

Comparative structural analysis of jaws of selected blood-feeding and predacious arhynchobdellid leeches (Annelida: Clitellata: Hirudinida)

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Abstract Morphological traits of jaws, denticles and salivary pores in jawed leeches are compared and an overview of their structural and functional properties is given. The species studied include *Hirudo medicinalis*, *H. verbana*, *H. orientalis*, *H. nipponia*, *H. troctina*, *Limnatis nilotica*, *Haemopsis sanguisuga* and *Whitmania laevis*. Morphological descriptions are based on scanning electron microscopy and translucent light microscopy. All the species possess denticles arranged in one or two rows on muscular jaws with salivary pores between neighboring denticles. Structural differences of the denticles occur between the genera *Hirudo*, *Limnatis* and *Haemopsis*, while within a genus, denticle structure is similar. In *Hirudo* spp., denticles are complex organs consisting of two subunits. Denticles of *Limnatis nilotica* are simple in their structure. Denticles and salivary pores of *Haemopsis sanguisuga* have the largest size and the most complex structure as compared with the other species. Those denticles are heart-shaped; two rows of them coalesce into a single row. Salivary canals open through multiple pores arranged in a number of patches and leading into large common openings located between the denticles. The denticle sizes and numbers were found to correlate negatively: species with larger denticles have a fewer number of them.

Keywords *Hirudo* spp. · Medicinal leech · *Haemopsis* · *Limnatis* · *Whitmania* · Jaw · Blood-feeding

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Introduction

The medicinal leeches (genus *Hirudo*) have been studied for centuries and have become model organisms in evolutionary biology, invertebrate neurophysiology, ecology and biochemistry (Dickinson and Lent 1984; Lent et al. 1988; Trontelj et al. 1999; Phillips and Siddall 2009; Trontelj and Utevsky 2012). Due to their unique saliva compounds (Baskova et al. 2008; Kvist et al. 2013), they are commonly used in medicine for treatment of various diseases since the ancient times (Whitaker et al. 2004; Elliott and Kutschera 2011; Kutschera 2012). Although leech jaws are essential parts of the digestive tract and may affect bloodletting, they have received relatively little attention concerning their denticle morphology and location of salivary gland apertures (Hildebrandt and Lemke 2011; Orevi et al. 2000). The interest in the European medicinal leeches and their detailed anatomy and physiology has been increased due to recent findings in the field of their taxonomy and phylogeny. Thus, a new species of medicinal leeches was described in 2005 (Utevsky and Trontelj 2005), and based on molecular analyses, the neglected *Hirudo verbana* Carena, 1820 was proved to have a species status since 2004 and later (Trontelj et al. 2004; Trontelj and Utevsky 2005; Siddall et al. 2007). The phylogeny of arhynchobdellids and their relationships with other leech taxa were also extensively studied (Borda and Siddall 2004a, b). The geographical distribution and ecological requirements of *Hirudo* spp. appeared to be quite different (Utevsky et al. 2010; Kovalenko and Utevsky 2012). All this must be closely associated with feeding habits and food preferences of the leeches. The investigation of these traits, however, requires a detailed description of denticles and jaw morphology.

In general, there are only two main types of feeding organs in Hirudinida. One of them is a proboscis of the paraphyletic rhynchobdellids that pierces the skin of fishes and other aquatic animals. Another one is a pharynx that possesses or lacks muscular jaws with sharp denticles in the monophyletic Arhynchobdellida (Sawyer 1986; Nese-mann and Neubert 1999; Trontelj et al. 1999). Depending on the presence and development of muscular jaws and denticles, arhynchobdellids can be divided into blood-feeders and predators. It is considered that distichodont jaws are typical to predaceous leeches and unable to cut the skin for bloodsucking. Usually, jaws of this type are weak, with small denticles or hard platelets or without any denticles, e.g., *Whitmania laevis* (Lukin 1976). Monostichodonts have only one row of denticles per jaw and are blood-feeding leeches. Their denticles are sharp and able to cut the skin or mucosa. Their number and size vary and are associated with a species-specific feeding style (Sawyer 1986).

The unicellular salivary glands are spread in parenchyma on the outer surface of the pharynx around the jaws (Marshall and Lent 1988). Each gland has its own duct leading to the pharynx lumen (Hildebrandt and Lemke 2011). The ducts open on the surface of the jaws. Several views about exact locations of the apertures have been proposed: on tips of the denticles (Damas 1972; Sawyer 1986) or subterminally on the denticles (Orevi et al. 2000), between the denticles (Hildebrandt and Lemke 2011) or even on jaw papillae that are commonly spread on the jaw surface, e.g., in *Hirudinaria manilensis* (Sawyer 1986). This problem is particularly important for the further experiments in the field of the bite physiology of blood-feeding leeches.

Recently, three species of medicinal leeches (*H. medicinalis*, *H. verbana* and *H. orientalis*) have been compared for the biochemical composition of their saliva (Baskova et al. 2008). Those authors showed that *H. medicinalis* and *H. orientalis* are closest to each other in terms of their saliva composition, which correlates with their phylogenetic relationships (Utevsky and Trontelj 2005). However, the comparison of jaws and denticles has not been undertaken for these species before. Five species of medicinal leeches were studied in this research: *H. medicinalis*, *H. verbana*, *H. orientalis*, *H. troctina* and *H. nipponia*. Also the Nile leech *Limnatis nilotica* and two species of predaceous distichodonts, *Haemopsis sanguisuga* and *Whitmania laevis*, were compared with the blood-sucking species. Herein, we analyzed jaw and denticle fine morphology and sizes, the number of denticles per jaw and the arrangement of denticles in a row according to their sizes in different species of jawed leeches.

Materials and methods

Eight species of leeches were examined in this research. Specimens of different populations that were collected during several expeditions were analyzed. *Hirudo medicinalis*, *Hirudo verbana* and *Haemopsis sanguisuga* were collected in lakes of the Kharkiv Region (Ukraine). Specimens of *Hirudo orientalis* and *Limnatis nilotica* were collected in Azerbaijan, Kazakhstan and Uzbekistan. *Hirudo troctina* originated from Tunisia, *Hirudo nipponia* and *Whitmania laevis* were obtained from Yunlin country (Taiwan). Samples were fixed in 96 % with the preliminary narcotizing in 10 % ethanol to prevent contraction. Altogether, 12 specimens of *H. medicinalis*, 14 of *H. verbana*, 12 of *H. orientalis*, 7 of *H. troctina*, 4 of *H. nipponia*, 10 of *L. nilotica* and 7 of *H. sanguisuga* were examined for jaws morphology.

