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Diversity of ciliates of the family Nyctotheridae in cockroaches

Diverzita nálevníků čeledi Nyctotheridae ve švábech

Diploma thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Matyáš Soviš

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Abstrakt

Švábi jsou nesmírně zajímavou skupinou hmyzu, jak rozmanitostí životních strategií, tak morfologickou diverzitou. Je známo, že ve svých střevech hostí spousty fascinujících protist nejrůznějších skupin od exkavátních bičíkovců, přes hromadinky a měňavky, až po pozoruhodné nálevníky ze skupiny Armophorea. O těchto nálevnících je toho ale, narozdíl například od zmíněných bičíkovců, známo jen velice málo. Data, která jsou k dispozici, jsou povětšinou jen morfologická a není známo nic o jejich hostitelské specifitě. Proto jsme tyto nálevníky hledali ve švábech z chovů, které jsou k dispozici na Katedře Zoologie PřF UK i ve sběrech z přírody. Nálevníky jsme molekulárně charakterizovali za pomoci sekvencí genu pro 18S rRNA a poodkryli jsme jejich vzájemné příbuzenské vztahy. Soustředili jsme se i na rozšíření jednotlivých linií v různých skupinách švábů. Prováděli jsme také barvení buněk protargolovou technikou, abychom mohli charakterizovat morfologii některých nově objevených linií.

Klíčová slova: Anaerobióza, symbióza, nálevníci, Clevelandellida, *Nyctotherus*, diverzita, 18S rDNA, hostitelská specifita

Abstract

Cockroaches are tremendously interesting group of insects with broad morphological diversity and a wide range of lifestyles. They are known to host a plethora of fascinating protists ranging from excavate flagellates through gregarines and amoebozoans to extraordinary ciliates of the group Armophorea. There is however, in contrast to the flagellates, only scarce information on these intestinal ciliates. The available data are mostly only morphological and there is a limited information on their host specificity. Therefore, we chose to inspect the diversity of ciliates in cockroaches, both in the stock cultures of Department of Zoology on Charles University as well as in those collected in nature. We studied their presence in various cockroach lineages, obtained 18S rRNA gene sequence data, and assessed their phylogenetic relationships. We also performed protargol staining of the cells to characterize the morphology of individual lineages.

Keywords: Anaerobiosis, symbiosis, ciliates, Clevelandellida, *Nyctotherus*, diversity, 18s rDNA, host specificity

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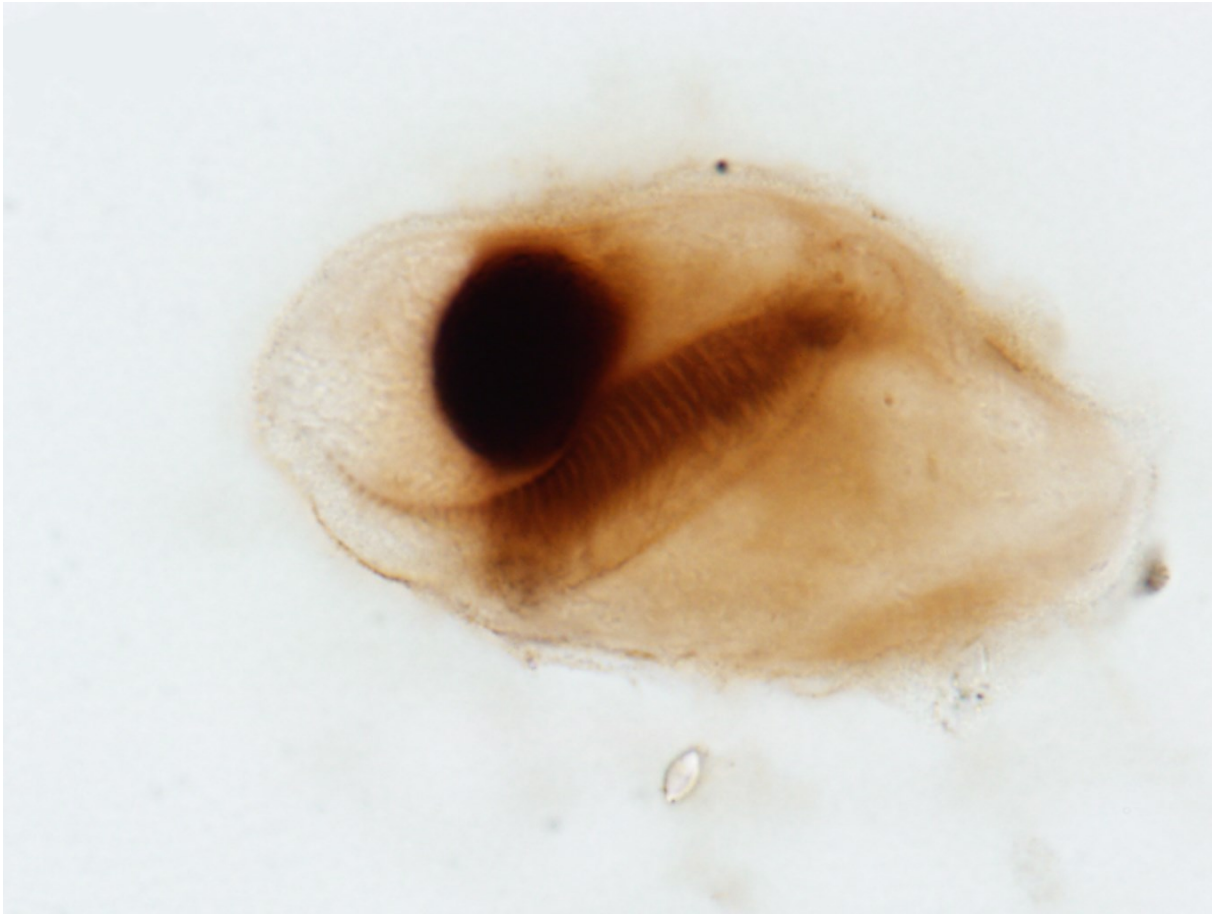


Nyctotherus sp. From intestine of the cockroach *Archimandrita tesselata* after protargol stainig

1. Introduction and goals

1.1. Introduction

Cockroaches (Blattodea) are a truly interesting group of insects – although they are not as rich in species (about 7500, Beccaloni, 2014) as more famous insect orders such as Coleoptera od Diptera, they often make up for it in abundances. Among the most (in)famous representatives are termites and synanthropic pest cockroaches which both are known to host a rich hindgut fauna.

Termites form a highly diverse monophyletic clade of cockroaches. They are so diverse and morphologically different from the rest of their kin that they were once thought to be a separate insect order, but currently are firmly considered as an inner lineage of cockroaches, being a sister group of another a wood-eating cockroaches: Cryptocercidae (Inward et al., 2007). There are two traditional groups – higher and lower termites – which differ in diet. Higher termites grow mushrooms serving as their food in their nests, and lower termites consume cellulose rich material such as grass, dry or damp wood or even lumber (depending on the family). Some species of mainly lower termites are economically important pests because they damage wooden structures (e.g., some representatives of families Rhinotermitidae, Kalotermitidae and Archotermopsidae) or crops (e.g., some Termitidae) (Su and Scheffrahn, 2000). Lower termites are generally known for their intestinal flagellate fauna. Higher termites do not harbour symbiotic flagellates but there were some ciliates found in them.

Synanthropic cockroach species such as notoriously known *Blattella germanica*, *Blatta orientalis*, *Supella longipalpa* or *Periplaneta americana* can make colonies of thousands. They are known to be vectors of many diseases (Gałęcki and Sokół, 2019) and they are therefore nightmare in almost everyone living in bigger cities where they can rapidly spread. These synanthropes are the first thing that everybody remembers when the word cockroach is mentioned, even though they represent only a small fraction of cockroach diversity – only about thirty out of more than 4500 species (excluding termites) are considered pests. Synanthropic cockroach pests do not form a single clade, but are scattered along the cockroach phylogenetic tree, belonging mostly to families Blattellidae, Blattidae and

Blaberidae. They are the most studied cockroaches and there is a considerable number of gut symbionts found to be living inside them. Among them there are again those pretty interesting ciliates.

The rest of cockroaches (non-synanthropic and non-termite Blattodea - about 4500 species) live their peaceful lives in leaf litter, caves, among the grass blades, under tree bark and in rotting wood. They are often found in narrow crevices where they hide from predators, frequently using spines on their legs to get stuck in the crevice and to make pulling them out much more difficult. Some of the lineages are omnivores, some are highly specialized regarding their diet.

At least some of these cockroaches are known to host a plethora of commensal, mutualistic or parasitic organisms in their intestines. They acquired the consortium of tiny tenants that they now host in their insides throughout millennia of evolution. These little creatures inhabit various parts of their host's digestive system. They belong to a wide array of unrelated lineages such as Excavata, Amoebozoa, and Alveolata. This thesis concerns only the latter group, a small ciliate order called Clevelandellida in the phylum Ciliophora, that happens to appear in termites and synanthropic pests as well as in various free-living cockroaches. There are two families of those ciliates reported from roaches – Nyctotheridae and Clevelandellidae. Nyctotheridae are widespread among many cockroach species, but Clevelandellidae only inhabits intestines of wood-feeding cockroaches of the cockroach subfamily Panesthiinae.

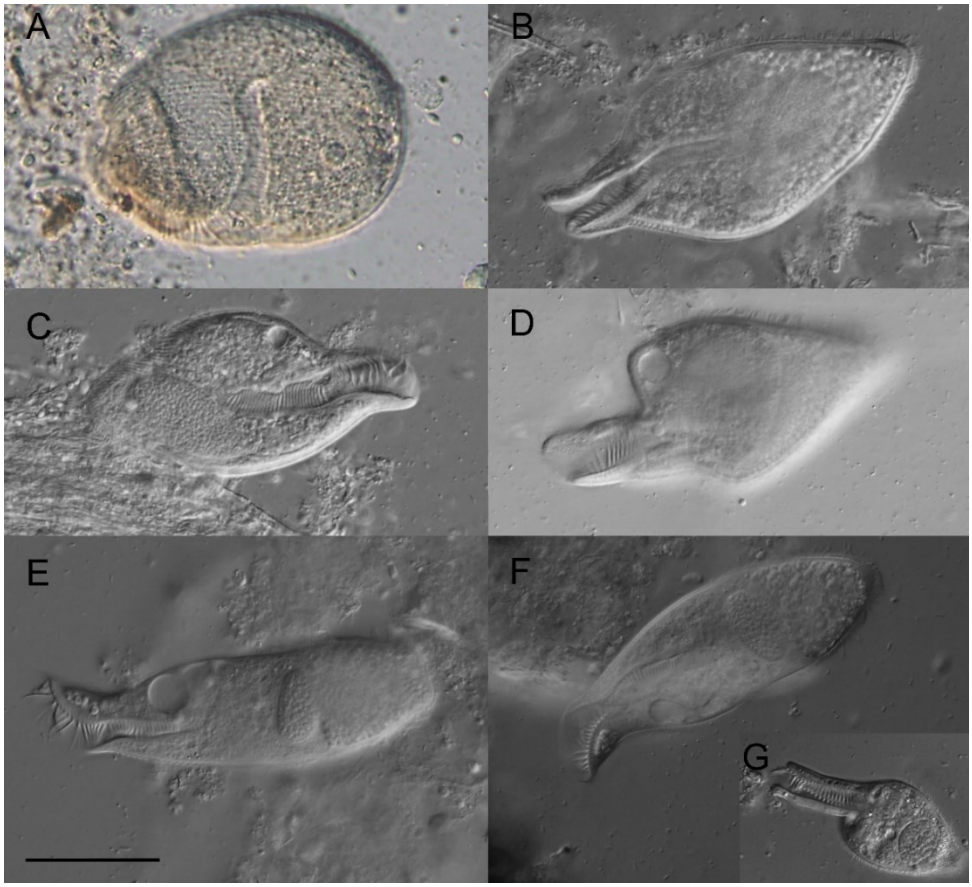
Clevelandellida are thought to be either beneficial symbionts or commensals, although some authors regard them as parasites (Affa'a, 1983; Affa'a et al., 2004; Lian-Xiang et al., 2002). At present, we have insufficient data, to know for sure whether they are beneficial for their host organisms in any way. The cockroaches can survive without their symbionts quite well, even though there is some evidence that roaches thrive better when the ciliates are present (Gijzen and Barugahare, 1992; Gijzen et al., 1994; Scrivener and Slaytor, 1994; Wharton et al., 1965).

Considering that these ciliates are relatively common in various animals, it is surprising how neglected they are in terms of research into their diversity by molecular methods. Most of them were described a long time ago, using only their morphological features. Molecular methods would certainly shed more light on the validity of already described species and

potentially reveal undiscovered diversity. Therefore, we decided to obtain sequences of genes coding 18S rRNA from ciliates living in cockroaches and to conduct a phylogenetic analysis in order to extend the knowledge on these, without any doubt interesting and important, organisms. We focused mainly on the clevelandellid family Nyctotheridae because it is much more widespread in cockroaches than the second cockroach-inhabiting family, Clevelandellidae, and there are other people in our lab that do research on Clevelandellidae.

1.2. Thesis goals

a) To examine available cockroaches for presence of clevelandellid ciliates and to gain information about the prevalence of cockroach ciliates.
b) To characterize individual ciliate strains with molecular methods, formulate a hypothesis based on the 18S rRNA gene and to look for any undiscovered lineages.
c) To gain some knowledge about host specificity of Clevelandellids.



Known species of ciliates residing in cockroaches. **A**: *Nyctotherus* sp. From *Periplaneta australasiae*, **B, C**: *Clevelandella panesthiae* and **D**: *Clevelandella parapanesthiae* from Panesthia angustipennis cognata, **E, F**: *Clevelandella constricta* from Salganea raggei, **G**: *Clevelandella hastula* from Panesthia sp. Scale: 50 µm. A – courtesy of Kateřina Poláková, B-G courtesy of Michael Kotyk.

2. Literature overview

2.1. Hosts

Cockroaches (Blattodea) are hemimetabolous insects that together with mantids (Mantodea) form a clade called Dictyoptera. Blattodea split from the common ancestor of Dictyoptera in early Carboniferous with crown groups appearing in Early Jurassic (Evangelista et al., 2019). According to the current knowledge, they form two large groups. The first one is Solumblattodea, which comprises families Blattidae, Corydiidae, Nocticolidae, Anaplectidae, and Cryptocercidae, plus nine families of Isoptera (Evangelista et al., 2019; Krishna et al., 2013). The second one is Blaberoidea which includes Ectobiidae, Blattellidae, Nyctoboridae, Pseudophyllodromiidae, and, notably, Blaberidae, the most diverse family of cockroaches excluding Isoptera (Djernæs et al., 2020; Evangelista et al., 2020). Cockroaches have various lifestyles ranging from inquilines in ant nests (e.g., genus *Attaphila*) through individually foraging species (most cockroaches, e.g., *Blaberus* spp., *Polyphaga* spp.), gregarious cockroaches (mostly pests), solitary to gregarious species eating decaying wood (Tryonicidae, and some Blaberidae: Panesthiinae, and Zetoborinae), sub-social wood feeders (*Salganea*, Panesthiinae) to eusocial termites (Bohn et al., 2021; Grandcolas, 1993, 1997; Howard and Thorne, 2010; Pellens et al., 2002; Thorne, 1997). Even really closely related cockroaches can maintain completely different lifestyles. Traditional Blaberid subfamilies Geoscapheiinae, burrowing cockroaches that eat dry leaves, and Panesthiinae, which consume decaying wood, were recently shown to all be in fact members of Panesthiinae (Lo Pinto et al., 2016). Geoscaphein morphology and diet preference therefore appeared multiple times along the evolution of this subfamily (Lo et al., 2016).

