BIOLOGY AND EVOLUTIONARY HISTORY OF THE FACULTATIVE PARASITIC NEMATODE *HALICEPHALOBUS GINGIVALIS.*

A MULTIDISCIPLINARY APPROACH

PAMELA FONDERIE

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Biologie en evolutionaire geschiedenis van de facultatief parasitaire nematode *Halicephalobus gingivalis*.
Een multidisciplinaire benadering.

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I would like to dedicate this dissertation to my dad.
You are dearly missed.

“The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.”

Albert Einstein

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DANKWOORD

Het dankwoord-moment is aangebroken. Dat kan maar één ding betekenen: het boekje is bijna af! Dit gaat gepaard met een oneindig gevoel van opluchting, maar ook wel van trots. Het betekent tegelijkertijd het einde van een fascinerende reis met hoogtes en laagtes, met overwonnen tegenkantingen, steeds met het gevoel van het onbekende achter de volgende hoek en dit alles vergezeld door een onstilbare honger naar méér. Come to think of it, in many ways, a PhD is like a box of chocolates… Nu het eind in zicht is, wordt het tijd een aantal mensen uitvoerig te bedanken voor hun bijdrage aan het geheel, want het mag gezegd zijn: een doctoraat maken, doe je niet alleen.

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SUMMARY

SAMENVATTING

SUMMARY

Nematodes are one of the most diverse groups of organisms, have a high functional and taxonomical diversity, are ubiquitous distributed in high numbers, and are very successful as parasites. They show a high diversity in both parasitic lifestyles and possible hosts, *i.e*., invertebrates, vertebrates and plants, which reflects a high adaptability to new environments and an aptitude towards parasitism. Animal parasitism has arisen independently at least six times within the phylum (Blaxter *et al.* 1998; Dorris *et al.* 1999) and in secernentean nematodes, all intermediate stages between free-living saprobionts and obligatory parasites, *i.e.*, completely dependent on the host during at least a part of their life cycle, are present (Sudhaus 2010). From an evolutionary point of view, facultative parasites, *i.e*., adaptive to a parasitic way of life when placed in such a relationship but not absolutely depending on it, are thought to represent the intermediate stage in the acquisition or loss of parasitism (Sudhaus 2010).

Halicephalobus gingivalis, previously also referred to as *Rhabditis gingivalis*, *Micronema deletrix* or *Halicephalobus deletrix*, belongs to the family Panagrolaimidae and is a small (235 – 460 µm) free-living bacteriovorous nematode, which is capable of facultative parasitism in horses (Blunden *et al.* 1987; Nadler *et al.* 2003) and humans (Ondrejka *et al.* 2010). Single cases have been described in a donkey (Schmitz and Chaffin 2004), a Grévy's zebra (Isaza *et al.* 2000), in the scrotal skin of a bull (Georgi and Georgi 1990) and *H. gingivalis* like nematodes have been reported in the brain of a three year old black Angus cow (Montgomery and O'Toole 2006). Little is known about the epidemiology of *H. gingivalis*. Several possible infection routes have been described, *e.g*., an oral route through the ingestion of contaminated plant material that acts as a mechanical vector (Hermosilla *et al.* 2011), through the respiratory tract via the inhalation of nematodes (Spalding *et al.* 1990; Ruggles *et al.* 1993; Trostle *et al.* 1993; Bröjer *et al.* 2000), or cutaneous infections facilitated by recumbency (Dunn *et al.* 1993). After penetrating the host, the nematodes can either remain on the spot and cause local infections or enter the bloodstream and disseminate throughout the body. The haematogenous spread is supported by small parasitic granulomas that have occasionally been found in the walls of blood vessels, and by the occurrence of nematodes in the blood vessel lumina (Bröjer *et al*. 2000; Reiser *et al*. 2011). Subsequently, its ability for parthenogenetic reproduction enables *H. gingivalis* to rapidly increase in

number and cause massive tissue damage due to its migratory behaviour resulting in granulomatous inflammation of the affected organs (Pearce *et al.* 2001; Mandrioli *et al*. 2002; Müller *et al.* 2008). Equine infections of *H. gingivalis* mainly involve the brain, kidneys, oral and nasal cavities, but have also been reported in the liver, eyes and bone (Blunden *et al.* 1987; Mandrioli *et al*. 2002; Hermosilla *et al*. 2011). Given the different internal organs that can be affected, halicephalobiasis is very difficult to diagnose and is therefore only rarely diagnosed ante mortem. Of the approximately 75 equine cases only two report on a successful treatment (Pearce *et al*. 2001; Müller *et al*. 2008).

Although the species is categorized as bacteriovorous, only a few records of free-living *H. gingivalis* have previously been reported, *i.e.*, isolate JB128 from a vegetable compost heap in Riverside (California, USA) and JB043 from potting soil containing peat near Neustadt, Germany (Nadler *et al*. 2003) and even though cases of equine halicephalobiasis are known from most of our neighbouring countries, neither a parasitic nor a free-living record was ever reported in Belgium. In current study, a thorough sampling of manure heaps and soil at 73 equine facilities in East and West Flanders revealed the first records of free-living *H. gingivalis*. Additionally, the first Belgian record of equine halicephalobiasis was described from a euthanized 5-year-old anorexic and lethargic Connemara mare with severe facial swelling and dyspnoea. Histological evaluation of the lesion revealed a granulomatous reaction with numerous adult and juvenile nematodes, which were morphologically and molecularly identified as *H. gingivalis*.

The total sampling effort resulted in over 40 isolates of different origin, *i.e*., from manure heaps, compost heaps, fresh horse dung, and from the rectum of horses. All these isolates, complemented by three non-Belgian isolates (two free-living, one clinical), were all kept in culture allowing a thorough biological characterization of *H. gingivalis* based on a multidisciplinary approach. Firstly, since detailed information concerning the internal morphology of *H. gingivalis* -especially in relation to possible adaptations to its lifestyle as a facultative parasite- was lacking, the morphology of the intestine of *H. gingivalis* was investigated by means of transmission electron microscopy (TEM) and propidium iodide staining. Specimens were cultured under different conditions to determine if differences in ultrastructure were induced by culturing method. Although TEM analysis revealed that the general morphology of the intestine of *H. gingivalis* is similar to that of other studied free-living nematodes (*e.g*., Epstein *et al.* 1971), unusual dichotomously and trichotomously

branched microvilli were observed next to finger-like cylindrical microvilli, the latter being the most commonly described form in nematodes. Also, three different types of secretion vesicles, *i.e*., spherical, threadlike and enlarged, globular vesicles, occurred independently from each other along the intestinal tract. The branching of the intestinal microvilli observed in *H. gingivalis* is likely to be a function of food consistency than of food availability. Most remarkable, deviations of the usual finger-like intestinal microvilli have arisen independently several times within the phylum, their occurrence always being in parasitic or in parasitism-related nematodes.

Secondly, the medical history of equine halicephalobiasis reported a regular treatment with common anthelmintic drugs, thereby strongly indicating that *H. gingivalis* has some sort of tolerance to these anthelmintic drugs (Boswinkel *et al.* 2006; Ferguson *et al*. 2008). Therefore an *in vitro* study was performed on the anthelmintic tolerance of both free-living and horse-associated *Halicephalobus* isolates to thiabendazole and ivermectin using an adaptation of the Micro-Agar Larval Development Test (Coles *et al*. 2006), which focused on egg hatching and larval development. Two closely related species, *i.e*., *Panagrellus redivivus* and *Panagrolaimus superbus*, were included as a positive control. The results generally showed that the anthelmintic tolerance of *Halicephalobus* to both anthelmintic drugs was considerably higher than that of the included Panagrolaimidae and, comparing to other studies, than that of obligatory equine parasites. The results further revealed a remarkable trend of increasing tolerance from fully free-living isolates towards horse-associated isolates, which was ascribed to possible evolutionary lineages or cryptic species. Additionally, *H. gingivalis* was proposed as an experimental tool to deepen our understanding of the biology of anthelmintic tolerance since *in vitro* anthelmintic testing with free-living and facultative parasitic nematodes offers the advantage of observing drug effect on the complete life cycle as opposed to obligatory parasites which can only be followed until the third juvenile stage.

Thirdly, while maintaining *H. gingivalis* cultures, it became immediately apparent that a substantial morphological variation was present within the individual populations depending on cultivating temperature and general condition. Therefore, the progeny of a single parthenogenetic female of one isolate (WB0801) was cultured under different food (low and high food availability) and temperature (15, 30 and 37°C) conditions, and measured in different adult age groups, *i.e*., young adults with a developed vulva but before the onset

of oviposition, adults laying eggs, and old, post-reproductive adults near the end of their life cycle. Of 540 specimens in total, 15 morphometric characteristics and 13 indices were determined and subsequently analyzed using both univariate (analysis of variance) and multivariate (principal components and canonical discriminant analysis) techniques. The main results revealed that the morphometric characters most used in *Halicephalobus* identification keys had a huge variability within a single progeny, *i.e*., of a magnitude that had not been observed in nematodes before. Further, by changing the environmental factors, the morphometric characters were influenced to an extent that one could assign – with seemingly 'statistical support'– different 'species' of the genus to different subpopulations. Although stoma length, ratio V and ratio corpus/isthmus length had a low overall variability, ratio V was influenced by temperature and the small size and weakly developed posterior part of the stoma makes previously observed interspecific variation less than convincing (Geraert *et al*. 1988). Therefore, only ratio corpus/isthmus length remained potentially useful as a discriminating factor.

Finally, it was not clear whether isolates of *H. gingivalis* rarely and opportunistically infect hosts, which would be supported if no genetic differences exist between free-living and parasitic isolates, or whether a lineage of *H. gingivalis* is evolving towards equine host specificity, which would be supported by reciprocal monophyly of isolates from horses versus those from soil (Blunden *et al*. 1987; Nadler *et al.* 2003). To this end, a multidisciplinary study was performed combining morphological, morphometrical and molecular data of 17 *H*. *gingivalis* isolates, of both free-living and parasitic origin, complemented with 4 isolates of other species of *Halicephalobus* with a distinctly different biology. A phylogeny based on two nuclear loci (18S and D2D3 expansion segment) and two mitochondrial loci (COI and ND4), and on both maximum likelihood and Bayesian inference indicated that most *Halicephalobus* isolates were found both molecularly and morphometrically distinguishable from a distinct *H. gingivalis*-clade. Only for isolates within this *H. gingivalis*-clade, LSU and SSU sequence data revealed a high level of intra-genomic variability; next to single nucleotide polymorphisms, two polymorphic regions were observed with associated alterations in the secondary structure model. This has, to the best of our knowledge, never been described before for nematodes. Given that in *Plasmodium* the expression of two distinct types of SSU rDNA has been linked to different parasitic life stages (Gunderson *et al*. 1987; Li *et al*. 1994; Rogers *et al*. 1996), it is possible that the

presence of alternating life stages in *H. gingivalis* is the key to understanding the presence of the SSU heterogeneity. Hence, this trait could facilitate the survival of a single population of *H. gingivalis* in extremely different habitats, *e.g.*, temperature ranges of 4 to 40°C (Discussion Chapter 3), and consequently enables it to be opportunistically parasitic. Within the *H. gingivalis*-clade, two morphological not discernible lineages were indicated reciprocal monophyletic by several species distinctiveness measures, revealing cryptic speciation. Most remarkably, all isolates originating from inside horses were within only one of these lineages and a phylogeny based on D2D3 sequences complemented with GenBank sequences of clinical isolates demonstrated a more close relation of horse-associated isolates, which indicates an evolution towards equine host specificity.

In conclusion, the present study based on a multidisciplinary approach demonstrates that the facultative parasitic nematode *H. gingivalis* has some characteristics that enable it to opportunistically colonize vertebrate hosts: **1)** ultrastructural adaptations at the level of the intestine, **2)** an unseen tolerance for common used anthelmintic drugs, **3)** a remarkable temperature range, and **4)** parthenogenetic reproduction. Moreover, a phylogeny based on multiple genes revealed two distinct evolutionary lineages within *H. gingivalis* of which one appears to be evolving towards vertebrate specific parasitism.

SAMENVATTING

Nematoden zijn een van de meest succesvolle en diverse groepen van organismen. Ze hebben een hoge functionele en taxonomische diversiteit, zijn alomtegenwoordig verspreid in grote aantallen en zijn zeer succesvol als parasieten. Ze vertonen een hoge verscheidenheid aan parasitaire levenswijzen en mogelijke gastheren, zowel planten als ongewervelde en gewervelde dieren, dit resulteert vanuit hun enorme aanpassingsvermogen aan nieuwe omgevingen. Dierparasitisme is binnen het fylum minstens zes keer onafhankelijk van elkaar ontstaan (Blaxter *et al.* 1998; Dorris *et al.* 1999). In de "Secernentea" worden al de intermediaire stadia teruggevonden tussen vrijlevende saprofyten en obligate parasieten (Sudhaus 2010). Obligate parasieten zijn volledig afhankelijk van een gastheer tijdens een deel of de gehele levenscyclus, terwijl facultatieve parasieten zich kunnen aanpassen aan een parasitaire levenswijze, maar er niet noodzakelijk afhankelijk van zijn. Vanuit een evolutionair standpunt worden facultatieve parasieten vaak gezien als een tussenstadium bij het ontwikkelen of verliezen van een parasitaire levenswijze.

Halicephalobus gingivalis, voorheen ook *Rhabditis gingivalis*, *Micronema deletrix* of *Halicephalobus deletrix* genoemd, behoort tot de familie van de Panagrolaimidae en is een kleine vrijlevende bacterie-etende nematode die facultatief parasitair kan zijn bij paarden (Blunden *et al.* 1987; Nadler *et al.* 2003) en mensen (Ondrejka *et al*. 2010). Geïsoleerde gevallen zijn beschreven bij een ezel (Schmitz and Chaffin 2004), een zebra (Isaza *et al.* 2000), in de huid rond het scrotum van een stier (Georgi and Georgi 1990) en op *H. gingivalis* gelijkende nematoden zijn beschreven in de hersenen van een drie jaar oude Black Angus koe (Montgomery and O'Toole 2006). Er echter is weinig geweten over de epidemiologie van *H. gingivalis*. Er zijn verschillende mogelijke infectieroutes beschreven: langs de mondopening door het eten van besmet plant materiaal dat dan dienst doet als mechanische vector (Hermosilla *et al.* 2011), langs het ademhalingsstelsel door inademing van nematoden (Spalding *et al.* 1990; Ruggles *et al.* 1993; Trostle *et al.* 1993; Bröjer *et al.* 2000), of door infectie langs de huid door het neerliggen van het paard (Dunn *et al.* 1993). Na het binnendringen in de gastheer laat de parthenogenetische voortplanting toe om snel in aantal toe te nemen, waarna de nematoden ter plaatse blijven of via het bloedvaten- of lymfestelsel de verschillende organen bereiken. De verspreiding via het bloed wordt

ondersteund door het voorkomen van nematoden in de bloedvatholte (Bröjer *et al*. 2000; Reiser *et al*. 2011). Doordat de nematoden doorheen het weefsel migreren ontstaan granulomateuze ontstekingen in de aangetaste organen (Pearce *et al.* 2001; Mandrioli *et al*. 2002; Müller *et al.* 2008). Infecties bij paarden komen vooral voor in de hersenen, de nieren, de mond- en neusopening, maar zijn ook beschreven in de lever, de ogen en de beenderen (Blunden *et al.* 1987; Mandrioli *et al*. 2002; Hermosilla *et al*. 2011). Doordat verschillende organen aangetast kunnen zijn, is het zeer moeilijk een infectie met *H. gingivalis* vast te stellen waardoor de diagnose zelden *ante mortem* wordt gesteld. Van de ongeveer 75 beschreven gevallen bij paarden vermelden slechts twee een succesvolle behandeling (Pearce *et al*. 2001; Müller *et al*. 2008).

Hoewel de soort gekend is als bacterie-etend, waren er bij de aanvang van dit doctoraatsonderzoek slechts enkele meldingen van vrijlevende populaties: isolaat JB128 uit een composthoop in Riverside (Californië, VS) en JB043 uit teelaarde met turf nabij Neustadt (Duitsland, Nadler *et al*. 2003). Hoewel infecties met *H. gingivalis* bij paarden beschreven zijn in de meeste van onze buurlanden, waren er nog geen meldingen van parasitaire of vrijlevende populaties van deze soort in België. Een grondige bemonstering van mesthopen en grond rondom 75 Oost- en West Vlaamse maneges resulteerden in de eerste Belgische meldingen van vrijlevende *H. gingivalis* populaties. Daarnaast werd in de huidige studie eveneens het eerste Belgische geval van halicephalobiasis bij paarden beschreven. Dit nadat een 5-jaar-oude Connemara merrie aangeboden werd aan de faculteit Diergeneeskunde met een opvallende zwelling van het gezicht en kortademigheid. Histologisch onderzoek toonde een granulomateuze reactie aan met een grote hoeveelheid volwassen en juveniele nematoden, die morfologisch en moleculair geïdentificeerd werden als *H. gingivalis*.

De totale bemonstering leverde uiteindelijk meer dan 40 *H. gingivalis* isolaten op van verschillende origine: uit compost- en mesthopen, verse paardenmest en uit meststalen genomen uit het rectum van een paard. Al deze isolaten, aangevuld met drie niet-Belgische isolaten (twee vrijlevende en één uit een infectie bij een paard) werden allemaal in cultuur gehouden wat, gebaseerd op een multidisciplinaire benadering, een uitgebreide biologische karakterisering van *H. gingivalis* toeliet.

Aangezien de medische dossiers van paarden die gestorven zijn aan halicephalobiasis steeds weergaven dat de dieren op regelmatige basis ontwormd waren, werd een tolerantie voor deze medicijnen vermoed (Boswinkel *et al.* 2006; Ferguson *et al*. 2008). Daarom werd

er in een tweede luik van het doctoraatsonderzoek een *in vitro* studie uitgevoerd naar de tolerantie van vrijlevende en paardgeassocieerde isolaten van *H. gingivalis* voor thiabendazole en ivermectine, beide courant gebruikt bij het ontwormen van paarden, met behulp van een aangepaste Micro-Agar Larvale Ontwikkeling Test (Coles *et al*. 2006). Hierbij werd er vooral gekeken naar het ontluiken van de eitjes en de verdere ontwikkeling van juveniel tot adult. Twee nauw verwante soorten, *Panagrellus redivivus* en *Panagrolaimus superbus*, werden toegevoegd als positieve controle. De resultaten toonden dat de tolerantie van *Halicephalobus* voor beide ontwormingsmiddelen aanzienlijk hoger was dan die van de nauw verwante Panagrolaimidae en, in vergelijking met andere studies, hoger dan die van obligate paardparasieten. Bovendien onthulde de resultaten ook een opmerkelijke stijgende trend van toenemende tolerantie van vrijlevende tot paardgeassocieerde isolaten, wat toegeschreven werd aan de mogelijke aanwezigheid van evolutionaire lijnen of cryptische soorten binnen *H. gingivalis*. Aangezien *in vitro* experimenten met vrijlevende facultatief parasitaire nematoden toelaten het effect van medicijnen te testen op de volledige levenscyclus, dit in tegenstelling tot obligate parasieten die enkel gevolgd kunnen worden tot het derde juveniele stadium, werd *H. gingivalis* voorgesteld als een potentiële experimentele tool om inzicht te krijgen in de effecten van ontwormingsproducten en andere medicijnen.

In een derde luik werd de interne morfologie van de darm van *H. gingivalis* bestudeerd met behulp van transmissie elektronen microscopie (TEM) en een propidium jodide kleuring om na te gaan of er morfologische aanpassingen aanwezig zijn die geassocieerd zouden kunnen zijn met een facultatief parasitaire levenswijze. Hierbij werden de nematoden onder verschillende omstandigheden gewkeekt om de invloed van externe omstandigheden te kunnen uitsluiten. Hoewel de TEM analyse onthulde dat de algemene darm morfologie van *H. gingivalis* gelijkend is op die van andere reeds bestudeerde vrijlevende nematoden (zie bijvoorbeeld Epstein *et al.* 1971), werden er naast cilindrische microvilli, die het meest beschreven zijn bij nematoden, ook dichotoom en trichotoom vertakte microvilli waargenomen. Ook werden drie verschillende soorten van uitscheiding beschreven, zijnde sferische, draadvormige en opgezwollen blaasjes, die onafhankelijk van elkaar gevonden werden doorheen de darm. Het vertakken van de darm microvilli kan mogelijk worden toegeschreven aan een verscheidenheid van de consistentie van voedsel bij een vrijlevende en parasitaire levenswijze. Opvallend is dat afwijkingen van normale

cilindrische microvilli verschillende keren in het phylum Nematoda ontstaan is en dit steeds in parasitaire of in aan parasitisme gerelateerde nematoden.

Een vierde luik was gebaseerd op de observatie dat er een aanzienlijk morfologische variatie aanwezig was binnen individuele populaties afhankelijk van de kweektemperatuur en de algemene conditie van de cultuur. Nakomelingen van één parthenogenetisch vrouwtje van isolaat WB0801 werden gekweekt bij een verschillende hoeveelheid beschikbaar voedsel (weinig en veel voedsel) en bij verschillende temperaturen (15, 30, en 37°C). Vervolgens werden verschillende volwassen leeftijdscategorieën (jonge adulten met een ontwikkelde vulva maar zonder eileg, adulten in eileg, en oude, postreproductieve adulten nabij het einde van hun levenscyclus) morfo-metrisch opgemeten. Van de in totaal 540 specimens werden 15 verschillende afmetingen genomen en 13 verhoudingen berekend die vervolgens geanalyseerd werden met behulp van zowel univariate (analysis of variance) als multivariate (principal components and canonical discriminant analysis) analyse technieken. De belangrijkste resultaten onthulden dat de morfometrische kenmerken die doorgaans gebruikt worden in sleutels om soorten van *Halicephalobus* te identificeren allemaal een ongeziene mate van variatie vertonen. Door verandering van de omgevingsfactoren werden de morfometrische kenmerken in die mate beïnvloed dat foutief – maar met statistische ondersteuning- verschillende soorten van het genus zouden kunnen toegekend worden aan verschillende subpopulaties afkomstig van één enkel vrouwtje. Hoewel stoma lengte, de verhouding V en de verhouding corpus/isthmus lengte een lage algemene variatie vertoonden, werd verhouding V sterk beïnvloed door temperatuur, en de kleine afmeting van het stoma en het slecht ontwikkelde achterste deel ervan hebben dan weer tot gevolg niet overtuigend te zijn om verschillende soorten van het genus van elkaar te onderscheiden (Geraert *et al*. 1988). Daardoor blijkt enkel de ratio corpus/isthmus lengte weerhouden als potentieel bruikbaar als betrouwbaar identificatie kenmerk.

Ten slotte was het niet duidelijk of isolaten van *H. gingivalis* eerder zeldzaam en opportunistisch gastheren infecteren of dat er een genetische lijn bestaat die evolueert naar gastheerspecifiek parasitisme, wat bevestigd zou kunnen worden door een afzonderlijke vrijlevende lijn en een parasitaire clade (Blunden *et al*. 1987; Nadler *et al.* 2003). Om hier klaarheid in te scheppen werd een multidisciplinaire studie uitgevoerd waarin morfologische, morfometrische en moleculaire data gecombineerd werden van 17 *H. gingivalis* isolaten en 4 andere soorten binnen het genus. Een gecombineerde fylogenetische

analyse van twee nucleaire (18S en D2D3) en twee mitochondriale merkers (COI en ND4), zowel gebaseerd op maximum likelihood als op Bayesiaanse analyse methoden, gaven aan dat de andere *Halicephalobus* isolaten moleculair te onderscheiden zijn van een duidelijke *H. gingivalis*-clade. Enkel voor isolaten binnen deze *H. gingivalis*-clade vertoonde de D2D3 en de 18S sequenties een hoge mate van variabiliteit binnen het genoom: naast single nucleotide polymorfs, werden er twee polymorfe regio's onderscheiden in 18S, met geassocieerde veranderingen in de secundaire structuur. Dit werd tot op heden, voor zover wij weten, niet eerder beschreven bij nematoden. Aangezien bij *Plasmodium* de expressie van twee verschillende kopijen van het 18S rDNA geassocieerd werd met verschillende parasitaire levensfasen (Gunderson *et al*. 1987; Li *et al*. 1994; Rogers *et al*. 1996), is het mogelijk dat in de aanwezigheid van de wisselende levensfasen geassocieerd met het facultatief parasitaire karakter van *H. gingivalis* wat een aannemelijke verklaring is voor deze rDNA heterogeniteit. Binnen de *H. gingivalis*-clade bleken verder twee goed ondersteunde clades morfologisch en morfometrisch niet te onderscheiden maar wel duidelijke afzonderlijke evolutieve lijnen te zijn op basis van verschillende maatstaven voor het onderscheiden van soorten, wat duidt op de aanwezigheid van cryptische speciatie. Meest opmerkelijk is dat al de isolaten afkomstig uit paarden teruggevonden werden binnen één clade. Verder toont een afzonderlijke fylogenie (enkel gebaseerd op D2D3 sequenties) aangevuld met sequenties van klinische isolaten afkomstig van GenBank een nauwere verwantschap aan tussen paardgeassocieerde isolaten wat opnieuw een evolutie naar gastheer specificiteit aangeeft.

Tot besluit, het huidige doctoraatsonderzoek gebaseerd op een multidisciplinaire aanpak demonstreert dat de facultatief parasitaire nematode *H. gingivalis* een aantal karakteristieken heeft die het mogelijk maken op opportunistische wijze gewervelde gastheren te infecteren: **1)** ultrastructurele aanpassingen van de darm, **2)** een buitengewone tolerantie voor courant gebruikte ontwormingsproducten, **3)** een opmerkelijke temperatuursrange waarbij ze kunnen overleven, en **4)** parthenogenetische voortplanting. Bovendien toont een fylogenie gebaseerd op 4 merkers de aanwezigheid aan van twee opvallende evolutionaire lijnen binnen *H. gingivalis* waarvan één lijkt te evolueren naar gastheer specificiteit bij zoogdieren.