Preparation techniques

After fixation, leeches were dissected under a stereomicroscope, and all three jaws were removed for the further examination. We examined all three jaws to verify a possible difference between jaws of every specimen. For easier distinction, we lettered the dorsal jaw as *a*, and ventral right and left jaws as *b* and *c*, respectively. All interspecific comparisons have been done for jaws of the same position only, i.e., dorsal jaws have not been compared with ventral jaws and the right ventral ones have not been compared with the left ventral jaws.

For measurements, permanent preparations of the jaws were made. Each jaw was squeezed between a slide and a cover slip to make it thin and transparent for light microscopy. After that, the jaws were dehydrated in the ethanol of the increasing strength: 70, 96 and 100 %. Xylene was applied before embedding samples in mounting medium to enhance the translucence of the muscle tissue. Thereby, denticles turned out clearly visible on the preparations and accessible for measurement and morphological observation. Photographs were taken with a BUC2-500C digital camera. Measurements were made using Axio Vision Software with calibration at appropriate microscope magnifications. The observations were made with a microscope Granum Lux R6052.

Scanning electron microscopy (SEM) images were made in the Tokyo Boeki Technology Ltd. laboratory of the Kiev Polytechnic Institute (Ukraine). Jaws were isolated and examined separately. Samples were preliminary prepared by the standard procedure that includes drying of the samples for SEM, mounting on the sample stub with the graphite paste and sputter-coating with platinum. Figures were processed and labeled in Photoshop CS2.

Results

General anatomy of jaw complex

The morphology of the jaws was analyzed for eight species of the arhynchobdellid leeches. The jaw complex is trigonathous in all species examined. The jaws are muscular, with cutting edges bearing denticles arranged in one (*Hirudo* spp. and *Limnatis nilotica*) or two rows (*Haemopsis*

sanguisuga) (Fig. 1). The jaws of *Hirudo* spp. are comparatively larger than those of *Limnatis* and *Haemopsis*, and the jaws of *Whitmania* are smallest comparatively to the other species and lacking any denticles or folds that could be identified as a cutting edge (Fig. 2). In all the species, muscle tissue is rather hard and coated with cuticle that covers the jaw including its cutting edge and denticles (Fig. 3). The internal tissues of the jaws are spongy and softer than the muscular layer. Jaws of *L. nilotica* appeared

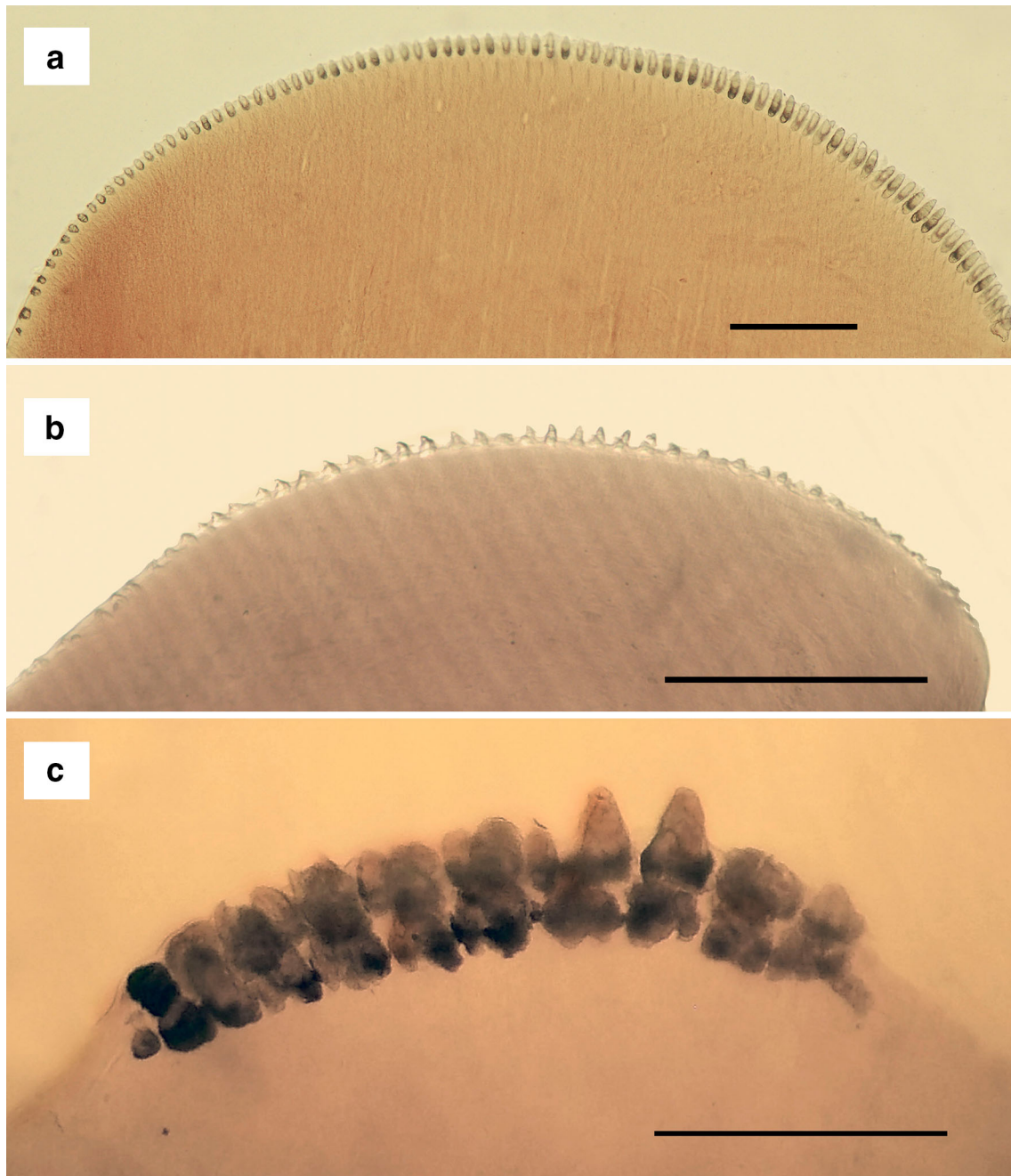


Fig. 1 Individual jaws viewed using translucent microscopy. **a** *Hirudo medicinalis*, **b** *Limnatis nilotica*, **c** *Haemopsis sanguisuga*. Scale bars 200 μ m