Most of the cockroaches are omnivores that eat almost any organic material they can find with preference for nutritious food such as ripe fruit, resin from injured plants, flower pollen, dead insects and so on. They are, however, also able to digest leaf litter, faeces of other animals and even unwholesome laboratory food-mixtures or paper as long as they can synthetize tryptophan and methionine from them (Noland and Baumann, 1951; Scrivener et al., 1989). There are also specialists adapted to digestion of decaying wood, which is surprising, because cockroach cellulases are quite inefficient and they make up for it by secreting large amounts (Scrivener and Slaytor, 1994). One clevelandellid family, the one

after which the whole order was named – Clevelandellidae –lives exclusively in the intestines of wood-feeding cockroach subfamily, Panesthiinae (Blaberidae).

2.2. General information about Clevelandellida

The order Clevelandellida de Puytorac & Grain, 1976, comprises five families: Clevelandellidae Kidder, 1938, Inferostomatidae Ky, 1971, Neonyctotheridae Affa'a, 1987, Nyctotheridae Amaro, 1972, and Sicuophoridae, Amaro, 1972 (see Adl et al., 2019).

Sicuophoridae contains seven genera: *Albaretia* Affa'a, 1986, *Geimania* Albaret, 1975, *Metasicuophora* Albaret, 1973, *Parasicuophora* Albaret, 1968, *Prosicuophora* de Puytorac & Oktem, 1969, *Spiroperistomatus* Amaro & Sena, 1967 and finally *Sicuophora* de Puytorac & Grain, 1969 The family Inferostomatidae comprises three genera, *Ichthyonyctus* Jankowski 1974, *Inferostoma* Ky, 1971 and *Nathella* Singh, 1953 (*nomen nudum* according to Aescht, 2001 for lack of type species fixation). Neonyctotheridae includes only one genus, *Neonyctotherus* Affa'a, 1983. Currently the largest family, Nyctotheridae comprises 13 genera and is currently the most genus (and species as well) rich family of Clevelandellida. The genera are: *Cichlidotherus* Affa'a, 1989, *Cryptonyctus* Jankowski, 1978, *Falconyctus* Jankowski, 1978, *Indonyctus* Jankowski, 1978, *Metanyctotherus* Albaret, 1970*, Micronyctus* Jankowski 1978*, Nyctositum* Affa'a, 1979, *Nyctotheroides* Grassé, 1928, *Nyctotherus* Leidy, 1849*, Paracichlidotherus* Grim, 1992, *Pronyctotherus* Albaret & Njiné, 1976, *Pygmotheroides* Affa'a, 1980 and *Vesonyctus* Jankowski, 1978. Finally, Clevelandellidae contains three genera: *Clevelandella* Kidder, 1938, *Metaclevelandella* Uttangi & Desai, 1963 and *Paraclevelandia* Kidder, 1937.(Aescht, 2001; Lynn, 2008)

Clevelandellida supposedly diverged from free-living metopids sometime between the Carboniferous (Paleozoic) and Jurassic (Mesozoic), and their crown diversification occurred during the Mesozoic (Fernandes and Schrago, 2019; Vďačný et al., 2019).

The order was named after the family, Clevelandellidae, which got this name as a tribute to Lemuel Roscoe Cleveland, a protistologist working with parabasalids (Excavata: Metamonada: Parabasalia) living in the hindgut of the wood-feeding cockroaches of the genus *Cryptocercus* and their termite relatives. He obtained some panesthiin cockroaches from one of his colleagues and since they harboured no flagellate symbionts that Cleveland

would be interested in, he gave them over to George W. Kidder in order to research the diversity of ciliates hidden inside those cockroaches (Kidder, 1937).

All clevelandellids live in intestines of a quite wide variety of hosts. All genera of the family Sicuophoridae as well as the representatives of the family Neonyctotheridae were described from intestine of frogs (Affa'a, 1983; Albaret, 1973). Inferostomatidae were described from fish (Ky, 1971). Some genera of family Nyctotheridae live in various vertebrates - *Nyctotheroides* inhabit the intestines of fish, amphibians, and reptiles (Affa'a, 1979a; Affa'a et al., 2004; Galavíz-Silva and Jiménez-Guzmán, 1986), *Cichlidotherus*, *Paracichlidotherus* and *Metanyctotherus* were all found in the bowels of fish (Affa'a, 1989; Grim, 1992; Grim et al., 1996), *Pygmotheroides* and *Nyctositum* were described from the intestine of frogs (Affa'a, 1979b, 1979a) - while the genus *Nyctotherus* with its type species, *N. velox*, isolated from julid millipede, resides in many invertebrate clades such as millipedes, earthworms and some insect orders including cockroaches, orthopterans, and beetles (Fokam et al., 2014; Lalpotu, 1980; Leidy, 1849, 1850; Zeliff, 1933). At least one *Nyctotherus* species (*N. teleacus*) was also described from a vertebrate (tortoise)(Geiman and Wichterman, 1937; Suzuki et al., 2020). The situation is completely different in case of family Clevelandellidae. Genera *Clevelandella* and *Paraclevelandia* are mostly strongly host-specific organisms living only in the hindguts of wood feeding cockroaches of subfamily Panesthiinae, often together with their *Nyctotherus* relatives which, at least in most cases, do not appear to show any strong host-specificity signal. The only known representative of the genus *Metaclevelandella* was described from a termite *Dicuspiditermes incola* (Uttangi and Desai, 1963).

All known species in Clevelandellida are endobiotic and anaerobic. Their lifestyle drove significant adaptations of their organelles and their metabolism. Hydrogenosomes, modified mitochondria that produces hydrogen, have been well studied in the species *Nyctotherus ovalis* (De Graaf et al., 2011). The [FeFe] hydrogenase of its hydrogenosome, is crucial for H_2 production, was likely obtained by lateral gene transfer (Boxma et al., 2007; Lewis et al., 2020). Hydrogen is then used by symbiotic methanogenic bacteria that live inside Clevelandellids to produce methane that then diffuses out of the ciliate cell (Gijzen et al., 1991).

2.3. Clevelandellida in cockroaches

All known clevelandellids that reside in cockroaches belong to families Nyctotheridae and Clevelandellidae. In the family Nyctotheridae there are at least 15 species of the genus *Nyctotherus* described from cockroaches including termites. All three genera of Clevelandellidae live only inside cockroaches. Table 2.1 shows clevelandellid species discovered in cockroaches. Figure 2.1 shows examples of in vivo captured cells encountered in various cockroaches

It is thought that they infect new hosts as cysts in faeces that other roaches occasionally eat, but it can work differently in e.g., sub-social genus *Salganea*, where the adults were observed exhibiting stomodeal trophallaxy – feeding their younglings from mouth to mouth (Hoyte, 1961a; Nalepa, 1991)

Family	Species	Type host	Author
C.	*Clevelandella parapanesthiae*	*Panesthia angustipennis angustipennis*	(Kidder, 1937)
C.	*Clevelandella panesthiae*	*P. a. angustipennis + P. a. spadica*	(Kidder, 1937)
C.	*Clevelandella hastula*	*P. a. angustipennis*	(Kidder, 1937)
C.	*Clevelandella nipponensis*	*P. a. spadica*	(Kidder, 1937)
C.	*Clevelandella elongata*	*P. a. angustipennis*	(Kidder, 1937)
C.	*Clevelandella contorta*	*P. a. angustipennis + P. a. spadica*	(Kidder, 1937)
C.	*Clevelandella constricta*	*P. a. angustipennis + P. a. spadica*	(Kidder, 1937)
C.	*Clevelandella kidderi*	*Panesthia* sp.	(Mandal and Nair, 1974)
C.	*Clevelandella lynni*	*P. a. angustipennis*	(Pecina and Vďačný, 2020)
C.	*Paraclevelandia brevis*	*P. a. angustipennis + P. a. spadica*	(Kidder, 1937)
C.	*Paraclevelandia simplex*	*P. a. angustipennis + P. a. spadica*	(Kidder, 1937)
C.	*Metaclevelandella termitis*	*Dicuspiditermes incola*	(Uttangi and Desai, 1963)
N.	*Nyctotherus ovalis*	*Blatta orientalis*	(Leidy, 1850)
N.	*Nyctotherus uichancoi*	*P. a. angustipennis*	(Kidder, 1937)
N.	*Nyctotherus panesthiae*	*P. a.* unknown ssp.	(Yamasaki, 1938)
N.	*Nyctotherus macrotermitis*	N/A *(Macrotermes bellicosus, M. natalensis)*	(Gisler, 1967)
N.	*Nyctotherus regalis*	N/A *(M. bellicosus, M. natalensis)*	(Gisler, 1967)
N.	*Nyctotherus basidentitermes.*	N/A (*Basidentitermes mactus*)	(Gisler, 1967)
N.	*Nyctotherus ebriensis*.	N/A (*Allognathotermes hypogeus*)	(Gisler, 1967)
N.	*Nyctotherus* sp.	*Cubitermes* spp.	(Gisler, 1967)

N.	*Nyctotherus termitis*	*Kalotermes militaris*	(Dobell, 1910)
N.	*Nyctotherus silvestrianus*	*Amitermes beaumonti + A. coachhelae, A. minimus, A. wheeleri, A. medius*	(Kirby, 1932)
N.	*Nyctotherus polyphagae*	*Polyphaga indica*	(Lalpotu, 1980)
N.	*Nyctotherus periplanetae*	*Periplaneta americana*	(Lalpotu, 1980)
N.	*Nyctotherus indica*	*Therea petiveriana*	(Bhaskar Rao, 1969)
N.	*Nyctotherus viannai*	"Wood-feeding roach"	(Pinto, 1926)
N.	*Nyctotherus buissoni*	"Wood-feeding roach"	(Pinto, 1926)
N.	*Nyctotherus hormeticae*	*Hormetica laeoigata*	(Carini, 1935)
N.	*Nyctotherus galerus*	*Panesthia a. cognata*	(Pecina and Vďačný, 2020)

Table 2.1: List of clevelandellid species described from cockroaches with type host or type plus additional hosts if mentioned in the works. "N." in first column stands for Nyctotheridae, "C." for Clevelandellidae. In Kiddder's (1937) paper, the species of cockroaches mentioned are *Panesthia javanica* and *P. spadica*, which were later synonymized with *P. angustipennis angustipennis* and *P. a. spadica*, respectively. *Nyctotherus macrotermitis* and *Nyctotherus regalis* are reported from *Bellicositermes natalensis* and *B. bellicosus*, which are younger synonyms for *Macrotermes natalensis* and *M. bellicosus*, respectively. Bhaskar Rao described *Nyctotherus indica* from *Corydia petiveriana*, which is currently a synonym of *Therea petiveriana*.Unfortunately, I could not find any information about type hosts of five species of *Nyctotherus* described from termites by Gisler, (Gisler, 1967), their occurrence is mentioned only in an old report from the parasite catalogue of Medical and Veterinary Zoology (1974).

2.4. Morphology of cockroach clevelandellids

Species of Nyctotheridae and Clevelandellidae, the two clevelandellid families living in cockroaches have notably distinct morphological features.

All Nyctotheridae are small to large (<80 µm to >200 µm) free-swimming ciliates, with a broadly ellipsoidal to ovoidal shape, and slightly laterally compressed. Longitudinal rows of ciliated dikinetids (doubled basal bodies) cover the whole surface of the cell (Grim, 1998; Paulin, 1967). Leidy's initial description (1849) of the type species, *N. velox*, was rather vague, and it took many years for better a description to appear (Ten Kate, 1927). *Nyctotherus* possesses a karyophore (fibres between the cortex and the macronuclear envelope) that divides the cell into anterior and posterior parts. The contractile vacuole is located at the posterior end of the cell near cytoproct (cell anus). It should be noted that different authors use different, and sometimes conflicting, terms of cell morphology and orientation for Nyctotheridae (Albaret, 1973; Kidder, 1937). Albaret (1973) provides a useful summary of morphologic terms and cell orientation for the Clevelandellida including Nyctotheridae which is followed in this study. The oral opening in nyctotheroids is on the "ventral" surface near the anterior end and continues with a channel (infundibulum), enclosing ciliary adoral membranelles that is curves dorsally and ends at the cytostome (the

opening into the cell pharynx at the end of which food vacuoles form). In most illustrations from the literature, Nyctotheridae are shown with their "right" side facing the viewer, thus the dorsal and ventral margins are on the viewers left and right respectively. Individual species differ in cell size, length, the shape of the infundibulum, the configuration and number of adoral membranelles, the macronuclear and karyophore morphology, and general shape of the cell.

The ciliates grow bigger when cockroaches are fed a carbohydrate-rich diet (Armer, 1944).They can, occasionally, grow to gigantic proportions. These giant forms also appeared in cockroach colonies being fed a balanced diet and even in those fed food deficient in carbohydrates. They were believed to be unable to initiate division because of some unknown factor (Hoyte, 1961b). It was later shown that these giant cells can normally undergo cell-division and give rise to normal ones (Lom, 1956). However, the exact factors leading to the development of these giant cells are unknown.

Clevelandellidae is a much more morphologically distinctive and diverse group. One of the strangest features of this group is that their oral opening has shifted to the posterior end of the cell. The most important morphological differences between the three genera of Clevelandellidae include the posterior peristomial projection in *Clevelandella* species, absence of such a projection in *Paraclevelandia* and absence of both peristomial projection and a karyophore in *Metaclevelandella*.(Kidder, 1937; Uttangi and Desai, 1963). Especially in the genus *Clevelandella* we see a striking morphological diversity in comparison to the family Nyctotheridae. W. Bourland (personal communication) re-emphasized the lack of cilia in the posterior body part of all Clevelandellidae of panesthiin hosts, already noted by Albaret (1973) but overlooked by other authors (Kidder 1937; Pecina and Vďačný 2020a,b), but overlooked by other authors (Kidder 1937; Pecina and Vďačný 2020a,b).

2.5. Current taxonomic position of Clevelandellida and phylogenetic relationships among them

Clevelandellids, according to both morphological and molecular data, belong to the phylum Ciliophora (commonly referred to as “ciliates”), part of the SAR clade where ciliates are one of subgroups of the clade Alveolata (others being dinoflagellates and apicomplexans) (Burki et al., 2020; Grattepanche et al., 2018).

Alveolates are defined by presence of “alveoli” (hence the name) - specialized vesicles underlying the plasmatic membrane and forming the so-called pellicle. Alveoli contain many unique proteins that do not appear elsewhere (Gould et al., 2011). In ciliates alveoli support the shape of the cell, although they can be greatly modified in other alveolate lineages (Hausmann and Hülsmann, 2010). In clevelandellids, they are greatly reduced. Ciliates are characterized by the presence of hair-like organelles, cilia, covering the outer membrane of their bodies and their oral cavities and by the presence of two types of nuclei – the vegetative, transcriptionally active macronucleus and transcriptionally inactive germinal micronucleus.

Within Ciliophora, Clevelandellids belong to subphylum Intramacronucleata characterized by assembly of microtubules inside of the macronucleus during division of the cell (Hammerschmidt et al., 1996; Lynn and Small, 1997)

Clevelandellida, together with a small order called Armophorida and the order Metopida, traditionally form the class Armophorea (Jankowski, 1968). It is one of the so called “riboclasses”, which means that they do not share any morphological traits but according to molecular methods they cluster together (Affa’a et al., 2004; Lynn, 2003). Clevelandellida was shown to form an internal branch of Metopida which makes metopids paraphyletic with respect to Clevelandellida (Bourland et al., 2017a, 2020). It seemed for a short time, that Armophorida, which comprises caenomorphids, could be actually more closely related to Litostomatea and Spirotrichea than to the rest of Armophorea (Bourland et al., 2017a). Phylogenenomic analysis however showed that they are close to Clevelandellida (Wang et al., 2021).

Clevelandellids are an internal branch of paraphyletic order Metopida (see Bourland et al., 2017b, 2017a, 2020). These are usually free-living bacteriovores mostly found in hypoxiuc sediment, although there is one endocommensal species, *Parametopidium circumlabens*, which lives in the intestines of sea urchins (Biggar and Wenrich, 1932; Bourland et al., 2020; Da Silva-Neto et al., 2016).