CHAPTER I

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

PARASITISM IN THE PHYLUM NEMATODA

Parasitism is a widespread evolutionary strategy that has arisen independently several times throughout the tree of life. Generally, in exploitative interactions between organisms, *i.e*., when one partner is benefitted and the other one experiences some form of disadvantage or harm, the aggressor is considered a parasite when typically one host is attacked, but not killed (Bush *et al.* 2001). Obligatory parasites are completely dependent on the host during a part or all of their life cycle, while organisms that are adaptive to a parasitic way of life when placed in such a relationship -but do not absolutely depend on it- are called facultative parasites. From an evolutionary point of view, facultative parasites are thought to represent the intermediate stage in the acquisition or loss of parasitism (Sudhaus 2010).

Nematodes are one of the most successful and diverse groups of organisms in the world. They show a high functional and taxonomical diversity and are ubiquitously distributed in high numbers. Cobb (1914) once wrote that if all matter in the universe was swept away, the world and its structure of mountains, valleys, lakes and oceans would still be dimly recognizable by the film of nematodes that was left behind. Although most nematodes are free-living forms found in a wide variety of aquatic (both marine and freshwater) and terrestrial habitats, most research focuses on parasites of agricultural crops, livestock and humans and on nematodes as model organisms, *e.g*., *Caenorhabditis elegans* and *Pristionchus pacificus*. Nematodes are very successful as parasites. They show a high diversity in both parasitic lifestyles and diversity of possible hosts, *i.e*., invertebrates, vertebrates and plants, which reflects a high adaptability to new environments and an aptitude towards parasitism. Animal parasitic nematodes are of great importance, both from an ecological, *i.e*., playing a significant role in regulating the productivity of wild populations, and a human point of view (Anderson 1984; Blaxter 2003). They can limit agricultural efficiency as parasites of domestic animals and result in a high morbidity and mortality as human parasites (Bush *et al*. 2001). Most vertebrates have associated nematode parasites, which can exploit almost any tissue in the vertebrate body (Bundy 1997).

It has been proposed that the earliest animal-parasitic nematodes probably were luminal dwellers feeding on the intestinal microflora of their host, with cutaneous penetration or passive ingestion as primary modes of infection (Anderson 1984). According to a molecular phylogenetic framework based on the SSU DNA gene such a single

mechanism for the evolution of parasitism seems unlikely since animal-parasitic taxa are not grouped in one distinct clade but occur within a radiation of free-living taxa (Dorris *et al.* 1999) revealing that the acquisition of an animal parasitic mode of life has arisen independently at least six times within the phylum (Blaxter *et al.* 1998; Dorris *et al.* 1999). Additionally, there appears to be an association between invertebrate and vertebrate parasitism since invertebrate-pathogenic and -parasitic clades are all placed sister to vertebrate-parasitic clades (Dorris *et al*. 1999).

Based on clade naming by Blaxter *et al*. (1998) and following the classification of De Ley and Blaxter (2002) (figure 1.1), within the class Enoplea, 'clade I' (*i.e*., subclass Dorylaimia) includes -next to plant-parasitic and free-living orders- the animal parasitic orders Trichinellida (in vertebrates), Mermithida (in arthropods, molluscs, polychaetes, echinoderms), Marimermithida (in deep-sea bottom-dwelling invertebrates), Muspiceida (in mammals), Dioctophymatida (birds and mammals), whereas 'clade II' (*i.e*., subclass Enoplia) does not include animal parasitic taxa. Within the class Chromadorea, three major clades are identified. 'Clade III' (*i.e*., suborder Spirurina) comprises only arthropod- and vertebrate-parasitic taxa, *i.e*., the infraorders Ascaridomorpha (large gut roundworms of vertebrates), Spiruromorpha (filarial nematodes), Oxyuridomorpha (pinworms) and Rhigonematomorpha (millipede-gut parasites). 'Clade IV' (*i.e*., suborder Tylenchina) groups the infraorder Panagrolaimomorpha, encompassing -next to free-living bacteriovorous taxa- the vertebrate-parasitic family Strongyloididae and the entomopathogenic genus *Steinernema*, together with the plant parasitic infraorder Tylenchomorpha, and the free-living bacteriovorous infraorder Cephalobomorpha. In 'clade V' (*i.e*., suborder Rhabditina), the superfamily Strongyloidea[1] include vertebrate-parasitic taxa within the mainly free-living infraorder Rhabditomorpha, the entomopathogenic genus *Heterorhabditis*, and the infraorder Diplogasteromorpha).

[1] Note the difference between the family Strongyloididae in clade IV (Strongyloidoidea: Panagrolaimorpha: Tylenchina), which contains *Strongyloides* spp., and the family Strongylidae in clade V (Strongyloidea: Rhabditomorpha: Rhabditina), which contains *Strongylus* spp.; a potential source of confusion.

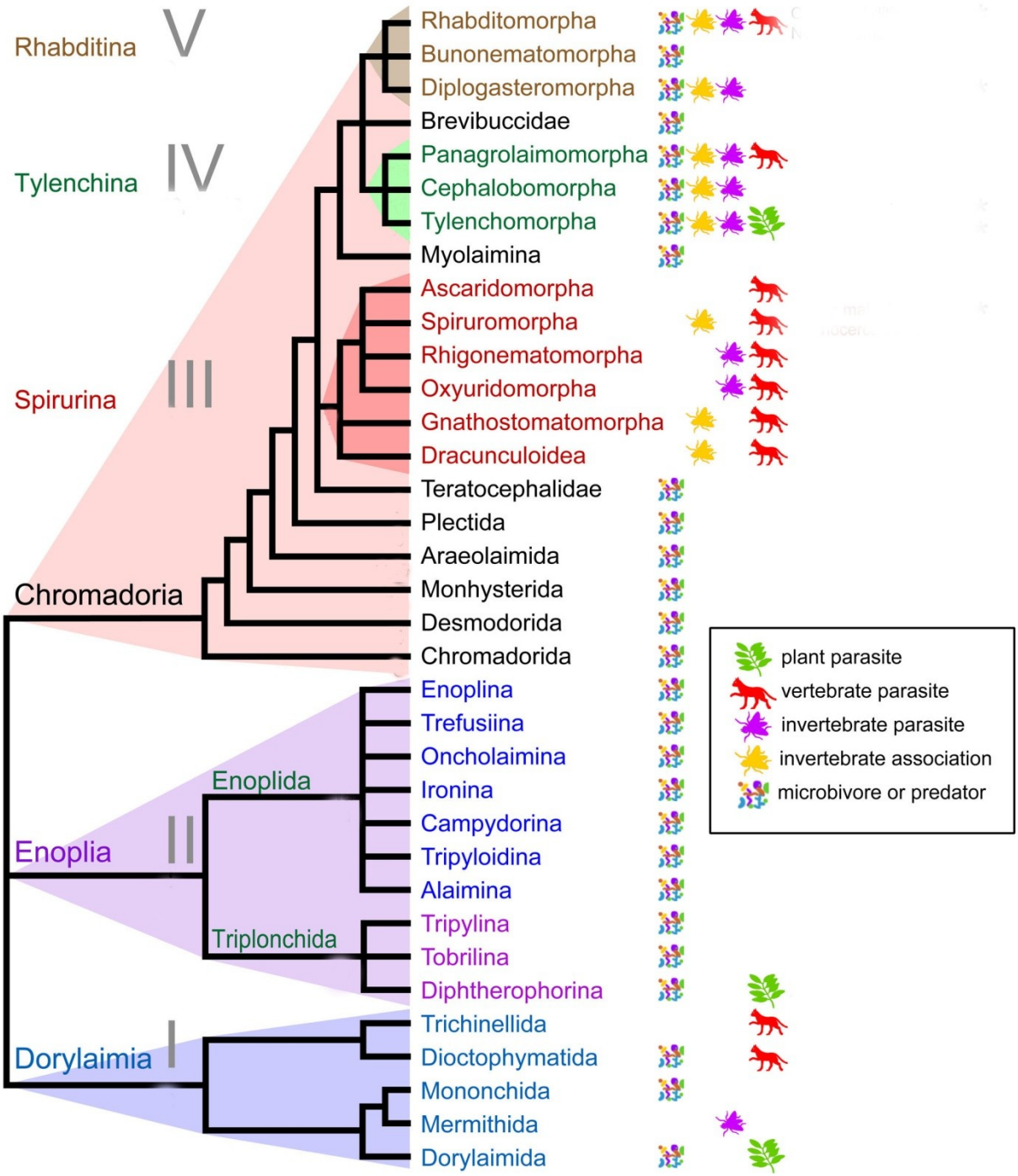

Figure 1.1. Phylogeny based on molecular data of the small subunit ribosomal RNA gene. Systematic names are based on De Ley and Blaxter (2002); clade naming is based on Blaxter *et al.* (1998). Feeding mode, animal or plant parasitism, and vector associations are indicated by icons. Figure taken from Blaxter (2011).

The evolution towards parasitism has long been thought irreversible since it requires the acquisition of many novel traits (overview in Siddall *et al.* 1993). Additionally, obtaining a parasitic way of life is often related to a loss of genetic information, *e.g*., in prokaryotic obligatory intracellular parasites (Sakharkar *et al.* 2004) and in parasitic red algae (Hancock *et al.* 2010), due to a loss of biosynthetic pathways because the parasites scavenge essential molecules from their host. Therefore the reduction of genetic information has been used as proof of an obligatory parasitic mode of existence (Hauser *et al.* 2010). However, genomic simplification is not necessarily true for parasitic nematodes due to an often complicated life cycle including multiple hosts, rendering reversibility of parasitism possible in nematodes (Blaxter *et al.* 2004).

NEMATODES AS EQUINE PARASITES

Although only a few of the more than 60 possible internal parasites of horses cause significant health problems, they remain one of the most important problems affecting their health and well-being. Of all domestic livestock, horses have the largest collection of parasites of which the vast majority are nematodes. The most common internal parasitic nematodes of horses are ascarids, threadworms, pinworms, and small and large strongyles (Bowman and Georgi 2009).

The ascarid species *Parascaris equorum* (GOEZE, 1782) YORKE AND MAPLESTONE, 1926 primarily affects younger horses under the age of six months. Adult nematodes reside in the small intestine, where they can cause intestinal obstruction when worm burdens are numerous (Laugier *et al.* 2012). Damage by *P. equorum* is mostly caused by the migration of the juveniles. They penetrate the intestinal wall, disseminate hematogenously to the liver, heart and lungs, after which they are coughed up and ingested to mature in the intestine. Prognosis are good when rapidly treated. In rare cases the lungs are damaged through the migration of juveniles, consequently making the horse more susceptible to secondary bacterial pneumonia. By 2 years of age most horses develop a natural immunity against this parasite (Reinemeyer 2012).

The pinworm[2] *Strongyloides westeri* IHLE, 1917 also primarily infects foals, causing diarrhoea and indigestion when present in large numbers. Species of the genus *Strongyloides* have a complex life cycle that has elements of both free-living and parasitic stages (Harvey *et al.* 1999). Parthenogenetic adult females reside in the intestine and form the parasitic stage. When the produced eggs pass with the faces, L1 juveniles emerge as chromosomally determined males or females (Harvey and Viney 2001). The male juveniles subsequently develop into functional, free-living males, whereas the female juveniles can either develop into free-living amphimictic females or develop directly into infective L3 juveniles (Harvey *et al.* 2000). Because arrested juveniles become activated and migrate into the mammary tissue with the onset of lactation, infection generally occurs when foals suckle the mares' contaminated milk (Lyons *et al.* 1973). Treatment consists of the administration of ivermectin to the mare shortly after birth or to the suckling foals (Ludwig *et al.* 1983).

Adult stages of the threadworm *Oxyuris equi* SCHRANK, 1788 occupy the dorsal and descending colons of horses of all ages, however, are mostly found in young horses up to two-years-old. Gravid female nematodes protrude from the anus and deposit their eggs in the perianal region which causes irritation and itching. The infection occurs through larvated eggs that are ingested from the environment and hatch in the intestine, whereupon they develop into adults and migrate to the distal gut (Reinemeyer 2012). Additionally, the rubbing of the tail because of irritation may cause secondary infection of the anus, tail and surrounding skin.

Equine parasites of the family Strongylidae are divided into small (subfamily Cyathostominae) and large strongyles. They have a direct life cycle without an intermediate host. All adult strongyles reside in the large intestine, where they produce eggs that are passed out into the horse's environment through the faces. Subsequently, the eggs develop into free-living infective third stage (L3) juveniles and infection occurs when contaminated grass, food, or water is ingested by the horse (Lyons *et al.* 1999; Stratford *et al.* 2011). There are three main species of large strongyles in horses, *i.e.*, *Strongylus vulgaris* LOOSS, 1900, *S. equinus* MÜLLER, 1780, and *S. edentatus* LOOSS, 1900. Most large strongyles have a migratory life cycle. The juveniles penetrate the intestinal wall, migrate to the branches of the intestinal arteries and various organs where they can cause damage, irritation and possibly

[2] In British usage, which is followed here, pinworms refer to *Strongyloides* and threadworms refer to *Oxyuris*, whereas in US usage nematodes of the genus *Enterobius* (family Oxyuridae) are known as pinworms.

fatal haemorrhage. Eventually, blood clots can dislodge and occlude the intestinal arteries causing colic, or thrombo-embolism of the arteries supplying the hind legs causing lameness. The bloodsucking adults can cause anemia, debilitation, and damage to the intestinal mucosa. There are approximately 50 equine species of small strongyles, of which about 10 species are most commonly reported (Corning 2009). As opposed to large strongyles, cyathostomins have a non-migratory life cycle. Ingested juveniles penetrate the intestinal wall, undergo development and, subsequently, re-emerge and mature to adults in the intestinal lumen (Stratford *et al.* 2011). The penetration of the intestinal wall by the juveniles and the emergence of the L4 juveniles can cause considerable damage to the intestinal mucosa (Corning 2009). However, the maturation of the L3 juveniles can be delayed in which case the L3 juveniles encyst and remain in the intestinal wall for up to two years. When these encysted juveniles re-emerge simultaneously into the intestinal lumen in large numbers, which is known as 'larval cyathostominosis', they can cause severe damage to the intestinal wall leading to diarrhoea, potentially serious colic and even the death of the horse (Corning 2009; Bodecek *et al.* 2010). Although cyathostomins mostly affect young horses under the age of six-years-old (Reid *et al.* 1995), they can cause clinical disease in horses of any age (Corning 2009).

ANTHELMINTIC RESISTANCE IN EQUINE PARASITIC NEMATODES

The second half of the 20th century has witnessed the advent of new classes of anthelmintic drugs with the first benzimidazoles developed in the 1960's, the pyrimidines (pyrantel) in the 1970's, ivermectin released during the 1980's and moxidectin during the 1990's (Nielsen 2009). With the arrival of new drugs came a new anthelmintic program, first described by Drudge and Lyons (1966), which was based on the complete suppression of the parasites through worming every other month all year round. Such high-frequency usage of the same drug is, however, one of the main causes of anthelmintic resistance known today (*e.g.*, Prichard *et al.* 1980; Martin *et al.* 1984; Sangster 1999; Kaplan 2004). First reports of resistance to benzimidazoles in equine parasites came to light in 1966 (Drudge and Lyons). Since then, resistance to this group of anthelmintics has been widely reported in cyathostomins (*e.g.*, Lyons *et al.* 1999). For years, resistance seemed to be restricted to Cyathostominae to benzimidazoles. This changed with reports of resistance of cyathostomes

to other anthelmintic drugs such as pyrantel (*e.g.*, Chapman *et al.* 1996; Lyons *et al.* 1999; Tarigo-Martinie *et al.* 2001), of large strongyles to benzimidazoles (Brady and Nichols 2009) and of *Parascaris equorum* to ivermectin and moxidectin (*e.g.*, Boersema *et al.* 2002; Edward and Hoffmann 2008; Reinemeyer 2009; Veronesi *et al.* 2009; Laugier *et al.* 2012). Today, reports of anthelmintic resistance of all important equine parasites to at least one class of anthelmintic drug have been published (Reinemeyer 2012).

NEMATODES AS FACULTATIVE PARASITES OF MAMMALS

In secernentean (*i.e.*, class Chromadorea) nematodes, all intermediate stages between free-living saprobionts and obligatory parasites are present, *i.e.*, those living in close relationship with a host, those capable of living temporarily inside a host, and those that are partially or facultative parasitic (Sudhaus 2010). Following are the most frequently occurring facultative parasitic nematodes of vertebrates that belong to the order Rhabditida, which includes the research organism. *Diploscapter coronatus* (COBB, 1893) COBB, 1913 (Rhabditidae, order Rhabditida) is a cosmopolitan and free-living species that has first been described from decaying banana roots in Fiji and generally reproduces parthenogenetically (Lahl *et al.* 2006). *D. coronatus* has been described as an occasional facultative parasite of humans as it has been reported in the urinary sediment of an old Japanese woman with a severe kidney infection (Yokogawa 1936), in the stomach of nine people who were diagnosed with low levels of hydrochloric acid (Chandler 1938), in the faces of a 73-year-old Thai woman (Watthanakulpanich *et al.* 2005) and of a 61-year-old Iranian man (Athari and Mahmoudi 2008) who had both developed gastrointestinal discomfort including diarrhoea. Additionally, *D. coronatus* has been reported from necrotic nodules in the skin of snakes (Sabu *et al.* 2002). Generally, an infection with *D. coronatus* causes limited discomfort and is easily overcome with anthelmintic drugs.

A second example, *Pelodera* (*Rhabditis*) *strongyloides* (SCHNEIDER, 1860) SCHNEIDER, 1866, also belonging to the order Rhabditida, is a small free-living inhabitant of decaying organic matter occasionally causing significant dermatitis in several mammalian species, including cattle, swine, dogs, sheep and horses (Bowman and Georgi 2009). Additionally, four human infections have been reported in an 11-year-old girl (Pasyk 1978), a six-month-old infant (Ginsburg *et al.* 1984), an 18-year-old male (Jones *et al.* 1991) and a 20-year-old

male (Tanaka *et al.* 2004), all showing varying degrees of dermatitis. Although sometimes difficult, infections have always been treatable with ectoparasiticides (Tanaka *et al*. 2004).

The final example, *Halicephalobus gingivalis* (STEFAŃSKI, 1954) ANDRÁSSY, 1984, is a free-living bacteriovorous panagrolaimid which is a known facultative parasite, especially of horses. It can be considered the most important of the facultative parasitic nematodes due to the high mortality rate it causes in both horses and humans. Since this species is our research organism, it will be introduced more thoroughly below.

INTRODUCTION TO THE RESEARCH ORGANISM

THE GENUS HALICEPHALOBUS

The genus *Halicephalobus* TIMM, 1956 belongs to the family Panagrolaimidae, which is placed in 'Clade IV' (Blaxter *et al*. 1998; De Ley and Blaxter 2002). The genus originally consists of 11 species and is morphologically characterized by a short body, a tuboid stoma narrowing at the posterior end, a pharynx consisting of a corpus with a median bulb, an isthmus and a terminal bulb with valves, and a monodelphic-prodelphic female reproductive system which is usually dorsally reflexed (fig. 1.2). Males have never been described in any of the species, suggesting that they all reproduce parthenogenetically. *Halicephalobus* is a cosmopolitan genus, which is known for its wide environmental range, *i.e.*, in compost, humus, soil, rotten wood, in water enclosures in mines up to approximately 1 km belowground, and in association with insects and chelicerates (table 1.1). Especially the facultative parasitic behaviour of *H. gingivalis*, which is bacteriovorous when free-living, is remarkable.

The genus includes species which were originally described as *Phytorhabditis palmaris* LORDELLO AND OLIVEIRA, 1963, *Cephalobus* (*Tricephalobus*) *similigaster* ANDRÁSSY, 1952, *Halicephalobus limuli* TIMM, 1956, *Micronema minutum* KÖRNER, 1954 and *Rhabditis gingivalis* STEFAŃSKI, 1954. Since the oldest available genus name *Micronema* is a homonym of a genus of sheatfishes (Bleeker 1858 in Blunden *et al.* 1987), *Halicephalobus* was proposed by Andrássy (1974) to classify these species.

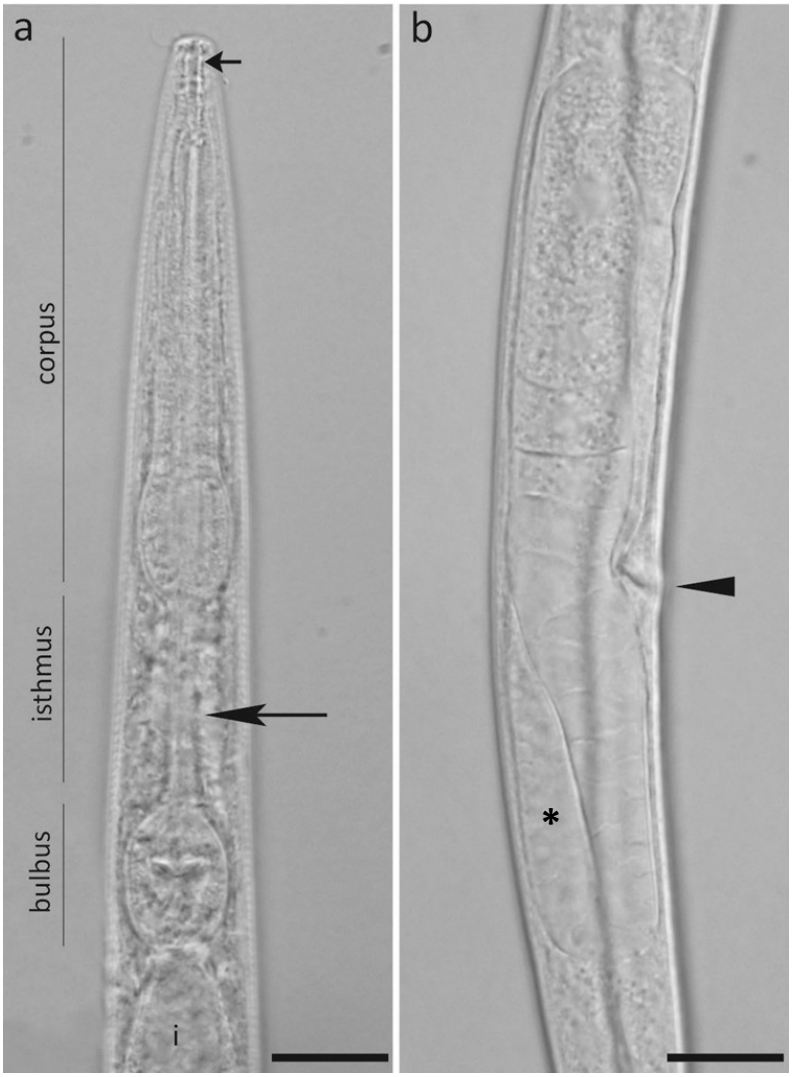

Figure 1.2. Morphological characterizations of *Halicephalobus*. a. Head region showing the tuboid stoma (small arrow), different parts of the pharynx, and the position of the nerve ring (large arrow), i = intestine; b. Monodelphic-prodelphic female reproductive system with dorsally reflexed ovary (asterisk). Position of the ventral vulva is indicated (arrowhead). Scale bars = 10 µm.

The species of the genus have very few discriminating morphologic traits. *H. laticauda* has a broad tail with distinct inner sclerotisations at the tip (Geraert *et al.* 1988), whereas the reproductive system of *H. mephisto* is not dorsally reflexed (Borgonie *et al.* 2011), as opposed to all other species of the genus. Of the remaining species, four have a distinctly longer tail, *i.e*., *H. minutus*, *H. parvus*, *H. similigaster* and *H. persicus*, and one has a distinctly shorter tail, *i.e*., *H. brevicauda*. The four other species, *i.e*., *H. gingivalis*, *H. intermedius*, *H. limuli*, and *H. palmaris*, have a similar morphology and are delineated based only on slight morphometrical differences. These characteristics show a relatively high intraspecific variation which may surpass species boundaries (Geraert *et al*. 1988). Further,

H. minutus and *H. parvus* were proposed as junior synonyms of *H. similigaster* based on overlapping morphometrical characters (Köhler 2011). A thorough revision of the genus appears necessary.

HALICEPHALOBUS GINGIVALIS

Halicephalobus gingivalis, previously also referred to as *Rhabditis gingivalis*, *Micronema deletrix* or *Halicephalobus deletrix*, is a small (235 – 460 µm) free-living bacteriovorous nematode, which is capable of facultative parasitism in horses (Blunden *et al.* 1987; Nadler *et al.* 2003) and humans (Ondrejka *et al.* 2010). Single cases have also been described in a donkey (Schmitz and Chaffin 2004) and a Grévy's zebra (Isaza *et al.* 2000), in the scrotal skin of a bull (Georgi and Georgi 1990), and *H. gingivalis*-like nematodes have been reported in the brain of a three-year-old black Angus cow (Montgomery and O'Toole 2006). The species was first described as *Rhabditis gingivalis* STEFAŃSKI, 1954 as an inhabitant of a tumour in the gingiva of a horse. Anderson and Bemrick (1965) described a similar tumourous inclusion of the nares of a horse and proposed the name *Micronema deletrix*, because they considered the description of Stefański unsatisfactory and claimed the species a 'species inquirenda'. Andrássy (1974) subsequently synonymised the two species under the valid genus name *Halicephalobus* and assigned it the oldest species epithet, *i.e.*, '*gingivalis'*. However, *H. deletrix* is still often wrongfully used.

Little is known about the epidemiology of *H. gingivalis*. Many possible infection routes have been described such as an oral route through the ingestion of contaminated plant material that acts as a mechanical vector (Hermosilla *et al.* 2011), or through the respiratory tract via the inhalation of nematodes (Spalding *et al.* 1990; Ruggles *et al.* 1993; Trostle *et al.* 1993; Bröjer *et al.* 2000). Other possibilities are infection through the gingiva, cutaneous infections through entry of free-living specimens facilitated by recumbency (Dunn *et al.* 1993) or open wounds, or infections via the oral cavity by first colonizing ingesta embedded in the gums and subsequently penetrating lacerations of the buccal mucosa, thereby invading the mandible or maxilla (Anderson *et al.* 1998; Ferguson *et al.* 2008). Halicephalobiasis is only rarely diagnosed ante mortem (Payan *et al.* 1979; Dunn *et al.* 1993; Trostle *et al.* 1993; Pearce *et al.* 2001; Müller *et al.* 2008). The geographical distribution of the equine infections further reveals that the species is cosmopolitan (table 1.2, figure 1.3).