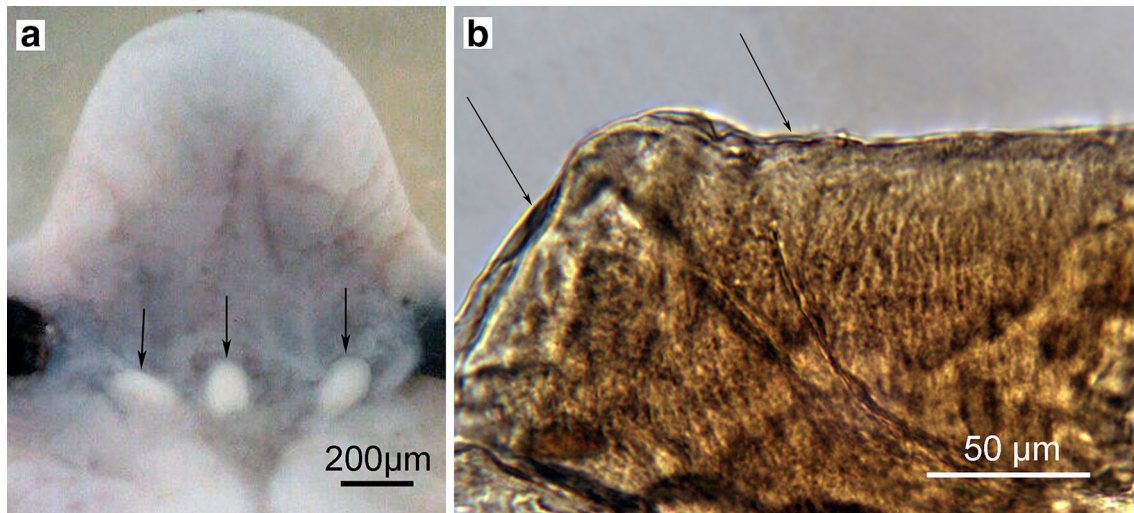


Fig. 2 **a** Three jaws of *Whitmania laevis*. **b** Entire jaw of *Whitmania laevis*. Arrows show the jaw edge without denticles

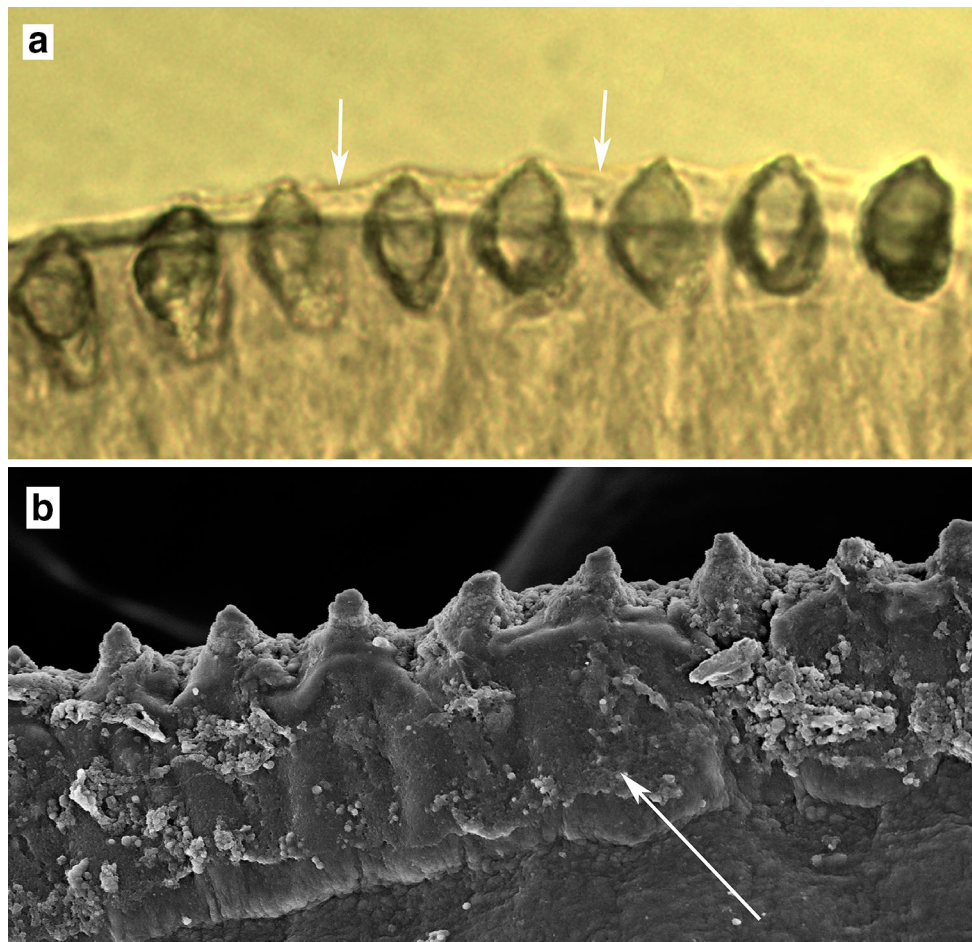


Fig. 3 Rows of denticles in *H. verbana*, translucent light micrograph (a) and SEM picture (b). White arrows point cuticle folds that cover basic parts of the denticles and leave their tips uncovered