Most of clevelandellids were only described morphologically and we have molecular data for only a very small number of clevelandellid species, so the composition of families, genera, the delimitation of species and phylogenetic relationships are yet to be validated.

We do not have any molecular data for families Inferostomatidae and Neonyctotheridae and only one sequence (*Sicuophora multigranularis*) for Sicuophoridae (Li et al., 2018). Within Nyctotheroidae there are only two genera with only a few species molecularly characterized (three *Nyctotherus,* and six *Nyctotheroides* species sequenced). In Clevelandellidae the situation is better – seven species of two genera (*Clevelandella*, *Paraclevelandia*) are already sequenced (with sequences available through GenBank database) and only one monotypic genus, *Metaclevelandella*, still remains to be molecularly characterized (Lynn and Wright, 2013; Pecina and Vďačný, 2020). The available data however seem to be showing that Sicuophoridae branches at the base of Clevelandellida while Clevelandellidae is an internal branch of Nyctotheridae (Bourland et al., 2017b, 2020; Li et al., 2018).

3. Material and methods

3.1. Cockroaches

Protists were obtained by dissecting cockroaches from both the stock cultures of Department of zoology and from fresh material obtained from field trips made by us or our colleagues.

Until to dissection cockroaches were kept in stock cultures of Department of Zoology at 25 ± 2 °C, ambient relative humidity, and under L12:D12 photoperiod. They were housed in plastic boxes with appropriate substrate, shelters, and humidity. Every box was supplied with food (dog chow, oat flakes, fish flakes, apples) and water (toilet paper plugged tube) ad libitum. Members of the subfamily Panesthiinae were given logs of decayed wood as their primary food source, even though they occasionally eat the same food mixture as other roaches.

Prior to dissection, the cockroaches were euthanized by ethyl acetate vapours in a killing jar. The abdomen of killed animals was opened by a scissor cut through the pleural membrane connecting tergites and sternites on each side and pulling ventral and dorsal parts apart with tweezers. The hindgut was then removed from the abdomen using tweezers and scissors and cleaned off the remnants of fat body. Subadult nymphs were used when available, as those have shown to usually host more ciliates than smaller nymphs and adults (especially females), even though other developmental stages usually harboured some protists as well.

Two individuals per population were usually dissected with some exceptions (four of *Henschoutedenia flexivittata*, *Diploptera punctata*, both without any ciliates in any specimen, and *Pycnoscelus femapterus*, one of *Arenivaga tonkawa, Compsodes schwarzii, Episymploce sundaica, Ectobius sylvestris, Pseudomops septentrionalis*, *Deropeltis madecassa* and *Deropeltis paulinoi*, more than four of *Panesthia angustipennis angustipennis, P. a. cognata, Ancaudelia serratisima serratisima* and *Salganea ternatensis hirsuta*).

Current scientific names of cockroaches were adopted from Cockroach Species File webpage (Beccaloni, 2014). Cockroach phylogeny and taxonomy was used according to most recent studies (Djernæs et al., 2020; Evangelista et al., 2020).

3.2. Single-cell manipulation

After dissection, the hindgut was put into Dobell's medium and homogenized with tweezers. Then it was examined under an inverted light microscope (Motic AE2000). During the inspection of gut contents, individual ciliate cells were caught using glass micropipettes, washed in Ringer's solution, and then transferred to separate vials with 30 μl of Ringer's solution. They were then stored in a freezer at -80 °C to preserve DNA. After picking at least four cells, the rest of the gut content was also stored in -80 °C for later use. Each picked cell was photographed. Dobell's medium was prepared by adding 50 ml of sterile egg whites to 500 ml of Ringer's solution.

3.3. DNA isolation

Genomic DNA from ciliate single cell specimens was isolated using the MasterPure™ Complete DNA & RNA Purification Kit (Lucigen) with a slightly modified protocol (volumes of chemicals used in the protocol were divided by five to match the volume of Ringer's solution in samples; DNA was eluded into 30 μl of elution buffer). DNA from faecal samples was isolated using Quick-DNA™ Fecal/Soil Microbe MiniPrep Kit (Zymo Research) according to manufacturer's instructions.

3.4. 18S rRNA gene amplification (PCR)

After isolation we performed PCR to amplify the nuclear 18S rRNA gene. All components needed for the reaction excluding the primers were already present in the EconoTaq® PLUS Green (Lucigen) master mix used for the reaction. For each set of samples one negative control sample was added. Details of the PCR thermocycler program are shown in table 3.1.

All PCR was done using T100™ Thermal Cycler (BioRad).

No. of repeats	Temperature	Time	Phase
1x	94 °C	4 min	Initial denaturation
30x	94 °C	1 min	Denaturation
	55 °C	1 min	Annealing
	72 °C	4 min	Elongation
1x	72 °C	15 min	Final elongation
∞	4 °C	∞	Resting temperature

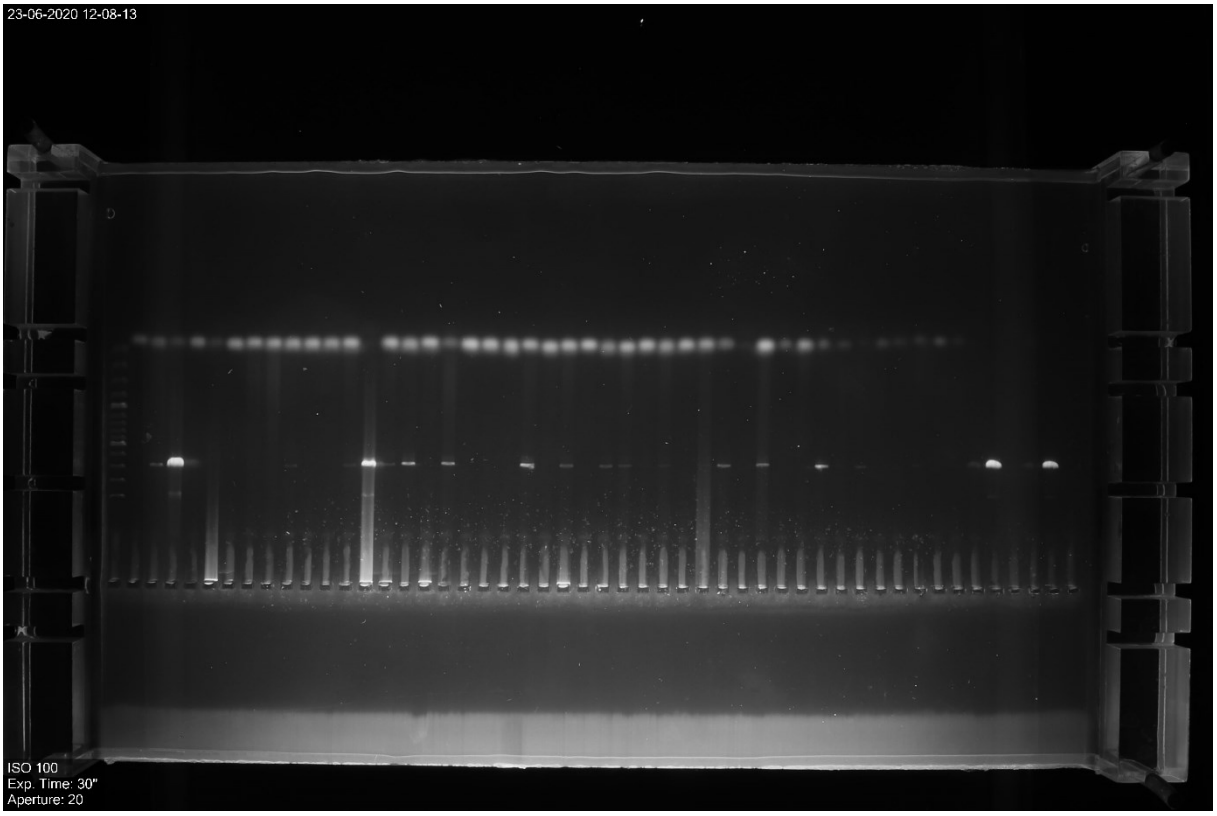
Table 3.1: Details of individual steps of PCR cycles.

3.5. Electrophoresis

Following PCR, the samples were checked by 1% agarose gel electrophoresis to validate if there was any signal. 3 µl of sample were used for every run. We used GeneRuler™ DNA Ladder mix as a ladder.

The composition of the gel was 1 g of agarose per 100 ml of tris-borate buffer and approximately 2,5 µl of SYBR green dye for each 50 ml of gel. The voltage and timer of every run was set to 100 V and 60 min, respectively. For all electrophoresis runs we used PowerPac™ Basic power supply (BioRad).

Results were visualized with green and blue LED light in the “FAS-Digi PRO” gel imager (Nippon Genetics). Gels were photographed with a Cannon EOS 200D and Camera Studio 1.0 software (Nippon Genetics).



Picture 3.1: Example of electrophoresis gel. Glowing bands in the middle row indicate that DNA is present in the sample and thus all the steps up until now were successful. columns without-bands indicated no DNA amplification and these samples were therefore omitted from further analysis

3.6. PCR product purification

After confirming DNA amplification by electrophoresis, we removed excess nucleotides and other remnants of chemicals used for PCR. The DNA was purified using ExoSap-IT™ (Applied Biosystems): We diluted the ExoSap-IT™ solution with nuclease-free water (ratio 1:10). We then mixed it with PCR product according to manufacturer's description (2 µl of diluted ExoSap-IT™ per 5 µl of sample – in total 9 µl of diluted ExoSap-IT™ and 22 µl of PCR product). We also prolonged the durations of individual parts of the run in the cycler (table 3.2.).

Purification was done using T100™ Thermal Cycler made by BioRad into T100™ Thermal Cycler made by BioRad.

Phase	Temperature	Duration
Degradation of PCR remnants	37 °C	60 min
Exo-Sap Inactivation	80 °C	15 min
Resting temperature	4°C	∞

Table 3.2: details of individual phases of the enzymatic PCR purification process.

3.7. Sequencing

Enzymatically purified PCR products were sequenced at the DNA sequencing laboratory of the Charles University Biotechnology and Biomedicine Center in Vestec (BIOCEV) using an Applied Biosystems Genetic Analyzer system. Each sample sent to them consisted of 4 µl of the diluted DNA, 3 µl of H_2O (pure, for molecular work) and 1 µl of corresponding primer (concentration 3,2 nmol). As a quality control, some sequences were also sent to Macrogen. Those samples were prepared by mixing 5 µl of diluted DNA and 5 µl of primer (concentration 5 nmol) No significant differences were detected between the two facilities.

For sequencing ciliates 18S rRNA I used five primers: – "ArmF1" also used for PCR, forward primers "1055F" and "577F", and two additional reverse primers either "577R" or 1055R for instances where 577R gave poor results. Primer sequences are written in table 3.4.

Phase	Temperature	Author
577F	5'-GCCAGCMGCCGCCGT-3'	(Elwood et al., 1985)
577R	5'-ACCGCGGCKGCTGGC-3'	(Elwood et al., 1985)
1055F	5'-GGTGGTGCATGGCCG-3'	(Elwood et al., 1985)
1055R	5'-CGGCCATGCACCACC-3'	(Elwood et al., 1985)
ArmF1	5'-GCGAYATRTCATTCAAGT-3'	(Bourland et al., 2017a)
MedlinB	5'-TGATCCTTCTGCAGGTTCACCTAC-3'	(Medlin et al., 1988)

Table 3.3: primers used for polymerase chain reaction and for sequencing with their respective sequences.

3.8. Sequence editing

Primer sequences were trimmed, and sequences were combined into assembly sequences (contigs). Then the quality of individual bases in each chromatograph was manually checked for errors and finally they were converted to consensus sequences.

3.9. Phylogenetic analysis

All available 18S rDNA sequences for Clevelandellida from the GenBank database were used for the tree construction (*Clevelandella* spp: 8., *Sicuophora: 1, Nyctotheroides* spp.: 8, *Nyctotherus* spp.: 15, 32 sequences in total) to which we added 70 new sequences.

Metopids of the IAC clade (representatives of genera *Atopospira, Idiometopus, Metopus* and *Parametopidium,* 12 in total) were chosen as the outgroup because recent phylogenetic studies suggested that Clevelandellida is an inner branch of IAC clade of metopids (Bourland et al., 2020; Li et al., 2018).

Our sequences used for the analysis came from various sources that can be identified using given codes (Fig. 4.1). Those starting with "Z" (e.g., "Z58ET") were sequences obtained from frozen gut contents of various cockroaches excluding the family Panesthiinae. Sequences labelled with letter "B" (e.g., "B72BAN") were obtained from single cells from a variety of representatives of Blattodea (except the subfamily Panesthiinae), and sequences labelled with "P" (e.g., "P36PAC") were obtained from single cells from members of the wood-feeding family Panesthiinae.

Sequences were put into Geneious prime (version: 2021.2.2) where they were aligned using the MAFFT algorithm, version: 7.450 with default settings (Katoh and Standley, 2013; Katoh et al., 2002).

The analysis was performed by using both maximum likelihood (ML) and Bayesian inference (BI) methods. Maximum likelihood analysis was conducted using the program RAxML version 8.0.0 (Stamatakis, 2014). Maximum likelihood trees (100 independent analyses - model GTRGAMMAI) were bootstrapped with 100 replicates. Bayesian analysis was performed using MrBayes 3.2.2. (Ronquist et al., 2012) using the GTR + G + I model. Four MCMCs were run for 4000000 generations until the mean standard deviation of split frequencies based on

the last 75% generations was lower than 0.01, with a sampling frequency of 1000 generations. The first 25% of trees were removed as burn-in.

We consider ML values <70 as "low" statistical support, 70–94 as "moderate" support, and ≥95 as "high" support (Hillis and Bull, 1993). For Bayesian posterior probabilities, we consider values <0.9 as low, 0.9-0.94 as moderate, and ≥0.95 as high support (Alfaro et al., 2003).

3.10. Light microscopy

Cells were observed using two different microscopes: an Olympus BX51 for both detailed observation of living cells and for examining protargol preparations. An inverted microscope, Motic AE2000 was used for picking the ciliates. Ciliates were observed in gut content diluted in Ringer's solution. For examining the fixed cells, we used Olympus BX 51 microscope.

3.11. Protargol staining

The morphology of ciliates was examined in protargol stained slides. We used the following protocol for their preparation.

Protargol staining protocol following Foissner (2014) **and modified by Bourland** (Pan et al., 2013)**:**

1) Cell fixation

a) Using the inverted microscope, cells were picked with a micropipette and fixed in 10% aqueousl formalin (approximately 4% formaldehyde).

b) Fixed cells were washed three times in tap water. Afterwards they were transferred into dilute Mayer's albumen (1% aqueous solution).

c) Cells (about five per slide) in a drop of diluted Mayer's albumen were then mounted on a clean slide and dried at room temperature overnight.

2) Impregnation

a) After drying, slides with dried drop of albumen with cells inside were first put into 95% isopropanol for 15 minutes to firmly attach cells to the slide.

b) Then they were placed into 70% isopropanol for 5 minutes.

c) Afterwards the slides were put into tap water for five minutes two times in a row to rehydrate. (Note: We have discovered that, although tap water has been recommended (Foissner, 2014; Pan et al., 2013), the "hardness" of water varies widely between communities and Prague tap water is unsuitable for use in protargol impregnation due to precipitation of calcium salts during heating. We were able to interpret our preparations using tap water despite these precipitates, but we have now reverted to using only NanoPure™ Water for all steps).

d) Slides were then soaked in aqueous 0.2% potassium permanganate for exactly two minutes.

e) After bleaching in potassium permanganate, they were rinsed with tap water (see note above in step c).

f) Slides were then transferred into aqueous 2.5% oxalic acid for 3 minutes

g) The next step was to rinse them twice in tap water for three minutes and then finally for another 3 minutes in NanoPure™ Water.

h) Finally, the slides were placed in pre-warmed 0.4% protargol (Merk) (60°C) for 10-30 minutes.