After invading the host, the nematodes can either remain on the spot and cause local infections, such as cutaneous swelling or anomalies (Payan *et al*. 1979; Dunn *et al*. 1993; Pearce *et al.* 2001; Müller *et al*. 2008), or enter the bloodstream or lymphatic system and disseminate throughout the body. The haematogenous spread is supported by small parasitic granulomas that have occasionally been found in the walls of blood vessels, and by the occurrence of nematodes in the blood vessel lumina (Bröjer *et al*. 2000; Reiser *et al*. 2011), whereas the affected lymph nodes confirm the involvement of the lymphatic system. Both haematogenous and lymphatic transport of nematodes would explain the often disseminated nature of *H. gingivalis* infections and the high variability of organs that can be affected.

Equine infections of *H. gingivalis* have mostly been described from different parts of the central nervous system, *i.e*., all regions of the brain, nerves and the spinal cord. Renal infections and infections of the prepuce, the lymph nodes, and the oral and nasal cavities also occur frequently, whereas only occasional infections have been reported in the liver, heart, myocardium, lungs, optic nerve, eyes, testicles, mammary glands, stomach and bone, *i.e*., mandible, maxilla, femur, humerus and nasal bones. In case of renal or testicular involvement, juvenile nematodes have been detected in the urinary sediment or in the semen (Kinde *et al*. 2000). Additionally, evidence of prenatal, perinatal or transmammary transmissions of the infection between mares and their foals has been reported. The first case reports on two half sibling foals, born a year apart, which both died within three weeks after birth (Spalding *et al*. 1990). In the second case, a foal born from a mare with a known mammary gland infection of *H. gingivalis* died of halicephalobiasis three weeks after birth, whereas the mare only perished from a disseminated infection approximately one year later (Wilkins *et al*. 2001).

Of the approximately 75 equine cases only two have reported a successful treatment (Pearce *et al*. 2001; Müller *et al*. 2008), and two cases fail to report further detail on the fate of the horse (Payan *et al*. 1979; Dunn *et al*. 1993), while the other infections were lethal. Strikingly, the medical history of the infected horses reveals a regular treatment with common anthelmintic drugs, thereby indicating that *H. gingivalis* may have some sort of resistance to these medicines (Boswinkel *et al.* 2006; Ferguson *et al*. 2008).

Table 1.1 *Halicephalobus* species list.

Species		Environment	Occurrence
H. brevicauda	(Mavljanov, 1976) Andrássy, 1984	soil	Uzbekistan
*H. deletrix**	(Anderson and Bemrick, 1965) Andrássy, 1974	tumourous inclusion in nares of horse	United States
H. gingivalis	(Stefański, 1954) Andrássy, 1984	compost, facultative parasitic in horses	cosmopolitan
H. intermedius	(Pokrovskaja, 1964) Andrássy, 1984	gall on the roots of cucumber plant	Russia
H. laticauda	Geraert, Sudhaus, Lenaerts and Bosmans, 1988	water supply in mine (600 m deep)	Belgium
H. limuli	Timm, 1956	in association with chelicerates (*Limulus*)	Bangladesh
H. mephisto	Borgonie, Garcia-Moyano, Litthauer, Bert, Bester, van Heerden, Moller, Erasmus and Onstott, 2011	water enclosure in mine (1.3 km deep)	South-Africa
H. minutus‡	(Körner, 1954) Andrássy, 1974	moulder of pine and plane tree	Germany
H. palmaris	(Lordello and De Oliveira, 1963) Andrássy, 1974	dead wood of imperial palm	Brasil
H. parvus‡	(Körner, 1954) Andrássy, 1974	moulder of oak; gnarl of lime tree	Germany
H. persicus	Shokoohi, Abolafia and Zad, 2007	soil containing humus of pine and plane tree	Iran
H. similigaster	(Andrássy, 1952) Andrássy, 1974	dark brown water in tree stump; dead wood live beech tree	Hungary; Germany

*Synonym of *H. gingivalis* (Andrássy 1974); ‡ Synonym of *H. similigaster* (Köhler 2011).

Table 1.2. Chronological overview of all described infections of *H. gingivalis* in horses, humans, and other vertebrates; the country of origin of the case, the principal organs affected, and the reference of the clinical description.

	Origin	Principal organs affected	Reference
horse	Poland	head, gingiva	Stefański 1954
	United States	head, maxilla and maxillary sinuses	Anderson and Bemrick 1965; Johnson and Johnson 1966
	United States	brain and meninges	Stone *et al.* 1970
	Netherlands	brain, meninges, kidneys, lymph nodes, maxilla, nasal cavity	Linde-Sipman and Gruys 1970
	Egypt	brain and cervical spinal cord	Ferris *et al.* 1972
	United States	brain and kidneys	Rubin and Woodard 1974
	United States	brain	Jordan *et al.* 1975
	United States	brain, cervical spinal cord, meninges and pituitary gland	Powers and Benz 1977
	United States	brain, pituitary gland, kidneys, cervical spinal cord	Alstad *et al.* 1979
	United Kingdom	brain, kidneys, heart	Khalil *et al.* 1979; Ingram and Khalil 1980
	Colombia	prepuce (no detail on fate of horse)	Payan *et al.* 1979
	Switzerland	brain, renal lymph node, lung, kidneys	Pohlenz *et al.* 1981
	Netherlands	head, maxilla, premaxilla, mandible, brain, kidneys	Keg *et al.* 1984
	United States	mandible, mandibular lymph nodes	Cho *et al.* 1985
	Japan	brain	Yoshihara *et al.* 1985
	United Kingdom	brain, meninges, kidneys, wall of arterioles	Blunden *et al.* 1987
	United States	cerebral spinal fluid, brain, meninges	Darien *et al.* 1988
	United States	cerebral spinal fluid, brain, meninges	Darien *et al*. 1988
	Italy	kidneys	Marocchio and Mutinelli 1988
	United States	femur, stomach, lungs, kidneys, adrenal gland, lymph nodes	Simpson *et al.* 1988
	Philippines	kidneys	Alejandro-Matawaran and Peneyra 1989
	Germany	kidneys	Liebler *et al.* 1989
	Canada	kidneys	Chalmers *et al.* 1990
	United States	brain, spinal cord, meninges	Spalding *et al.* 1990
	United States	brain	Spalding *et al*. 1990
	United States	kidneys, brain, meninges, lungs	Buergelt 1991
	Scotland	kidneys, brain, meninges	Angus *et al.* 1992
	United States	prepuce (no detail on fate of horse)	Dunn *et al.* 1993
	Austria	brain, meninges	Reifinger 1993
	United States	kidneys, mandible, lungs, brain, meninges	Ruggles *et al.* 1993
	Germany	kidneys	Schelz 1993
	United States	cerebellum, lymph nodes, maxilla, nasal sinuses	Trostle *et al.* 1993
	United States	brain, optic chiasm, eye, kidney	Rames *et al.* 1995
	United States	humerus	Kreuder *et al.* 1996
	Italy	brain	Cantile *et al.* 1997
	Germany	gingiva, maxilla	Teifke *et al.* 1998
	Ireland	brain	Weaver *et al.* 1999
	Norway	kidney, brain, meninges, lungs, pericardium and arterial wall	Aleksandersen *et al.* 2000
	Norway	kidney, brain, meninges and maxilla	Aleksandersen *et al*. 2000
	Norway	kidney, brain, meninges, uvea, retina and optic nerve	Aleksandersen *et al*. 2000
	Norway	kidney, brain and meninges	Aleksandersen *et al*. 2000

Table 1.2 Continued

	Origin	Principal organs affected	Reference
	Canada	brain, mandible	Bröjer *et al.* 2000
	United States	cerebellum, optic chiasm, kidney	Kinde *et al.* 2000
	United States	brain, testicle	Kinde *et al*. 2000
	Austria	kidneys, lymph nodes, eye	Majzoub *et al.* 2000
	Austria	kidney, lymph nodes, spinal cord	Majzoub *et al*. 2000
	Ireland	kidneys	Sturgeon and Bassett 2000
	Austria	kidneys, central nervous system, eye	Wlaschitz *et al.* 2000
	Germany	cerebellum, kidney	Wollanke *et al.* 2000
	United States	spinal cord, meninges, nerves	Johnson *et al.* 2001
	Canada	in a mass above the left eye (horse survived)	Pearce *et al.* 2001
	United States	mandible	Snider *et al.* 2001
	United States	brain, lungs	Wilkins *et al.* 2001
	United States	brain, cervical spinal cord, mammary gland, kidney	Wilkins *et al*. 2001
	Germany	kidneys, lymph nodes, brain and spinal cord	Grosche *et al.* 2002
	Italy	brain	Mandrioli *et al.* 2002
	Japan	kidney, perirenal lymph nodes, meninges and brain	Shibahara *et al.* 2002
	Japan	kidney, brain, cervical lymph nodes	Takai *et al.* 2005
	Netherlands	kidney, uvea, optic nerve	Boswinkel *et al.* 2006
	United States	cerebellum	Bryant *et al.* 2006
	Japan	kidney, brain, perirenal lymph nodes	Akagami *et al.* 2007
	Brasil	brain and meninges	Vasconcelos *et al.* 2007
	Canada	mandible, kidney, heart, brain, pituitary gland	Ferguson *et al.* 2008
	Switzerland	prepuce (horse survived)	Müller *et al.* 2008
	Czech Republic	brain	Halouzka *et al.* 2010
	Costa Rica	kidneys and brain	Berrocal and Oliveira 2011
	Honduras	kidneys	Berrocal and Oliveira 2011
	Iceland	cerebellum	Eydal *et al.* 2012
	Iceland	cerebellum, brain stem, meninges and cervical spinal cord	Eydal *et al*. 2011
	Austria	brain, spinal cord, blood vessel walls, heart, kidneys	Reiser *et al.* 2011
	Canada	kidneys, perirenal lymphnodes, lungs	Sponseller *et al.* 2011
	France	maxilla, kidneys, aorta wall	Deniau *et al.* 2012
	Italy	brain	Di Francesco *et al.* 2012
	Brasil	cerebellum, thalamus, brain stem and meninges	de Sant'Ana *et al.* 2012
	United States	kidneys, perirenal lymph nodes, brain	Umlauf *et al.* 2012
	United States	abdominal aorta, kidneys	Rodriguez *et al.* 2013
zebra	United States	kidneys, heart, eyes, uterus, lymph nodes	Isaza *et al.* 2000
donkey	United States	Kidney (survived after resection affected kidney)	Schmitz and Chaffin 2004
cattle	United States	scrotal skin bull	Georgi and Georgi 1990
	United States	brain	Montgomery, O'Toole 2006
human	Canada	brain, meninges, spinal cord	Hoogstraten and Young 1975
	United States	brain, meninges	Shadduck *et al.* 1979
	United States	brain, blood vessels, liver, myocardium	Gardiner *et al.* 1981
	United States	brain	Ondrejka *et al.* 2010

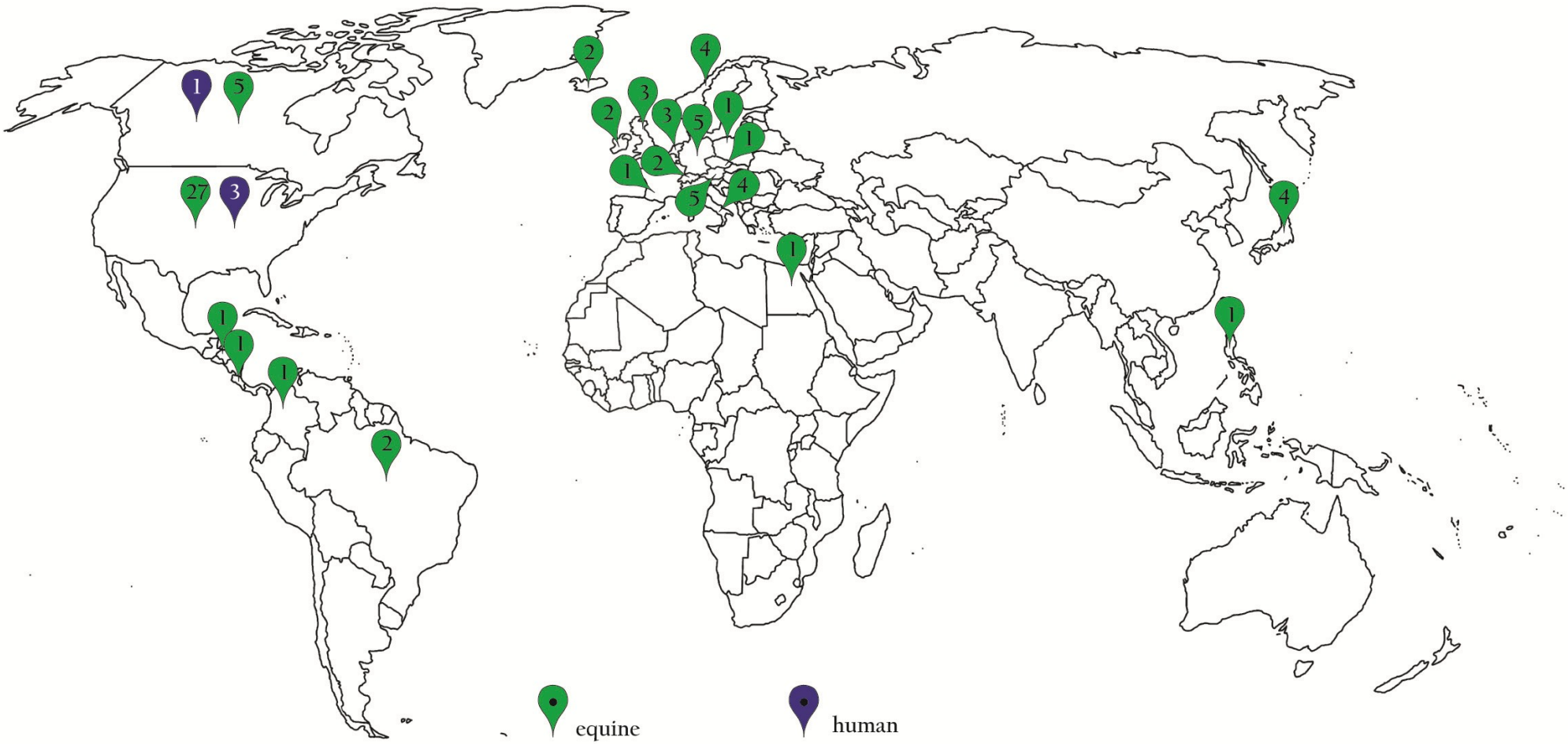

Figure 1.3. Geographical distribution of equine and human infections of *Halicephalobus gingivalis*, which are listed in table 1.2. Given within each balloon is the number of described cases for each country.

The difficulty in diagnosing halicephalobiasis lies in the lack of specific clinical symptoms when different internal organs are affected, as a result of which diagnosis is often only made post-mortem. The migration of the nematodes through the tissue causes proliferative, firm, grey-white granulomatous lesions (Johnson *et al.* 2001). The histological findings include replacement of normal tissue architecture by dens collagen and fibroblasts with infiltration of tissue by lymphocytes, plasma cells, epitheloid macrophages, multinucleate giant cells, eosinophils, and intralesional juvenile and adult nematodes (Johnson *et al.* 2001).

Several other nematode species can cause a similar kind of inflammation in horses. *Strongyloides* sp. and *Pelodera strongyloides* can occasionally infect the horse's skin causing granulomatous lesions. They are, however, easily differentiated from *H. gingivalis* by a distinctly different morphology and/or by the absence of adults and eggs (Dunn *et al*. 1993). Further, *Cephalobus* sp. has been reported from verminous mastitis in a mare (Greiner *et al*. 1991), and can be differentiated from *H. gingivalis* by its blunt tail, different shape of stoma, different ratios of pharynx, and presence of males (Dunn *et al*. 1993). Further, encephalomyelitis caused by the migration of parasites is a rare, yet important cause of neurological disease in horses. Besides *H. gingivalis*, also *Strongylus vulgaris*, *S. equines*, *Angiostrongylus cantonensis*, *Setaria* spp. and *Draschia megastoma* have been identified from the equine central nervous system (CNS) (Tanabe *et al*. 2007).

Depending on the affected organs, halicephalobiasis should be considered in case of ocular disease, granulomatous nephritis or renal failure, acute neurological signs, especially in conjunction with cutaneous, osteolytic, or renal sings (Umlauf *et al*. 2012), and radiographies show aggressive osteolytic changes in the mandible or maxilla (Ruggles *et al*. 1993). However, a clinical examination of granulomatous infections followed by histopathological examinations are necessary to establish a final diagnosis (Müller *et al*. 2008).

The four reported human cases of halicephalobiasis were all fatal within three weeks after the onset of clinical symptoms. Human infection probably occurs through wounds or the oral cavity, after which the nematodes probably disseminate haematogenously and reproduce within the tissue. In humans, *H. gingivalis* shows a predilection for the central nervous system, where it causes a massive inflammatory response, generally resulting in fever, neurological signs, mental confusion, headache, coma and finally death. The first case

(Hoogstraten and Young 1975) reports on a five-year-old boy who fell into a manure spreader, which resulted in deep lacerations contaminated with manure. Eighteen days after the accident the boy developed a fever and began to exhibit lethargy. He died 24 days after the farming accident and was clinically diagnosed with meningoencephalitis of unknown aetiology. Post-mortem examination revealed adult and juvenile nematodes in his brain. In the second case (Shadduck *et al*. 1979), involving a 47-year-old white male, no route of infection was determined. The man was a small ranch owner, who had little to no contact with his horses. Upon admission to the hospital he complained of acute pain in his leg and showed mental confusion, which progressed to lethargy and finally death 19 days later. The third case (Gardiner *et al*. 1981) reports on a 54-year-old African American male who entered the hospital for diarrhoea and sudden weight loss. He had a fever and appeared disoriented. He died of meningoencephalitis caused by *H. gingivalis* 11 days after admission. The infection is believed to have occurred through the decubitus ulcers that were present on each buttock. The final case describes a case of meningoencephalomyelitis caused by *H. gingivalis* in a 39-year-old female (Ondrejka *et al*. 2010). She was admitted with an acute onset of headache, altered mental status and neck pain. The route of entry is unknown since the woman had no known contact with horses and never did much gardening.

The absence of males in both free-living isolates and in examined tissue suggests that this species always reproduces parthenogenetically, *i.e.*, without males (Stefański 1954; Andrássy 1984; Akagami *et al.* 2007), which contradicts the hypothesis that *H. gingivalis* has a separate, gonochoristic cycle when free-living (Blunden *et al*. 1987). More accurately, since there are no indications of a specialized lifecycle, the host tissue can be interpreted as an alternative habitat in which *H. gingivalis* is able to survive and reproduce efficiently.

It is not clear whether isolates of *H. gingivalis* rarely and opportunistically infect hosts, which would be supported if no genetic differences exist between free-living and parasitic isolates, or whether a lineage of *H. gingivalis* is evolving towards equine host specificity, which would be supported by reciprocal monophyly of isolates from horses versus those from soil (Blunden *et al*. 1987). The phylogenetic relationship between two free-living isolates and four clinical isolates was investigated based on their large subunit (LSU) rDNA sequences and provided evidence for the existence of distinct genetic lineages (Nadler *et al*. 2003). However, these genetic lineages did not reflect that one of them is evolving towards mammalian host specificity, thus confirming the expectation that free-

living isolates are capable of equine infections. Furthermore, two *H. gingivalis* isolates from fatal equine cases in Ontario and Tennessee proved genetically homogeneous, while two fatal equine cases, both from Tennessee, showed 18 fixed differences in their LSU sequences (Nadler *et al*. 2003). This shows that genetically homogeneous isolates are not necessarily restricted in geographical distribution, and single geographic regions may contain a diverse pool of isolates capable of equine infection (Nadler *et al.* 2003). However, only one locus was used for the inference of the trees and data from pooled individuals were used. To fully test the hypothesis of cryptic species within the morphotype *H. gingivalis*, a multilocus phylogenetic analysis based on single individuals and supported by a detailed study on the morphology of the indicated lineages is necessary (Nadler *et al.* 2003).

RESEARCH AIMS

The general aim of this study is to provide an improved insight in the evolutionary history and general biology of the facultative parasitic nematode *Halicephalobus gingivalis*. Following research questions were formulated:

- **i.** Infections with *H. gingivalis* have been described in many of our surrounding countries, but does the species occur in Belgium?
- **ii.** Considering the high mortality rate it causes in animals treated by anthelmintics, does it show resistance to commonly used anthelmintic drugs?
- **iii.** Does *H. gingivalis* have morphological adaptations on an ultrastructural level that enables its facultative parasitic life style?
- **iv**. Since morphometrical characteristics are very important in *Halicephalobus* species delineation, what is the degree of intraspecific morphometrical variability within the progeny of a single female?
- **v**. Do clinical and free-living isolates form separate phylogenetic lineages, *i.e*., is *H. gingivalis* merely an opportunistic invader or is it evolving towards parasitism in mammalian hosts? And do morphological and morphometrical data corroborate these results?

OUTLINE OF THE THESIS

The thesis is divided into seven chapters. **Chapter I** encompasses a general introduction to the research organism *Halicephalobus gingivalis* and to (facultative) animal parasitism in nematodes in general.

Chapters II to VI represent the actual results and are designed in accordance with publications in SSCI ranked journals, *i.e.*, containing a specific introduction, a materials and methods section, a results section and a specific discussion:

- **Chapter II** is divided into two parts. The first part describes an extensive sampling in East- and West-Flanders which resulted in the first record of free-living *H. gingivalis* in Belgium and lists all *Halicephalobus* isolates used in this thesis and their origin. The second part presents the first Belgian case of equine halicephalobiasis.

- In **Chapter III**, the resistance or tolerance of *H. gingivalis* to the commonly used anthelmintic drugs ivermectin and thiabendazole is analyzed using a modification of the Micro-Agar Larval Development test. Herein the suitability of *H. gingivalis* as an additional model for anthelmintic resistance testing is discussed

- **Chapter IV** comprises the results of a study on the morphology of the intestine of *H. gingivalis* using transmission electron microscopy and propidium iodine staining, in order to investigate possible morphological adaptations to a facultative parasite lifestyle. Specimens cultured under different conditions were included to determine if the ultrastructural morphology is influenced by culturing method. Finally, the relationship of morphological adaptations of the microvilli to parasitism in nematodes is discussed.

- **Chapter V** encompasses the results of a study on the intraspecific morphometrical variation in *H. gingivalis*. In order to understand the remarkable variation within one species, the progeny of a single female was cultured under varying temperature and food conditions. The morphometrical characters of 540 specimens were analyzed using both univariate (analysis of variance) and multivariate (principal components and canonical discriminant analysis) techniques. An attempt is made to forward morphometrical characters that are useful for *Halicephalobus* species delineation.

- In **Chapter VI**, *H. gingivalis* isolates of different origins, both free-living and parasitic, were used together with at least two other species of the genus in an integrative approach to disentangle their mutual relation. To this end, a thorough phylogenetic analysis was performed using both nuclear (18S and 28S) and mitochondrial (COI and ND4) markers. Subsequently, morphological (both light and scanning electron microscopic data), morphometrical characteristics and biological characteristics were mapped on the obtained molecular framework. The evolutionary lineages are discussed in relation to parasitism.

Finally, **Chapter VII** concludes the thesis with a general discussion of the results and formulates future research prospects.

CHAPTER II

SEARCHING FOR A NEEDLE IN A HAYSTACK:

FIRST RECORD OF *HALICEPHALOBUS GINGIVALIS* IN BELGIUM

Part II modified from:

FONDERIE P., DE VRIES C., VERRYKEN K., DUCATELLE R., MOENS T., VAN LOON G., BERT W. (2013). Maxillary granulomatous inflammation caused by *Halicephalobus gingivalis* (Nematoda) in a Connemara mare in Belgium. *Journal of Equine Veterinary Science* 33 (3), 186-190.

PART I

FIRST RECORD OF FREE-LIVING *HALICEPHALOBUS GINGIVALIS* IN BELGIUM

ABSTRACT

Halicephalobus gingivalis is a free-living bacterivorous nematode which is also known as a facultative parasite of primarily horses. Although equine cases of halicephalobiasis have been reported for most neighbouring countries of Belgium, there had never been reports of the species in Belgium. Presented here are the results of a thorough sampling of 75 horse riding schools in West and East-Flanders, which led to the first report of free-living *H. gingivalis* in Belgium. Several isolates of different origin (*i.e.*, manure, compost and soil) were brought into culture for future research on the biology of this nematode species.

INTRODUCTION

The distribution of infections with *H. gingivalis* (table 1.2, Chapter I) indicates that the species is cosmopolitan. However, although this species is categorized as bacteriovorous, only a few records of free-living *H. gingivalis* have previously been reported, *i.e.*, isolate JB128 from a vegetable compost heap in Riverside (California, USA) and JB043 from potting soil containing peat near Neustadt, Germany (Nadler *et al.* 2003).

Even though equine infections with *H. gingivalis* are known from most of our neighbouring countries, *i.e.*, France, United Kingdom, Netherlands and Germany (table 1.2, Chapter 1), neither a parasitic nor a free-living record has ever been reported in Belgium. Presented here are the first records of free-living *H. gingivalis* in Belgium established by taking samples of manure heaps and soil at 73 equine facilities in East and West Flanders. The initial sampling was followed by a more directed sampling at the facilities that tested positive for the presence of the species. Additionally, an overview is given of the origin of all *Halicephalobus* spp. isolates used throughout the following chapters.

MATERIALS AND METHODS

SAMPLING

Since *H. gingivalis* is known as a facultative parasite of horses, sampling was performed especially in environments associated with horses. These sites, which were commercial enterprises, were sampled with permission of the owners. Initially, both soil samples and samples from manure heaps were taken. Sampling was done by means of bulk samples, which were composed of 10 individual samples of approximately 100 g each. The bulk samples were homogenized, whereafter three subsamples of approximately 50 g were taken for nematode extraction. The remainder of the samples was stored at 4°C.