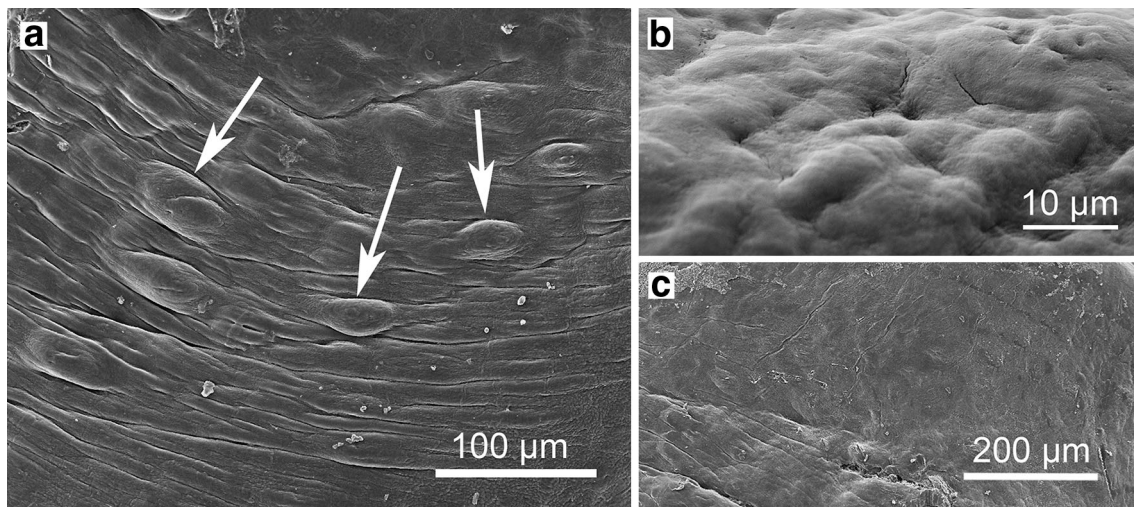


Fig. 4 Surface of a jaw in different leech species. **a** jaw of *H. orientalis* bearing papillae (white arrows) on its sides. Papillae do not have any pores or fissures. **b** surface of a *L. nilotica* jaw is tuberos, with no discernible papillae. **c** smooth surface of a *H. verbana* jaw

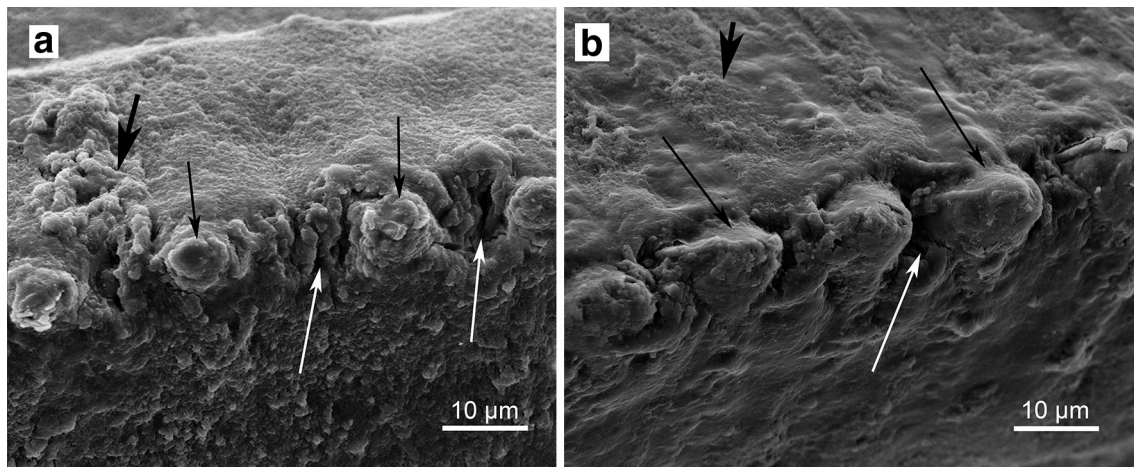


Fig. 5 Denticle rows of *H. medicinalis* (a) and *H. verbana* (b) viewed by SEM. Salivary pores (white arrows) are situated between neighboring denticles (thin black arrows). Thick black arrows indicate the outflow of saliva from the salivary pores

much softer than those of *Hirudo* spp. and even of *H. sanguisuga*. Numerous papillae are scattered on the sides of the jaws in *Hirudo* spp., but the papillae lack any pores that could be associated with salivary glands (Fig. 4a). *Haemopsis sanguisuga*, *L. nilotica* and *W. laevis* have smooth jaws with no papillae (Fig. 4b, c).

Salivary pores

The location of salivary gland canals has been studied quite scantily. Some investigations in this area have been already undertaken for the medicinal leech *Hirudo medicinalis*, but there have been no observations for other species of the jawed leeches. The application of SEM technique enabled us to find out the arrangement of canal

openings on the jaw surface in *Hirudo* spp. and other species examined. We found out well-discernible pores between denticles on the cutting edge of the jaw (Fig. 5). The salivary apertures of *Hirudo* spp. are fissured; their size is about $6 \times 2 \mu\text{m}$.

The jaws of *Haemopsis sanguisuga* also possess the canal apertures between the denticles (Fig. 6a, b). However, these pores have a more complicated structure than those in *Hirudo* spp. The opening is of irregular shape and rather large with a diameter approximately $8 \mu\text{m}$. The bottom of the hole is well seen through the opening. On the bottom, a number of regularly arranged pores $2.5 \mu\text{m}$ in diameter are discernible (Fig. 6c). We suppose that those small pores are apertures of secondary salivary canals assembled in groups with one larger opening for each.