3) Developing

a) Slides with several drops of protargol solution were put under BX51 microscope (the solution was left on the slide to prevent it from drying out).

b) Two or three drops of acetone developer (see table 3.4) were added onto each slide and mixed with the protargol while observing development of the ciliature.

c) After oral ciliature started to develop, the fluid was poured off.

d) Afterwards, the slides were dipped in tap water 3 times, then in 2,5% sodium thiosulphate (3 s) to stop the development and then again 3 times in tap water to wash off the sodium thiosulphate.

e) Slides were then put into tap water for 3 minutes, then into 70% isopropanol for 5 minutes and finally into 100% isopropanol for another 5 minutes

4) Mounting

a) After dehydration in isopropanol, slides were transferred to xylene for 10 minutes

b) They were then mounted with DPX mounting medium (Sigma-Aldrich) and covered with cover glass and left flat to harden.

c) After hardening, the slides were ready for examination.

Order	Component	Volume
1.	H_2O – distilled water	80 ml
2.	H_3BO_3 – boric acid	1,4 g
3.	$C_6H_6O_2$ - hydroquinone	0,3g
4.	Na_2SO_3 – sodium sulphite	2g
5.	$(CH_3)_2CO$ - acetone	15ml

Table 3.4: Composition of acetone developer. Individual components were added by order noted in the first column. Each step followed only after the chemical from the previous one was fully dissolved.

3.12. Photo documentation

Images of both living cells and protargol slides were taken using the Olympus DP71 digital camera with QuickPHOTO CAMERA 2.3 (PROMICRA) at a magnification of 400x - 1000x.

4. Results

4.1. Phylogenetic tree

Phylogenetic tree of Clevelandellida made of 114 sequences of 18S rDNA gene is shown in figure 4.1. We also used all available sequences of Clevelandellida and some closely related metopids from GenBank database.

Trees that resulted from both methods, Maximum likelihood, and Bayesian analysis, were highly congruent. They only differed in the topology of four sequences in clade X. Relationships of these four sequences were poorly supported in both analyses. We chose to use the topology inferred from ML tree.

We chose to divide *Nyctotherus* spp. into clades and we chose to accept individual clades as monophyletic on bootstrap value over 75. This resulted in formation of 12 individual *Nyctotherus* clades plus Clevelandellidae. Six clades (II, III, VI, VIII, IX and XI) were composed solely of new sequences. Clade interrelationships were mostly unsupported. The branch comprising *Nyctotherus*, *Nyctotheroides* and Clevelandellidae was moderately supported in ML and fully supported in BI (BS: 76, PP: 1), *Nyctotherus teleacus* clustered with *Nyctotheroides* but with low support. The analysis included *Nyctotherus velox* from a julid millipede into our cockroach clades.

Sicuophora multigranularis (Sicuophoridae) turned out to be a sister taxon to Nyctotheridae + Clevelandellidae, and monophyly of Clevelandellida was strongly supported (BS:99, PP:1).

Monophyly of clades I and II was not supported well by bootstrap values, but Bayesian analysis yielded better result (BS: 67, PP: 0.98). **Clades II** and **III** alone, however, had maximum support (BS:100, PP: 1). **Clade IV**, which includes *Nyctotherus galerus*, was moderately well supported in ML and fully supported in BI (BS: 82, PP: 1) with high support values of inner branches overall.

Clades V to XII (plus Clevelandellidae) formed a highly supported lineage (BS: 99, PP: 1). Relationships between most of its inner clades were however not resolved. **Clade V** was highly supported as a sister branch to all the others (BS: 95, PP 1). Two GenBank sequences that were labelled as *Nyctotherus ovalis* appeared in this clade.

The monophyly of branch splitting into **clades VI** and **VII** had low support in ML but maximum support in BI (BS: 66, PP: 1). Since it did not exceed bootstrap value of 75, we regarded both sequences as separate clades.

Clade VIII had maximum support (BS:100, PP 1). The internal relationships of this clade are also very well supported. **Clade IX** was represented by only a single sequence and their split with clade X had a low support in ML.

Clade X was robustly supported by both ML and Bayseian methods (BS: 99, PP: 1). The topology of first four sequences in clade X varied between the Maximum likelihood and Bayesian tree. Further internal branches are relatively well supported. Most of the GenBank sequences labelled as *Nyctotherus ovalis* turned out to belong to this clade. **Clade XI**'s monophyly was highly supported (BS: 79, PP: 1) and its short internal branches were mostly robustly supported as well.

Monophyly of Clevelandellidae was robustly supported with almost maximum values (BS: 98, PP: 1). With high support, it appears to be a sister taxon to *Nyctotherus* clade XII (BS: 79, PP: 1). **Clade XII** was very well supported by both methods (BS: 90, PP: 1) and its internal branches were also well supported. It was the clade with the most representatives found by our team.

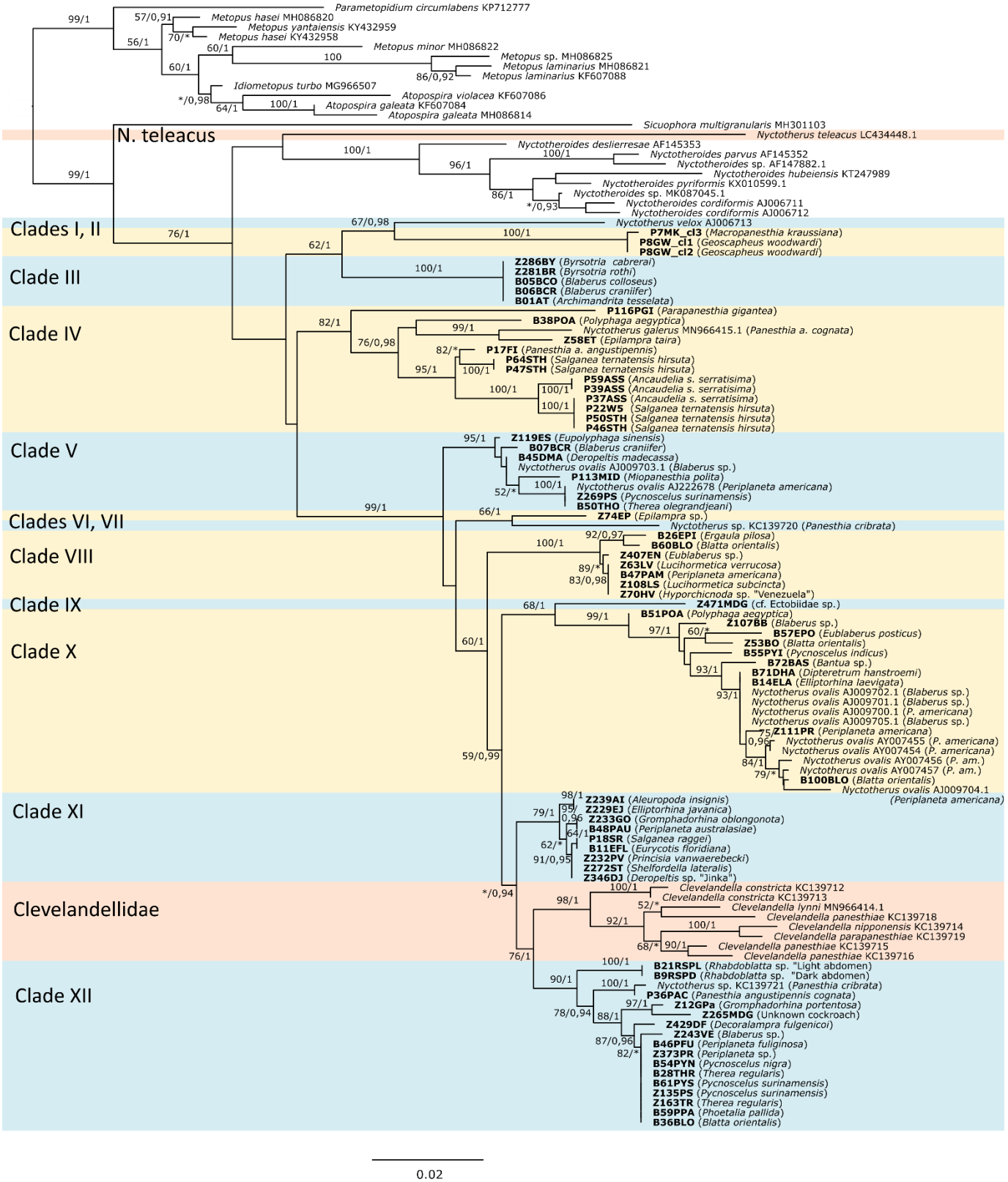


Figure 4.1: Phylogenetic tree inferred from 18S rRNA gene sequences. Only bootstrap values >50 and posterior probabilities > 0,9 are shown. "*" marks ML values <50 and BI values <0,9. New sequences from the current study are in bold. Host names appear in brackets following the identification code.

4.2. Presence of ciliates in cockroach families

First, we looked at which clades of ciliates were present in which group of cockroaches. There was no clade appearing exclusively in Solumblattodea. Representatives of clades II, III, VI, VII and IX were found only in cockroaches of the group Blaberoidea.

Clade I has not been reported from cockroaches.

Clade II appeared in two members of Blaberidae: Panesthiinae, *Geoscapeheus woodwardi* and *Macropanesthia kraussiana*.

Clade III is only present in Blaberidae: Blaberinae. There is no record of it from any other cockroach family and it is the only clade (out of families that have more than two representatives with infected individuals) with this trait. It was found in *Archimandrita tesselata*, *Blaberus colosseus*, *B. craniifer*, *Byrsotria rothi,* and *B. cabrerai*.

Clade IV was mostly found in cockroaches belonging to Blaberidae: Panesthiinae. They include *Ancaudelia serratisima serratisima, Parapanesthia gigantea, Panesthia a. angustipennis, P. a. cognata* (GenBank – MN966415), and *Salganea ternatensis hirsuta*. It was however also found in Blaberidae: Epilamprinae (in an unknown species of the genus *Epilampra*) and in Corydiidae (*Polyphaga aegyptica*).

Nyctotherus spp. belonging to clade V were scattered throughout the cockroach families, having one representative in Blaberidae: Panesthiinae (*Miopanesthia polita*), one in Blaberidae: Pycnoscelinae (*Pycnoscelus surinamensis*), two in Blaberidae: Blaberinae (*Blaberus craniifer* and *Blaberus* sp. from GenBank – AJ009703), two in Blattidae (*Deropeltis madecassa* and *Periplaneta americana* – GenBank - AJ222678) and finally two in Corydiidae (*Eupolyphaga chinensis,* and *Therea olegrandjeani*).

Clades VI, VII and IX were each represented in only single cockroach population. The record of a *Nyctotherus* clade VI was from *Epilampra taira* belonging to Blaberidae: Epilamprinae. Clade VII did not appear in our cockroaches at all, but was reported from *Panesthia cribrata* (Blaberidae, Panesthiinae) (Lynn and Wright, 2013). Clade IX only appeared in one unidentified ectobiid species (Blaberidae: Ectobiidae).

Clade VIII was found in representatives of three families: Blattidae (*Periplaneta americana* and *Blatta orientalis*), Corydiidae (*Ergaula pilosa*) and Blaberidae: Blaberiinae (*Eublaberus* sp., *Hyporhicnoda* sp. "Venezuela", *Lucihormetica verrucosa,* and *L. subcincta*).

Clade X was quite widely distributed among the cockroach families. We found it in Blattidae (six GenBank sequences of ciliates from *Periplaneta americana* - AJ009700, AY007454, AY007455, AY007456, AY007457, and AJ009704, as well as from protists from one of our *P. americana* colonies and from two separate populations of *Blatta orientalis*), Corydiidae (*Polyphaga aegyptica*), Ectobiidae (*Dipteretrum hanstroemi*), also in Blaberiidae: Pycnosceliinae (*Pycnoscelus indicus*) and in Blaberiidae: Blaberiinae (*Bantua* sp., our *Blaberus* sp., *Blaberus* sp. from GenBank – AJ009701, AJ009702, and AJ009705, and *Eublaberus posticus*)

Clade XI appeared in three families: Blattidae (in *Periplaneta australasiae*, *Deropeltis* sp. "Jinka" and in *Eurycotis floridiana*), Blaberidae: Panesthiinae (in *Salganea raggei*) and Blaberidae: Oxyhaloinae (*Aeluropda insignis, Elliptorhina javanica, Gromphadorhina oblongonota,* and *Princisia vanwaerebecki*).

The widest distribution was recorded for clade XII. It inhabited representatives of seven families: Blattidae (in *Periplaneta fuliginosa, Periplaneta* sp., and *Blatta orientalis*), Corydiidae (in *Therea regularis*), Blaberidae: Blaberinae (in *Blaberus* sp. and *Phoetalia* pallida), Blaberidae: Oxyhaloinae (in *Gromphadorhina portentosa*), Blaberidae: Panesthiinae (in *Panesthia angustipennis cognata,* and *Panesthia cribrata* from GenBank - KC139721), Blaberidae: Pycnosceliiane (*Pycnoscelus* cf. *surinamensis*, *P. surinamensis,* and *P. nigra*) and Epilamprinae (in *Decoralampra fulgenicoi*, *Rhabdoblatta* sp. "Light abdomen, and *Rhabdoblatta* sp. "Dark abdomen").

We did not deal with Clevelandellidae in this study, but we found multiple species of those ciliates in every wood feeding panesthiin cockroach we dissected.

The only cockroach family where we found only two *Nyctotherus* clades (clade IV and VI) was Ectobiidae.

There were three (sub)families that hosted three different *Nyctotherus* clades. Blaberidae: Oxyhaloinae hosted clades X, XI and XII, Blaberidae: Epilamprinae hosted clades IV, VI and XII and finally Balebridae: Pycnoscelinae with clades V, X and XII.

4.3. Distribution of clevelandellid lineages among cockroaches

We examined 108 different populations of cockroaches belonging to 62 genera and 91 species. We found *Nyctotherus* spp. in 65 populations in 53 species representing 35 genera.

Table 4.1 shows species and, when available, various populations of individual species that we searched for presence of clevelandellids. At least two cockroach specimens from each population were examined, only the first one is always noted in the table to keep it shorter and easier to navigate through. All but one population contained only one ciliate lineage.

"Captivity" as the locality is used for populations bred in captivity for many years with a high chance of contamination among individual populations in hobby, because most hobby breeders did not distinguish among different populations of the same species until recently.

There were only 3 cockroach populations that harboured two separate *Nyctotherus* lineages. *Blaberus craniifer* from Key West in Florida was the only species of the ones examined by us harbouring members of two *Nyctotherus* clades in members of one colony. Lynn (2013) found two separate *Nyctotherus* lineages in *Panesthia cribrata*. *Salganea ternatensis hirsuta* from Wanang in Papua New Guinea hosted two *Nyctotherus* spp. that appeared somehow distant on the phylogenetic tree but belonged to the same clade.

We found clevelandelids in two families of Solumblattodea. According to our findings, members of Blattidae harbour *Nyctotherus* members of clades V, VIII, X, XI and XII with some cockroach species hosting more than one clade in colonies that originated in different locations (e.g., clades V, VIII and X in *Periplaneta americana*, clades VIII, X and XII in *Blatta orientalis*). Across the genus *Periplaneta* we found protists belonging to five separate *Nyctotherus* clades (V, VIII, X, XI, and XII).

The only species of the family Nocticollidae we studied did not contain any ciliates.

Cockroaches of the family Corydiidae harboured *Nyctotherus* spp. belonging to clades IV, V, VIII, X and XII with two populations of *Polyphaga aegyptica* hosting two different clades (IV and X).

In Blaberoidea we searched for ciliates in members of four families. Subfamilies of Blaberidae were treated separately because the whole family is the largest among

cockroaches. One specimen was a frozen intestine of a cockroach for which all the data beside locality was lost.

There were no clevelandellids in any cockroach species belonging to families Blattellidae and Phyllodromiidae and in four subfamilies of Blaberidae: Gyninae, Paranauphoetiinae, Diplopterinae and Perisphaeriinae.