SAMPLE *ANALYSIS*

Preliminary analyses indicated that more traditional extraction methods (*e.g.*, adaptation of the Baermann-funnel, extraction by flotation using Ludox) have a low success ratio in the retrieval of *H. gingivalis* from life samples (data not shown). Therefore, and since its preference for higher temperatures, the extraction of the nematodes was done by

incubating the samples at 30°C on part of a Petri dish containing 2% bacteriological agar (Oxoid Ltd., Hampshire, UK) enriched with cholesterol (final concentration 1 mg ml^{-1}). If present in the sample, specimens of the species became visible within a few days as they migrated out of the sample onto the agar. As such, the detection of *H. gingivalis* is possible even if only a limited number of eggs or specimens of the species are present in the incubated subsample. The plates were closed with Parafilm® M Sealing film (Pechiney Plastic Packaging, Chicago, USA) to avoid dehydration and were checked daily. When putative *Halicephalobus* specimens were observed using a stereomicroscope (Leica MZ95), they were picked up and mounted for identification.

NEMATODE IDENTIFICATION

Specimens were identified using a light microscope (Olympus BX 51 DIC, Olympus Optical, Tokyo, Japan) and identified as the morphospecies *Halicephalobus gingivalis* using current identification keys for the genus (Andrássy 1984; Geraert *et al.* 1988; Shokoohi *et al.* 2007). Although considerable mutual sequence differences in the D2D3 expansion region of LSU rDNA (28S) have been observed between isolates (Chapter 3), a thorough molecular analysis based on 4 markers (Chapter 6) revealed that all isolates but one (*i.e.*, WB0701) belong to a highly supported *H. gingivalis*-clade. Therefore, all isolates established within the framework of current research were appointed as *H. gingivalis*[3], with the exception of isolate WB0701 which was appointed *H.* cf. *gingivalis* (Chapter 6).

CULTURE OF THE NEMATODES

When specimens were positively identified as *H. gingivalis*, one individual was taken to start a culture. No males have ever been observed, confirming that this species reproduces parthenogenetically (Andrássy 1952; Stefański 1954; Akagami *et al.* 2007). Hence, each isolate represents the progeny of a single female. Cultures are maintained on 1% agar enriched with cholesterol (final concentration 1 mg ml^{-1}), with a bacterial lawn of *Escherichia coli* OP50 as a food source and generally handled as described by Brenner (1974).

[3] Although the isolates found at the beginning of this PhD morphologically resembled *H. gingivalis*, their relationship was not yet clear based on a considerable D2D3 sequence difference. Therefore, they were referred to and published as *Halicephalobus* confer (cf.*) gingivalis* until their relationship was clarified based on a multi-gene phylogeny (Chapter 6), causing some discrepancy between published papers and the present thesis.

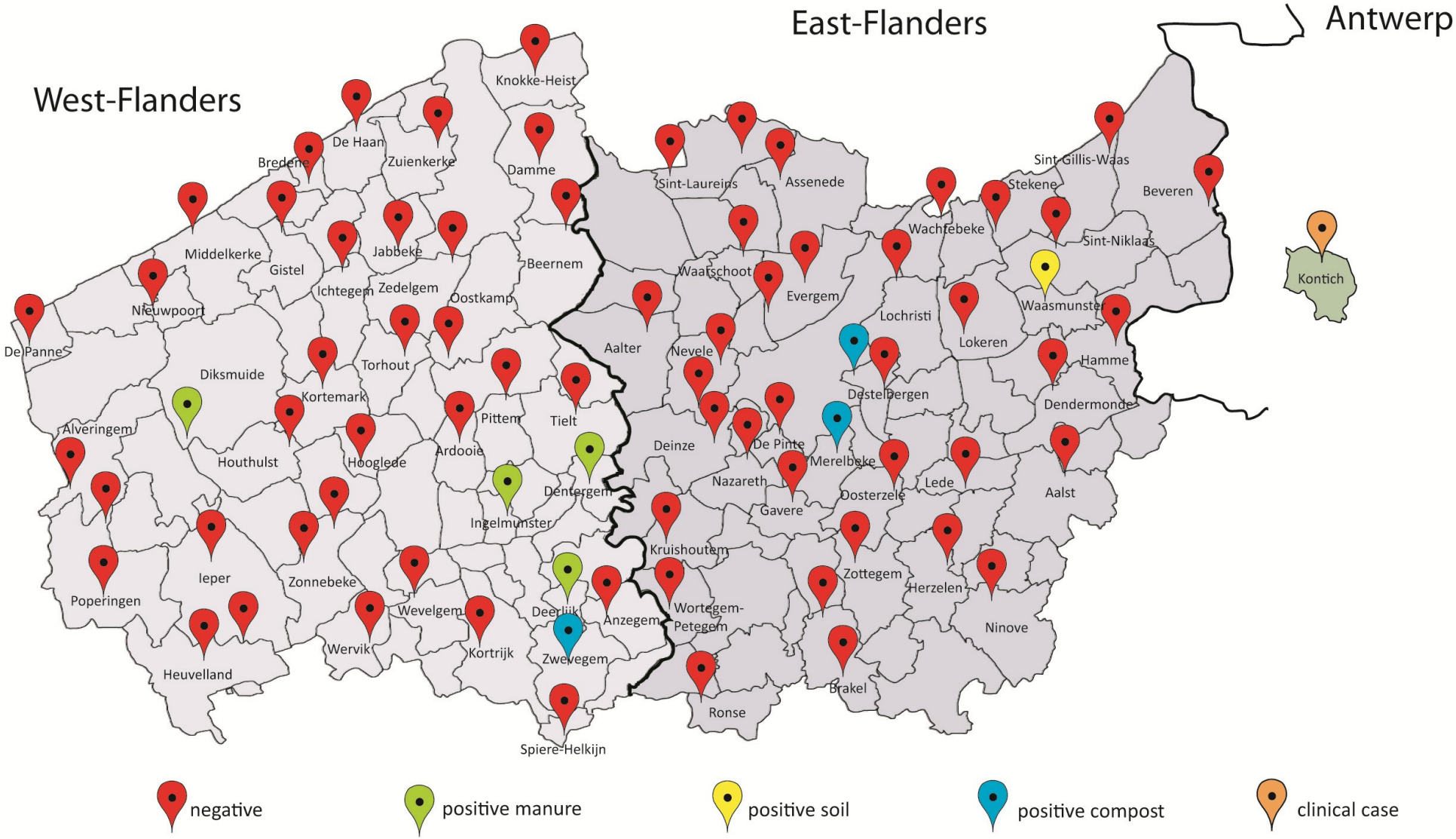

Figure 2.1. Map of East and West Flanders, with indication of the sampling sites. Red indicates sampling sites that tested negative for the presence of *H. gingivalis* in both soil and manure sample, whereas yellow or green indicates the presence of *H. gingivalis* in soil or manure samples, respectively. Blue indicates the location of compost heaps, which tested positive for the presence of *H. gingivalis*. Orange indicates the origin of the first clinical case in Belgium in the province of Antwerp.

RESULTS

FIRST SAMPLING – SOIL AND MANURE HEAPS

Equestrian facilities were chosen based on their distribution in East and West Flanders in an attempt to have a sample site within each 100 km^2. At each of the 73 selected sites (fig. 2.1) two bulk samples were taken, *i.e.*, one bulk soil sample from the paddocks and meadows surrounding the stables and one bulk manure sample from the manure heap which was mostly situated outside and based on a concrete floor.

Five of the 73 equestrian facilities tested positive for the presence of *H. gingivalis*. Positive manure heap samples were found from four equestrian facilities in West Flanders and one positive soil sample from an equestrian facility in East Flanders (fig. 2.1). These populations are the first records of free-living *H. gingivalis* in Belgium. An isolate from each site was brought successfully into an agar culture, *i.e.*, WB0701 - 0705 (table 2.1).

SECOND SAMPLING – FRESH DUNG FROM STABLES

The five sample sites that tested positive for the presence of *H. gingivalis* (fig. 2.1) in either their soil or manure sample, were inspected for a second time by taking bulk samples composed of fresh dung taken from ten individual horse stables. This resulted in one positive sample from an equestrian facility in West Flanders and also in an additional isolate in culture, *i.e.*, WB0801 (table 2.1).

Subsequently, this facility was investigated more thoroughly by analyzing a single sample from each individual stable at two consecutive moments (2 weeks apart). A subsample of each sample was used for nematode extraction. The first stable sampling revealed the presence of *H. gingivalis* in 18 individual stables, whereas, at the second stable sampling 21 stables tested positive. Only 8 stables were tested positive for the presence of *H. gingivalis* at both sample moments. In other words, *H. gingivalis* was found in 31 out of the 56 stables sampled over a period of two weeks. Nevertheless, the equestrian facility never had horses showing symptoms that could indicate an infection with *H. gingivalis.*

THIRD SAMPLING – RECTAL FAECAL SAMPLE

Since *H. gingivalis* specimens were isolated from fresh horse dung in several individual stables during the second sampling (table 2.1), the question arose whether these specimens originated from the intestine of the horse or whether their presence was caused by external contamination. To this end, rectal faecal samples were taken from all 8 horses that tested positive for the *H. gingivalis* specimens in their fresh dung during both previously described sampling moments in the stables. All rectal dung samples tested negative for the presence of *H. gingivalis*.

After the occurrence of the first clinical case in Belgium (Part II Chapter 2), a final attempt was made to prove the presence of *H. gingivalis* in the intestine of horses. Previous to admission, the infected mare was part of a small group of 11 ponies that were stabled together in an equestrian facility in the Belgian province of Antwerp. Also, a horse originating from the same French farm as the infected mare, although not housed with the other ponies, was included in the examination.

These rectal samples of 11 horses finally rendered two rectal samples that tested positive for the presence of *H. gingivalis*, *i.e.*, one pony housed together with the infected mare and the horse that originated from the same French farm (isolates WB1101 and WB1102). To ensure that not only eggs are transferred through the faces, another subsample was analyzed by means of a modification of the Baermann funnel during 48 hours at the most. Since eggs of *H. gingivalis* do not develop into adults at 20°C during this time[4], the adult specimens found indicate the presence of juveniles or adults in the rectal samples.

[4] At 20°C, eggs hatch after approximately 50 hrs and subsequently develop into adults in oviposition after 5 days (unpublished results, not shown)

Table 2.1 An overview of all *Halicephalobus* spp. isolates that, at this moment, are kept in culture at the Nematology Unit of Ghent University.

Species	Code	Origin		Isolated by
Halicephalobus	WB0702	soil	East Flanders, Belgium	Fonderie P.
gingivalis	WB0703	manure heap	West Flanders, Belgium	Fonderie P.
	WB0704	manure heap	West Flanders, Belgium	Fonderie P.
	WB0705	manure heap	West Flanders, Belgium	Fonderie P.
	WB0707	compost	ILVO, Merelbeke, Belgium	Steel H.
	WB0708	compost	ILVO, Merelbeke, Belgium	Steel H.
	WB0709	compost	ILVO, Merelbeke, Belgium	Steel H.
	WB0801	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/02	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/03	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/04	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/05	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/09	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/11	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/12	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/15	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/17	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/18	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/21	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/22	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/29	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/31	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/35	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/38	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/39	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 06/01/44	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/01	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/02	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/05	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/06	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/07	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/10	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/15	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/16	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/17	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/18	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/19	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/20	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/22	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/25	fresh horse dung	West Flanders, Belgium	Fonderie P.

Table 2.1. Continued

	PF 19/01/34	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/35	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/37	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/40	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/44	fresh horse dung	West Flanders, Belgium	Fonderie P.
	PF 19/01/50	fresh horse dung	West Flanders, Belgium	Fonderie P.
	WB1101	rectal dung sample	Antwerp, Belgium	Fonderie P.
	WB1102	rectal dung sample	Antwerp, Belgium	Fonderie P.
	JB128	compost	Riverside, USA	Baldwin J.
	SAN100	clinical isolate	Orlando, Canada	Nadler S.
H. cf. *gingivalis*	WB0701	manure heap	West Flanders, Belgium	Fonderie P.
H. similigaster	WB1103	beech tree	Berlin, Germany	Fonderie P.*
H. mephisto	not available	water enclosure in mine	South Africa	Borgonie G.
Halicephalobus spp.	RGD838	association with termites	USA	Giblin-Davis R.
Halicephalobus spp.	RGD892	association with termites	USA	Giblin-Davis R.

* isolated from sample from trunk of beech tree provided by Köhler A.

DISCUSSION

The primary aim of the described sample effort was to demonstrate the presence of free-living *H. gingivalis* in Belgium and not to provide a complete picture of the distribution of the species in Belgium. Five of the 73 equestrian facilities tested positive for the presence of this facultative parasitic species, and, additionally, a parallel study on nematode succession during the composting process revealed a prevalence of this species in compost (Steel *et al*. 2010). These findings strongly indicate that the distribution of free-living populations of *H. gingivalis* is highly underestimated. Conversely, the incidence of *H. gingivalis* in 31 out of the 56 stables of one equestrian facility without any of the horses presenting symptoms which could be related to an infection with *H. gingivalis*, suggests the possibility that not all *H. gingivalis* isolates are able to cause infections in horses.

ADDITIONAL ISOLATES

Parallel to this research, a study on nematode succession during the composting process (Steel *et al.* 2010) provided us with three additional *H*. *gingivalis* isolates from compost, *i.e.*, WB0707 – 0709 (table 2.1, figure 2.1). Further, the analysis of a sample of rotten

wood from the trunk of a live beech tree provided by Köhler A., yielded an isolate of *H. similigaster* (WB1103) (described in Köhler 2011).

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Finally, we would like to thank Steel H. for providing us with isolates WB0707, WB0708 and WB0709, Baldwin J. for isolate JB128, Nadler S. for isolate SAN100, Giblin-Davis R. for isolates RGD838 and RGD892, Borgonie G. for providing *H. mephisto*, and Köhler A. for providing us with a sample of a beech tree from Berlin, thereby giving us the opportunity to bring *H. similigaster* into culture.

PART II

MAXILLARY GRANULOMATOUS INFECTION CAUSED BY *HALICEPHALOBUS GINGIVALIS* IN A CONNEMARA MARE

ABSTRACT

A 5-year-old anorexic and lethargic Connemara mare presented with severe facial swelling and dyspnea. No distinct central nervous symptoms were present. Radiographs of the skull showed diffuse radiolucency with loss of definition of the periodontal lamina dura and swelling of the decalcified nasal bones. Given the severe bone damage and its poor general condition, the pony was euthanized. Histological evaluation of the lesion revealed a granulomatous reaction with numerous sections of adult and juvenile nematodes, which were morphologically and molecularly identified as *Halicephalobus gingivalis*. The position of this facultative parasite within its free-living congeners is reviewed in this article, and the possible infection routes are discussed. This report presents the first record of equine halicephalobiasis in Belgium.

INTRODUCTION

Halicephalobus gingivalis, previously referred to as *Micronema deletrix* and *Halicephalobus deletrix*, is a small (235 – 460 µm) free-living bacteriovorous nematode belonging to the family Panagrolaimidae, which is capable of facultative parasitism in horses (Blunden *et al.* 1987; Nadler *et al.* 2003) and humans (Ondrejka *et al.* 2010), causing generally fatal infections. Single cases have also been described in a donkey (Schmitz and Chaffin 2004) and a Grévy's zebra (Isaza *et al.* 2000) and *H. gingivalis*-like nematodes have been reported in the brain of a three year old black Angus cow (Montgomery and O'Toole 2006). All four human cases had a fatal outcome within three weeks after the occurrence of symptoms (Ondrejka *et al*. 2010), and of the approximately 60 equine cases only two have reported a successful treatment (Dunn *et al.* 1993; Pearce *et al.* 2001). The geographical distribution of the infections further reveals that the species is cosmopolitan (Blunden *et al*. 1987; Nadler *et al.* 2003). However, it is not clear if all reported cases of halicephalobiasis are caused by the species *H. gingivalis*, since molecular data are usually missing (Nadler *et al.* 2003) and the limited morphological traits discriminating the different species of the genus are even more difficult to discern in histological sections.

In addition to its fascinating abilities as a facultative parasite, the genus *Halicephalobus* is known for its wide environmental range, *i.e.*, as an inhabitant of compost (Steel *et al*. 2010), humus (Shokoohi *et al*. 2007), soil (Andrássy 1952), rotten wood (Köhler 2011), water enclosures in mines up to 1 km deep belowground (Geraert *et al*. 1988; Borgonie *et al*. 2011), and in association with insects (Geraert *et al.* 1988; von Lieven and Sudhaus 2008; Powers *et al.* 2009) and chelicerates (Timm 1956). The genus *Halicephalobus* is morphologically characterized by its short body, a tuboid stoma narrowing at the posterior end, a pharynx consisting of a corpus with a median bulb, an isthmus and a terminal bulb with valves, and a monodelphic-prodelphic female reproductive system which is usually dorsally reflexed (Stefański 1954; Anderson *et al.* 1998). The absence of males in both free-living isolates and in examined tissue suggests that this species always reproduces parthenogenetically, *i.e.*, without males (Stefański 1954; Andrássy 1984; Akagami *et al.* 2007), which contradicts the hypothesis that *H. gingivalis* has a separate, gonochoristic cycle when free-living (Blunden *et al*. 1987).

Although *H. gingivalis* has all the morphological characteristics of a free-living nematode, it has some ultrastructural adaptations which could emanate from its facultative parasitic life style (Chapter 4). *H. gingivalis* appears to be a soil nematode 'preadapted' for massive proliferation inside vertebrate hosts. Further, the species has a very high tolerance to the common anthelmintics ivermectin and thiabendazole (Chapter 3).

Equine infections of *H. gingivalis* mainly involve the brain, kidneys, oral and nasal cavities, but have also been reported in the liver, eyes and bone (Blunden *et al.* 1987; Mandrioli *et al.* 2002; Hermosilla *et al.* 2011). Bone infections mainly involve the mandible and maxilla, but also the femur and nasal bones (Blunden *et al.* 1987; Mandrioli *et al.* 2002; Ferguson *et al.* 2008; Hermosilla *et al.* 2011). Consequently, it is likely that infections are overlooked when affected animals without clear symptoms are not subjected to a histological examination.

This report presents the first record of an equine infection with *Halicephalobus gingivalis* in Belgium and presents conclusive evidence of the parasite's identity based on both morphological and molecular data.

CASE REPORT

CASE HISTORY

A 5-year-old Connemara pony mare was presented at the faculty of Veterinary Medicine, Ghent University, for marked facial swelling and dyspnoea. The mare had been imported from France 6 months earlier. First symptoms started 2 weeks prior to admission and included a mild facial swelling and loss of appetite. A complete blood cell count and biochemistry profile had been performed by the referring veterinarian, revealing marked elevation of beta globulines and liver enzymes. The veterinarian suspected a severe helminth infection and the horse was treated intramuscularly with doramectin (Dectomax®, Pfizer, Belgium, dosage unknown). Subsequently, the horse received fenylbutazone 2 g orally once daily for 10 days, penicillin 15 mg kg^{-1} intramuscularly once daily for 1 week and dexamethasone orally for 10 days. The mare's

temperature was normal and no cough or nasal discharge were observed. Other horses on the same farm were normal. Two days prior to admission the symptoms severely aggravated.

CLINICAL EXAMINATION

The horse was anorexic and severe facial swelling was apparent. The buccal mucosae were swollen and had a cyanotic colour (fig. 1A). The horse was lethargic and showed severe dyspnoea. No distinct central nervous symptoms were present. Tachycardia (heart rate 80 beats minute^{-1}) and tachypnoea (respiration rate 40 breaths minute^{-1}) were present. Auscultation revealed enforced breathing sounds. No other abnormalities were detected. Thoracic and abdominal ultrasound did not show abnormalities. A complete blood cell count was normal (6,1x10^{9} L^{-1} white blood cells). Blood biochemistry showed marked elevation in total protein (91 g L^{-1}, reference: 60-80 g L^{-1}) and beta globulin fraction (40%, reference: 10-21%). Muscle enzymes were slightly elevated (lactate dehydrogenase 1318 mU ml^{-1}, reference: 246-658 mU ml^{-1} and creatine kinase 189 mU ml^{-1}, reference: 10-146 mU ml^{-1}). Radiographs of the skull showed diffuse radiolucency with loss of definition of the periodontal lamina dura, most obvious within the rostral aspects of the skull and swelling of the decalcified nasal bones. The crowns of the incisors in the maxilla were not attached to the bone and a large amount of soft-tissue swelling was present. At this point differential diagnoses included a neoplastic or chronic inflammatory process. Given the severe bone damage and the poor general condition of the horse, the owners declined further treatment and elected euthanasia.

NECROPSY

At necropsy, gross findings included a diffuse severe bilateral swelling of the maxillary region, extending to the infraorbital region. The oral mucosa showed multifocal petechiae and there was cyanosis and multifocal ulceration of the marginal gingiva. The upper incisive teeth were displaced due to the space-occupying swelling, which resulted in multiple diastemata. Median sagittal section of the skull revealed diffuse replacement of the incisive bone, the processus palatinus maxillae and the processus alveolaris maxillae, and partial replacement of the maxillary bone by soft tissue swelling (fig. 1B). A

complete transverse section of the skull was made cranial to the 1st premolar teeth. The swelling extended to the maxillary bone, the ventral and medial meatus, the ventral conchal sinus and the ventral concha. On cut surface the swelling was homogeneous yellow-to-white and had a firm consistency. There were no macroscopic abnormalities in other organs.

Multiple samples of the swelling between the two upper 1st incisors and 10 cm caudal to the 3rd incisor were fixed in 10% phosphate-buffered formalin and embedded in paraffin wax. Approximately 5-µm thick tissue sections were made using routine histological techniques. The sections were stained with haematoxylin and eosin. Histopathological examination revealed a partially encapsulated, moderately demarcated granulomatous inflammation in the submucosa. The inflammatory reaction mainly consisted of multinucleated Langerhans type giant cells and macrophages, but also contained a moderate amount of lymphocytes and plasma cells. There was multifocal fibrosis and the capsule was infiltrated by macrophages, lymphocytes and plasma cells. The incisive bone was largely replaced by the granulomatous inflammation, with only a small part of the bone still visible. Within the granulomatous reaction there were numerous tangential and cross-sections of juvenile and adult nematodes. Adult specimens were measured from mashed tissue, *i.e.*, 274 ± 21 µm (n=16) in length and 17 ± 1 µm (n=16) in diameter, and were morphologically identified as members of the genus *Halicephalobus*. Occasionally the characteristic rhabditiform pharynx with a corpus, isthmus and terminal bulbus (fig. 2A) was visible. Some specimens showed the genus-characteristic large, single, dorsally retroflexed ovary (fig. 2B), sometimes containing an egg. Multiple developing zygotes (fig. 2C) were present indicating reproduction within the tissue.

A molecular characterisation was done based on specimens recovered from frozen maxillary tissue. Amplification and sequencing of the D2D3 expansion region of LSU rDNA (441 bp, GenBank JQ838156) was done as in Múnera Uribe *et al*. (2010) and confirmed the morphological identification : Bayesian phylogenetic analysis including GenBank sequences of different *H. gingivalis* isolates, placed isolate JQ838156 within a *H. gingivalis* clade (results not shown), differing 6 (1.3%), 7 (1.6%), 12 (2.7%) or 15 (3.4%) nucleotides from other clinical isolates of *H. gingivalis* available in GenBank, *i.e.*, AY294180, AY294177, AY294181 and AY294182, respectively.

Figure 2.1. Gross examination of the head. (**A**) Severe bilateral swelling of the maxillary region with cyanosis and multifocal ulceration of the marginal gingiva and multiple diastemata of the upper incisive teeth; (**B**) Median sagittal section of the head. Diffuse replacement of the processus palatinus maxillae (asterisk); (**C**) Transverse section of the head, cranial to the 1st premolar. Severe swelling extending to the sinus conchalis ventralis (1), the ventral concha (2) and the maxillary bone (3).

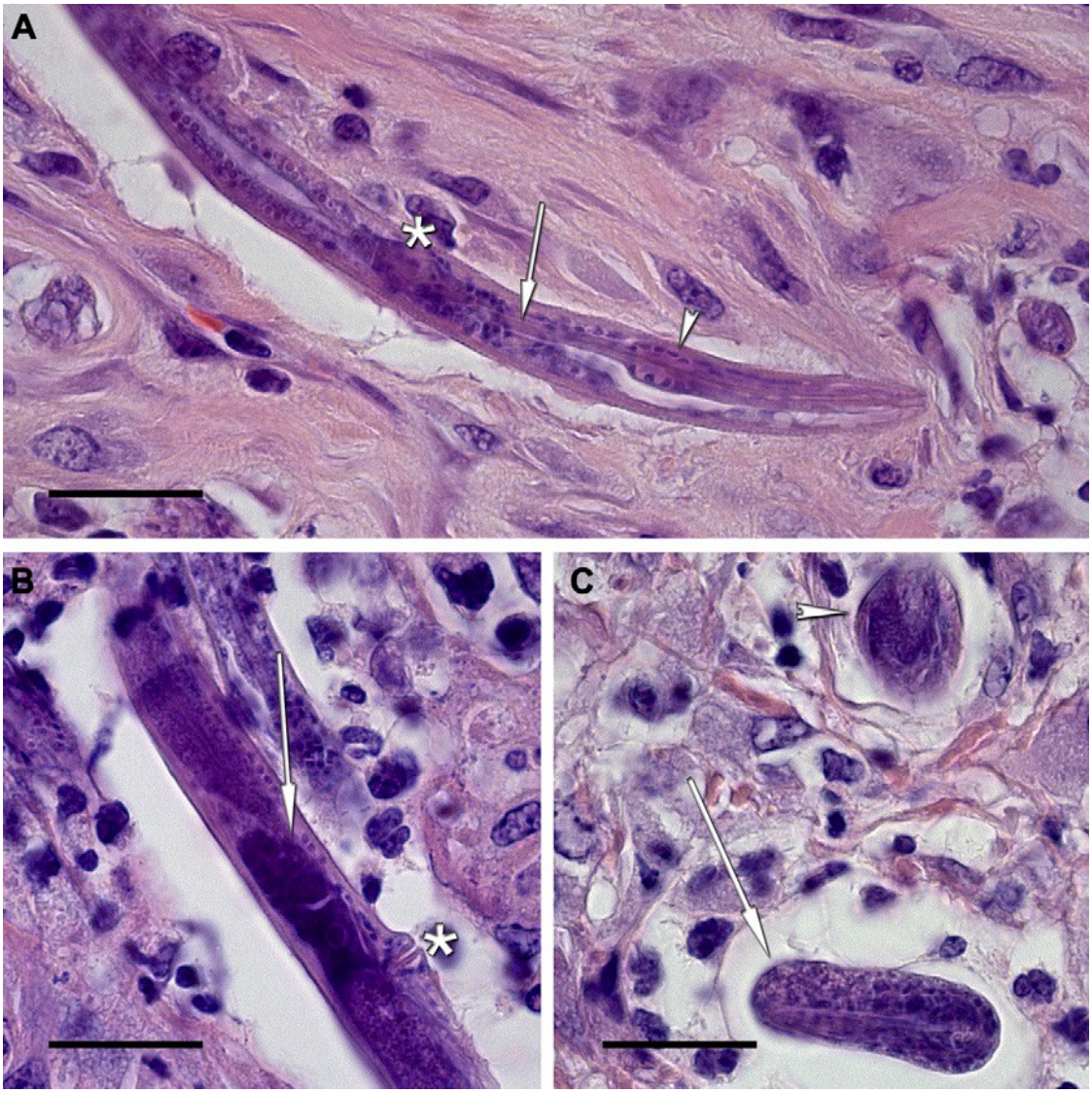

Figure 2.2 Histopathological examination of the maxillary granulomatous inflammation revealing sections of *Halicephalobus gingivalis* (haematoxylin and eosin staining, scalebar=20 µm). (**A**) *H. gingivalis* specimen showing the characteristic pharynx with a corpus with a medial bulb (arrowhead), isthmus (arrow) and a terminal bulb (asterisk); (**B**) *H. gingivalis* adult showing characteristic female reproductive system with vulva (asterisk) and dorsally reflexed ovary (arrow); (**C**) Developing zygote (arrow) and a transverse section of a *H. gingivalis* specimen (arrowhead).

