensis, Notochelys platynota* and *Siebenrockiella crassicollis*) have been documented to harbour polystomatids (see Combes and Rohde 1979, Rohde 1963, 1965). One would expect, given the number of chelonians in Africa and the rest of the world, that there will be many more polystomatids. Only when we know more about the diversity and geographical distribution of the turtles and their polystomatids will we be able to draw some conclusions as to how these parasites speciated and radiated. A molecular analysis of the genetic material of the parasites could shed some light on this.

The egg production for *Neopolystoma liewi* was found to be 1.5 eggs per worm per day. In the laboratory most eggs did not develop and only two eggs hatched

naturally. Pichelin (1995) reported that the rate of egg production was 4.3 eggs per worm per day for *Neopolystoma macleayi* and 0.7 for *N. cribbi.* She also reported that eggs of *N. cribbi* failed to develop while eggs of *N. macleayi* failed to hatch naturally. In spite of the low rate of egg production, these parasites survive and manage to infect hosts successfully, indicating that they must have a very effective mode of transmission. This phenomenon together with the apparent poor success rate of egg development in the laboratory awaits further investigations.

**Acknowledgements.** We are indebted to: The University of the Free State (South Africa) and the University of Malaya (Malaysia) as well as the South African National Research Foundation and a R&D grant by the Malaysian government for financial support. Mr Liew Kim-Seng George provided technical as well as field assistance.

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Received 3 March 1999

Accepted 17 September 1999