One representative of clade IX and one of clade X were present in Ectobiidae.

The family to which most of our specimens belonged was Blaberidae. It is the biggest family They served as hosts for five different *Nyctotherus* clades (III, V, VIII, X, and XII). Across four examined species of genus *Blaberus* we found four separate *Nyctotherus* lineages (clades I, III, VI and VIII).

Most representatives of the family Oxyhaloinae hosted *Nyctotherus* belonging to clade XI. Two other *Nyctotherus* clades (X and XII) were present in cockroaches of this family.

Panesthiinae mostly harbour *Nyctotherus* clade IV, although we also found representatives of clades II, V, XI, and XII.

Pycnoscelinae hosted mostly *Nyctotherus* clade XII although clades V and X were also present in two different species examined.

Most representatives of family Epilamprinae that we dissected harboured *Nyctotherus* of clade XII, although different clades were also present (IV, VI). *Epilampra* sp. from Nouragues Inselberg in French Guyana was the only host we dissected that harboured *Nyctotherus* clade VI.

According to other studies and descriptions of GenBank sequences, there were nyctotherids present in three or more populations of *Periplaneta americana*, two of *Blaberus* sp., one of *Panesthia angustipennis cognata* and one of *Panesthia cribrata.* Sequences from *Panesthia cribrata* (two ciliate lineages) clustered into clades VII (the only record of that clade) and XII. Sequences from ciliates of *Periplaneta americana* fell into clades V and X as well as sequences from symbionts of *Blaberus* sp. *Nyctotherus galerus* described from *Panesthia angustipennis cognata* clustred in clade IV.

Finally in four families we found five clades in each. Blattidae harboured clades V, VIII, X, XI, and XII. Corydiidae hosted *Nyctotherus* clades IV, V, VIII, X, and XII. Blaberidae: Blaberinae

comprised cockroaches hosting representatives of clades III, V, VIII, X, and XII. Blaberidae: Panesthiinae hosts clades II, IV, V, XI, and XII.

Figure 4.2.: Living cells of *Nyctotherus*. Examples of encountered lineages. **A**: *Nyctotherus* sp from *Miopanesthia polita*, **B**: *Nyctotherus* sp. From Salganea ternatensis hirsuta **C**: *Nyctotherus* sp. from *Salganea raggei*, **D**: cyst of *Nyctotherus* sp. from *Elliptorhina laevigata*, **E, F**: small and large specimens of *Nyctotherus* from *Parapanesthia gigantea*, **G, H, I**: *Nyctotherus* sp. from *Rhabdoblatta* sp. Scale 10 µm. Images courtesy of Michael Kotyk.

Cockroach families				*Nyctotherus*
Genus/Clade	Species	Code	origin of colony	clade
Solumblattodea				
Blattidae				
Periplaneta	*americana*	B47PAM	Nyassosso village, Cameroon	VIII
Periplaneta	*americana*	Z111PR	Fuzhou, China	X
Periplaneta	*australasiae*	B48PAU	Mamba village, Cameroon	XI
Periplaneta	*fuliginosa*	B46PFU	Emei Shan, China	XII
Periplaneta	sp.	Z373PR	Mt. Malambo, Phillipines	XII
Neostylopyga	*rhombifolia*	B63NRH	Madagascar	N/A
Deropeltis	*paulinoi*	B44DPA	Namibia	N/A
Deropeltis	*madecassa*	B45DMA	Madagascar	V
Deropeltis	sp. "Jinka"	Z346DJ	Jinka, Ethiopia	XI
Pseudoderopeltis	sp.	B73PDE	RSA, Oribi	N/A
Melanozosteria	*nitida*	B19MEN	Philippines	N/A
Eurycotis	*floridiana*	B25EFL	Torch Key, Florida, USA	N/A
Eurycotis	*floridiana*	B11EFL	Sarasota, Florida, USA	XI
Blatta	*orientalis*	Z53BO	Porto, Portugal	X
Blatta	*orientalis*	B36BLO	Liege, Belgium	XII
Blatta	*orientalis*	B60BLO	Baku, Azerbaijan	VIII
Blatta	*orientalis*	B100BLO	Varna, Bulgaria	X
Shelfordella	*lateralis*	Z272ST	captivity	XI
Nocticollidae				
Nociticola	sp.	B22NOC	Malaysia	N/A
Corydiidae				
Arenivaga	*tonkawa*	B17ART	San Antonio, Texas, USA	N/A
Compsodes	*schwarzii*	B24CSW	Pena Blanca, Arizona, USA	N/A
Ergaula	*pilosa*	B26EPI	captivity	VIII
Eucorydia	*yasumatsui*	B15EYA	Ryuku archipelago, Japan	N/A
Eupolyphaga	*sinensis*	119ES	Xian, Shaanxi, China	V
Polyphaga	*aegyptica*	B38POA	Georgia	IV
Polyphaga	*aegyptica*	B51POA	Korfu	X
Polyphaga	*aegyptica*	B43POA	Tunisia	N/A
Polyphaga	*aegyptica*	B40POA	Sinai, Egypt	N/A
Therea	*bernhardti*	B31THB	captivity	N/A
Therea	*regularis*	B28THR	captivity	XII
Therea	*regularis*	Z163TR	captivity	XII
Therea	*olegrandjeani*	B50THO	captivity	V
Blaberoidea				
Ectobiidae				
cf. Ectobiidae	sp.	Z471MDG	Madagascar	IX
Ectobius	*sylvestris*	B35ESY	Czech Republic	N/A
Dipteretrum	*hanstroemi*	B71DHA	captivity	X
Parcoblatta	*fulvescens*	B75PFU	captivity	N/A
Blattellidae				

cf. *Symploce*	sp.	B79SYM	Xianmen, China	N/A
Episymploce	*sundaica*	B30ESU	captivity	N/A
Blatella	*vaga*	B32BVA	captivity	N/A
Blatella	*germanica*	B95BGE	Nachichevan, Azerbaijan	N/A
Blatella	*germanica*	B101BGE	Ethiopia	N/A
Paratemnopteryx	*coulloniana*	B88PCO	captivity	N/A
Pseudomops	*septentrionalis*	B34PSE	RSA, Oribi	N/A
Loboptera	*decipiens*	B103LOD	Brač, Croatia	N/A
Ischnoptera	*rufa*	B107ISR	captivity	N/A
Pseudophylodromiidae				
Balta	*notulata*	B12BAN	Japan	N/A
Supella	*longipalpa*	B90SUL	Athens, Greece	N/A
Supella	sp.	B85SUS	RSA	N/A
Supella	cf. *dimidiata*	B83SUD	Somaliland	N/A
Blaberidae - Blaberinae				
Archimandrita	*tesselata*	B01AT	captivity	III
Bantua	*sp.*	B72BAS	RSA, Hogsback	X
Blaberus	*colosseus*	B05BCO	captivity	III
Blaberus	*craniifer*	B06BCR	Key West, Florida	III
Blaberus	*craniifer*	B07BCR	Key West, Florida	V
Blaberus	sp.	Z243VE	Venezuela	XII
Blaberus	sp.	Z107BB	Rurenabaque, Bolivia	X
Byrsotria	*cabrerai*	Z286BY	captivity	III
Byrsotria	*fumigata*	B58BFU	captivity	N/A
Byrsotria	*rothi*	Z281BR	captivity	III
Cyrtotria	sp.	B67CYR	Malawi	N/A
Derocalymma	sp.	B77DEC	RSA	N/A
Eublaberus	*sp.*	Z407EN	Nouragues, French Guyana	VIII
Eublaberus	*posticus*	B57EPO	captivity	X
Hyporhicnoda	sp. "Venezuela"	Z70HV	Venezuela	VIII
Phoetalia	*pallida*	B59PPA	captivity	XII
Lucihormatica	*subcincta*	Z108LS	captivity	VIII
Lucihormatica	*verrucosa*	Z63LV	captivity	VIII
Blaberidae - Gyninae				
Gyna	*lurida*	B65GLU	captivity	N/A
Gyna	*caffrorum*	B81GYC	captivity	N/A
Blaberidae - Paranauphoetinae				
Paranauphoeta	*formosana*	B91PAF	captivity	N/A
Blaberidae - Diplopterinae				
Diploptera	*punctata*	B49DIP	captivity	N/A
Blaberidae - Oxyhaloinae				
Aeluropoda	*insignis*	Z239AI	captivity	XI
Elliptorhina	*javanica*	Z229EJ	captivity	XI
Elliptorhina	*laevigata*	B14ELA	captivity	X
Henschoutedenia	*flexivittata*	B69HFL	Cameroon	N/A

Gromphadorhina	*oblongonota*	Z233GO	captivity	XI
Gromphadorhina	*portentosa*	Z2GPa	captivity	XII
Oxyhaloa	*deusta*	B109OXD	RSA	N/A
Princisia	*vanwaerebecki*	Z232PV	captivity	XI
Rhyparobia	*maderae*	B98RHM	captivity	N/A
Nauphoeta	*cinerea*	B105NAC	captivity	N/A
Blaberidae - Geoscapheinae	(Panesthiinae)			
Parapanesthia	*gigantea*	P116PGI	Australia	IV
Geoscapheus	*woodwardi*	P8GW	Australia	II
Macropanesthia	*kraussiana*	P07MK	Australia	II
Blaberidae - Panesthiinae				
Ancaudellia	*s. serratisima*	P36ASS	Wanang, PNG	IV
Miopanesthia	*polita*	P113MID	Malaysia	V
Panesthia	*a. angustipennis*	P17FI	Mt. Malambo, Phillipines	IV
Panesthia	*a. cognata*	P36PAC	Tam Dao, Vietnam	XII
Salganea	*raggei*	P18SR	Huang Kong, Laos	XI
Salganea	*taiwanensis*		captivity	N/A
Salganea	*ternatensis hirsuta*	P22W5_7	Wanang, PNG	IV
Salganea	*ternatensis hirsuta*	P47STH	Wanang, PNG	IV
Blaberidae - Pycnosceliinae				
Pycnoscelus	cf. *surinamensis*	B61PYS	Mt. Malambo, Phillipines	XII
Pycnoscelus	*indicus*	B55PYI	Xiamen, China	X
Pycnoscelus	*nigra*	B54PYN	China	XII
Pycnoscelus	*striata*	B62PYR	captivity	N/A
Pycnoscelus	*surinamensis*	Z269PS	Ecuador	V
Pycnoscelus	*surinamensis*	Z135PS	captivity	XII
Pycnoscelus	*surinamensis*	B52PYS	Mamba village, Cameroon	N/A
Pycnoscelus	*tenebrigera*	B56PYT	Pakistan	N/A
Blaberidae - Perisphaeriinae				
Corydidarum	*pygmaea*	B96COP	captivity	N/A
Blaberidae - Epilamprinae				
Decoralampra	*fulgenicoi*	Z429DF	Polillo island, Phillipines	XII
Epilampra	*taira*	Z58ET	Nouragues, French Guyana	IV
Epilampra	sp.	Z74EP	Nouragues, French Guyana	VI
Rhabdoblatta	sp. "light abdomen"	B08RSPL	Wanang, PNG	XII
Rhabdoblatta	sp. "dark abdomen"	B09RSPD	Wanang, PNG	XII
Unknown				
Unknown	sp.	Z2656MDG	Madagascar	XII

Table 4.1: List of species examined for presence of *Nyctotherus* spp. Species underlined with light red colour were withoutf any clevelandellid cilliates. In species underlined by light green, a representative of *Nyctotherus* spp. was found.

The other armophorean group present in cockroaches, the family Clevelandellidae, was only encountered in wood-feeding, panesthiin cockroaches (Blaberidae, Panesthiinae) and they were present in every species examined.

4.4. Geographic distribution of *Nyctotherus* clades

After assigning the hosts to their respective families, we mapped the origins of each *Nyctotherus* spp. we obtained from cockroaches with known original locality to the world map to see if there is any geographical pattern regarding their phylogeny. Table 4.1 and figure 4.3 show the locations of individual collections. In the picture, each *Nyctotherus* clade is marked in different colour in the picture. Only clades with more than three representatives were chosen to be shown on the map.

We only used original locations of sequences for *Panesthia cribrata* and *Panesthia angustipennis cognata* fr the GenBank sequences because the rest were either synanthropes or unknown *Blaberus* species without any additional location information (presumably in captivity for a very long time).

We also tried to add the localities in which the cockroaches from captivity are reported to live to our map (Figure 4.4.). We only did this for non-synanthropic species, since synanthropic species are distributed worldwide.

Clade I is not represented in cockroaches and the only record from a julid millipede comes from an unidentified locality (Van Hoek et al., 1998a).

Clade II was found in two species of burrowing panesthiin cockroaches, *Geoscapheus woodwardi* and *Macropanesthia kraussiana* from Australia.

The only species that hosted protists of clade III and for which we knew the locality of origin was *Blaberus craniifer* from Key West, Florida in America. The other four were all from captivity. The origin of all those cockroaches from captivity lies in Central and South America. All species of genus *Byrsotria* live only in Cuba, *Blaberus colosseus* is native to countries from Mexico to Venezuela and *Archimandrita tesselata* lives in northern part of South America.

Clade IV was discoveredin three continents. It was found in panesthiins *Parapanesthia gigantea* from Australia and *Ancaudelia serratisima serratisima* and *Salganea ternatensis hirsuta* from Papua New Guinea. Then in *Panesthia a. angustipennis* from Mt. Malambo in Philippines. It also appeared in *Polyphaga aegyptica* from Georgia in Europe and in *Epilampra taira* from Nouragues Inselberg in French Guyana in South America.

Clade V was noted from three continents: Madagascar, Africa in *Deropeltis madecassa*, China, Asia in *Eupolyphaga sinensis*, Malaysia, Asia in *Miopanesthia polita*, Florida, USA, America in *Blaberus craniifer* and from Ecuador, America in *Pycnoscelus surinamensis*. One examined species comes from captivity. It was *Therea olegrandjeani* originally from India.

Clade VI was only recorded from a single unidentified cockroach species belonging to the genus *Epilampra* from Nouragues Inselberg in French Guyana in South America.

Clade VII was not represented in any of our cockroaches and the only record of this clade comes from the eastern Australia where it was found in *Panesthia cribrata* (GenBank - KC139720) (Lynn and Wright, 2013).

Clade VIII is represented in three continents. We found its representatives in two cockroach species from South America – *Eublaberus* sp. and *Hyporhicnoda* sp. "Venezuela", one in *Blatta orientalis* from the capital of Azerbaijan in Asia and finally one in Nyassosso village in African Cameroon. There were three other captive populations that contained this clade.

Clade IX was only found in one unidentified ectobiid species from Madagascar.

Clade X was discovered in cockroaches originating from four continents. Two records were from Europe - from Porto in Portugal and Varna in Bulgaria (both in *Blatta orientalis*) and from Korfu (*Polyphaga aegyptica*). One was from Bolivia (*Blaberus* sp.) in South America and two from Africa – one from Madagascar (in an unidentified ectobiid species) and one from Hogsback in the eastern part of Republic of South Africa (*Bantua* sp.). Finally, the two remaining infected cockroach populations were from Asia, from Fuzhou and from Xiamen in China (*Periplaneta americana* and *Pycnoscelus indicus*, respectively). We also found *Nyctotherus* of this clade in three colonies from captivity. Those three species with *Nyctotherus* inside from captivity are originally from south Africa (*Dipteretrum hanstroemi*), Madagascar (*Elliptorhina laevigata*) and South America (*Eublaberus posticus*).

Clade XI was recorded from three continents – one record came from Sarasota in Florida, USA (*Eurycotis floridiana*), two from Africa (*Deropeltis* sp. "Jinka" from Ethiopia and in *Periplaneta australasiae* from Mamba village in Kenya) and one from Asia, particularly from Huang Kong in Laos (*Salganea raggei*). It was also present in four species of the subfamily Oxyhaloiinae obtained from captivity that all originate in Madagascar (*Aeluropoda insignis,*

Elliptorhina javanica, Gromphadorrhina oblongonota and Princisia vanwaerebecki, all originally from Madagascar).