DISCUSSION

Little is known about the epidemiology of *H. gingivalis*. Multiple possible infection routes have been described such as through open wounds, nasal or oral cavities, through the ingestion of contaminated food or via the respiratory tract (Spalding *et al.*

1990; Ruggles *et al.* 1993; Trostle *et al.* 1993; Bröjer *et al.* 2000). Other possibilities are cutaneous infections through entry of free-living specimens facilitated by recumbency (Dunn *et al*. 1993), or infections via the oral cavity by first colonizing ingesta embedded in the gums and subsequently penetrating lacerations of the buccal mucosa, thereby invading the mandible or maxilla (Anderson *et al.* 1998; Ferguson *et al*. 2008). However, since (endo)phoretic behaviour associated with ants and flies has been described for *H. similigaster* (Köhler 2012), another species of the genus, insects may play an important role in the distribution of *H. gingivalis*. This is especially true for infections through lesions in the skin or orbital infections (Anderson *et al.* 1998). Thus, *H. gingivalis* may not have one specific infection route and may be able to opportunistically colonize the host in different ways. Moreover, this species has a predilection for warm and moist environments as shown by its optimal culture temperature of 38°C (unpubl. data) and its natural environment, including compost (Steel *et al.* 2010) and manure heaps (Chapter 2).

After penetrating the host, the nematodes can either remain on the spot and cause local infections or enter the bloodstream and disseminate throughout the body. The hematogenous spread is supported by small parasitic granulomas that have occasionally been found in the walls of blood vessels, and by the occurrence of nematodes in the blood vessel lumina (Bröjer *et al.* 2000; Reiser *et al.* 2011). Subsequently, its ability for parthenogenetic reproduction enables *H. gingivalis* to rapidly increase in number and cause massive tissue damage through its migratory behaviour resulting in granulomatous inflammation of the affected organs (Pearce *et al.* 2001; Mandrioli *et al.* 2002; Müller *et al.* 2008).

Given the maxillary involvement in the presented case, it is very likely that infection occurred through lesions in the buccal mucosa and subsequently spread to the incisive bone, the processus palatinus maxillae, the processus alveolaris maxillae and the maxillary bone. Since the macroscopic postmortem examination revealed no additional abnormalities, no histological sections were made of other internal organs. Therefore, renal or neurological involvement cannot be excluded.

Equine infections of *H. gingivalis* have mostly been described to affect the brain, kidneys, oral and nasal cavities, prepuce, spinal cord and skin, but have occasionally also been reported in the liver, heart, lungs, lymph nodes, optic nerve, eyes, testicles,

mammary glands, stomach and bone, *i.e.*, mandible, maxilla, femur and nasal bones (table 1.1, Chapter 1). In case of renal or testicular involvement, juvenile nematodes have been detected in the urinary sediment or in the semen (Kinde *et al.* 2000). Especially when infections involve the central nervous system or the kidneys the disease progresses extremely rapidly and is generally fatal. However, when the infection is restricted to non-vital organs it can asymptomatically be present in the horse over a longer period of time. This is corroborated by the description of prenatal, perinatal or transmammary transmissions between mares and their foals (Spalding *et al.* 1990; Wilkins *et al.* 2001), in which the foals always perished before the mares.

Only two equine cases have reported a successful treatment of an infection with *H. gingivalis*. The first case discussed a horse with multiple nodules of approximately 1 cm diameter on the external lamina of the prepuce and without any further symptoms (Dunn *et al.* 1993). This horse was treated with ivermectin and diethylcarbamazine which resulted in regression of the nodules. The second case involved a horse with a large granuloma on the head, which was surgically removed followed by a high dosage of ivermectin administered locally (Pearce *et al.* 2001). However, none of these papers reported on long-term follow-up of the horses involved.

Although halicephalobiasis is rare, it should be suspected in case of acute neurological disease and renal dysfunction as well as when aggressive osteolytic space-occupying lesions are present in the mandible or maxilla. Antemortem diagnosis is possible after biopsy of infected tissue and, depending on the organ involved, the therapy of choice for local infections is surgical debulking and administration of high doses of ivermectin. However, a recent *in vitro* study on both free-living and facultative parasitic isolates of *H. gingivalis* showed that this species has an unseen high tolerance for both thiabendazole and ivermectin (Chapter 3), rendering it unlikely that these anthelmintics will be sufficient in treating halicephalobiasis, even when concurrently administered as proposed by (Ferguson *et al*. 2008). These findings are supported by the medical history of horses which suffered lethal infections of this nematode species in spite of regular treatment with common anthelmintics (Boswinkel *et al.* 2006; Ferguson *et al.* 2008).

The present case reports on the first record of an equine infection with *H. gingivalis* in Belgium. Since halicephalobiasis is known to occur in France (Deniau *et al.*

2012) and since the Connemara mare was imported from France 6 months earlier, the infection was potentially already present upon importation. However, since the presence of free-living, but horse-associated *H. gingivalis* has been demonstrated in Belgium (Chapter 2), it is equally possible that the infection occurred on the Belgian farm.

There is no clarity on the phylogenetic relationship between free-living and clinical isolates of *H. gingivalis* (Blunden *et al*. 1987; Nadler *et al.* 2003). Existence of reciprocal monophyly of clinical isolates versus free-living isolates would support the idea that a lineage of *H. gingivali*s is evolving towards parasitism in mammalian hosts. Conversely, if there is no genetic distinction between free-living and clinical isolates (Nadler *et al.* 2003), then *H. gingivalis* is merely an opportunistic invader that can cause massive damage due to its parthenogenetic reproduction and migratory abilities.

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CHAPTER III

ANTHELMINTIC TOLERANCE IN FREE-LIVING AND FACULTATIVE PARASITIC ISOLATES OF *HALICEPHALOBUS* (PANAGROLAIMIDAE)

Modified from:

FONDERIE P., BERT W., HENDRICKX F., HOUTHOOFD W., MOENS T. (2012). Anthelmintic tolerance in free-living and facultative parasitic isolates of *Halicephalobus* (Panagrolaimidae). *Parasitology* 139 (10), 1301-1308.

ABSTRACT

Studies on anthelmintic resistance in equine parasites do not include facultative parasites. *Halicephalobus gingivalis* is a free-living bacteriovorous nematode and a known facultative parasite of horses with a strong indication of some form of tolerance to common anthelmintic drugs. This research presents the results of an *in vitro* study on the anthelmintic tolerance of several isolates of *Halicephalobus* to thiabendazole and ivermectin using an adaptation of the Micro-Agar Larval Development Test hereby focusing on egg hatching and larval development. *Panagrellus redivivus* and *Panagrolaimus superbus* were included as a positive control. The results generally show that the anthelmintic tolerance of *Halicephalobus* to both thiabendazole and ivermectin was considerably higher than that of the closely related Panagrolaimidae and, comparing to other studies, than that of obligatory equine parasites. Our results further reveal a remarkable trend of increasing tolerance from fully free-living isolates towards horse-associated isolates. *In vitro* anthelmintic testing with free-living and facultative parasitic nematodes offers the advantage of observing drug effect on the complete life cycle as opposed to obligatory parasites which can only be followed until the third larval stage. We therefore propose *Halicephalobus gingivalis* as an experimental tool to deepen our understanding of the biology of anthelmintic tolerance.

INTRODUCTION

To date, studies on anthelmintic resistance or tolerance in equine parasites only include obligatory parasites, not facultative parasites. *Halicephalobus gingivalis* (STEFAŃSKI, 1954) ANDRÁSSY 1984, also referred to as *H. deletrix* or as *Micronema deletrix* (Anderson *et al.* 1998), is a small (235 - 460 µm) free-living bacteriovorous nematode (Panagrolaimidae) and a known facultative parasite of horses (Blunden *et al.* 1987; Nadler *et al.* 2003) and zebra (Isaza *et al.* 2000). In addition, four cases of human infection, all with a fatal outcome, have been described (Ondrejka *et al.* 2010). *H. gingivalis* has all the characteristics of a free-living nematode, only on ultrastructural level some potential adaptations to facultative parasitism can be observed (Chapter 4). Infection probably occurs through open wounds and oral or nasal cavities (Pearce *et al.* 2001). Subsequently nematodes most likely invade the bloodstream and lymphatic system and thus reach different organs (*e.g.*, kidneys, liver and brain) where the number of nematodes increases rapidly through parthenogenetic reproduction (Akagami *et al.* 2007). The clinical symptoms vary depending on which organs are infected (Blunden *et al.* 1987; Spalding *et al.* 1990; Rames *et al.* 1995; Johnson *et al.* 2001; Müller *et al.* 2008). A few cases have been described in which the infection was recognized in time and the horse was successfully treated (Dunn *et al.* 1993; Pearce *et al.* 2001; Müller *et al.* 2008). Still, most infections were only recognized post-mortem after a thorough autopsy. Most importantly, the clinical histories of all reported equine infections show that the horses had been regularly treated with common anthelmintics (*e.g.*, Boswinkel *et al.* 2006; Ferguson *et al.* 2008). This strongly indicates that *H. gingivalis* either has a high tolerance or some form of resistance to these anthelmintic drugs.

The current paper presents the first research on anthelmintic tolerance of the facultative parasitic nematode *Halicephalobus gingivalis*. Several isolates were tested for tolerance to common anthelmintic drugs through *in vitro* experiments focusing on egg hatching and larval development. Both free-living and parasitic isolates were included to examine whether tolerance is restricted to parasitic isolates or whether it also holds for free-living isolates. The results on the *H. gingivalis* isolates were compared with those on the closely related free-living nematode species *Panagrellus redivivus* and

Panagrolaimus superbus, allowing us to discriminate species- or strain-specific tolerance from any more general tolerance in the free-living Panagrolaimidae.

MATERIALS AND METHODS

MAINTAINING CULTURES

We selected four isolates for our experiments. They were all light-microscopically identified as the morphospecies *Halicephalobus gingivalis* using different identification keys (Geraert *et al*. 1988; Shokoohi *et al*. 2007). No males were observed confirming that this species is parthenogenetic (Stefański 1954; Andrássy 1984; Akagami *et al.* 2007). The JB128 isolate was obtained from a vegetable compost heap in Riverside (California, USA). The WB0708 isolate was obtained from a large-scale compost heap (Steel *et al.* 2010) at the Institute for Agricultural and Fisheries Research in Merelbeke (Belgium). The WB0801 isolate was obtained from fresh horse droppings from an individual stall on a stable in the province of West-Flanders (Belgium). The SAN100 isolate is a clinical isolate originating from an infection in a horse (Guelph, Ontario, Canada) described by Anderson *et al*. (1998). SAN100 has been maintained in culture on plain bacteriological agar with a bacterial food source since its isolation. Hence, we used two compost isolates from horse independent habitats and two isolates from horse associated habitats including one parasitic isolate and one isolate found in the near vicinity of horses. Two closely related free-living species, *Panagrellus redivivus* PS1163 and *Panagrolaimus superbus* DF5050, were included in the experiments to discriminate species- or strain-specific tolerance from a possible more general tolerance in the otherwise free-living Panagrolaimidae.

Stock cultures of all species were maintained on 1% bacteriological agar (Oxoid, Basingstoke, UK) plates containing cholesterol (1 mg ml^{-1}) and *Escherichia coli* OP50 as a food source. The stock cultures were incubated at 20°C and generally handled as described by Brenner (1974). To provide enough eggs to start the experiments, the *Halicephalobus* isolates were subcultured and incubated at 37°C for 3 to 4 days. At this temperature the cultures grow fast and numerous eggs can be generated over a short

period of time. The *P. redivivus* and *P. superbus* isolates were subcultured two weeks beforehand and incubated at 20°C to yield a sufficient amount of eggs.

ANTHELMINTIC DRUGS

The anthelmintic drugs used in the experiments were thiabendazole (TBZ; Sigma-Aldrich, Bornem, Belgium) and ivermectin (IVM) which represent members of two important anthelmintic groups, the benzimidazoles and the avermectin/mylbecins, respectively. They are selected because of the use of products of these groups on the location where the WB0801 isolate was found. TBZ is the most soluble member of the benzimidazole group which facilitates *in vitro* experiments. The IVM used in the experiments are dilutions of Ivomec® Injection (Merial, Brussels, Belgium), a commercially used form of the drug.

EXPERIMENTAL SETUP

The technique used was a modification of the micro-agar larval development test (MALDT) (Coles *et al.* 2006). The MALDT method was originally designed as a larval development test (LDT) on a solid instead of in a liquid medium. MALDT was chosen over LDT because of the ease of culturing *Halicephalobus* on solid medium and because this very small nematode is easier to discern on solid than in liquid medium. The main objective of any larval development test is to follow the development of nematode eggs onto third stage larvae, which generally is the infective stage in obligatory animal parasites. Here we mainly focused on quantitative hatching data and development to the adult stage.

The experiments were performed on 24-well plates (Greiner Bio-One, Frickenhausen, Germany). The anthelmintics were dissolved in 100% dimethylsulfoxide (DMSO; Carl Roth GmbH, Karlsruhe, Germany). Five stock solutions of TBZ (100, 1000, 2000, 5000, 10000 µg ml^{-1}) and five stock solutions of IVM (1, 10, 50, 100, 200 µg ml^{-1}) were prepared. Stock solutions were diluted 100x by adding 49.5 ml 1% bacteriological agar at approximately 45°C to 0.5 ml of drug solution in a 50 ml falcon. The final solution was carefully homogenized before adding 3 ml to each well. The control consisted of 1%

bacteriological agar with a final concentration of 1% DMSO. We chose to keep the concentration of DMSO in the final solutions at 1% to exclude any influence on the mortality rate as reported for *Caenorhabditis elegans* by Ura *et al.* (2002) for DMSO concentrations in excess of 5%. Final drug concentrations in the wells were 1, 10, 20, 50 and 100 µg ml^{-1} for TBZ and 0.01, 0.1, 0.5, 1.5, and 2 µg ml^{-1} for IVM. Initially, the *Halicephalobus* isolates were tested against a range of drug concentrations based on Várady *et al.* (2009). Because only small to no effects were noticed, higher concentrations were chosen in the present experiments.

The tests were performed in three replicates for each anthelmintic concentration with the zero concentration as a negative control. Approximately 50 nematode eggs were transferred into each well. The exact number of eggs was counted for each well. The plates were subsequently incubated at an optimal temperature for development, *i.e.*, 30°C for the *Halicephalobus* isolates and 20°C for *P. redivivus* and *P. superbus*. Hatching was quantified at the time eggs normally develop into the adult stage; which is after 48 h incubation at 30°C for the *Halicephalobus* isolates and after 7 days incubation at 20°C for *P. redivivus* and *P. superbus*. In order to assess the reproducibility of our bioassay, we repeated the entire experiment 15 months after the first trial.

The hatching proportion (HP) is calculated for each well as follows: the number of hatched eggs and surviving larvae or adults is divided by the number of eggs originally transferred onto the agar. This proportion is determined at each concentration.

In contrast to TBZ which prevents both embryonation and hatching of nematode eggs (Taylor *et al.* 2002), IVM mainly has an effect on the larval stage and only prevents hatching at very high concentrations (Patel 1997). Therefore, an experiment was performed to verify whether larval stages surviving high IVM concentrations but initially not developing into the adult stage, can overcome the effect of drug treatment and resume development. To this end, three replicas of 40 eggs of each *Halicephalobus* isolate were transferred on 1% bacteriological agar containing 1.5 µg ml^{-1} IVM and were incubated at 30°C. After 76 h, 20 surviving larvae of each replicate were transferred onto plain 1% bacteriological agar and observed for several days. Their recovery rate is defined as the number of transferred larvae that develop into the adult stage divided by the number of initially transferred larvae x100.

EFFECT OF PRE-EXPOSURE ON ANTHELMINTIC TOLERANCE

Anthelmintic tolerance can be caused or increased by contact of the nematodes with the anti-parasitic drug in question. In order to verify the short-term effect of a prior anthelmintic treatment on the anthelmintic tolerance of *Halicephalobus gingivalis*, all four isolates (JB128, WB0708, SAN100 and WB0801) were cultured for approximately 12 ± 2 generations at 30°C on 1% bacteriological agar containing a low dose of anthelmintics, *i.e.*, 10 µg ml^{-1} TBZ or 0.01 µg ml^{-1} IVM. After this period, the modification of the MALDT method was performed as described above, leaving out the lowest TBZ concentration of 1 µg ml^{-1}.

STATISTICAL ANALYSIS

To test for differences in the response of the isolates towards each anthelmintic across the two trials, the data were modeled by means of a generalized linear mixed model (PROC GLIMMIX in SAS® v.9.3, SAS Institute Inc., Cary, NC, USA). As we are merely interested in the effect of 'isolate' and 'concentration' and their interaction, these factors were treated as fixed effects in the model. Yet, as the whole experiment was replicated in two trials, the fixed effects were assessed across both trials by including the factor 'trial' as random effect in the model. As the response variable includes number of hatched or survived individuals on the total number of individuals, a binomial error distribution was assumed and a logit link was incorporated to relate the predictive part of the model to the mean response. Standard error and degrees-of-freedom were estimated according the method described by Kenward and Roger (1997). Significance of the fixed effects and their interactions were tested by means of Type III tests. Differences in tolerance between the isolates were post-hoc tested by comparing the expected hatching success and survival probability at different concentrations (least square means), using a Tukey-Kramer-adjustment to correct for multiple testing.

Given that generalized linear mixed models are large sample tests (Agresti 2002), we did not rely on this procedure to compare the effect of the anthelmintics between the *Halicephalobus* isolates and *Panagrellus redivivus* and *Panagrolaimus superbus* as their hatching proportion approached zero at higher concentrations. Therefore, a Fisher

exact test procedure was used for these comparisons as implemented in StatXact® v.5.0 (Cytel Inc., Cambridge, MA, USA).

The effect of pre-exposure to anthelmintics was analyzed using Statistica 7 (StatSoft Europe GmbH, Hamburg, Germany) for each *Halicephalobus* isolate separately using two-way analysis of variance (ANOVA) with the factors anthelmintic concentration and pre-exposure, followed by a post-hoc Tukey HSD test. The assumptions for ANOVA (normality and homogeneity of variances) were tested using a Kolmogorov-Smirnov test and a Bartlett test, respectively.

RESULTS

THIABENDAZOLE (TBZ)

Figure 4.1 shows the mean hatching proportions (HPs) of the *Halicephalobus* isolates and of *Panagrellus redivivus* and *Panagrolaimus superbus* for both trials at different TBZ concentrations, with 0 µg ml^{-1} being the negative control. The mean HPs of the negative controls were comparable for the parasite isolate, the droppings isolate, *P. redivivus* and *P. superbus*, with an average ranging from 0.96 to 0.99. However, the HPs of the negative controls of the compost isolates, with an average ranging from 0.91 to 0.93, were significantly (Tukey post hoc, $P<0.0001$) lower than those of the parasite isolate and the droppings isolate.

The response of *P. redivivus* and *P. superbus* to TBZ concentration was similar in both trials; they had an initially high HP with an average ranging from 0.85 to 0.94 at 1 µg ml^{-1} followed by a steep decrease towards zero hatching at 10 µg ml^{-1} and higher TBZ concentrations. From 10 µg ml^{-1} onwards, the HPs of both *P. redivivus* and *P. superbus* were significantly lower than those of all *Halicephalobus* isolates (fig. 4.1; Fisher's exact test: P all <0.0001).

Besides an overall significant negative effect on the HPs of all the *Halicephalobus* isolates, significant differences were observed in their mean HP ('Isolate' effect), as well as in their response towards TBZ concentration across trials (Isolate*concentration effect) (table 4.1). Comparison of the mean HPs across trials revealed that the effect

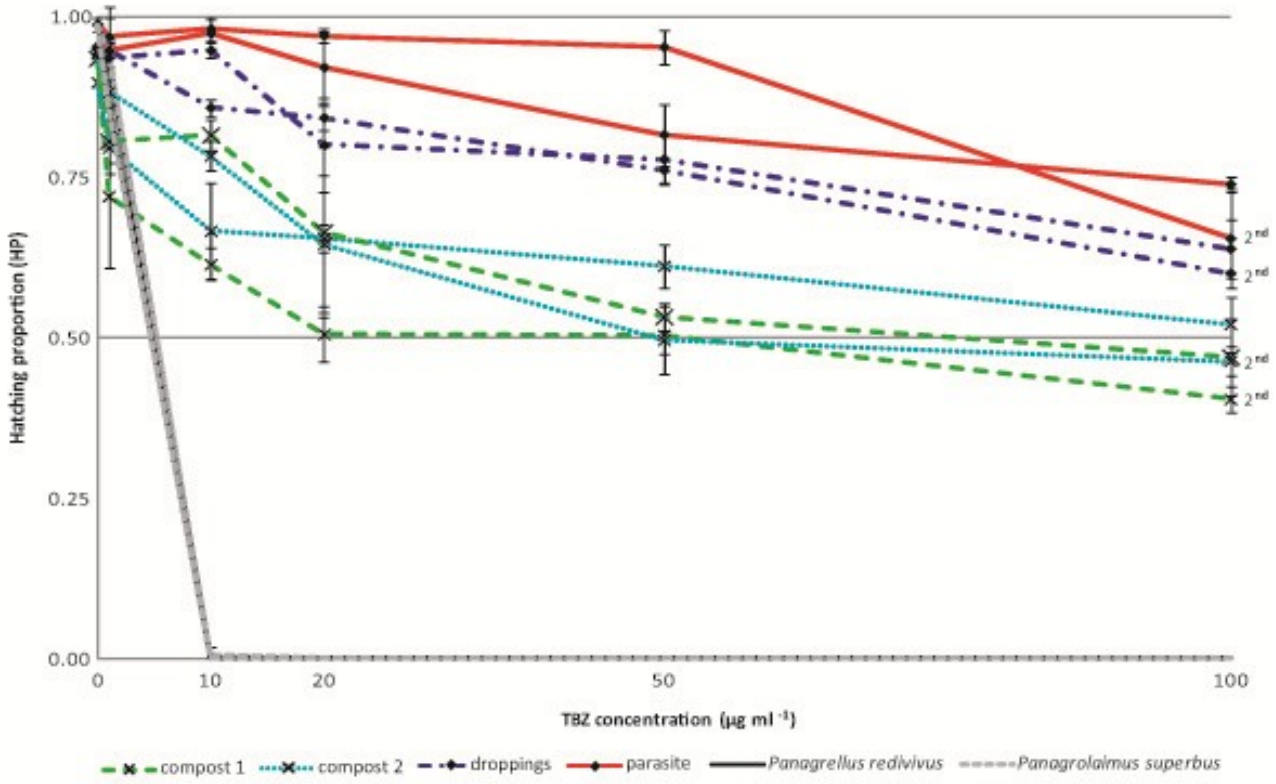

Figure 4.1. The hatching proportion (HP) for all *Halicephalobus gingivalis* isolates[†] and for *Panagrellus redivivus* (PS1163) and *Panagrolaimus superbus* (DF5050) at different thiabendazole (TBZ) concentrations in µg ml^{-1}. Data represent the mean of three replicates ±1 stdev for two independent and consecutive trials. Data from the second trial are indicated (2^{nd}). [†]compost isolates, *i.e.*, compost 1 (WB0708) and compost 2 (JB128); horse associated isolates, *i.e.*, parasite (SAN100) and droppings (WB0801).

Table 4.1. Type III statistics of fixed effects generated by means of a generalized linear mixed model on the average hatching proportions (HPs).

	TBZ		IVM	
Effect	*F* value	*P*	*F* value	*P*
isolate	85	<0.0001	64	<0.0001
concentration	604	<0.0001	275	<0.0001
concentration*isolate	5.8	<0.001	7.3	<0.0001

parasite*isolate had the highest average HPs, which were significantly different from the average HPs of the droppings isolate (Tukey post hoc, *P* all <0.05) and from both

compost isolates at all TBZ concentrations (*P* all <0.0001). The droppings isolate had the second highest average HPs, which were also significantly higher than the average HPs of both compost isolates at all drug concentrations (*P* all <0.0001). Compost isolates 1 and 2 had the overall lowest average HPs of the *Halicephalobus* isolates, without significant (*P* all >0.07) mutual HP differences.

