Redescription of *Protoopalina pingi* Nie, 1935 inhabiting the recta of *Hylarana guentheri* and *Pelophylax nigromaculatus* in China

Weidong Li¹,a, Chong Wang²,a, Feng Huang¹, Ming Li¹,³,⁴,* Frank Nilsen³, Huiyu Liu¹, and Jianlong Xu¹

¹ Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China
² Institute of Hydroecology, Ministry of Water Resources & Chinese Academy of Sciences, Wuhan 430079, China
³ Sea Lice Research Centre, Department of Biology, University of Bergen, Bergen 5020, Norway
⁴ Hubei Collaborative Innovation Centre for Freshwater Aquaculture, Wuhan 430070, China

Received 18 February 2014, Accepted 1 May 2014, Published online 12 September 2014

**Abstract** – A redescription of *Protoopalina pingi* Nie, 1935 is presented in this paper to complete Nie’s description at both light and scanning electron microscope levels. These organisms were collected from the recta of the frogs *Hylarana guentheri* Boulenger, 1882 and *Pelophylax nigromaculatus* Hallowell, 1861 from Jialing River, Sichuan Province and Honghu Lake, Hubei Province, respectively, in China. This is the first record of its occurrence in *H. guentheri* and *P. nigromaculatus*. The body of *P. pingi* is elongated and somewhat spindle-like in shape, slightly narrowed and bluntly rounded at the anterior extremity, while the posterior end is tapering or sharply pointed. The body surface is thickly flagellated, with the caudal tip being barren. The falx, located at the margin of the anterior end, is composed of a narrow band of kinetosomes. Four round or oval-shaped nuclei, usually arranged in a straight line, are situated in the middle region of the body. Comparisons are made between *P. pingi* and its congeners.

**Key words:** *Protoopalina pingi*, flagellate, frog, *Hylarana guentheri*, *Pelophylax nigromaculatus*.

**Introduction**

Opalinids, originally discovered by Leeuwenhoek in 1683 [7], are multinuclear, mouthless, osmotrophic flagellated protozoa. They live as commensals in the digestive tracts of different poikilothermic vertebrates, especially anuran amphibians [15]. The opalinids were for a long time regarded as the astomatous (no cytostome) ciliates because of their superficial similarities with the ciliates and were given the status “protociliates” as opposed to “euciliates” since the monomorphic nuclei, in contrast to dimorphic nuclei, were suggested to be an ancestral state of ciliates [1, 13, 14, 23]. Then the hypothesis of opalinid-ciliate affinity was abandoned since other characteristics, such as the structure of the nucleus, the mode of cell division and the reproductive cycle, differed remarkably from those of ciliates and these organisms were deemed to be either an isolated taxon in the phylum Zoolophylla or were treated as a separate phylum: Opalinata [3, 4, 8, 24]. Now, it has been convincingly shown that opalinids belong to heterokonts as a sister group to *Proteromonas* within the order Slopalinida based on detailed ultrastructural study and believable phylogenetic analyses [2, 6, 10, 11, 16, 17, 19, 20, 22].
The family Opalinidae can be separated into two subfamilies, Protoopalininae and Opalininae, based on the shape of the cell body and the number of nuclei. The subfamily Opalininae is comprised of the genera *Cepedea* Metcalf, 1920 and *Opalina* Duskinje and Valentin, 1835, while the subfamily Protoopalininae contains the genera *Protoopalina* Metcalf, 1918 and *Zelleriella* Metcalf, 1920.

*Protoopalina* is the most common genus of opalinids inhabiting anuran amphibians [5, 21]. It was established by Metcalf in 1918. Thereafter, many new species of *Protoopalina* have been found from the anuran amphibians. *Protoopalina pingi* was first discovered and named by Nie in 1935 from the intestines of *Rana plancyi* Lataste, 1880 [18]. Although discovered more than 70 years ago, many biological aspects of *P. pingi* are still unknown. After simple morphological information, no further data about this opalinid have been reported. The previous morphological data, however, are incomplete, and some descriptions of important taxonomic structures also need revision. This study adds to Nie’s description and attempts to contribute to the knowledge of this genus.

### Materials and methods

Host frogs, including 256 *H. guentheri* and 104 *P. nigromaculatus*, were captured from Jialing River in Pengan county (31°15’–31°29’ N; 106°12’–106°25’ E), Sichuan Province, China, in August 2011 and Honghu Lake (29°40’–29°58’ N; 113°12’–113°26’ E), Hubei Province, China, in June 2012, respectively. They were transported alive to the laboratory for further examination. We obtained the permits allowing us to capture and sacrifice these specimens. All frog samples were dissected, with the intestines and recta being opened and put into Petri dishes for examination. Then a 0.65% saline solution was added to the samples and we waited for a few minutes to allow *P. pingi* to swim out of the gut contents. The flagellates were collected with a Pasteur micropipette and washed twice in distilled water.