Finally, the most prevalent clade, XII, was obtained from four continents. There are several records from Asia (*Periplaneta americana* from Emei shan, China, *Periplaneta* sp. From Mount Malambo and *Decoralampra fulgenicoi* from Polillo island both in Phillipines and *Panesthia angustipennis cognata* from Tam Dao in Vietnam). One was from Europe (*Blatta orientalis* from Liege in Belgium), one from South America (*Blaberus* sp. from Venezuela). Two identical *Nyctoteherus* spp. were found in two species of *Rhabdoblatta* from Papua New Guinea (taken as a part of Australia) and finally one was reported from Madagascar (Africa, in an unknown cockroach). We also found ciliates of this clade in five populations from captivity. Those were *Therea regularis* and *T. olegrandjeani* that originate from India, *Phoeatalia pallida* and *Pycnoscelus surinamensis* (both synanthropic and thus not useful for mapping) and in *Gromphadorrhina portentosa* originally from Madagascar.

Figure 4.3: Geographic distribution of individual *Nyctotherus* clades from cockroach specimens with known original locality. Orange – clade III. Yellow – clade IV. Blue – clade V. Magenta – clade VIII. Green – clade X. Pink – clade XI. Red – clade XII.

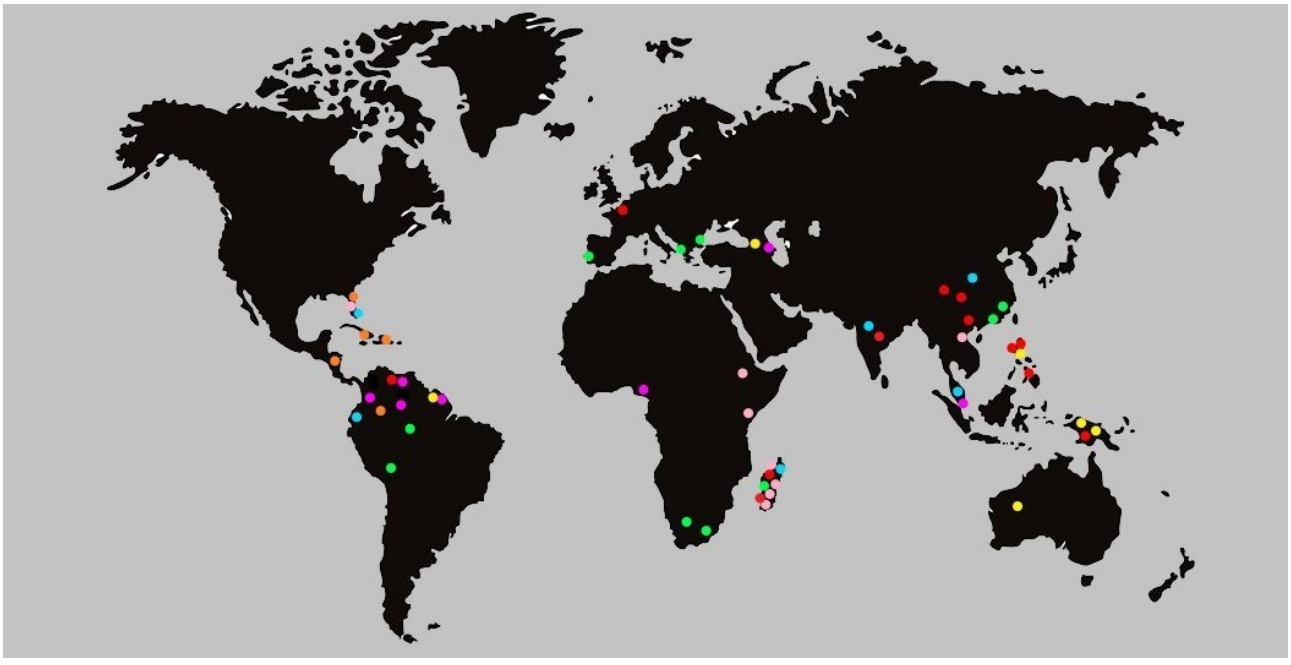


Figure 4.4: Geographic distribution of *Nyctotherus* clades with localities in which cockroaches from captivity originally lived added to the localized ones. Orange – clade III. Yellow – clade IV. Blue – clade V. Magenta – clade VIII. Green – clade X. Pink – clade XI. Red – clade XII.

4.5. Morphology of selected *Nyctotherus* strains

We performed protargol staining of slides with ciliate cells. Only a few slides were usable for analysis as they often got overcoloured and we were not able to see important features. The best looking protargol slides were selected and the protists on those slides were morphometrically analysed. Table 4.2 shows morphometrical characteristics of each examined population. Figures 4.5 to 4.9 show pictures of some of the measured cells, while figure 4.2 shows living cells of *Nyctotherus* spp.

All *Nyctotherus* cells that we measured, except protists from *Parapanesthia gigantea*, were roughly of the same shape, mostly being ovoidal or kidney shaped (may depend on the angle of view). The cells appeared to be slightly constricted in the location of macronucleus. Micronucleus was visible only in two cells from *Salganea ternatensis hirsuta* and two from *Elliptorhina laevigata*. Karyophore was visible only in few cells. All those ciliates had slightly curved end of the cytopharynx, just before the cytostome.

Strain from *Salganea ternatensis hirsuta* was clearly distinguishable by the angle of adoral zone entering the cell. Ciliates from *Archimandrita tesselata* and *Blaberus colloseus* tended to have the macronucleus located slightly more anteriorly than the rest, but they differed from each other in size and in macronucleus proportions. The biggest cells were found in *Elliptorhina laevigata.* These ciliates also had the highest adoral membranelle count.

Cells originating from the gut of *Parapanesthia gigantea* were at first glance clearly distinct from all other ciliates that we encountered. Their shape was rather ellipsoid to ovoid and there was no constriction near the macronucleus. Cytopharynx was short, straight, and conical and there was no curve at its end. The shape of the macronucleus also differed as it was the only *Nyctotherus* with macronucleus much longer than wide. It also had the highest density of membranelles in the adoral zone, although their numbers were the lowest among all examined lineages.

Cells from *Salganea ternatensis hirsuta* (N=7)	mean	median	st. dev	min	max
Body length	48.4	49.5	3.5	40.9	52.2
Body width	29.3	29.,1	1.1	27.8	31
Body length/body width	1.7	1.7	0.1	1.4	1.8
Adoral membranelle zone length	27.2	27.6	2.4	22.4	30.6
Adoral membranelle zone total length	43.8	44.2	4.1	36.1	49.8
Anterior end to macronucleus	9.4	10	1.8	6.1	11.8
Anterior end to macronucleus - % of body length	19 %	20 %	3 %	12 %	23 %
Posterior to macronucleus	30.1	31.2	3.3	22.9	34.1
Posterior end to macronucleus - % of body length	62 %	62 %	4 %	56 %	69 %
Macronucleus length	8.8	8.4	1.1	7.3	10.6
Macronucleus width	12.5	12.2	1.6	10.5	15.5
Macronucleus length/macronucleus width	0.7	0.7	0.1	0.6	0.9
Adoral membranelle count	39.1	39	1.7	36	42
Adoral membranelle count/adoral zone total length	0.9	0.9	0.1	0.8	1.1
Cells from *Archiandrita tesselata* (N=20)	mean	median	st. dev	min	max
Body length	67.2	67.2	7	53.2	79.4
Body width	38.5	39	3.7	31.9	45.3
Body length/body width	1.7	1.7	0.1	1.6	2
Adoral membranelle zone length	38.6	38.5	4.2	32.1	46.7
Adoral membranelle zone total length	45.6	45.7	5	36.7	56.5
Anterior end to macronucleus	8.7	8.8	2.5	2.6	13.9
Anterior end to macronucleus - % of body length	13 %	14 %	3 %	4 %	18 %
Posterior to macronucleus	46.3	46.4	6.5	34.6	58.2
Posterior end to macronucleus - % of body length	69 %	69 %	4 %	61 %	77 %
Macronucleus length	12.2	12.1	2.2	8.6	18.7
Macronucleus width	17.9	17.8	2.6	11.9	22.3
Macronucleus length/macronucleus width	0.7	0.7	0.1	0.5	1
Adoral membranelle count	36.5	37	2.2	34	42
Adoral membranelle count/adoral zone total length	0.8	0.8	0.1	0.7	0.9
Cells from *Blaberus colloseus* (N=20)	mean	median	st. dev	min	max
Body length	80.5	83.5	10.4	57.9	102.2
Body width	49.4	47.4	7	35.8	61.2
Body length/body width	1.6	1.7	0.2	1.4	2
Adoral membranelle zone length	39.9	40.8	4.7	29.8	48.5
Adoral membranelle zone total length	46.1	46.3	5.6	37.3	58.9
Anterior end to macronucleus	16.1	15.7	3.1	11.1	23.3
Anterior end to macronucleus - % of body length	20 %	20 %	3 %	14 %	27 %
Posterior to macronucleus	52.7	52.5	8.5	37.1	74.7
Posterior end to macronucleus - % of body length	65 %	65 %	4 %	58 %	73 %

Macronucleus length	11.9	11.4	2.1	9	15.9
Macronucleus width	20.1	20.3	3.3	14.7	29.5
Macronucleus length/macronucleus width	17.9	17.8	2.6	11.9	22.3
Adoral membranelle count	38.2	38	3.4	33	46
Adoral membranelle count/adoral zone total length	0.8	0.8	0.1	0.7	1
Cells from *Parapanesthia gigantea* (N=21)	**mean**	**median**	**st. dev**	**min**	**max**
Body length	61.4	60.3	7.5	52.3	86
Body width	35.9	35.8	6.3	28.5	56.9
Body length/body width	1.7	1.7	0.1	1.5	2.1
Adoral membranelle zone length	23.1	22.2	2.7	20	30.9
Adoral membranelle zone total length	23.1	22.2	2.7	20	30.9
Anterior end to macronucleus	7	6.9	1.7	4.2	10.8
Anterior end to macronucleus - % of body length	11 %	12 %	3 %	6 %	17 %
Posterior to macronucleus	33.8	31.9	6.7	26.9	57
Posterior end to macronucleus - % of body length	55 %	55 %	5 %	48 %	66 %
Macronucleus length	25.2	25.4	3.9	17.6	34.3
Macronucleus width	9	8.8	2.4	5.4	15.5
Macronucleus length/macronucleus width	18	18.3	2.6	11.9	22.3
Adoral membranelle count	25.1	25	3	20	32
Adoral membranelle count/adoral zone total length	1.1	1.1	0.1	0.9	1.3
Cells from *Elliptorhina laevigata* (N=20)	**mean**	**median**	**st. dev**	**min**	**max**
Body length	83.9	81.8	10	68.9	107.6
Body width	49.4	49.7	7.2	40.1	66.2
Body length/body width	1.7	1.7	0.2	1.1	2
Adoral membranelle zone length	50	48.5	7.4	38.2	63.5
Adoral membranelle zone total length	60.5	59.4	8.6	47.6	74.8
Anterior end to macronucleus	24.7	24.8	2.6	20.1	28.3
Anterior end to macronucleus - % of body length	30 %	239 %	2 %	25 %	35 %
Posterior to macronucleus	45.6	46	6	35.6	57.5
Posterior end to macronucleus - % of body length	54 %	54 %	3 %	48 %	60 %
Macronucleus length	14.5	13.7	3.2	9.8	22.2
Macronucleus width	22.8	21.8	3.6	18.1	32.7
Macronucleus length/macronucleus width	17.9	17.8	2.6	11.9	22.3
Adoral membranelle count	60.5	58	6.9	52	76
Adoral membranelle count/adoral zone total length	1	1	0.1	0.9	1.2

Table 4.2: Morphometric data of measured *Nyctotherus* spp. **st. dev** = Standard deviation. Adoral zone membranelle count/adoral zone total length = **adoral membranelle density**.

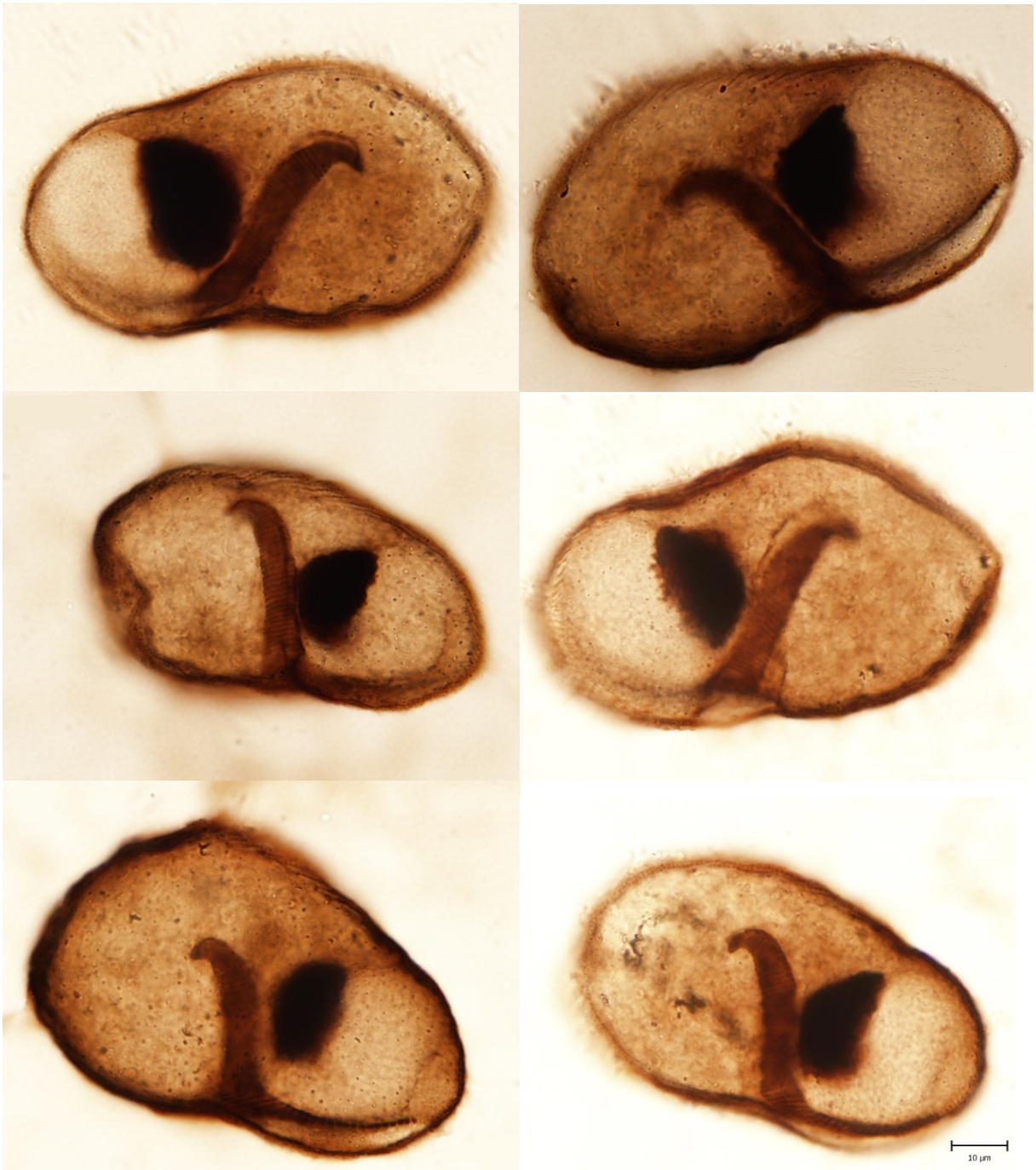


Figure 4.5.: Examples of protargol stained nyctotheridcells obtained from the cockroach *Elliptorhina laevigata* and used for morphometrical analysis. Note the curvature of the proximal (posterior) end of infundibulum. Scale 10 µm.