IVERMECTIN (IVM)

The mean HPs of the *Halicephalobus* isolates and of *P. redivivus* and *P. superbus* of both trials at the different IVM concentrations are shown in figure 4.2, with 0 µg ml^{-1} being the negative control. The average HPs of the negative controls were similar for all *Halicephalobus* isolates and for *P. redivivus* and *P. superbus* with an average HP ranging from 0.92 to 1.

As for TBZ, the HPs of both *P. redivivus* and *P. superbus* soon dropped significanty lower (at 0.1 µg ml^{-1}) compared to those of the *Halicephalobus isolates* (Fisher's exact test: *P* all < 0.0001). As the HPs for both *P. redivivus* and *P. superbus* even approached zero for IVM concentrations that were higher than 0.5 µg ml^{-1}, they were significantly lower compared to the HPs of the *Halicephalobus* isolates for all the remaining IVM concentrations (Fisher's exact test: *P* all <0.0001).

Also for IVM, within the *Halicephalobus* isolates significant differences were observed in both the mean hatching rate and in their response to IVM concentration across both trials (table 4.1). The average HPs of the parasite isolate were also the highest at all IVM concentrations, followed by those of the droppings isolate and subsequently those of compost isolate 1. Although they were not clearly seperated, they were significantly different from each other (*P* all <0.02), except for the average HPs of the droppings isolate and compost isolate 1 from 1.55 µg ml^{-1} onwards (*P* all >0.06). Compost isolate 2 had the overall lowest average HPs, which were significantly lower than the average HPs of the other *Halicephalobus* isolates at all IVM concentrations (Tukey post hoc, *P* all <0.0001).

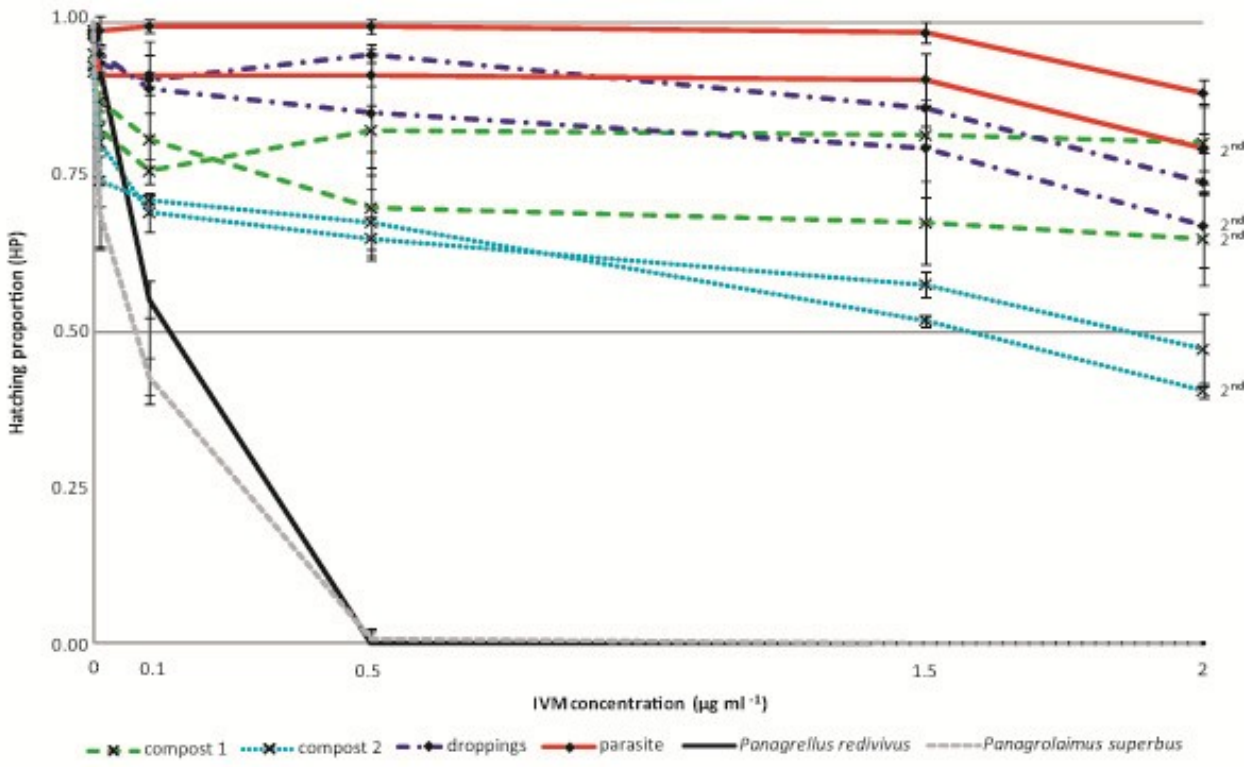

Figure 4.2. The hatching proportion (HP) for all *Halicephalobus gingivalis* isolates(†) and for *Panagrellus redivivus* (PS1163) and *Panagrolaimus superbus* (DF5050) at different ivermectin (IVM) concentrations in µg ml^{-1}. Data represent the mean of three replicates ±1 stdev for two independent and consecutive trials. Data from the second trial are indicated (2^{nd}). (†)compost isolates, *i.e.*, compost 1 (WB0708) and compost 2 (JB128); horse associated isolates, *i.e.*, parasite (SAN100) and droppings (WB0801).

RECOVERY CAPACITY AFTER DRUG TREATMENT

TBZ treatment showed a dose-related inhibitory effect on egg hatching for the *Halicephalobus* isolates and for *P. redivivus* and *P. superbus*. However, the eggs that hatched show no discernible delay in developmental rate at all TBZ concentrations, and almost all the hatched larvae developed into the adult stage. In contrast, for the tested IVM concentrations the inhibitory effect on egg hatching was less explicit. Only little influence on hatching was observed compared to the negative controls and the developmental rate of the eggs at the different IVM concentrations was the same. However, there was a dose-related effect on the survival of the juveniles and at higher IVM concentrations there was a noticeable delay in the development of the juveniles

into the adult stage. At 0.01 µg ml^{-1} IVM concentration the adult stage of all *H. gingivalis* isolates and *P. redivivus* and *P. superbus* was attained with a delay of 24 to 48 h. At 0.5 to 2 µg ml^{-1} IVM, the larvae of the *Halicephalobus* isolates survived, however with a reduced motility, but did not develop into the adult stage. However, this negative effect on larval development was found to be reversible for the parasite isolate, the droppings isolate and compost isolate 1. Hatched larvae incubated for 76 h on wells containing 1.5 µg ml^{-1} IVM had a survival rate of 85.4 ± 4.5% (mean ± 1 stdev) for the parasite isolate, 71.6 ± 2.5% for the droppings isolate and 75.8 ± 4.6% for compost isolate 1. Surviving larvae were subsequently transferred onto 1% plain bacteriological agar whereupon they reached the adult stage after 3 days. The recovery rate was 94.1 ± 2.3% (mean ± 1 stdev) for the parasite isolate, 77.9 ± 5.7% for the droppings isolate and 91.1 ± 5.1% for compost isolate 1. Compost isolate 2 only had 16.5 ± 4.5% surviving larvae, which did not develop into the adult stage after transfer onto plain 1% bacteriological agar. Finally, this recovery capacity could not be tested for *P. redivivus* and *P. superbus* at 1.5 µg ml^{-1} IVM since there were no surviving larvae at higher IVM concentrations.

INFLUENCE OF PRE-EXPOSURE ON ANTHELMINTIC TOLERANCE

The effect of pre-exposure with anthelmintics for ca. 12 generations was very similar for all isolates and is therefore only illustrated for compost isolate 1 (fig. 3). For both anthelmintics, the HPs of all *Halicephalobus* isolates in the control treatment were significantly lower (Tukey post hoc, $P<0.001$) upon pre-exposure as opposed to the HPs of eggs deposited by nematodes which had not been pre-exposed, *i.e.*, 10-20% and 37-47% lower for IVM and TBZ, respectively. The dose-response upon pre-exposure was nevertheless different between TBZ and IVM.

Upon pre-exposure to TBZ, the HPs exhibited limited (compost isolate 2) to no (compost isolate 1, parasite isolate, droppings isolate) concentration dependence, which was demonstrated by the lack of significant differences (Tukey post hoc, P all >0.05) between the HPs at different drug concentrations, including the control.

Upon pre-exposure to IVM the HPs still showed a similar concentration dependence as opposed to non pre-exposure. However, for most isolates (compost isolate 1, parasite isolate, droppings isolate) the HPs at all concentrations were

significantly lower (*P* all <0.05) than in the original experiment, except the HPs of compost isolate 2 that showed no significant difference (*P*>0.05) at all concentrations.

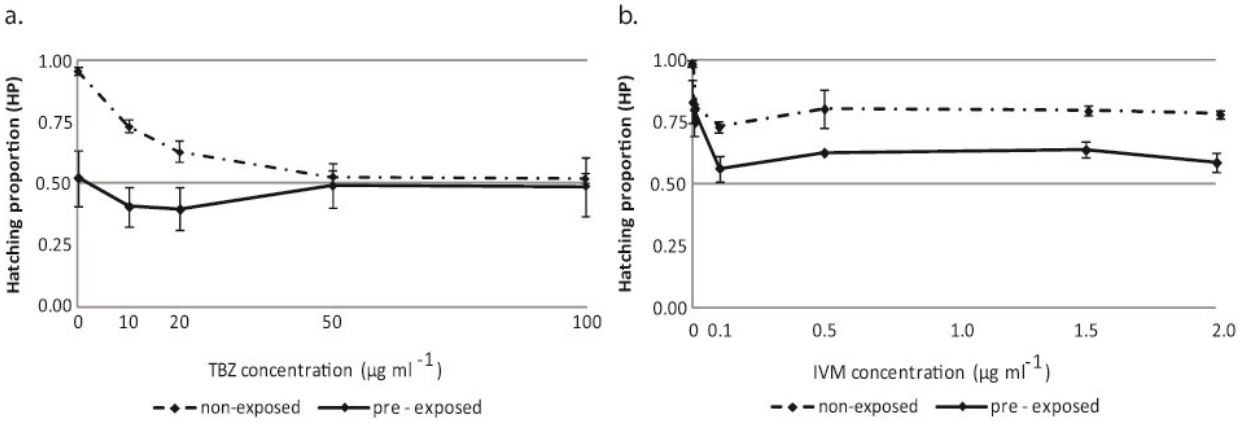

Figure 4.3. The mean hatching proportion (HP) of pre-exposed eggs (dotted line) is compared with the mean HPs of non-exposed eggs (full line) for *Halicephalobus* compost isolate 1 (WB0708) at different TBZ (a) and IVM (b) concentrations in µg ml^{-1}. Data represent the mean of three replicates ±1 stdev.

DISCUSSION

METHODOLOGICAL CONSIDERATIONS

Several methodological aspects may bear upon the results of dose-effect studies like the present one. We are, however, convinced that the methods used here allow an accurate assessment of the effect of the anthelmintics used in this study. The MALDT method has been proven earlier to give reliable results concerning the detection of benzimidazole resistance (Várady *et al.* 2009), and by using this agar based method the insolubility problem of IVM is eliminated (Lacey *et al.* 1991). Moreover, former studies have revealed that the activity of IVM incorporated in agar is higher than in aqueous solutions (Várady *et al.* 2009). Secondly, our negative controls of the *Halicephalobus* isolates, *Panagrellus redivivus* and of *Panagrolaimus superbus* have HPs close to 100%. Therefore it can be assumed that the incubation conditions used here are adequate. Thirdly, the steep decrease of the HPs of *P. redivivus* and *P. superbus* compared to the relatively high HPs of the *Halicephalobus* isolates at higher concentrations of both TBZ and IVM confirms that effective drug treatment is detectable using this method. Finally, the

highly concordant results of two independent experimental trials demonstrate the reproducibility of our bioassay.

TOLERANCE VERSUS RESISTANCE

It is very important to distinguish between an original low, or lack of, effectiveness of an anthelmintic drug to a population and the presence of actual resistance to that same anthelmintic (Brady and Nichols 2009). The lack of effectiveness can be seen as an existing natural tolerance to an anthelmintic even before the parasite has come in contact with the drug (Fallon *et al.* 1996; Coles 2006), whereas acquired resistance is the conversion within a species of a low or absent tolerance towards a higher tolerance which is initiated by contact with the anthelmintic (James *et al.* 2009). The overall high hatching proportions of the *Halicephalobus* isolates at all concentrations tested in this study suggest the presence of some kind of natural tolerance to IVM and TBZ. This tolerance appears specific for the facultative parasitic species *H. gingivalis*, since in at least some Panagrolaimidae (*P. redivivus* and *P. superbus*) no tolerance was observed. The stunning tolerance of the *Halicephalobus* isolates to TBZ and IVM is further confirmed by the considerably higher concentrations (roughly 75 times the maximum dose used for TBZ and roughly 45 times the maximum dose used for IVM) used in the present study as compared to TBZ and IVM concentrations used in another *in vitro* study using the MALDT method for testing anthelmintic resistance of the obligatory parasite *Haemonchus contortus* (Várady *et al.* 2009).

Our results reveal that the horse associated *Halicephalobus* isolates are highly tolerant for both tested anthelmintic drugs and that the *Halicephalobus* compost isolates show an anthelmintic tolerance that is generally lower. Thus, our results also reveal a remarkable trend of increasing tolerance from fully free-living isolates towards horse-associated isolates, which is especially true for TBZ. This difference in tolerance to anthelmintics between the *Halicephalobus* isolates may be associated with earlier contact to these anti-parasitic drugs. However, none of the *Halicephalobus* strains have been found to be fully susceptible to either anthelmintic. Since there is no fully susceptible strain available, no actual acquired resistance can be proven (Brady and Nichols, 2009). In addition, the pre-exposure experiments did not show a decreased

susceptibility to the tested anthelmintics. The average HPs of all pre-exposed isolates in the control treatment (no anthelmintic added) were considerably lower (by 10-47%) than those of non pre-exposed nematodes, which indicates that the fitness of all isolates is negatively affected by prolonged exposure to the anthelmintics, resulting in a lower egg viability. This type of negative effect of a chemical compound on nematode egg viability has been shown earlier for, *e.g.*, tannins on gastro-intestinal parasites (Min *et al.* 2003). Further, the HPs under anthelmintic exposure exhibited only limited (compost isolate 2) to no (the other isolates) concentration dependence for TBZ and similar (compost isolate 2) or generally lower HPs (the other isolates) for IVM. This is contrary to the idea that the high tolerances observed in short exposure experiments and the differences between the horse-associated and the other isolates would be due to a true resistance. Moreover, differences in the D2D3 expansion segment of the LSU rDNA region (data not shown) shows a remarkable interpopulation variation. However, phylogenetic analyses, including GenBank (Benson *et al.* 2008) sequences, appointed our *Halicephalobus* isolates (WB0801, GenBank HQ697251 and WB0708, GenBank JF706244) within an internally unresolved *H. gingivalis* clade (data not shown). Additionally, a thorough molecular analysis based on 4 markers revealed that all isolates belong to a highly supported *H. gingivalis*-clade (Chapter 6).

Finally, since even the very high anthelmintic concentrations used in the present study appear ineffective to control the *Halicephalobus* isolates and since IVM administered to horses at the recommended dosage has a maximum plasma persistence of 4 to 62 ng ml^{-1} (Gokbulut *et al.* 2010), it is very unlikely that *in vivo* anthelmintic treatments are effective for infections with this facultative parasite. This is supported by the medical history of horses which suffered lethal infections of this nematode species in spite of regular treatment with common anthelmintics (*e.g.*, Boswinkel *et al.* 2006, Ferguson *et al.* 2008).

HALICEPHALOBUS *AS A MODEL ORGANISM*

In research on the effects of anti-parasitic drugs, using free-living nematodes for *in vitro* experiments has the advantage of allowing observations on their complete life cycle, including survival and (delayed) development. In contrast, obligatory animal parasites

can only be followed until the infective stage. *Caenorhabditis elegans* has been used as a model for studies on the development of anthelmintic resistance and the testing of the efficiency of new drugs (*e.g.*, Simpkin and Coles 1981; Sangster *et al.* 2002; James and Davey 2009). Since its complete genome is known, *C. elegans* is especially suitable for studying the effects of anthelmintics at the gene level (Holden-Dye and Walker 2007). However, the usefulness of *C. elegans* as a model for parasitic nematodes has been questioned (Geary and Thompson 2001), among other reasons simply because it is not capable of parasitism in its natural environment. *Halicephalobus gingivalis* shares several of the advantages of *C. elegans* as a model organism: it is amenable to culture under laboratory conditions; it has a very short generation time (approximately 48 hrs at 30°C), produces a lot of offspring, can be cultured in liquid (monoxenic as well as axenic) as well as on solid media (Chapter 4) and at temperatures ranging from 4°C to more than 40°C (personal observations, unpublished). Additionally, *H. gingivalis* is capable of parasitism in its natural environment. Moreover, since complete genome sequencing is nowadays relatively fast and easy (Elsworth *et al.* 2011), the 'genetic barrier' can easily be overcome. Although the lack of a susceptible isolate is a drawback to the use of *Halicephalobus gingivalis* as a model organism for testing new anthelmintics, the presence and ease of cultivation of susceptible close relatives such as *Panagrellus* and *Panagrolaimus* provides great potential as an experimental tool for testing the effects of various drugs on a model system encompassing a range of tolerances and including an organism with a life history intermediate between that of obligatory parasites and of fully free-living nematodes.

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CHAPTER IV

INTESTINE ULTRASTRUCTURE OF THE FACULTATIVE PARASITE *HALICEPHALOBUS GINGIVALIS*

Modified from:

FONDERIE P., WILLEMS M., BERT W., HOUTHOOFD W., STEEL H., CLAEYS M, BORGONIE G. (2009) Intestine ultrastructure of the facultative parasite *Halicephalobus gingivalis* (Nematoda: Panagrolaimidae). *Nematology* 11 (6), 859-868.

ABSTRACT

Halicephalobus gingivalis, classified as a free-living nematode, is a known facultative parasite of horses, zebras and humans. However, detailed information concerning its internal morphology is lacking, especially in relation to possible adaptations to its lifestyle as a facultative parasite. The research presented here uses TEM and PI staining to analyse the morphology of the intestine of *H. gingivalis*. Specimens cultured under different conditions were included to determine if differences in ultrastructure are induced by culturing method. TEM analysis revealed that the intestinal tract comprises a single layer of cells in which nine pairs of nuclei can be distinguished. Further, unusual dichotomously and trichotomously branched microvilli were observed next to finger-like cylindrical microvilli, the latter being the most commonly described form in nematodes. Finally, three different types of secretion vesicles, *i.e.*, spherical, threadlike and enlarged, globular, vesicles, occurred independently from each other along the intestinal tract. The relationship of morphological adaptations of the microvilli to parasitism in nematodes is discussed.

INTRODUCTION

Halicephalobus gingivalis (STEFAŃSKI 1954) ANDRÁSSY 1974, formerly referred to as *H. deletrix* or as *Micronema deletrix* (Anderson *et al*. 1998), is a small (235-460 µm) free-living bacteriovorous nematode (Panagrolaimidae) which is also a known facultative parasite of horses (Blunden *et al*. 1987; Nadler *et al.* 2003) and zebras (Isaza *et al*. 2000). Little is known about the epidemiology of *H. gingivalis*. Infection can occur through open wounds or oral or nasal cavities (Pearce *et al.* 2001) whereupon the nematodes probably invade the bloodstream or the lymphatic system, thereby reaching the different organs (Akagami *et al.* 2007). Subsequently the number of nematodes increases rapidly through parthenogenetic reproduction. This massive proliferation usually causes death of the infected animal. Most infections in horses are only recognised post-mortem after a thorough autopsy in which the extremely small nematode is found. The clinical symptoms are very variable depending on which organs are infected (Blunden *et al.* 1987; Spalding *et al.* 1990; Rames *et al.* 1995; Johnson *et al.* 2001; Grosche *et al.* 2002; Mandrioli *et al.* 2002; Müller *et al.* 2008). In only a few cases does the infected animal survive (Dunn *et al*. 1993; Pearce *et al*. 2001; Müller *et al*. 2008). Infection in three human cases has also been described, all with a fatal outcome (Hoogstraten and Young 1975; Shadduck *et al.* 1979; Gardiner *et al*. 1981).

The ability of facultative parasitism renders *H. gingivalis* an interesting subject for further research on the ultrastructural diversity of the intestine within the Nematoda. When free-living, *H. gingivalis* is bacteriovorous, but when parasitic, the exact food source is unknown, although as it reproduces and proliferates in the tissue of different organs of the host (Müller *et al.* 2008), some kind of food uptake, such as blood and host tissue, is likely. The question, therefore, arises as to whether this species has ultrastructural adaptations enabling it to thrive in two very different circumstances with two very different food sources. Detailed information on the internal structure of *H. gingivalis* is missing, *i.e*., whether the ultrastructural morphology resembles other known parasitic nematodes of mammals (*e.g*., *Ascaris suum*) or is more similar to that of other free-living bacteriovorous species (Borgonie *et al.* 1995). Various ultrastructural analyses of the intestine have been done on plant parasitic (reviewed in (Geraert 1992; Endo *et al.* 1997), animal parasitic (Kessel *et al.* 1961; Jenkins and Erasmus 1969; Smith

and Harness 1972; Wright *et al.* 1985; Endo and Nickle 1991; Frantova and Moravec 2004), marine (Deutsch 1978; Vandevelde and Coomans 1989; Miljutin and Tchesunov 2001; Miljutin *et al.* 2006), predatory (Arpin and Kilbertus 1981)and terrestrial bacteriovorous (Epstein *et al.* 1971; Borgonie *et al.* 1995) nematodes. The general structure of the intestine appears quite uniform, with exception of some marine (*e.g*. Miljutin and Tchesunov 2001; Miljutin *et al*. 2006) and plant parasitic taxa (Geraert 1992). The intestine is a straight tube which comprises a limited number of cells organised in a single layer with a luminal surface that is lined with microvilli. The microvilli mostly have a regular, cylindrical, shape although some exceptions have been observed in *A. suum* (Kessel *et al.* 1961), *Metastrongylus* sp. (Jenkins and Erasmus 1969), and *Hexatylus viviparus* (Shepherd and Clark 1976).

The ultrastructural analysis of the intestine of *H. gingivalis* is presented here and reveals unusual dichotomously branched microvilli and a form of intestinal secretion not previously described in nematodes.

MATERIALS AND METHODS

Three *Halicephalobus gingivalis* isolates were used in this study, *i.e.*, JB128 originating from a vegetable compost heap (Riverside, California, USA), JB043 originating from potting soil containing peat (Neustadt, Germany), and SAN100 isolated from an equine clinical case (Orlando, Canada). The species reproduces parthenogenetically and no males have been observed.

All products were obtained from Sigma-Aldrich (St. Louis, USA) unless mentioned otherwise.

NEMATODE CULTURE

Different culturing methods were used to determine whether ultrastructural characteristics were induced when specimens were cultured differently over a longer period of time, *i.e*., during minimal 20 generations. The nematodes were cultured both monoxenically and axenically. Monoxenic cultures were maintained on both solid and

liquid medium with *Escherichia coli* OP50 as a food source. The solid cultures were maintained on 1% bacteriological agar (Oxoid Ltd., Hampshire, UK) plates containing cholesterol (80 µl 5 mg ml^{-1} in 400 ml medium). The cultures were kept in an incubator at 20°C and generally handled as described by Brenner (1974). The liquid culture was grown in an Erlenmeyer with S-buffer (0.1 M NaCl, 50 mM potassium phosphate buffer in distilled water) and closed with cotton wool. The liquid cultures were kept at room temperature and placed in a shaker.

The axenic medium consisted of 4 ml haemoglobin (5% in 0.1M KOH), 12 g soy peptone (Oxoid Ltd., Hampshire, UK) and 12 g yeast extract (Oxoid Ltd., Hampshire, UK) in 400 ml distilled water. Eggs of *H. gingivalis* were sterilised by chloroxing twice in 5.5 ml sterile bidi, 0.5 ml 10 M NaOH and 4 ml 5% NaOCl. The first chlorox of 2 min was followed by rinsing the eggs twice with sterile S-buffer and the second chlorox of 1.5 min was followed by rinsing three times with sterile S-buffer. The eggs were then placed in the axenic medium under sterile conditions. Axenic cultures were kept in culture flasks in an incubator at 20°C and renewed every month.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Three specimens from each culturing method were used for the TEM study. Both longitudinal and transverse sections were taken at different levels of the intestine. Approximately 50 sections of each specimen were studied. Only young adults were used for TEM because the eggshell in gravid females can cause poor fixation and tissue damage when sectioning. The nematodes were fixed in Karnovsky's solution (2% paraformaldehyde, 2.5% glutaraldehyde and 0.5% CaCl2 in 0.2 M sodium cacodylatebuffer pH 7.2) (Bert *et al.* 2003) at 60°C for 30 min. The specimens were kept overnight in Karnovsky's solution at 4°C, during which time the solution was stirred. The following day the nematodes were rinsed in a 0.134 M sodium cacodylatebuffer (pH 7.2) for 8 h at room temperature. Post-fixation took place overnight in reduced osmium at 4°C. After rinsing with double distilled water the specimens were dehydrated in a 50%, 70%, 90%, and 100% ethanol series at room temperature, each stage being repeated three times at 20 min each. Subsequently, the specimens were infiltrated with a low-viscosity medium (Spurr 1969) and finally polymerised at 70°C for 8 h. Longitudinal

sections 70 nm thick were made using a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome Ltd., Biel, Switzerland) and mounted on formvar coated single slot copper grids (Agar Scientific, Stansted, UK). Sections were stained (EM stain, Leica) with uranyl acetate and lead citrate. Electron microscopy was done using a Jeol JEM 1010 (Jeol Ltd., Tokyo, Japan), operating at 60 kV. The first micrographs were taken on Kodak electron image film (Agar Scientific, Stansed, UK). Later pictures were digitised using a DITABIS system (Pforzheim, Germany). Plates were composed using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA).