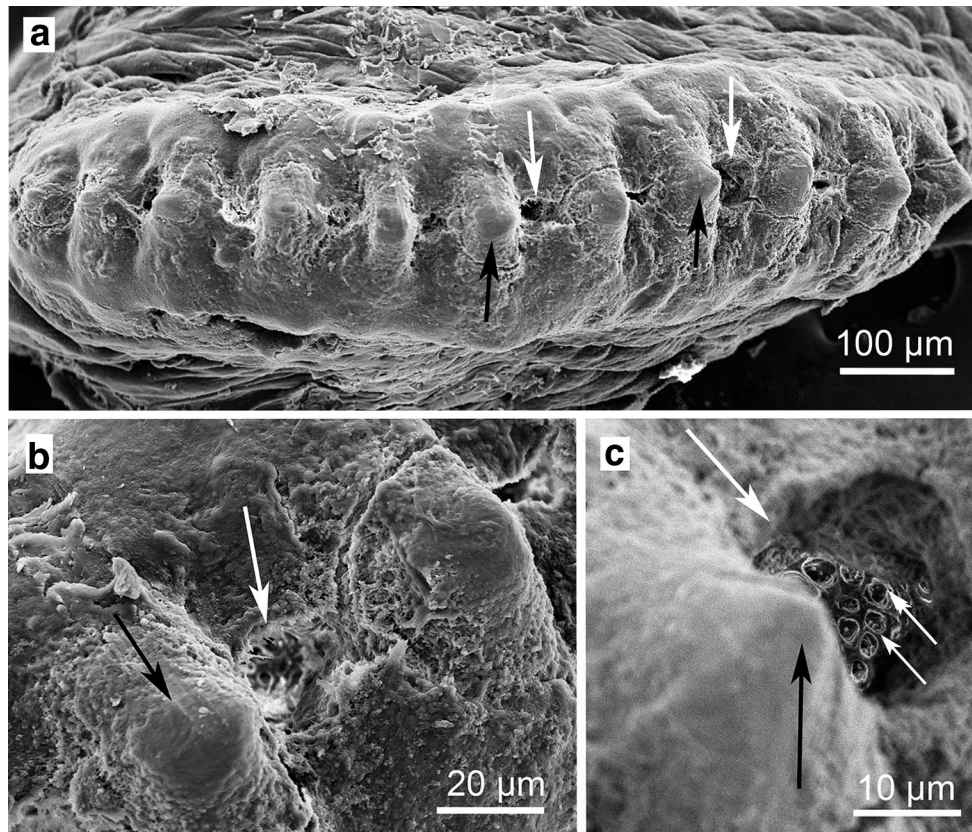


Fig. 6 Entire jaw (a), salivary pores and denticles (b, c) of *H. sanguisuga* viewed by SEM at different magnifications. Black arrows point denticle tips, white arrows point salivary pores. c short white

arrows point secondary pores on the bottom of a salivary canal viewed through a common opening (long white arrow)

Denticle structure

During this research, we revealed a number of morphological characters that have not been described before. The shape of the denticles appeared to be most discernible in permanent preparations of jaws using transparent light microscopy (Fig. 7). The contrast of the images was improved by mounting medium that increased transparency of soft jaw tissues and enabled to analyze the fine structure of the denticles. As was mentioned above, a jaw is coated by cuticle. As shown in Fig. 3, roots of denticles are covered with a cuticle layer, so that only sharp tips of the denticles are uncovered that can be seen both in SEM and light microphotographs.

Denticles arranged in a row were numbered from the posterior margin of the jaw to its anterior one. The first denticle of all jaws appeared morphologically distinct from the other denticles in the row. The former was approximately twice broader and blunter than following denticles and, in the most cases, inclined to its neighbors. The denticles were arranged in the row from the large at the beginning to the small at the end (Fig. 1), though the first denticle was often slightly shorter than the second.

The denticle structure was described and compared in different species. Every denticle of *Hirudo* spp. is heart-shaped with its sharp tip turned up (Fig. 7a, b). A few denticles were divided into two halves lying in parallel (Fig. 7d). In *Haemopsis sanguisuga*, a row contains only about ten denticles unlike the jaws of *Hirudo* spp. possessing 70–90 denticles (Figs. 1, 8). However, their sizes were much larger than those of medicinal leeches. Light microscopy (Fig. 8a) revealed two rows of large denticles in *H. sanguisuga*. SEM technique found one row of denticle tips per jaw (Fig. 8b). These images suggest that the rows in distichodont leeches conjugate rather strongly and denticles stay paired into one row in living animals, being separated only because of the slide preparation. Denticles of *Haemopsis sanguisuga* appeared to be rather large with specific morphological features. Thus, we noted that every individual denticle of *H. sanguisuga* is heart-shaped as in *Hirudo* spp. (Fig. 8a, c), but in *Haemopsis*, this heart-shaped structures lay in two parallel rows of a distichodont jaw and conjugate forming units that consist finally of four tear-shaped structures.

Unlike the denticles of the species described above, those of the Nile leech *Limnatis nilotica* seem to be rather