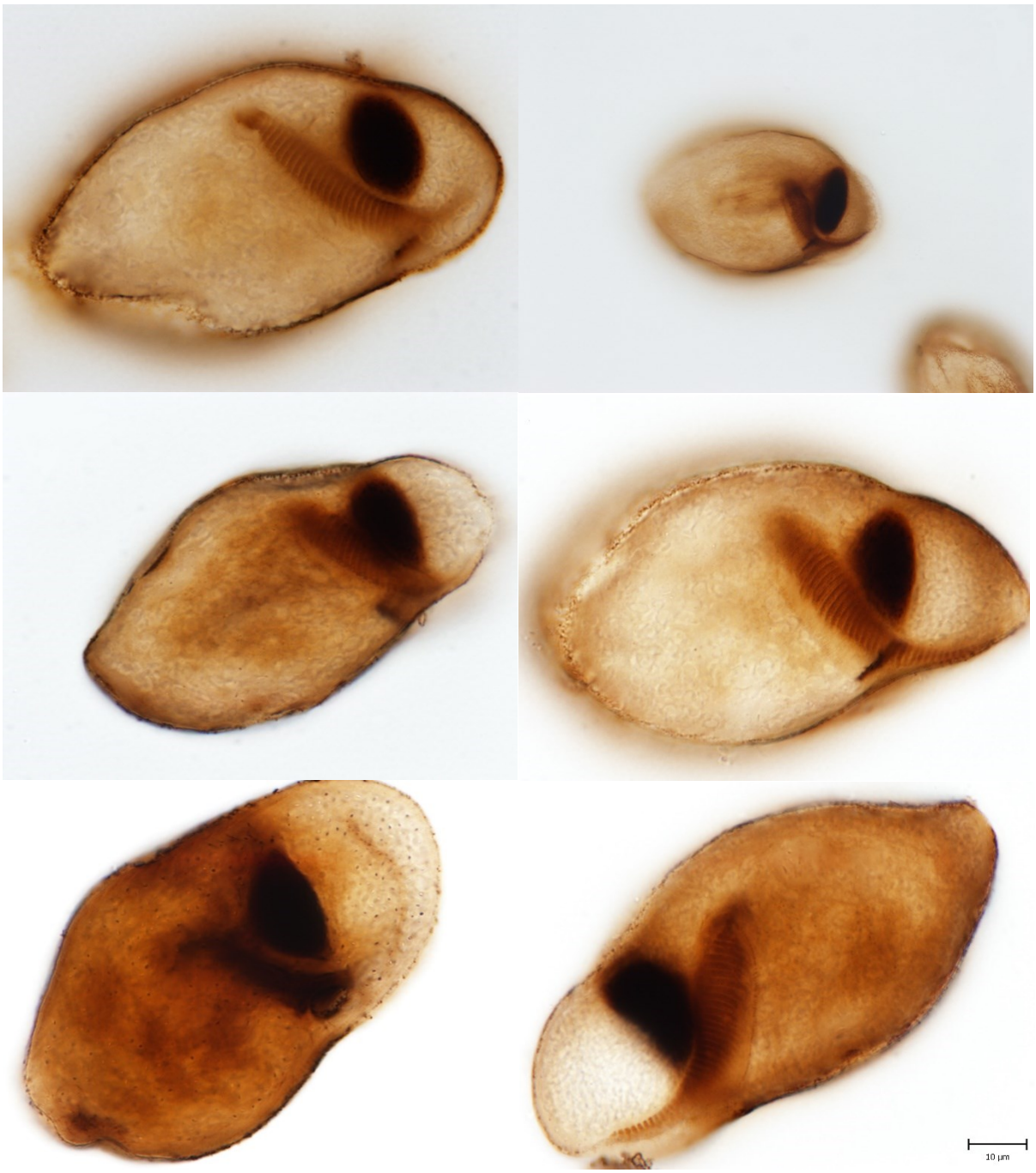


Figure 4.6.: Examples of Protargol stained cells obtained from the cockroach *Blaberus colloseus* and used for morphometrical analysis. Note the anterior position of macronucleus and only slightly curved end of infundibulum. Scale 10 µm.

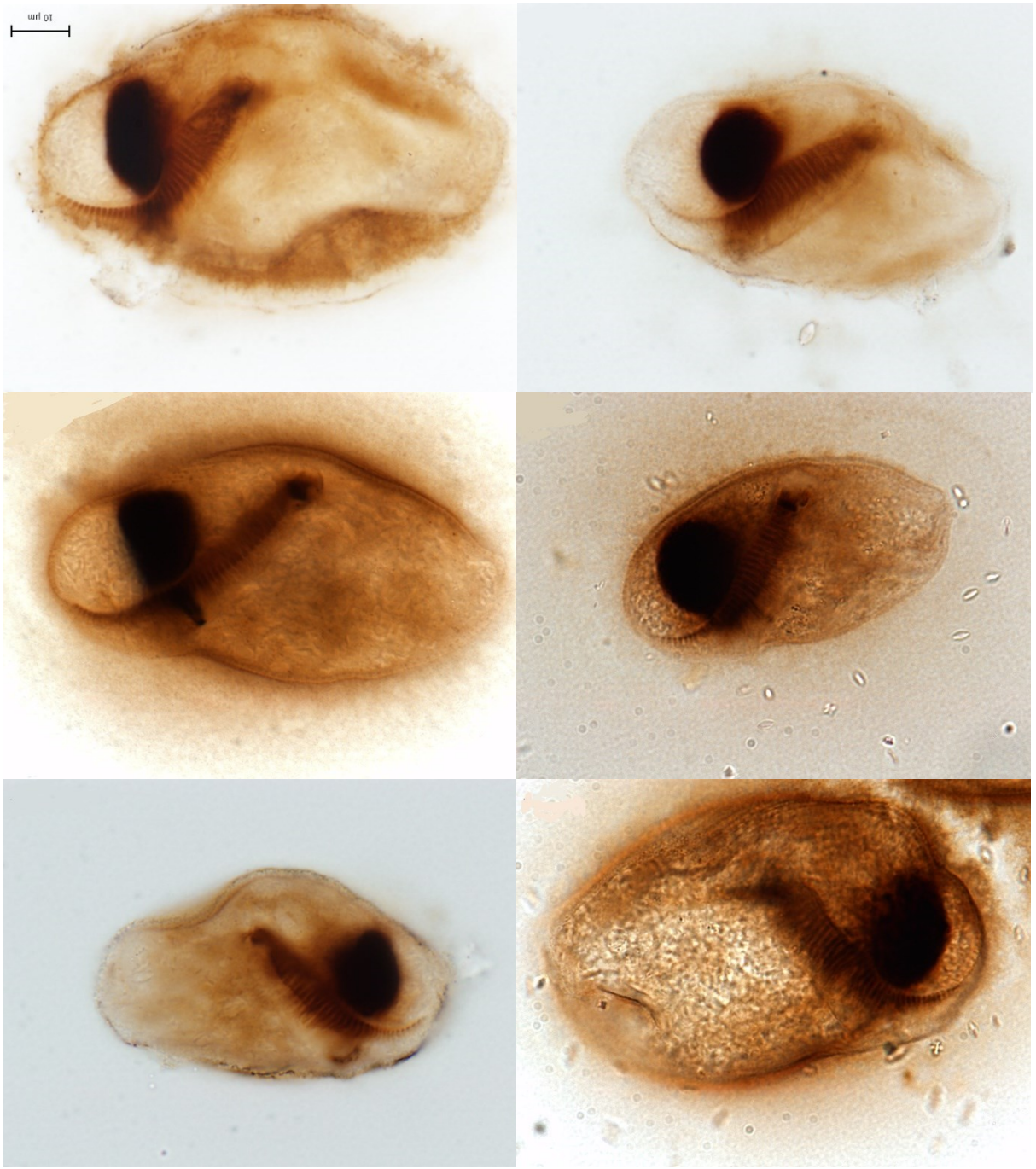


Figure 4.7.: Examples of Protargol stained cells obtained from the cockroach *Archimandrita tesselata* and used for morphometrical analysis. Note the anterior position of macronucleus and only slightly curved end of infundibulum. Scale 10 µm.

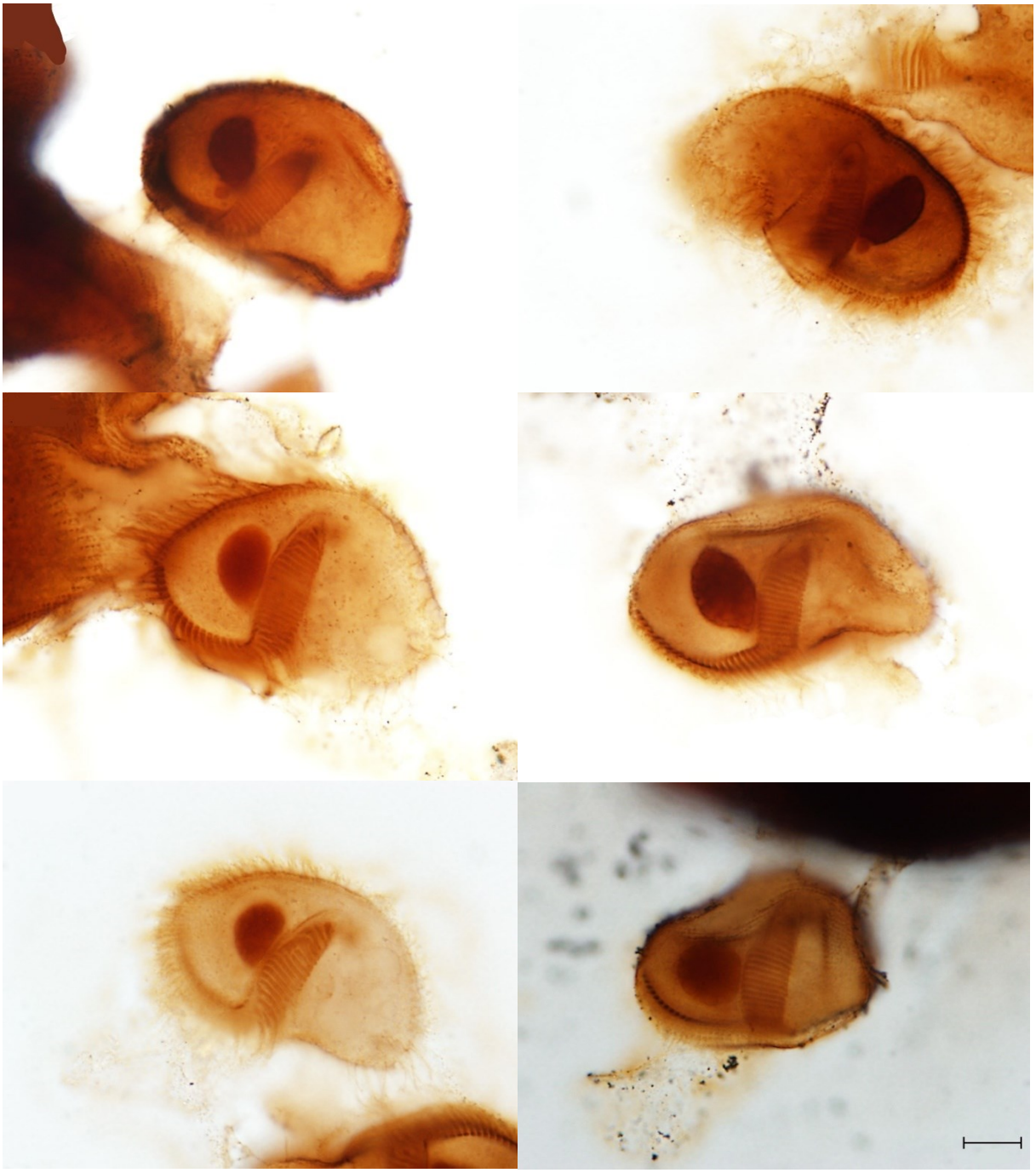


Figure 4.8.: Examples of Protargol stained cells obtained from the cockroach *Salganea ternatensis hirsuta* and used for morphometrical analysis. Note small size of cells and the angle under which the infundibulum enters the cell. Scale 10 µm.

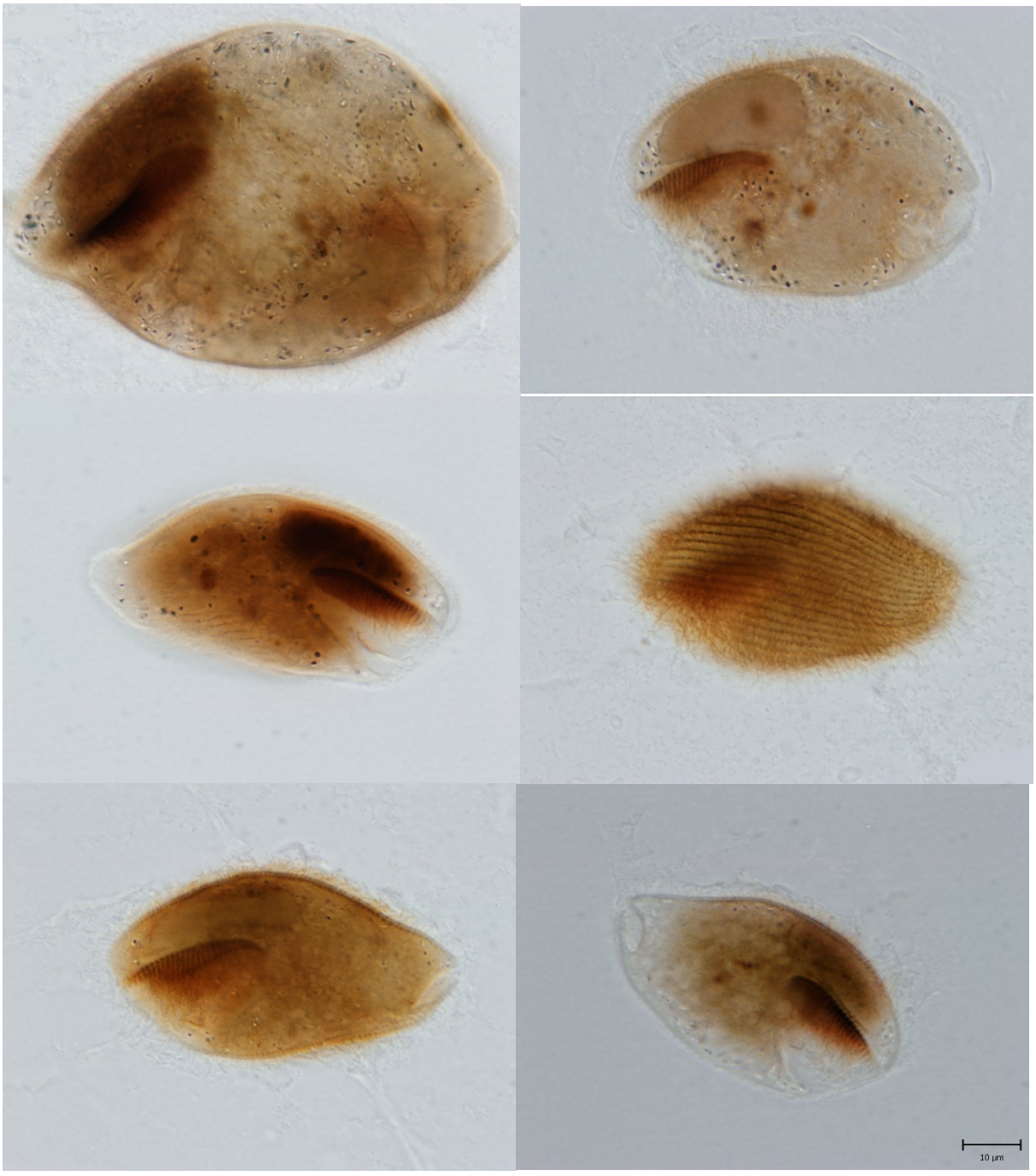


Figure 4.9.: Examples of Protargol stained cells obtained from the cockroach *Parapanesthia gigantea* and used for morphometrical analysis. Note overal differences from other strains: different shape of infundibulum, adoral zone not present on the cell surface and different orientation of macronucleus. Scale 10 µm.