NUCLEAR STAINING WITH PROPIDIUM IODIDE

To determine the number of intestinal cells along the alimentary tract, the nuclei of 30 young adult females were stained using propidium iodide (PI). First, the nematodes were fixed for 2 h in 4% formaldehyde at 70°C and washed with phosphate buffered saline (1x PBS). Subsequently they were incubated for 5 min in propidium iodide which was diluted 1:2500 in PBS. Finally the nematodes were washed in PBS and then ten specimens were mounted on glass slides in Vectashield mounting medium (Vector Laboratories Ltd., Peterborough, UK). The nuclei were visualised with a Nikon EZ-C1 confocal microscope.

RESULTS

Culturing method and origin of the isolate (free-living versus parasitic) did not influence the general ultrastructural morphology of *H. gingivalis*. As observed with TEM, the intestinal lumen is triradiate (Y-shaped) just posterior to the pharynx and becomes sausage shaped more posterior. The lumen narrows at the mid-body region as it is squeezed between the epidermis and the reproductive system (fig. 3.1A). The intestinal tract comprises a single cell layer. Although all nuclei were stained using propidium iodide, the intestinal nuclei could be easily discerned from other nuclei due to their large size. The intestinal cells surrounded the lumen two by two across the whole length of the tract (fig. 3.1B). Nine pairs of nuclei could be distinguished of which five pairs are situated anterior to the vulva and four pairs posterior.

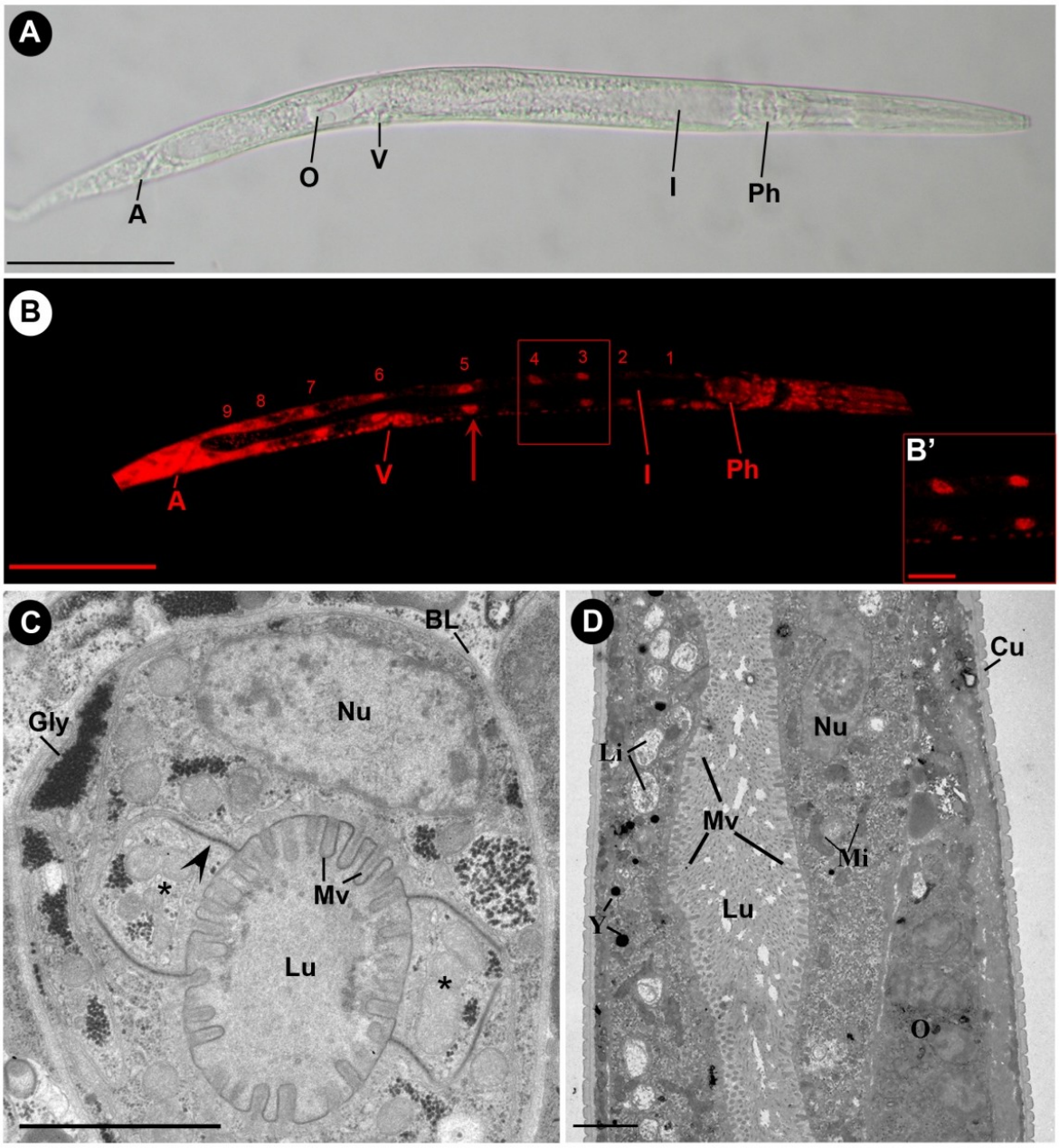

Figure 3.1. (A) Light microscopic image of *Halicephalobus gingivalis* from monoxenic culture; (**B**) Confocal image of *H. gingivalis* with propidium iodide staining showing nuclei (arrow) of nine pairs (1-9) of intestinal cells with five pairs anterior and four pairs posterior to vulva; (**B'**) Detail of two pairs of intestinal nuclei; (**C**) TEM image of transverse section through most anterior part of intestine of *H. gingivalis* showing intercalation (*) with posterior pharyngeal cells and desmosomes (arrowhead) between two adjacent cells. (**D**) TEM image of longitudinal section of *H. gingivalis* showing intestine and distribution of different cell organelles; Abbreviations: A = anus; BL = basal lamina; Cu = cuticula; Gly = glycogen; I = intestine; Li = lipid inclusions; Lu = intestinal lumen; Mi = mitochondria; Mv = microvilli; Nu = nucleus; O = ovarium; Ph = posterior bulbus of pharynx; V = vulva; Y = yolk inclusions. (Scale bars: A, B = 50 µm; B' = 10 µm; C = 2 µm; D = 1 µm.)

Adjacent intestinal cells are joined with membrane junctions or desmosomes (fig. 3.1C). The large and prominent nuclei are situated centrally in the intestinal cells. They are amoeboid shaped, approximately 3.5 ± 0.07 µm (n = 20) across and have an electron dense nucleolus. The cytoplasm is granulated and contains a large amount of endoplasmatic reticulum. Ovoid to long sausage-shaped mitochondria are present along the entire intestinal tract, being more numerous most anteriorly compared to at the mid-body region and more posteriorly. Golgi complexes are evenly distributed along the intestinal tract. Lipid vacuoles and yolk inclusions are present in all intestinal cells (fig. 3.1D). The number of yolk inclusions is highest at the mid-body where the reproductive system is situated. The number of lipid vacuoles increases at the mid-body and more posteriorly. Glycogen (fig. 3.1C) is present along the entire intestine with no discernible pattern of distribution.

At the apical surface of the intestinal cells, relatively short and blunt microvilli occur, each with an electron-dense cylinder of microfilaments at their core. The inner core of microfilaments extends into the terminal web which is located just beneath the brush border. The terminal web can be distinguished as an approximately 0.1 ± 0.01 µm (n = 20) thick layer, characterised by a seemingly absence of organelles.

The length of the microvilli was measured at the anterior end, at the mid-body and further posterior for both axenic and monoxenic cultures (fig. 3.2). No differences were observed between culturing methods. The length of the microvilli increases at the mid-body and decreases again more posteriorly. The shape of the microvilli appeared to be independent of both position in the intestine and culturing method. Most microvilli are straight and cylindrical and lie within the glycocalyx that is visible as a grey coat above and between the microvilli. Besides the cylindrical microvilli, numerous branched microvilli were also observed. They were also observed along the entire intestinal tract in specimens from all culturing methods. The majority of these branched microvilli are dichotomous, although some are trichotomous (fig. 3.3). Figure 3.4 shows a longitudinal section (fig. 3.4A) and several consecutive transverse sections (fig. 3.4B-D) through the microvilli in which both cylindrical as dichotomously branched microvilli can be seen.

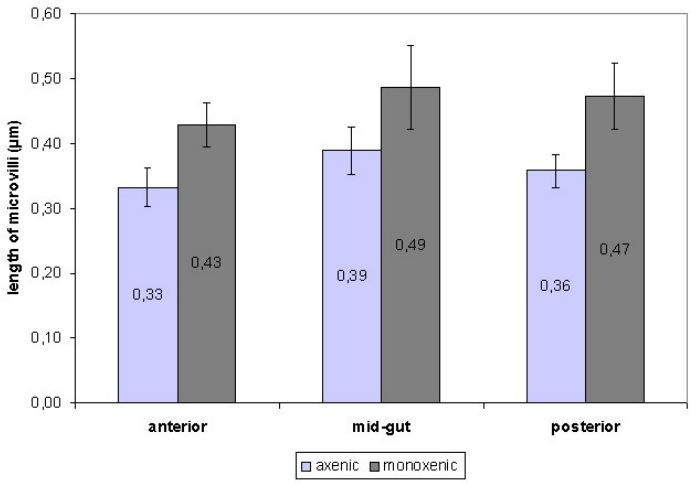

Figure 3.2. Bar graph of length of microvilli in µm (y-axis) of *Halicephalobus gingivalis* at different regions of the intestine (x-axis) for both axenic and monoxenic cultured specimens (n = 20). Standard deviations are shown on bars.

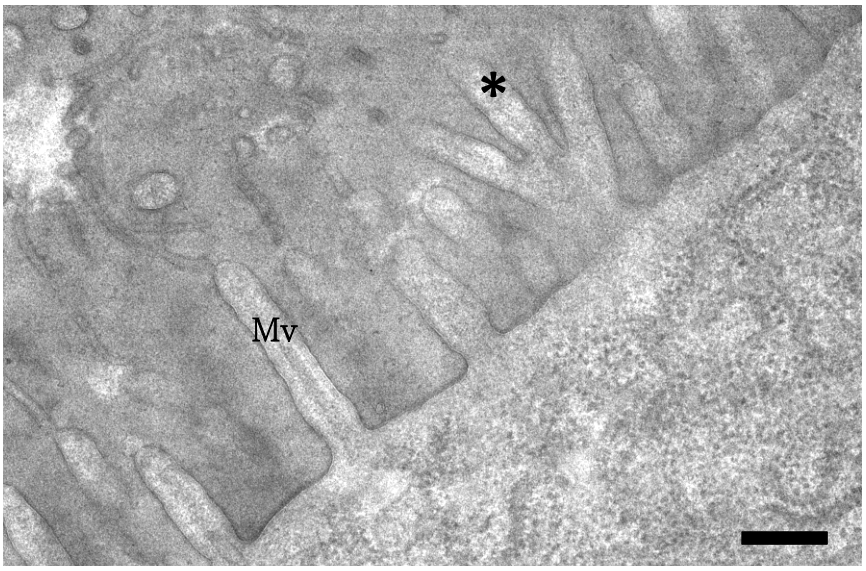

Figure 3.3. TEM image of longitudinal section showing both cylindrical microvilli (Mv) and a trichotomously branched microvillus (*) (Scale bar = 200 nm).

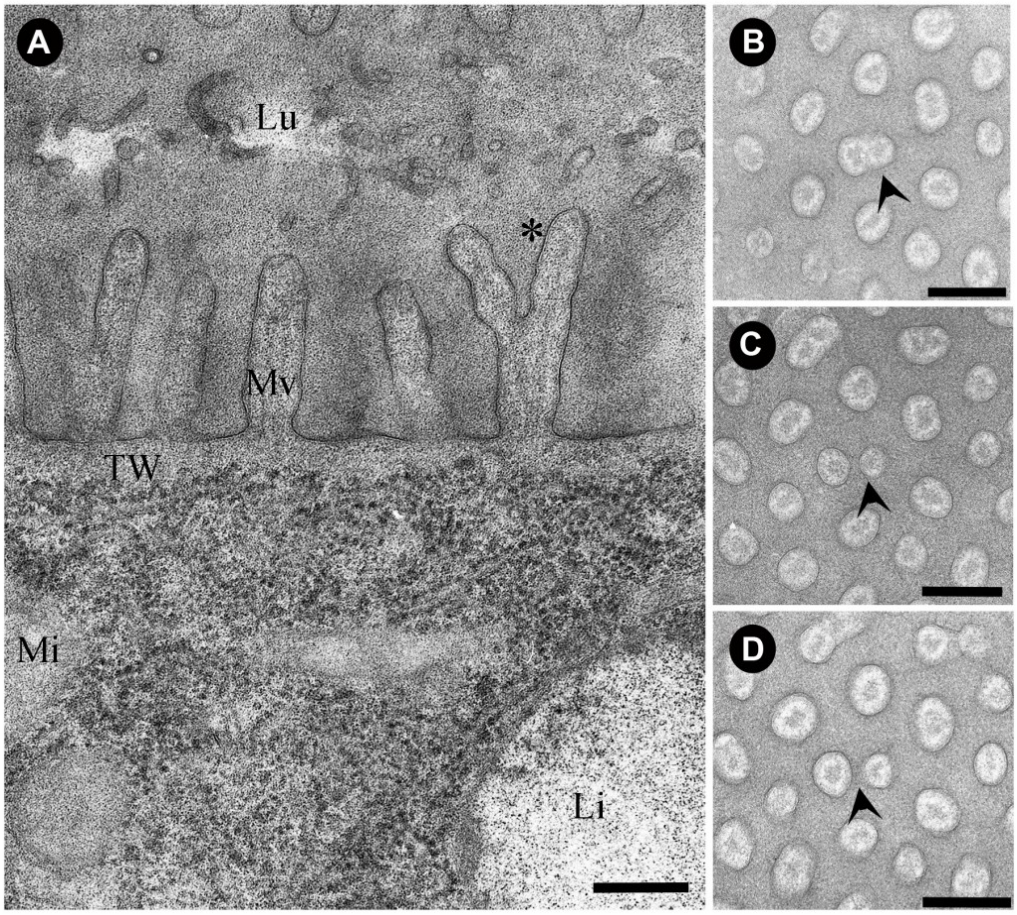

Figure 3.4. TEM images of intestine of *Halicephalobus gingivalis*. (**A**) Longitudinal section showing both cylindrical microvilli (Mv) and a dichotomously branched microvillus (*); (**B-D**): Consecutive transverse sections through cylindrical and dichotomously branched (arrowheads) microvilli. Abbreviations: Li = lipid inclusion; Lu = lumen; Mi = mitochondrion; Mv = microvillus; TW = terminal web. (Scale bars = 200 nm)

Three types of vesicles could be discerned along the entire length of the alimentary tract. The first type consists of small spherical vesicles that are visible in large numbers in the most anterior part of the intestine and decrease substantially further posterior. They occur between the microvilli as well as in the intestinal lumen (fig. 3.4A). The vesicles are small at first but enlarge towards the lumen. The second type is most common at the mid-body and in the more posterior end of the intestine and consists of thread-like vesicles that seem to emanate from the top of the microvilli (fig. 3.4A). These threads are released into the lumen where they disintegrate into smaller particles (fig. 3.4B). From the mid-body to further posterior this type of vesicle is the most common type observed in the intestine. The third type has only been observed at the mid-body region and consists of enlarged, globular, vesicles that remain attached to the

microvillus (fig. 3.4C). All three vesicle types have a double membrane and seem to be budded off from the microvilli. The first and second types of vesicle have been observed in all culturing methods, whereas the third type has only been observed in the monoxenic cultured specimens. The general number of vesicles decreased from anterior to posterior.

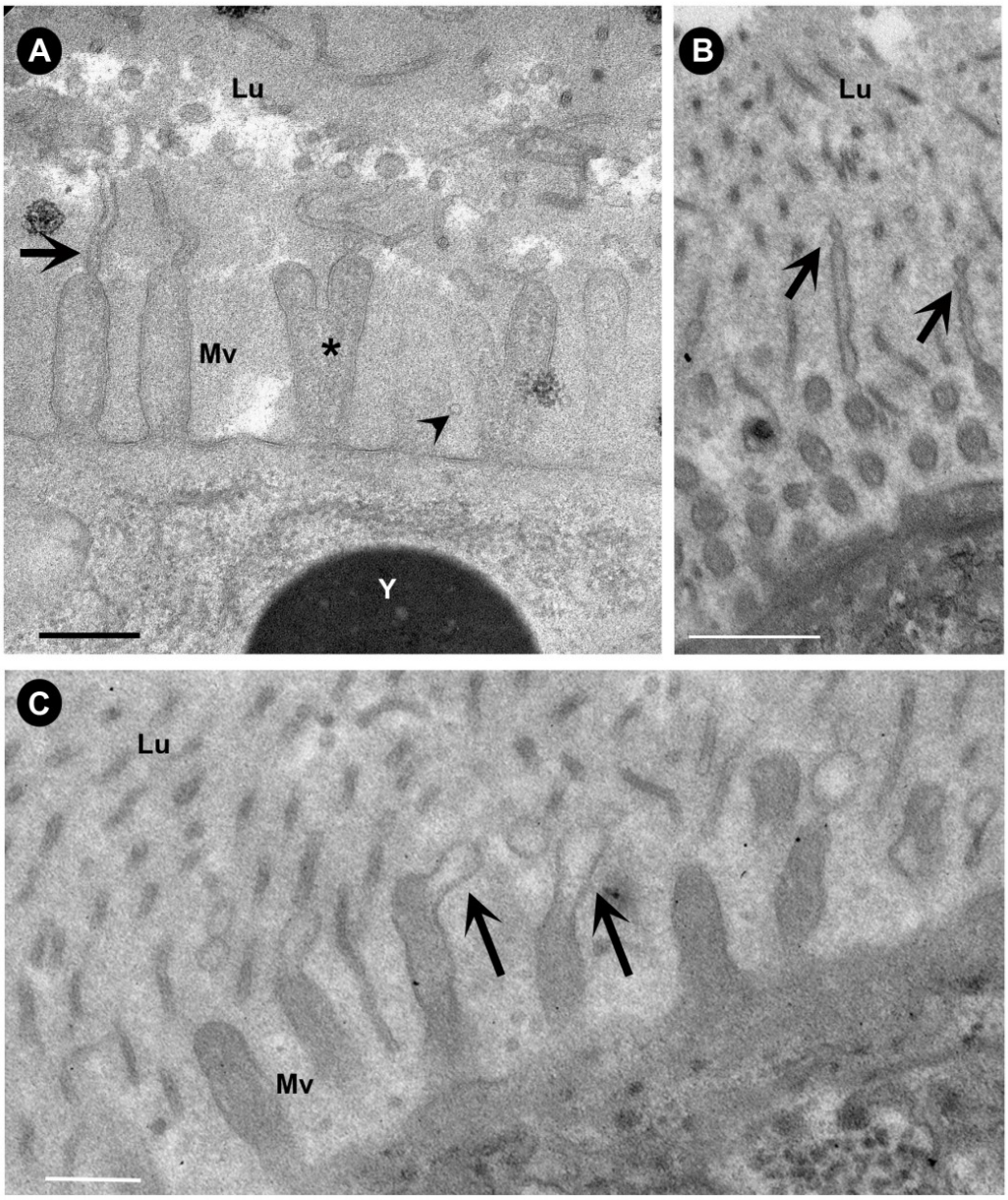

Figure 3.4. TEM images of longitudinal sections through intestine of *Halicephalobus gingivalis* showing different types of vesicles observed along gut. (**A**) Small spherical vesicle (first type) in-between the microvilli (arrowhead) and threadlike vesicles (second type) that emanate from the top of the microvilli (arrow). Note the dichotomously branched microvillus (*); (**B**) Thread-like vesicles (arrows) disintegrating into smaller vesicles in lumen of intestine; (**C**) Enlarged globular (third type) vesicles (arrows) that remain attached to top of microvilli. Abbreviations: Lu = lumen; Mv = microvillus; Y = yolk. (Scale bars: A, C= 200 nm; B = 500 nm.)

DISCUSSION

The general morphology of the intestine of *H. gingivalis* is similar to that of other studied free-living nematodes (*e.g*., Epstein *et al.* 1971; Borgonie *et al*. 1995). The 18 intestinal nuclei, visualised by means of propidium iodide staining, are organised two by two along the intestinal tract which is consistent with results of the embryonic cell lineage of *H. gingivalis* (Houthoofd *et al.* 2006). The ultrastructure of the *H. gingivalis* digestive tract and the distribution of the different organelles in the intestinal cells are similar to that of other terrestrial (*e.g*., Borgonie *et al.* 1995) and plant-parasitic nematodes (*e.g*., Geraert 1992).

Using only TEM, the nature of the three types of vesicle observed in *H. gingivalis* could not be exactly determined but, according to their position throughout the intestine, it is likely that they represent some form of secretion. The first type observed in this study has frequently been described in nematodes as secretion vesicles (Bird and Bird 1991; Borgonie *et al.* 1995; Willems *et al.* 2005) and is reported as having a double membrane (Borgonie *et al.* 1995). The thread-like vesicles, *i.e.*, the second type, that emanate from the top of the microvilli in the intestine of *H. gingivalis* have also been observed in *Hexatylus viviparus* (Shepherd and Clark 1976), a nematode species that has a fungivorous as well as an entomoparasitic life cycle. Shepherd and Clark (1976) described these threads in *H. viviparus* as fine extensions of the microvillar tips which were situated in the anterior intestine as well as in the mid-intestine. These extensions have a double membrane (see figure 11E in Shepherd and Clark, 1976), in accordance with the thread-like vesicles observed in the intestine of *H. gingivalis*. Because type I vesicles occur more anteriorly in the intestine of *H. gingivalis* than type II, it can be concluded that these two types occur independently from each other, *i.e*., the first type of vesicles are not merely a cross section of the second type. Finally, similar to the enlarged globular vesicles in this study (*i.e*., type III vesicles), dilated balloon-like tips to the microvilli have been observed in the intestine of *Metastrongylus* sp. (Jenkins and Erasmus 1969). These dilated tips budded from the main stem of the microvilli and were released into the lumen. The released vesicles had a granular content and were interpreted as a form of secretion (Jenkins and Erasmus, 1969). Kurosumi (Kurosumi 1961) named this type of secretion “micro-apocrine”. Shepherd and Clark (1976)

reported similar “blebs” associated with the mid-intestinal microvilli of *H. viviparus*. In *H. gingivalis*, the globular secretions were only observed in the monoxenically cultured specimens. Possibly, these globular secretions only occur when larger amounts of food need to be processed. All three types of vesicle are likely to be pieces that bud from the microvilli, an origin that explains the occurrence of a double membrane around all the observed vesicles. To our knowledge, this is the first time that all three types of secretion have been described in one species.

Microvilli are projections of the plasma membrane into the lumen of which the length, shape and number are known to vary slightly between the different regions of the intestine (*e.g.*, Endo and Nickle 1991; Borgonie *et al.* 1995). Although most nematodes have simple cylindrical, finger-like intestinal microvilli, *H. gingivalis* has both dichotomous and trichotomous (fig. branched microvilli located adjacent to cylindrical ones. This remarkable observation is confirmed by observations made on several specimens and also appeared to be independent of culture method.

A similar kind of branched microvillus has previously been observed in the animal parasitic *A. suum* (Kessel *et al.* 1961). According to Kessel *et al.* (1961) these branched microvilli only play a role in imposing geometry on the microvilli, with each microvillus equidistant to the six others that surround it (Palay and Karlin 1959). By branching, this organisation of the microvilli is preserved throughout the intestine. However, because of the relatively long size of the microvilli (6-7 µm) in *A. suum* (Sheffield 1964) and their entanglement with each other, we consider it unlikely that geometry requirements are a plausible explanation for branching. Shepherd and Clark (1976) also reported a few branched microvilli in the intestine of the insect-parasitic *H. viviparus*.

Willems *et al*. (2005) also reported microvilli that deviate from the usual finger-like microvilli in *Rhabditophanes*. Microlamellae were observed here instead of cylindrical microvilli. These lamellae formed a complex 3-D maze network with cavities of varying length. According to Willems *et al*. (2005) the function of the microlamellae network may be related to a maximisation of the intestinal absorption surface. Moreover, by slowing down the movement of the absorbed food through the intestine, more time is available for nutrient uptake and the species is able to survive in an environment with low food availability.

The branching of the intestinal microvilli observed in *H. gingivalis* is more likely to be a function of food consistency than food availability. Food in the intestinal tract of most nematodes moves along by the ingestion of more food and by the general movement of the nematode (Bird and Bird, 1991). However, when parasitizing a vertebrate, it is likely that *H. gingivalis* ingests blood and other tissues. While bacteriovores are thought to concentrate bacteria in their intestine while digesting, it is more difficult to concentrate a liquid food source which therefore passes faster through the digestive tract. According this hypothesis, *H. gingivalis*, when in the vertebrate parasitic phase, needs an increase of digestive surface in order to optimise the intake of nutrients. Although, based on TEM sections, it is impossible to give an exact count of the number of branched microvilli *vs.* the number of cylindrical microvilli, these branched microvilli have been observed frequently along the entire intestinal tract and in all studied sections. Therefore, it can be assumed that the branching of the microvilli represents an increase of abundance and density of the microvilli and thus an increase in the surface area of the intestinal epithelium.

The current study, in corroboration with Kessel *et al*. (1961), Shepherd and Clark (1976) and Willems *et al*. (2005), shows that the morphology of the intestinal microvilli within the phylum Nematoda is more diverse than initially thought. Most remarkable, deviations of the usual finger-like intestinal microvilli have arisen independently several times within the phylum, their occurrence always being in parasitic or in parasitism-related nematodes. To our knowledge, there are no reports of branched intestinal microvilli in other invertebrates. In vertebrates, however, studies on the intestine of salamander (Tilney and Cardell 1970) and chick intestine in organ culture (Burgess and Grey 1974) have shown that branching of microvilli can be induced by changing environmental conditions or by exposure to drugs. This implies that microvilli are dynamic structures that are liable to stress. However, studies on the intestinal ultrastructure of nematodes under stress conditions (*e.g.*, Borgonie *et al.* 1996) did not report any form of branched microvilli. Furthermore, in the present study branched microvilli were frequently observed independent from culturing method, *i.e.*, they were not observed to be induced by external factors. Therefore, it is most likely that variations in form of the intestinal microvilli represent adaptations to a parasitic lifestyle.