For light microscopy, individuals were observed, measured and photographed in vivo using both bright-field and differential interference contrast microscopy (Zeiss Axioplan 2 imaging and Axiophot 2, Oberkochen, Germany). The remaining specimens...
Figure 2. Light microscope images of Protoopalina pingi Nie, 1935. (A) Living specimens, showing the normal trophozoites of P. pingi. Scale bar = 20 μm. (B) Living specimens, showing the flagella covering the body (arrowhead). Scale bar = 5 μm. (C) Specimens stained with Protargol, showing the somatic kineties and the nuclei with distributed nucleoli. Scale bar = 10 μm. (D) Specimens stained with Protargol, showing the somatic kineties in the posterior extremity (arrowhead). Scale bar = 5 μm. (E) Specimens stained with Protargol, showing the falx region in the anterior extremity (arrowhead). Scale bar = 5 μm. (F) Specimens stained with Heidenhain’s haematoxylin, showing the nuclei (arrow) and the corpuscles of uneven size (arrowhead). Scale bar = 20 μm.
were placed directly on coverslips, fixed in a saturated HgCl₂ solution and stained with Heidenhain’s haematoxylin and a 1% Protargol solution. All measurements are in micrometres.

For scanning electron microscopy (SEM), the washed specimens were fixed in 2.5% glutaraldehyde in 0.2M phosphate buffered saline (PBS, pH 7.4) on a clean glass slide (1 cm × 1 cm), previously treated with 0.1% poly-L-Lysin and dried completely in air at room temperature (RT). After being washed with PBS three times, they were post-fixed in 1% osmium tetroxide at 4 °C for 1 h, followed by serial dehydration in acetone and critical point drying using a HCP-2 critical point dryer (Hitachi Science Systems, Ibaraki, Japan). Then the glass slide was mounted on an aluminium stub using double-sided adhesive tape and sputter-coated with a thin layer of gold in an IB-3 ion coater (Eiko Engineering, Ibaraki, Japan) before observing and photographing with a Quanta 200 SEM (FEI, Amsterdam, Netherlands).

Results

One hundred and thirty-five of the 256 H. guentheri examined and 42 of the 104 P. nigromaculatus examined were found to be infected with P. pingi. Large numbers of P. pingi were found in the recta of all frog hosts that contained them.

Protoopalina pingi Nie, 1935

Host: Hylarana guentheri Boulenger, 1882 and Pelophylax nigromaculatus Hallowell, 1861.

Prevalence: Total 135 (52.7%) out of 256 H. guentheri and 42 (40.4%) of 104 P. nigromaculatus were infected with this opalinid, respectively.

Habitat: Rectum.

Locality: Jialing River, in Pengan county, Sichuan Province, China; Honghu Lake, in Honghu City, Hubei Province, China.

Deposited specimens: Slides 2012W001-003 of Heidenhain’s haematoxylin-stained specimens, and slides 2012W004-010 of Protargol-stained specimens have been deposited in Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, China.

Description: The body is elongated and somewhat spindle-like in shape, slightly narrowed and bluntly rounded at the anterior extremity, while the posterior end is tapering or sharply pointed (Figs. 1A and 2A). The body length is 115.9 μm (93.6–144.0 μm, n = 20) and the width 31.1 μm (21.6–48.4 μm, n = 20) in vivo. The ratio of length to width is about 4:1. The body surface is thickly flagellated (Figs. 1A, B and 2B) with the caudal tip being barren (Figs. 1A, C). The flagella are arranged in the ridge running parallel to the longitudinal axis, most of which are fused into groups of two or three in our specimens (Figs. 1B, D). All the somatic kineties converge on the falk, anteriorly, and many extend to the posterior extremity (Figs. 2C, D and 3) and number 18–29 in total. The falk, composed of a narrow band of kinetosomes, can be observed in Protargol-impregnated specimens at a higher magnification. It is located at the margin of the anterior pole and extends to both dorsal and ventral sides (Figs. 2E and 3). Four
Table 1. Morphometric light microscopic characterisation of P. pingi.