5. Discussion

It must be noted that, before we obtained the specimens, most of the cockroaches studied spent some time in captivity with breeders who keep multiple species before we obtained the specimens, so infection with different strains of *Nyctotherus* spp. different from those they originally harboured cannot be completely excluded. More samples directly from the wild are clearly preferable, but current restrictions, such as the Nagoya make it increasingly difficult.

To rule out the possibility of inter-colony infection in captivity, more knowledge about the requirements that individual clades of *Nyctotherus* spp. have for their hosts would be necessary. Some clades that we established could for example prefer either acidic, neutral, or alkaline environment or be dependent on certain bacteria that require those conditions.

5.1. Prevalence of ciliates in cockroaches

Of 108 cockroach populations examined, 43 harboured no ciliates. Almost all populations had all examined specimens either uninfected or quite heavily infected with *Nyctotherus* spp. Only two populations showed both infected and uninfected individuals. These were *Pycnoscelus femapterus* and *Eurycotis Floridiana* from Sarasota, Florida, USA. Individuals of *Panesthia angustipennis angustipennis* always hosted ciliates of the family Clevelandellidae, but only three out of seven examined contained *Nyctotherus*.

Representatives of family Nyctotheridae were found in every family (and subfamily regarding Blaberidae) that we had more than two samples from, except Blattellidae and

Phyllodromiidae (both Blaberoidea). Literature however revealed that *Nyctotherus ovalis* was found at least in *Blattella germanica* (Blattelidae) (Hoyte, 1961b).Clade XII was the most prevalent and most widespread clade among cockroaches in our study.

Usually only one strain of *Nyctotherus* lives in a single colony. There were however some colonies that hosted two. We found two clades in one colony of *Blaberus craniifer* and the phylogenetic tree showed that the two isolates from *Panesthia cribrata* available through GenBank (KC139721, KC139720) also belong each to a different clade. Finally, *Salganea ternatensis hirsuta* hosted two strains of *Nyctotherus*, both belonging to clade IV.

Members of the family Clevelandellidae only appeared in cockroaches belonging to the blaberid subfamily Panesthiinae. They were observed in every species belonging among wood feeding Panesthiinae.

For better estimation of prevalence, it would be necessary to study more specimens, prefferably directly from the wild, since prolonged captivity with lots of cyst-laden faeces in a closed container may increase the prevalence of infection compared to the wild (Hoyte, 1961a; Nalepa, 1991).

5.2. Phylogenetic and interclade relationships

The phylogenetic tree that we constructed corroborates previous hypothesis and confirms that Clevelandellida are an endobiotic lineage of Metopida. Subsequently, Clevelandellidae are a monophyletic group which is an internal branch of Nyctotheridae. Nyctotheridae is thus paraphyletic in respect to Clevelandellidae

The analysis clearly shows that there are several clades of *Nyctotherus* spp. and that at least some of these lineages are well supported. This study characterized twelve clades of *Nyctotherus* spp. Six of these (clades II, III, VI, VIII, IX and XI) were unknown to science until now.

For clades I and II we have too few representatives to draw firm conclusions. Clade III represents molecularly new strain waiting to be compared with available morphological data to assess its taxonomic position.

With relatively strong support for monophyly, clade IV looks very much like a species complex, considering the length of its internal branches and presented morphological

diversity. At least five well supported internal branches are visible, and it is a matter of further research to shed more light on the diversity within this clade.

The superclade consisting of clades V to XII is strongly to fully supported (BS: 99, PP: 1). Clade V is strongly supported but its internal relationships are largely Further taxon sampling should help with further analyses.

Clades VI, VII and IX were each represented only by one sequence.

Clade VIII could consist of two species. There is some nonnegligible genetical variability, but the branches are quite short and detailed morphological and molecular study with more samples aree needed before any conclusions can be drawn.

Clade X likely includes some deeper diversity hidden inside it. Strongly supported branches emerging one after another suggest that this clade could actually be a complex of species.

Sequences forming clade XI showed low divergence. We expect those records to represent a single, but genetically variable species.

The last clade, clade XII again shows some interesting inner lineages well supported by both Maximum likelihood and Bayesian analysis methods and therefore will probably represent several species.

GenBank sequences labelled as *Nyctotherus ovalis* by their authors appeared in two separate and relatively distant clades (V and X). Almost all records of *Nyctotherus* from cockroaches in literature were determined as *N. ovalis*. These cockroaches were mostly *Periplaeta americana*, *Blatta orientalis* and *Blaberus* spp. (De Graaf et al., 2011; Van Hoek et al., 1998b; Hoyte, 1961b; Lucas, 1928). Since we showed that protists living in *Periplaneta americana* and *Blatta orientalis* each belong to at least three different *Nyctotherus* clades, and we found four clades in the genus *Blaberus*, there is a high chance that some of those protists were misidentified.

5.3. Morphology

Examined ciliates belonged to three clades defined above (III, IV X). Each of examined clades has shown significant morphological differences from the others. There was a striking morphological difference between two examined protist lineages belonging to clade IV.

Nyctotherus from *Archimandrita tesselata* and *Blaberus colloseus* both belonged to clade II and their 18S rDNA gene sequences were identical. They varied in size but displayed the same trait of greatly anteriorly located macronucleus and shared the overall shape of the cytopharynx. Morphological differences between the ciliate populations were present, mainly in proportions of macronucleus (ML/MW), but as they are genetically identical, we assume that it is just a matter of interpopulation variability of this species.

Protists from *Salganea ternatensis hirsuta*, as well as those from *Parapanesthia gigantea*, belonged to clade IV, but their morphology was surprisingly different. *Salganea ternatensis hirsuta* hosted the smallest ciliates from all examined lineages, clearly distinguishable by the sharp angle under which the cytopharynx enters the cell. The adoral zone continues from the cytopharynx across the right side of the cell up to the anterior end. *Nyctotherus* cells obtained from *Parapanesthia gigantea* have short, straight, and conical cytopharynx with densely packed adoral membranelles present only inside the pharynx. The surface part of the adoral zone present in every other examined strain was not present in this one. It also exhibited very long and slender macronucleus, compared to the rest of examined lineages. These findings support our hypothesis that clade IV is in fact a species complex and protists from Parapanesthia gigantea probably represent a new genus of the family Nyctotheridae.

Ciliates from *Elliptorhina laevigata* were the biggest of all examined ciliates. They exhibited the second highest adoral membranelle density. Aside from the size, it differed from the representatives of other clades in more curved end of the cytopharynx. Their nucleus was also located more posteriorly than in the other clades.

Examining the morphology showed that at least some *Nyctotherus* lineages are somehow morphologically plastic. On the contrary, among representatives of other clades, there are some differences too great to be accounted to intraspecies variability.

5.4. Geographic distribution of *Nyctotherus* clades

Mapping original sources of cockroaches on world map did not show any clear pattern in any single *Nyctotherus* clade (pictures 4.2, 4.3). Any results originating from adding approximate origin localities of species from captivity are non-plausible, because the cockroaches could have been infected during their time in captivity. The patterns that they show may however help us to build a hypothesis to test with more specimens in the future.

We obtained only a single cockroach population hosting clade III for which we surely know the original locality - Key West in Florida. If we consider cockroach species that host the protists that cluster in this clade, we can however see that they are all originally from central to northern South America. There are several possibilities. Either this could represent a geographically limited species, or they becameinfected somewhere during captivity (*A. tesselata* and *B. colosseus* were obtained from the same breeder as *B. craniifer*, but *B. craniifer* was the only one with locality noted, both lineages of species of genus *Byrsotria* are already in captivity for a long time). There may be a preference of *Nyctotherus* clade III for Blaberidae: Blaberinae (into which all the species belong) or we may just lack data from populations outside Central and South America.

Clade IV appeared mostly in panesthiin cockroaches from Australia, Papua New Guinea, and the Philippines, but were also found in specimens from other cockroach families (Corydiidae, Blaberidae: Epilamprinae) obtained from Nouragues Inselberg in French Guyana and from Georgia, a country between Europe and Asia. Most of the Australian and Indonesian members of Blaberidae: Panesthiinae, that live only in South Asia and Australia, host this clade. The phylogenetic tree topology and support of the branches suggest that there may be some internal, Panesthiinae-related, clade, as there is a well-supported branch (BS: 95, PP: 1) comprising 9 sequences obtained from panesthiin cockroaches. Australian Parapanesthia *gigantea* and species from continental Asia hosted different clades. This may suggest panesthiins expanding from Indo-Malaysian area where they originated crossed the sea to the south and brought with them, primarily, protists belonging to this clade. More research of panesthiins from this area is necessary (Beasley-Hall et al., 2021).

Clade VIII splits into two well supported internal branches. One part of seems to be present mainly in cockroaches from central and northern South America while the other part is noted from Europe and from Africa. The South American part may represent some geographically limited South American species.

Most records of clade XI as well as the primal origin of species from captivity show the main area of presence is Africa, but there is one record from Florida, USA and one from Asia. The bias towards African records could reflect under-sampling of other areas, but it could also mean that the centre of its distribution is in Africa with few populations colonizing more distant places.

The rest of the clades have either worldwide distribution or there is too little information to assess anything about them.

Geographic origin of individual records does not shed any light on most of the distribution of *Nyctotherus* diversity. If we only consider the infected species with known original locality, there are five clades that only appear in a single continent: Clades I, III, VI, VII and IX, but we cannot say that they really appear only in those, because they are all only represented by a single localized host species. However, if we also take into account those species from captivity and use the type localities and areas from which these species are reported, at least clade III really seems to be present only in the central and northern part of South America. It may however reflect lack of sampling from other regions. The clades suspected to actually be species complexes could comprise several geographically separated species.

Clevelandellidae only appeared in cockroaches from south Asia and Australia. It corresponds with the geographic distribution of their host subfamily, Panesthiinae.

5.5. Coevolution with hosts

Clevelandellids appeared at approximately the same time as crown groups of cockroaches. (Evangelista et al., 2019; Fernandes and Schrago, 2019; Vďačný et al., 2019). This could mean that the clades that now inhabit intestines of cockroaches specialized for these insects since their emergence.

The mechanism of transfer for clevelandellids between cockroaches, cysts in faeces waiting to be eaten by another cockroach, however, allows for massive host switching. Because more cockroach species often live at one spot, they can incidentally ingest the *Nyctotherus*-infected faeces from a different roach species. Coevolution with a single host species is therefore unlikely and it sounds more plausible that they specialized for the cockroaches as a group.

5.6. Host specificity

No clade (except those with only one representative) of *Nyctotherus* spp. shows any strong host specificity. The strongest signal for possible existence of some degree of host specificity appears in clade III that seems to prefer large species of Blaberidae: Blaberinae, but we would need more samples to prove this. Clade II was only found in Australian Blaberidae:

Panesthiinae, so there is some possibility for a certain degree of host specificity, but more data are needed to prove this claim. Panesthiins from Australia and Indonesia also mostly harboured one clade (IV) so there may exist some Panesthiinae-specific clade of ciliates. The recently described species, *Nyctotherus galerus*, is known only from *Panesthia angustipennis cognata* (Pecina and Vďačný, 2020). All currently described species are also known from only one to two hosts with one exception, *Nyctotherus silvestrianus* reported from five different termite species.

There is too little information regarding clades II, VI and IX and all other clades were found in multiple species of multiple families. It is however possible that the clades represent clusters of individual species that in reality have some host preferences.

Since there is no molecular data for *Nyctotherus* appearing in other insect orders and only one sequence of *Nyctotherus* from millipede (which is, however, closely related to symbionts of cockroaches), we cannot even say for sure that the lineages that we described are specific to cockroaches. Some clades can possibly infect wider range of hosts, given their prevalence among cockroach families.

5.7. Host size preference

We categorized cockroaches by their size (length of adult females) as following: large species – >3 cm, medium species – 1,5-3 cm, small species – <1,5 cm. Cockroach size was variable within the families with some encompassing medium to large species (Blattidae and Blaberidae: Blaberinae, Panesthiinae, and Epilamprinae), and the rest comprising medium sized and small roaches. Families comprising very small cockroaches were uninfected for most of the cases.

Clades III and XI were only found in large species of cockroaches. Clades II, VI, VII and IX had too few representatives to make an assessment.

Clade IV did not show any obvious hosts size preference, inhabiting large roaches such as *Salganea ternatensis hirsuta* as well as medium ones such as *Polyphaga aegyptica*.

Clades V, X and XII lived in all size categories of cockroaches. They were found in large species such as *Blaberus craniifer*, *Gromphadorhina portentosa* and *Eublaberus posticus,* medium ones such as *Therea olegrandjeani* and *Blatta orientalis* and small species like

Pycnoscelus surinamensis, Dipteretrum hanstroemi and *Phoetalia pallida*. Clade VIII seemed to prefer medium sized roaches, but it was also discovered in *Eublaberus* sp. which is a genus of large blaberid roaches

No clade preferred small cockroach species. Generally speaking, it is more likely to find clevelandellids in larger roaches, although not all of them contain ciliates.

The two families in which we did not find any ciliates, Phylodromiidae and Blattellidae, were both of small body proportions. Record of *Nyctotherus* from *Blattella germanica* in literature (Hoyte, 1961b) leave Phylodromiidae the only family without any known *Nyctotherus* infection. There just might not be enough place for the protists to live and thrive in. However, we found some *Nyctotherus* spp. in considerable number of small cockroach species such as *Dipteretrum hanstroemi* (Ectobiidae), *Decoralampra fulgenicoi* (Blaberidae: Epilamprinae) and *Pycnoscelus indicus* (Blaberidae: Pycnoscelinae), plus there were some faecal samples from and an unidentified ectobid (Ectobiidae), and *Diploptera punctata* (Blaberidae: Diplopterinae) that are almost as small as blattellids and phylodromiids, positive for clevelandellid DNA. It is however possible that they in fact do appear in blattellid and phyllodromiid cockroaches and we just did not manage to obtain any infected specimens.

6. Conclusions

Despite their description in the early 20th century, the research in the field of ciliate symbionts of cockroaches is still in its infancy. However, this study showed that the diversity of Nyctotheridae is surprisingly large and showed six lineages unknown to science up until now. Protists belonging to these clades still wait to be compared with known, morphologically described species. We inferred the 18S rRNA gene phylogeny of ciliated protists from cockroaches originating from all over the world and identified twelve fairly well supported clades of *Nyctotherus* spp., We also confirmed that Clevelandellidae is an internal branch of Nyctotheridae (BS: over 75, PP: 1). This proves Nyctotheridae, itself being previously shown to be an internal branch of paraphyletic order Metopida, to be paraphyletic regarding Clevelandellidae.

Morphology also showed significant differences among protists of various clades and even signalized inner diversity of at least one clade. Stated differences were backed by morphometrical data.

The prevalence of ciliate infections in cockroaches is rather high and it is possible, but uncommon, for cockroaches to host multiple *Nyctotherus* lineages.

Regarding the distribution of clevelandellid diversity we cannot say for sure if there is any geographical restriction of individual clades, but at least some clades suggest this is a possibility. More data is, however, needed. Nyctotheridae do not seem to be overly specialized for living in any particular cockroach taxon, although we encountered clades that only appeared in one family. Until more information and sequences are available, it is too soon to assess levels of host specificity.

We found clevelandellids in almost every cockroach family from which we had more than two specimens. There were two exceptions, families Blattellidae and Pseudophylodromiidae (although literature mentions infection in Blattellidae). It is possible that clevelandellids really do not prefer to live in those cockroaches, or, as is often the case, undersampling might be the explanation. As those two families comprise mostly small cockroach species and we did not find any ciliate clade to specialize in colonising intestines of small cockroach species, the first answer seems to make sense, because if there was any host size preference, it tended towards larger hosts.

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