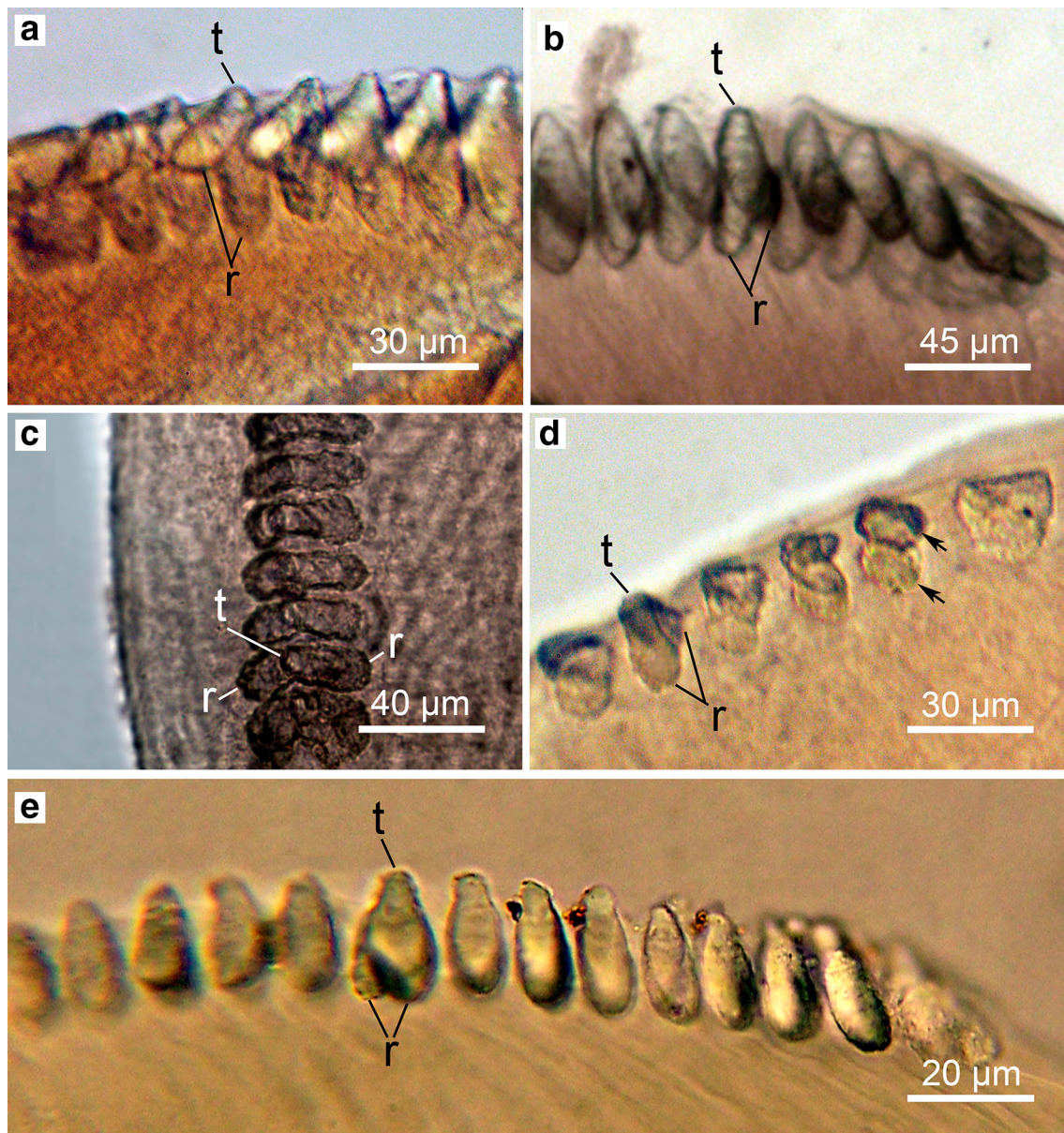


Fig. 7 Denticles viewed using translucent light microscopy. Jaws were processed through ethanol and xylene dehydration and embedded into mounting medium. **a** *H. medicinalis*: denticle consisting of two parts that unite at its tip; **b** *H. verbana*; **c** *H. troctina*: top view;

d *H. orientalis*: arrows show denticle parts disconnected at the tip and situated in parallel to each other; **e** *H. nipponia*. *t* denticle tips; *r* denticle “roots”

simply structured. These are small triangle denticles that form a single row on each jaw (Fig. 9). We did not find any other complex features of the denticles in *L. nilotica* in contrast to the other arhynchobdellids.

Measurements

The denticles of *Hirudo medicinalis*, *H. verbana*, *H. orientalis*, *H. troctina* and *Limnatis nilotica* were also counted, measured and compared statistically. The highest number of denticles was found in *H. medicinalis*, at the

average 82 (range 74–93) per jaw. *Hirudo orientalis* and *H. troctina* were rather similar to *H. medicinalis* with means 80 (range 71–91) and 79 (71–91), respectively, and jaws of *H. verbana* bear comparatively less denticles, at the average 75 (range 68–84). The smallest number of denticles was recorded in *L. nilotica*, at the average 40 (range 38–40).

For measurements and interspecific comparison, several denticles in each jaw were chosen: number 1, 2, 7 and 33. The first, second and seventh denticles are among the largest and the 33rd one is among medium-sized denticles,

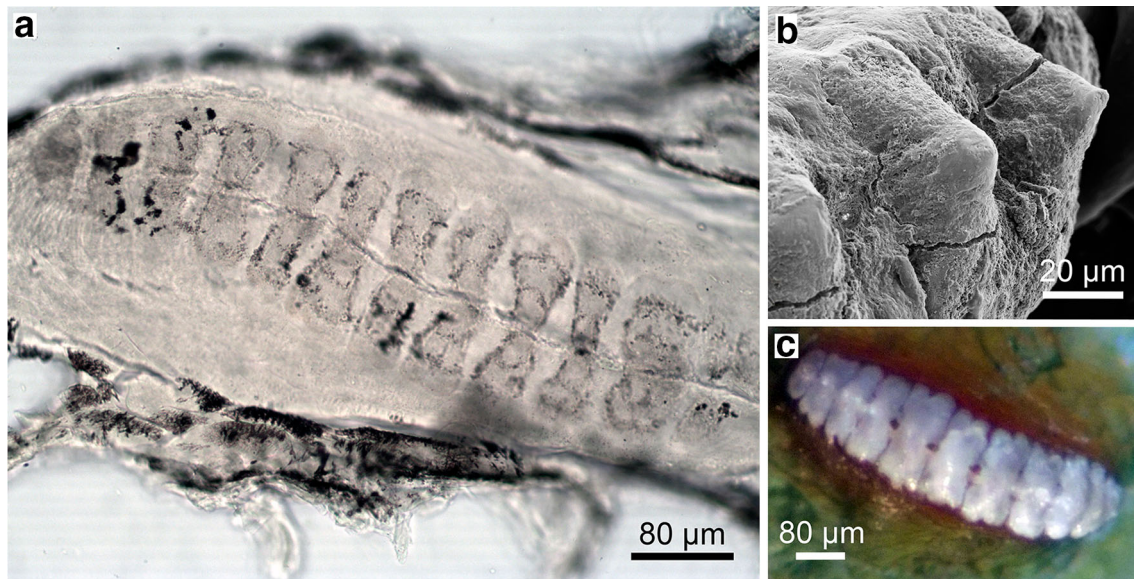


Fig. 8 *Haemopsis sanguisuga*: **a** top view of a jaw in a translucent light micrograph: two rows coalesce at denticle tips forming single row; every denticle is triangle with bifid base; **b** single row of denticle

tips in a SEM picture; **c** top view of denticles from a dried preparation: bifid bases of the denticles and salivary pores are visible