| Character                        | Min     | Max     | Mean    | SD      | CV (%)  | N  |
|---------------------------------|---------|---------|---------|---------|---------|----|
| Body length, in vivo            | 93.6    | 144.0   | 115.9   | 13.6    | 11.7    | 20 |
| Body width, in vivo             | 21.6    | 48.4    | 31.1    | 4.7     | 16.3    | 20 |
| Body length, Protargol          | 72.0    | 110.4   | 89.5    | 10.3    | 11.6    | 15 |
| Body width, Protargol           | 14.4    | 36.4    | 23.1    | 3.7     | 18.5    | 15 |
| Nucleus length, Protargol       | 7.0     | 15.0    | 10.8    | 2.1     | 19.8    | 15 |
| Nucleus width, Protargol        | 5.0     | 11.5    | 8.0     | 1.5     | 18.7    | 15 |
| Number of total somatic kineties| 18      | 29      | 23.6    | 3.3     | 13.9    | 10 |

Measurements in μm; Min = minimum, Max = maximum, Mean = arithmetic mean, SD = standard deviation, CV = coefficient of variation, N = number of individuals investigated.

Table 2. Morphological comparison among P. pingi and other similar species of Protoopalina.

| Species               | BL       | BW       | Nn     | NL       | NW       | Ns     | Source of data          |
|-----------------------|----------|----------|--------|----------|----------|--------|-------------------------|
| P. pingi              | 93.6–144.0 | 21.6–48.4 | 4      | 7–15     | 5–11.5   | 18–29  | Present paper           |
| P. caudata michyla    | 120–290  | 40–70    | 2      | 15–23    | 15–18.8  | –      | Nie (1935) [18]          |
| P. quadrinucleata     | 58–109   | 10–18    | 1–8    | –        | –        | –      | Lu (1945) [12]           |
| P. heleophrynes       | 21–54    | 5.7–12   | 2      | –        | –        | –      | Delvinguier et al. (1995) [5] |
| P. pomacantha         | 157.2    | 28.3     | 2      | 14.6     | 7.7      | 26.3   | Grim et al. (2000) [9]   |

Measurement in μm; BL = Body length, BW = Body width, Nn = Number of nuclei, NL = Nucleus length, NW = Nucleus width, Ns = Number of total somatic kineties.

round or oval-shaped nuclei are situated in the middle region of the body, usually with many nucleoli distributed within the karyoplasm (Figs. 2C, F and 3). Normally, the nuclei are arranged in a straight line running parallel to the longitudinal axis (Figs. 2C, F, and 3). The nuclei range in length from an average of 10.8 μm (7.0–15.0 μm, n = 15) and in width 8.0 μm (5.0–11.5 μm, n = 15) in Protargol specimens. Many apparent corpuscles of uneven size can be observed over the cytoplasm (Fig. 2F).

Data for measurements related to morphometric characteristics are given in Table 1.

Discussion

As mentioned above, P. pingi was first discovered and named by Nie from the intestines of Rana plancyn [18]. This is the first record of its occurrence in the recta of H. guentheri and P. nigromaculatus. The opalinids examined in the present study appear slightly bigger than Nie’s type specimens, since he gave ranges of 55–160 l by 12.5–57 l. The caudal tip of P. pingi is barren of flagella. Morphological comparison among P. quadrinucleata, P. heleophrynes, and P. pomacantha have two nuclei, while P. quadrinucleata has 1–8. Furthermore, P. caudata michyla discovered in Microhyla ornata has relatively longer and wider body dimensions (120–290 × 40–70 vs. 93.6–144 × 21.6–48.4 μm) and larger nuclei than P. pingi (15–23 × 15–18.8 vs. 7–15 × 5–11.5 μm). P. quadrinucleata, inhabiting Rana guentheri, is smaller than our present opalinids for body size (58–109 × 10–18 vs. 93.6–144 × 21.6–48.4 μm). P. heleophrynes reported in tadpoles of Heleophryne rosei also has relatively smaller body dimensions than P. pingi (21–54 × 5.7–12 vs. 93.6–144 × 21.6–48.4 μm) in this paper. P. pomacantha found in the rectum of Angelfishes most resembles P. pingi considering the body size (157.2 × 28.3 vs. 93.6–144 × 21.6–48.4 μm), and the phenomenon that both of their caudal tips are barren of flagella. Morphological comparison among P. pingi and other similar species of Protoopalina are presented in Table 2.

In conclusion, based on general morphological characteristics, P. pingi is recorded and redescribed in detail from H. guentheri and P. nigromaculatus. Future collections will be made at different stages of the hosts’ life cycles to determine if the trophonts always have four nuclei instead of the two usually found in Protoopalina, to determine if cysts are formed, to study its possible “infection” routes and further assess the host specificity.

Acknowledgements. Financial support for this study was provided by the National Natural Science Foundation of China (Grant
References

1. Calkins GN. 1933. The biology of the protozoa, 2nd edn. Lea & Febiger: Philadelphia.
2. Cavalier-Smith T. 1998. A revised six-kingdom system of life. Biological Reviews, 73, 203–266.
3. Corliss JO. 1955. The opalinid infusorians: flagellates or ciliates? Journal of Protozoology, 2(3), 107–114.
4. Corliss JO. 1979. Flagellates, opalinids and the search for the most primitive ciliate and its progenitor. Ceylon Journal of Science (Biological Sciences), 13(1/2), 65–78.
5. Delvinquier BLJ, Markus MB, Passmore NI. 1995. Opalinidae in African Anura. Genus Protoopalina. Systematic Parasitology, 30, 81–120.
6. Delvinquier BLJ, Patterson DJ. 2002. Order Slopalinida, in An illustrated guide to the Protozoa, 2nd edn. Lee JJ, Leedale GF, Bradbury P, Editors. Society of Protozoologists: Lawrence, KS. p. 754–759.
7. Dobell C. 1932. Antony van Leeuwenhoek and his “Little Animals”. John Bale, Sons and Danielson: London.
8. Grassé PP. 1952. Traité de Zoologie, vol 1, Masson: Paris.
9. Grim JN, Pérez-España H, Martínez-Díaz SF. 2000. The morphology of Protoopalina pomacantha, n. sp., symbiont in the rectum of the Angelfishes, Holacanthus passer and Pomacanthus zonipectus. Molecular Phylogenetics and Evolution, 16, 695–705.
10. Kostka M, Cepicka I, Hampl V, Flegr J. 2007. Phylogenetic relationships and perspectives. Green JC, Leadbeater BSC, Diver WL, Editors. Clarendon Press: Oxford. p. 357–379.
11. Kostka M, Cepicka I, Hampl V, Flegr J. 2004. Phylogenetic position of Protoopalina intestinalis based on SSU rRNA gene sequence. Molecular Phylogenetics and Evolution, 33(1), 220–224.
12. Lu K. 1945. On some parasitic ciliates from frogs of Pehpei Sinensia, 16, 65–72.
13. Metcalf MM. 1918. Opalina and the origin of the Ciliata. Anatomical Record, 14, 88–89.
14. Metcalf MM. 1923. The opalinid ciliate infusorians. Bulletin of the United States National Museum, 120, 1–484.
15. Mignot JP. 1994. Patterning in opalinids. I. Implications of new morphological and ultrastructural findings on the genesis of kinetics. European Journal of Protistology, 30(2), 196–210.
16. Mignot JP, Affa’a FM. 1994. Structural and ultrastructural study of Protoopalina drachi Tuzet & Knoepffler, 1968. Archiv für Protistenkunde, 144(2), 173–184.
17. Nishi A, Ishida KI, Endoh H. 2005. Reevaluation of the evolutionary position of opalinids based on 18S rDNA, and α- and β-tubulin gene phylogenies. Journal of Molecular Evolution, 60, 695–705.
18. Nie D. 1935. Intestinal ciliates of amphibian of Nanking. Contributions from the Biological Laboratory of the Science Society of China: Zoological Series, 11, 67–95.
19. Patterson DJ. 1985. The fine structure of Opalina ranarum (Family Opalinidae): opalinid phylogeny and classification. Protistologica, 21(4), 413–428.
20. Patterson DJ. 1989. Stramenopiles, chloroplasts from a protistan perspective. In The chromophyte algae, problems and perspectives. Green JC, Leibader BSC, Diver WL, Editors. Clarendon Press: Oxford. p. 357–379.
21. Sandon H. 1976. The species problem in the opalinids (Protozoa, Opalinata) with special reference to Protoopalina. Transactions of the American Microscopical Society, 95, 357–366.
22. Silberman JD, Sogin ML, Leipe DD, Clark CG. 1996. Human parasite finds taxonomic home. Nature, 380(6573), 398.
23. Stein F. 1860. Über die Eintheilung der holotrichen Infusionsthiere und stellte einige neue Gattungen und Arten aus dieser Ordnung auf. Sitzungsberichte der königlichen böhmischen Gesellschaft der Wissenschaften in Prague Juli, December, p. 56–62.
24. Wessenberg HS. 1978. Opalinita, in Parasitic Protozoa 2. Kreier JP, Editor. Academic Press: London. p. 551–581.