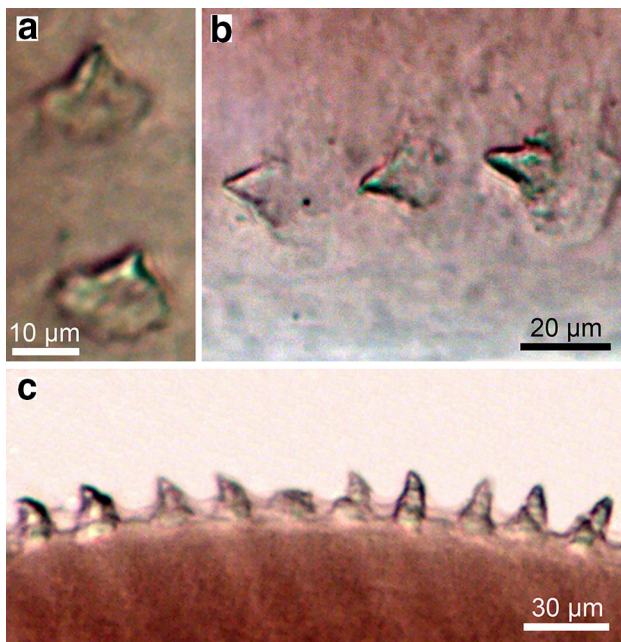


Fig. 9 *Limnatis nilotica*: sharp denticles viewed by translucent light microscopy: denticle of triangle shape with whole bases, not divided into parts (**a**, **b**); **c** denticle row

which enables to increase the accuracy of the measurements and comparison. *T* test was applied for interspecific comparisons. In most cases, the denticles differed significantly ($p < 0.05$). The correlation between the leech length and denticle sizes was not found.

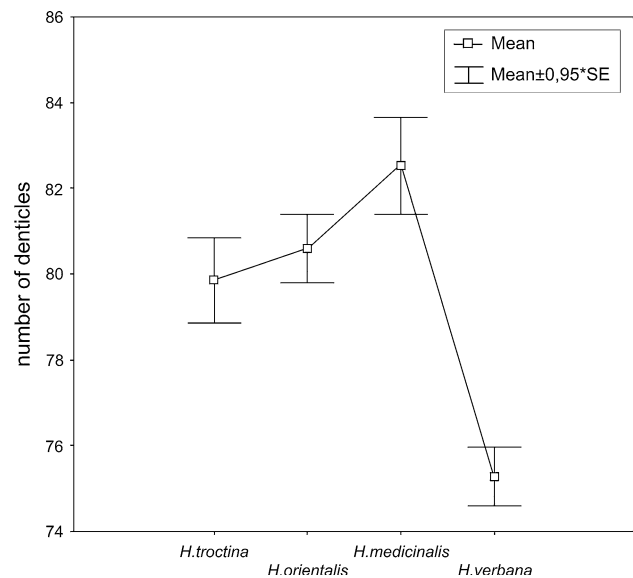


Fig. 10 Distribution of means of denticle numbers within *Hirudo* spp. ($n = 40$)

Figures 10 and 11 show two graphs that indicate a negative correlation between the size and the number of denticles for a jaw. The species with the largest denticles have the fewest number of denticles for a jaw. And conversely the species with small denticles have more denticles for a jaw. *Hirudo medicinalis*, *H. orientalis* and *H. troctina* are rather similar to each other in terms of the size

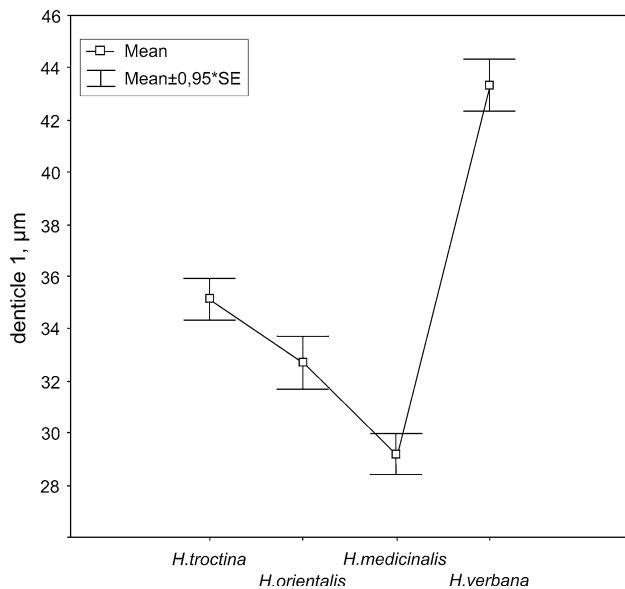


Fig. 11 Distribution of means of denticle sizes within *Hirudo* spp. ($n = 40$)

and number of denticles, whereas *H. verbana* is quite different from them, as the graphs show.

Discussion

This study compares the jaw morphology of the blood-feeding and predaceous jawed leeches. A phylogenetic analysis found that blood-feeding habit emerged more than once in the evolutionary history of the arhynchobdellid leeches (Borda and Siddall 2004a; Phillips and Siddall 2009). The leeches feeding on the mucosa (*Limnatis* spp.) form a separate group on the phylogenetic tree (Phillips and Siddall 2009) that is distinct from the medicinal leeches. *Hirudo* spp. (feeding by piercing skin) is closer to the carnivorous *Haemopsis* spp. than to the former group. The latter two genera are rather related phylogenetically in spite of the different feeding manner and behavior.

The further discussion encompasses the cutting edges and jaw morphology variation (1), remarks of the salivary gland pores location and (2), variation of denticle shape within the family Hirudinidae (3) and finally a comparison of denticle mean sizes and their correlation with denticle numbers for the different species of the genus *Hirudo* (4).

The leeches observed have similar jaw gross morphology: the structure of the pharynx with three muscular jaws situated equilaterally and forming a triangle. Those jaws possess denticles or lack them depending on the species. The fine morphology of the denticles and jaws is compared and analyzed herein for better understanding the

correlation between the feeding habits and jaw structure, and interspecific relationships of these traits. The jaw gross morphology is similar to that reported by Moquin-Tandon (1846), Lukin (1976) and Sawyer (1986): monostichodont jaws of blood-sucking leeches and distichodont jaws of the predaceous leeches (Fig. 1). The softness of the jaw tissues is correlated with food preferences of a particular species. *Hirudo* spp. have the largest and the most rigid jaws that are an adaptation for piercing thick skin of mammals and amphibians, being usual hosts of medicinal leeches in the wild (Elliott 2008). *Limnatis nilotica* has soft jaws that are enough for cutting delicate mucosa. Jaws of *Haemopsis sanguisuga* are smaller comparatively to the medicinal and Nile leeches, but are rather rigid and bearing blunt denticles. These features are useful for grinding and pushing large pieces and whole invertebrate preys that are main components of *Haemopsis* diet (Lukin 1976; Nesemann and Neubert 1999).

Location and structure of salivary gland pores

The location of salivary gland canals of jawed leeches is still a controversial question. Damas (1972) and Sawyer (1986) reported apertures to be situated on the tips of pyramidal denticles and supposed the denticles to be hollow with salivary canals inside. Orevi et al. (2000) denoted apertures on the denticles sides close to their tips. Hildebrandt and Lemke (2011) showed the canals openings between the neighboring teeth through the histological sections technique. We used SEM and translucent light microscopy for jaws of a number of species: *H. medicinalis*, *H. verbana*, *H. orientalis*, *H. troctina* and *L. nilotica*. The SEM technique shows the pores position in soft tissue between the denticles. In the Fig. 5, some fluid secretion from salivary pores is discernible and supposed to be saliva secretion. No openings were found on denticle tips that commonly have smooth surface without holes or fissures (Figs. 5, 6, 7, 8, 9).

The information on salivary pores of *Haemopsis sanguisuga* has been very scanty. Previous studies have provided a brief description of the general jaw morphology and number of denticles (Lukin 1976; Nesemann and Neubert 1999), but have not provided any details of the cutting edge structure and the shape of the denticles. The structure of the salivary pores of *H. sanguisuga* appeared more complex than that in medicinal leeches. Small pores are grouped (Fig. 6) at the bottom of a chamber, while every chamber opens outside via a broad hole. Similarly to the complex origin of the *H. sanguisuga* denticles, which is described below, we can suppose that the clustered salivary apertures could origin through converging those pores situated separately on the cutting edge of jaws in an ancestor.

Variation of the denticle shape within the family Hirudinidae

Denticles arranged in one row per jaw are in agreement with the previous studies that consider *Hirudo* spp. as monostichodonts (Sawyer 1986) (Fig. 1). However, Fig. 7 shows a more complex morphology of the denticles than considered before. The denticles forming a single row in *Hirudo* spp. are triangle with a bifid base, so they could be viewed as merged pairs of drop-like elementary denticles (Fig. 7). These results suggest a secondary origin of monostichodonty in *Hirudo* spp. from ancestral distichodonty. Paired denticles are typical to all species of the genus *Hirudo*. Even *H. nipponia* that is genetically rather distinct from the other *Hirudo* spp. (Borda and Siddall 2004a; Phillips and Siddall 2009) has heart-shaped denticles (Fig. 7e).

The examination of the jaw anatomy of the *Haemopsis sanguisuga* also showed a more complicated denticle structure than it was thought before. In the previous studies, denticles of *H. sanguisuga* have been described as small hard platelets that were tiny even in adult individuals (Lukin 1976). However, we found them to be 2–4 times larger than the denticles of the medicinal leeches. Also it is important to note that while *H. sanguisuga* is considered as a distichodont leech (Sawyer 1986) and Fig. 1 shows double row of denticles, both SEM and jaw anatomical examination (without squeezing them) show only one row of denticles that probably is formed by two merged rows (Figs. 6, 8). In addition, the denticles of every single row are like merged pairs of drop-like elementary denticles (Fig. 8b, c). Thus, we suppose that a denticle of *H. sanguisuga* is composed of four elementary denticles, while a denticle of *Hirudo* spp. is composed of two elementary denticles. The number of denticles per jaw in *H. sanguisuga* is only about 10–15, unlike the blood-feeding leeches that have 40–90 denticles per jaw. Due to these traits, a cutting edge of a *Haemopsis* jaw looks like a row of molar teeth of some herbivorous mammals (Fig. 8d). Functionally, both are good adaptations to grind hard food.

Measurements

Some studies on denticle sizes and numbers and entire jaw morphology were carried out for *H. medicinalis*, *H. verbana*, *L. nilotica* and *H. sanguisuga* (Lukin 1976; Sawyer 1986; Nesemann and Neubert 1999; Orevi et al. 2000). The jaws of *H. orientalis* are first described in this paper. Therewith, *H. orientalis* was recognized as a species much later than the aforementioned works were published (Utevsky and Trontelj 2005), and all these papers might deal with two and even three species under the name of *H. medicinalis*. The comparison of the denticle size and

number found that *H. verbana* has the significantly largest denticles, but their number is smallest in comparison with *H. medicinalis*, *H. orientalis* and *H. troctina*. The latter three species have more numerous and rather small denticles. The graphs show that the number of denticles is correlated with their size, so the smaller denticles, the bigger number of them on a jaw (Figs. 10, 11). This conclusion has something common with the distribution of the salivary components concentrations revealed by Baskova et al. (2008), where *H. medicinalis* and *H. orientalis* have similar traits, while *H. verbana* was quite distinct from them. Thus, in *H. verbana*, small number of large denticles provides an effective incision of thick skin of mammals that are considered as main hosts of this species (Utevsky et al. 2010). It is important to note that *H. verbana* occurs in steppe lakes (often in ephemeral pools), and their common food source is ungulates, whose skin is thick and hard (Utevsky et al. 2010). Unlike *H. verbana*, its congenitors *H. orientalis*, *H. medicinalis* and *H. troctina* inhabit permanent lakes, where they mainly feed on amphibians that have thin soft skin, so their denticles can be small. Denticles of *Limnatis nilotica* are comparatively small and not numerous, i.e., adapted for feeding on mucosa where large denticles are not necessary. Denticles of *Hirudo nipponia* appeared more similar in their size and number to *L. nilotica* than to other species of its genus, while morphologically, they are similar to those of *Hirudo* spp.

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