ACKNOWLEDGEMENTS

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CHAPTER V

EXPERIMENTAL INDUCTION OF INTRASPECIFIC MORPHOMETRIC VARIABILITY IN A SINGLE POPULATION OF *HALICEPHALOBUS GINGIVALIS* MAY SURPASS TOTAL INTERSPECIFIC VARIABILITY

Modified from:

FONDERIE P., STEEL H., MOENS T., BERT W. (2012). Experimental induction of intraspecific morphometric variability in a single population of *Halicephalobus gingivalis* may surpass total interspecific variability. *Nematology* available on line (doi:10.1163/15685411-00002699)

ABSTRACT

Although molecular techniques are revolutionising nematode taxonomy, morphological data still form the basis of nematode species descriptions. However, morphological characters show a natural variability that should be taken into account before describing new species. The current study presents the results of an elaborate morphometric study of *Halicephalobus gingivalis*, including 15 measurements and 13 indices of 540 specimens, the progeny of a single parthenogenetic female and cultured under different temperature and food conditions and measured in different adult age groups, *i.e*., young adults with a developed vulva but before the onset of oviposition, adults laying eggs, and old, post-reproductive adults near the end of their life cycle. The morphometric characteristics were analysed using both univariate (analysis of variance) and multivariate (principal components and canonical discriminant analysis) techniques. The main results reveal that the morphometric characteristics most used in *Halicephalobus* identification keys have a huge variability within a single progeny, *e.g*., body length 1.9 times longer than the shortest or ratio VA/tail length 3.9 times larger than the smallest. This variability has a magnitude that has not been observed in nematodes before. Further, by changing the environmental factors, the morphometric characteristics are influenced to an extent that one could assign – with seemingly 'statistical support' – different 'species' of the genus to different subpopulations. With this experimental study we provide convincing elements to advocate an integrative taxonomic approach and to discourage the description of new species based only on morphometric differences.

INTRODUCTION

Since molecular data are relatively easy to obtain and very useful in identifying relationships among species, large-scale application of molecular techniques is revolutionising nematode taxonomy (Powers 2004). However, molecular data can merely suggest the presence of a new species (Luc *et al.* 2010) and are therefore best used in combination with morphological and physiological characteristics in an integrative approach (De Ley 2000; Köhler 2007; Fonseca *et al.* 2008; Luc *et al.* 2010; Abebe *et al.* 2011). The importance of such an approach lies in the complexity of species biology that requires multiple perspectives to study species boundaries (Dayrat 2005). This is especially true in taxonomy, where species boundaries are often not clear.

Despite the importance of molecular data, morphological and morphometric data still form the basis of most nematode species descriptions (Luc *et al.* 2010). Whereas morphometrics generally encompass the quantitative analyses of size and shape, in nematodes this concept refers to measurements and derived ratios. These measurements and ratios play an important role in the identification and classification of nematodes, although they should not be used unconditionally since they can vary to different degrees among and within nematode populations (Geraert 1968, 1990). Some of the more extensive morphometric variations published in nematodes were recorded in adult body length within a single population of *Aphelenchus avenae* Bastian, 1865, where the largest adult specimen was 2.2 times longer than the shortest (Geraert 1990), and among the progeny of a single female of *Helicotylenchus dihystera* (COBB, 1893) SHER, 1961, where a variation in body length of 1 : 1.7 (*i.e.*, 1.7 times longer than the shortest) was recorded (Fortuner 1984). Variability between individuals can be strongly reduced by using ratios between well defined distances (Geraert 1968). However, the use of ratios, such as the de Man ratios a, b and c, in the identification of various nematode species has often been questioned because of their high variability or a low mutual correlation between both entities of the ratio (summarised in (Geraert 1968). Conversely, other ratios such as these related to the vulva position (V and V'), are considered more stable and thus useful diagnostic characteristics (Geraert 1968).

Morphometric variation has often been reported between geographically separated populations of a species (Brown *et al.* 1997; Stock *et al.* 2000; Hazir *et al.*

2001; Lax *et al.* 2004). Intraspecific variation is not only affected by geographical origin but also by other factors such as host, climate and food availability (Evans and Fisher 1970a, b; Poinar 1992; Stock *et al.* 2000). Because of environmental influence, variation in morphometric characteristics has often been explored experimentally by adjusting biotic and/or abiotic factors under controlled laboratory conditions. For example, a change of host plant has a significant effect on most measurements used as taxonomic criteria in *Paratrichodorus christiei* (= *minor*) (ALLEN, 1957) SIDDIQI, 1974 (Bird and Mai 1967) and *P. rhodesiensis* (Siddiqi and Brown, 1956) Siddiqi, 1974 (Wondirad *et al.* 2003), and causes a variability in tail shape within the progeny of a single female of *H. dihystera* such that the tail shape may correspond to that of multiple species in the genus (Fortuner and Quénéhervé 1980). Also, according to (Doucet *et al.* 2001) different temperatures induce significant variation in several morphometric characteristics in *Paratylenchus nanus* Cobb, 1923 (Fisher 1965) and *Pratylenchus vulnus* Allen & Jensen, 1951, and differences in nutrition, especially when measured over several generations, influences morphometric variation in *Aphelenchus avenae* (*e.g*., Kline 1976). Effects of differential food availability on body length and diameter have also been observed in several free-living nematodes. As an example, large variations in bacterial abundance causes remarkably similar variations in body length (by 1.3 to 1.4) and diameter (by 1.3 to 1.55) in three bacteriovore marine species, *i.e*., two Monhysteridae and one Rhabditidae (dos Santos *et al.* 2008). The above studies all point at ratio V as the most stable character.

De Coninck (1940) reported on this natural variability of measurements and ratios, thereby emphasising the importance of addressing this variability in the individual morphological characteristics before a morphospecies can be reliably proposed (Dayrat 2005). Characteristics that are prone to changes in environmental conditions can be identified experimentally and multivariate statistical methods can provide an insight into which morphological characteristics are the most discriminating between different morphological forms or species (Fonseca *et al.* 2008).

In this paper we experimentally demonstrate a remarkable variability in morphometric characteristics of a population that is morphologically most close to *Halicephalobus gingivalis* (Stefański 1954). *H. gingivalis* is a small (235-460 µm) free-living bacteriovorous nematode (Panagrolaimidae) which is known as a facultative

parasite of horses (Blunden *et al.* 1987; Nadler *et al.* 2003), zebra (Isaza *et al.* 2000), donkey (Schmitz and Chaffin 2004) and humans (Hoogstraten and Young 1975; Shadduck *et al.* 1979; Gardiner *et al.* 1981; Ondrejka *et al.* 2010). *Halicephalobus gingivalis* resembling nematodes have also been reported in the brain of a three year old black Angus cow (Montgomery and O'Toole 2006). The genus has further been found in compost (Steel *et al.* 2010), horse manure (see first part of Chapter 1), humus (Körner 1954; Shokoohi *et al.* 2007), soil (Andrássy 1952; Pokrovskaja 1964; Mavljanov 1976), rotten wood (Körner 1954; Lordello and De Oliveira 1963; Köhler 2011), water enclosures in mines up to 1 km deep belowground (Geraert *et al.* 1988; Borgonie *et al.* 2011), in (endo)phoretic association with insects (von Lieven and Sudhaus 2008; Powers *et al.* 2009; Köhler 2012), and in commensal association with chelicerates (Timm 1956). The different species of the genus have few discriminating morphological traits and therefore morphometric data are very important in species identification. By means of both univariate and multivariate analyses of 15 measurements and 13 indices from 540 specimens, all being the progeny of a single parthenogenetic female and cultured under different temperature and food conditions, we assess: *i*) the degree of variability in morphometric characteristics commonly used in *Halicephalobus* species identification; *ii*) whether this variability is significantly influenced by temperature, food availability or adult age; and *iii*) whether this variability is of such magnitude that it could affect a correct species diagnosis.

MATERIALS AND METHODS

ISOLATION OF THE NEMATODES

The *H. gingivalis* isolate WB0801 used in this study was obtained from fresh horse droppings on a stable in the province of Western-Flanders (Belgium). Several fresh horse droppings were collected from an individual stall and mixed into a bulk sample. Five subsamples were placed on Petri dishes containing 2% bacteriological agar (Oxoid Ltd., Hampshire, UK) enriched with cholesterol (final concentration 1 mg ml^{-1}). The plates were closed with Parafilm® M Sealing film (Pechiney Plastic Packaging, Chicago, USA) to

avoid dehydration and incubated at 30°C. The plates were checked daily and after one week putative *Halicephalobus* specimens were observed and picked up. Specimens were identified under the light microscope as the morphospecies *H. gingivalis.* No males were observed, confirming that this species reproduces parthenogenetically (Stefański 1954; Andrássy 1984; Akagami *et al.* 2007). The culture of the WB0801 isolate was started with one individual, thus all specimens used in the experiments are the progeny of a single female.

The morphometric variability of specimens (n = 30) from an *in situ* population of *Halicephalobus* was included for comparison. These specimens originated from a compost sample and were extracted by means of a modified Baermann funnel method (Steel *et al*., 2010).

MOLECULAR ANALYSIS AND SPECIES IDENTIFICATION

The D2D3 expansion region of LSU rDNA (28S) was sequenced for ten specimens from a single subculture derived from the original isolate as described in (Múnera *et al.* 2010) and all molecular analyses were started from single individuals. The sequences showed an intrapopulation difference of 0 to 1 nucleotides (0.2%). Phylogenetic analyses based on D2D3 sequences, including GenBank sequences, placed our *Halicephalobus* isolate WB0801 within an internally unresolved *H. gingivalis* clade (data not shown). Although the sequence differences between different *H. gingivalis* isolates were remarkable (*i.e*., 28S (partial) sequence of WB0801 (HQ697251) shows a 21 base (4.5%) difference with *H. gingivalis* compost isolate JB128 (AY294181), a 27 base (5.8%) difference with *H. gingivalis* parasitic isolate SAN100 (AY294177) and a 60 base (12.8%) difference with *H. gingivalis* pot soil isolate PDL0017 (DQ145637 a thorough molecular analysis based on 4 markers revealed that the isolate belongs to a highly supported *H. gingivalis*-clade (Chapter 6).

EXPERIMENTAL DESIGN

All the eggs used in the experiment originated from one plate that was subcultured from the original WB0801 culture. Approximately 100 eggs were placed on each of 18 Petri dishes containing 1% bacteriological agar enriched with cholesterol (1 mg ml^{-1}). To

determine if and to what extent morphological variability can be affected by environmental variability, the developing eggs were subjected to different temperature and food conditions. Six plates each were placed at 15°C, 30°C and 37°C, respectively. These temperatures are well within the temperature range found in the natural environments of *Halicephalobus* species. At each temperature, three out of six replicates contained a bacterial lawn as food source, obtained by adding 10 µl of *Escherichia coli* OP50 at a density of approximately 2.85×10^6 μl^{-1} to each plate. No food was added to the other three replicates except the bacteria (*E. coli* OP50) that were cotransferred with the eggs from the main culture. These were few since the eggs were first collected in distilled water. Ten specimens were collected from each plate at three different times corresponding to three age groups: young adults with a developed vulva but before the onset of oviposition, adults laying eggs, and old, post-reproductive adults near the end of their life cycle. Since development of *H. gingivalis* is faster at higher temperatures and under optimal food conditions (personal observations), the age groups occurred at different time intervals in different treatments. They were determined using a stereomicroscope (Leica MZ95) to observe the movement of the specimens, *i.e*., active for (young) adults or sluggish for old adults, and a microscope (Olympus BX 51 DIC, Olympus Optical, Tokyo, Japan) was used to confirm the full development of the vulva in young adults.

FIXATION AND MOUNTING

The nematodes were picked off the plates using a stereomicroscope (Leica MZ95) and collected in a very small drop of water in an embryo dish. An excess (4-5 ml) of heated (70°C) formaldehyde (4% with 1% glycerol) was added to instantaneously kill and preserve the nematodes (Seinhorst 1966). The use of hot formaldehyde has the advantage that nematodes are typically outstretched and not curled. The preserved nematodes were processed to anhydrous glycerin following the glycerin-ethanol method (Seinhorst 1959), as modified by (De Grisse 1969), subsequently mounted on glass slides and covered with a cover slip.

MEASUREMENTS

Measurements of 540 individuals were obtained directly from drawings manually prepared with a *camera lucida* on an Olympus BX 51 DIC microscope (Olympus Optical, Tokyo, Japan). Measuring was performed using a ruler for straight measurements and a curvimeter for curvilinear measurements. The measurements and ratios (table 5.1) were chosen based on their use in different identification keys for the genus (Andrássy 1984; Geraert *et al.* 1988; Shokoohi *et al.* 2007). In addition to morphometrics more generally used in nematode identification, in *Halicephalobus* identification these also include specific morphometric characteristics based on different parts of the female reproductive system (fig. 5.1): ratio ovary (= post-v/pre-v = distance vulva to posterior ovarian flexure/distance vulva to anterior ovarian flexure), ratio reflexed/ovary length (= length of dorsally reflexed part of ovary/total length ovary), ratio reflexed/post-v (= length of dorsally reflexed part of ovary/distance vulva to posterior ovarian flexure) and (VA/2)/post-v (= 0.5 × vulva to anus distance/distance vulva to posterior ovarian flexure).

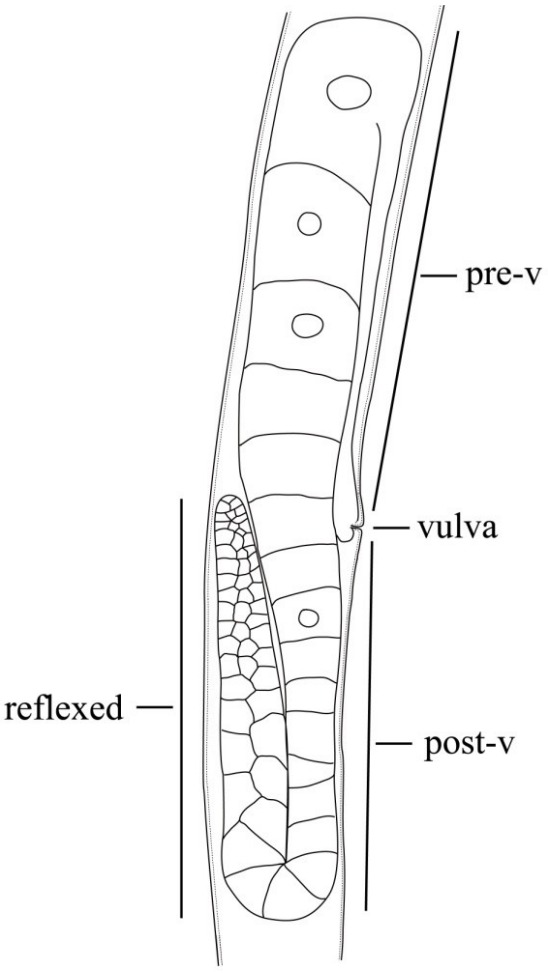

Figure 5.1. Detail of the female reproductive system with notation of the different measurements used to calculate specific ratios used in *Halicephalobus* identification keys. Reflexed = length of dorsally reflexed part of ovary, pre-v = distance vulva to anterior ovarian flexure, post-v = distance vulva to posterior ovarian flexure.

STATISTICAL ANALYSIS

First, for each morphometric character the measurements were normalised for size by subtracting its mean and dividing by its standard deviation (both mean and the standard deviation being based on 540 measurements). The *F*-values of all the morphometric characteristics were calculated by means of one-way analysis of variance (ANOVA) using Statistica 7 (StatSoft Europe GmbH, Hamburg, Germany) with the factor temperature or food availability or age. Residual plots indicated homogeneous variances and homoscedastic error distributions, but formal testing using a Kolmogorov-Smirnov test and a Bartlett test rejected the normality assumption and the assumption of homogeneity of variance, respectively. However, when based on a large sample size ($n > 30$) deviations from normality are negligible because of the central limit theorem, according to which the sampling distribution of the mean approximates the normal distribution, regardless of the distribution of the variable in the population (StatSoft Electronic Statistics Textbook 2012, http://www.statsoft.com/textbook/). Additionally, the *F*-statistic is remarkably robust to deviations from normality and homogeneity of variances (Lindman 1974).

The *F*-value represents the ratio between variances due to effect and variances due to error and fluctuates around 1 if the variances of the different populations are approximately the same (Sokal and Rohlf 1995). Consequently, the *F*-value can be used as an indication of the variability of the measurements and ratios under different culturing conditions and for different age groups. A correlation structure among the morphometric values was explored in SAS® 9.3 (SAS Institute Inc., Cary, NC, USA) by means of a Principal Component Analysis (PCA). Only the diagnostic characteristics commonly used in species identification keys (Andrássy 1984; Geraert *et al.* 1988; Shokoohi *et al.* 2007) were included: ratio a, ratio c, ratio c', ratio V, ratio ovary (= distance vulva to posterior ovarian flexure/distance vulva to anterior ovarian flexure), ratio corpus/isthmus, distance vulva to anus/tail length and the ratio dorsally reflexed part ovary/distance vulva to posterior ovarian flexure.

Finally, a canonical discriminant analysis (CDA) was performed including only those morphometric characteristics that have no significant correlation with each other (significant correlation at $P < 0.05$, $r > 0.8$) using the CANDISC procedure in SAS® 9.3 (SAS

Institute Inc., Cary, NC, USA) to find the sets of variables that discriminate most between the populations, based on the pooled within variance-covariance matrix, and to test whether or not the morphometric values of the subpopulations cultured under variable conditions are significantly different. Note that indices and quantitative data can be combined in a discriminant function analysis when they are not highly correlated (Fortuner 1990).

RESULTS

The measurements and the ratios of 540 individuals subcultured from a single female are presented in table 5.1. Measurements for each temperature and food condition are based on 90 individuals and include three replicates of ten individuals (n = 10) of each of the three different age classes, *i.e*., young adults with a fully developed vulva but before the onset of oviposition, actively moving egg-laying adults and old (sluggish moving) adults near the end of their life cycle.

MAXIMAL INTRASPECIFIC VARIABILITY OF MORPHOMETRIC CHARACTERISTICS

To determine the maximal variability, *i.e*., the range between the lowest and the highest value of a morphometric character, all 540 specimens were analysed together without consideration of the influence of temperature, food availability and age (first column of table 5.1). The maximal variability is presented (fig. 5.2) as a ratio of one (*i.e*., representing the minimum value) to the least common multiple (*i.e*., representing how many times the maximum value is larger than the minimum value), and the coefficients of variation (CV, a measure of relative variability) of all measurements and ratios are given in figure 5.2. The results reveal that nearly all morphometric characteristics show considerable variation: corpus length, pharynx length, stoma length, ratio V, ratio V' and ratio b show the lowest intraspecific variation, whereas the highest variability is found in the different parts of the reproductive system and the ratios derived from these measurements. The overall largest variation is found in the length of the dorsally reflexed part of the ovary (CV 57%) with the longest length measuring 31 times the

shortest (1 : 31). Ratio ovary, which is considered an important diagnostic character in *Halicephalobus* identification keys, shows a maximal variation of 1 : 9 (CV 23%) with values ranging between 0.12 and 1.10, thus overlapping ratio ovary values of all known *Halicephalobus* species. For the non-reproduction related characteristics, the putative diagnostic ratio VA/tail length (CV 16%; 1 : 4) and the isthmus length (CV 11%; 1 : 3) are the most variable. For comparison, such a large variation could also be observed in an *in situ* population of *Halicephalobus*. Measurements of 30 specimens originating from a natural population found in compost and their resulting CV values showed a similar variation compared to our cultured populations (table 5.2).

Table 5.1. Morphometrics of specimens cultured at different temperatures (15°C, 30°C or 38°C) and food availability (with or without additional *Escherichia coli* OP50). The first column represents all measurements pooled together without considering the different treatments. Measurements are given in μm and are represented as mean ± standard deviation with (ranges) and coefficient of variation expressed as percentage.

temperature	all	15°C		30°C		38°C	
character		*+ E. coli*	*- E. coli*	*+ E. coli*	*- E. coli*	*+ E. coli*	*- E. coli*
n	540	90	90	90	90	90	90
anal body width	11.80 ± 1.72	10.80 ± 1.53	10.70 ± 1.25	12.67 ± 1.99	11.54 ± 1.40	13.01 ± 1.31	12.08 ± 1.27
	(7.23 - 16.87)	(7.83 - 13.86)	(7.23 - 13.25)	(8.43 - 16.87)	(8.43 - 16.26)	(10.24 - 15.66)	(9.04 - 14.46)
		14	12	16	12	10	11
corpus length	47.81 ± 3.17	46.81 ± 2.99	47.11 ± 2.50	48.74 ± 2.90	49.72 ± 3.81	46.23 ± 2.24	48.23 ± 3.00
	(38.55 - 57.23)	(38.55 - 53.01)	(40.96 - 53.01)	(40.96 - 54.82)	(42.17 - 57.23)	(40.36 - 51.81)	(40.36 - 54.22)
		6	5	6	8	5	6
dorsally reflexed	60.28 ± 34.59	47.86 ± 20.94	38.63 ± 12.49	80.23 ± 46.53	43.25 ± 16.52	84.44 ± 36.53	67.28 ± 30.22
part ovary	(4.82 - 151.20)	(6.63 - 96.38)	(10.84 - 62.05)	(4.82 - 151.20)	(10.24 - 81.93)	(15.66 - 147.59)	(12.05 - 112.65)
		44	32	58	38	43	45
isthmus length	18.94 ± 2.06	18.47 ± 2.07	18.08 ± 1.65	19.62 ± 1.74	20.01 ± 2.07	18.42 ± 1.88	19.07 ± 2.20
	(8.43 - 25.30)	(8.43 - 22.89)	(13.86 - 21.69)	(13.86 - 22.89)	(13.86 - 25.30)	(13.86 - 22.29)	(14.46 - 24.70)
		11	9	9	10	10	12
L	389.58 ± 48.40	397.38 ± 55.51	381.31 ± 45.13	409.36 ± 56.52	374.69 ± 36.43	395.56 ± 36.70	379.20 ± 48.00
(total body length)	(262.04 - 504.81)	(262.04 - 476.50)	(273.49 - 455.41)	(286.74 - 504.81)	(312.65 - 456.62)	(305.42 - 463.25)	(267.47 - 452.40)
		14	12	14	10	9	13
L'	329.61 ± 42.85	340.47 ± 47.18	325.70 ± 38.27	344.52 ± 52.22	309.89 ± 32.35	337.49 ± 31.39	319.58 ± 41.87
(L – tail lentgh)	(224.70 - 439.75)	(224.70 - 407.82)	(234.94 - 391.56)	(232.53 - 439.75)	(251.80 - 384.33)	(258.43 - 393.97)	(228.31 - 384.93)
		14	12	15	10	9	13
max. body width	22.3 ± 4.5	20.96 ± 4.02	19.36 ± 2.54	24.55 ± 6.46	21.01 ±2.94	25.90 ± 3.15	22.02 ± 3.02
	(12.05 - 33.13)	(12.05 - 27.11)	(13.25 - 23.49)	(12.05 - 33.13)	(13.86 - 27.71)	(18.67 - 30.12)	(15.06 - 27.71)
		19	13	26	14	12	14
pharynx length	81.84 ± 5.27	80.29 ± 5.15	79.66 ± 3.75	84.13 ± 4.61	84.55 ± 6.22	80.61 ± 4.10	81.79 ± 5.41
	(60.24 - 98.19)	(60.24 - 92.17)	(71.08 - 88.55)	71.69 - 93.97)	(72.29 - 98.19)	(69.88 - 89.16)	(68.07 - 93.37)
		6	5	5	7	5	7
post-v	45.64 ± 14.30	47.42 ± 18.02	43.79 ± 9.44	47.75 ± 20.35	40.51 ± 9.60	47.17 ± 12.35	47.16 ± 11.08
(vulva to posterior	(7.23 - 80.12)	7.23 - 80.12)	(18.07 - 63.85)	(8.43 - 75.30)	(12.05 - 57.83)	(9.04 - 73.49)	(18.07 - 66.87)
ovarian flexure)		38	22	43	24	26	25
pre-v	72.16 ± 19.35	71.08 ± 20.83	62.85 ± 13.04	78.69 ± 27.74	63.67 ± 12.02	81.81 ± 14.37	74.89 ± 15.36
(vulva to anterior	(26.51 - 115.66)	(26.51 - 104.82)	(30.72 - 91.56)	(26.51 - 112.65)	(39.76 - 92.77)	(50.60 - 113.85)	(37.95 - 115.66)
ovarian flexure)		29	21	35	19	18	21

Table 5.1. Continued

PV	147.64 ± 25.75	155.04 ± 28.12	146.32 ± 24.22	153.54 ± 30.29	133.23 ± 19.67	153.89 ± 19.15	143.82 ± 24.72
(distance pharynx-	(86.75 - 192.77)	(86.75 - 191.56)	(90.36 - 184.94)	(89.16 - 192.77)	(96.77 - 176.50)	(104.22 - 188.55)	(94.58 - 178.91)
vulva)		18	16	20	15	12	17
stoma length	8.96 ± 0.85	8.61 ± 0.73	8.80 ± 0.70	9.19 ± 0.77	9.05 ± 1.14	9.08 ± 0.77	9.07 ± 0.79
	(7.23 - 12.05)	(7.23 - 10.24)	(7.23 - 10.24)	(7.23 - 10.84)	(7.23 - 12.05)	(7.23 - 12.05)	(7.23 - 10.84)
		9	8	8	12	8	8
tail length	59.97 ± 8.75	56.91 ± 10.10	55.61 ± 8.29	64.85 ± 5.83	64.79 ± 7.46	58.07 ± 8.26	59.62 ± 7.46
	(34.94 - 78.31)	(34.94 - 74.70)	(36.75 - 78.31)	(53.01 - 75.90)	(46.38 - 78.31)	(41.57 - 74.10)	(39.16 - 71.69)
		18	15	9	12	14	13
VA	100.13 ± 16.41	105.14 ± 18.06	99.72 ± 13.59	106.85 ± 21.94	92.11 ± 12.20	103.00 ± 11.10	93.97 ± 13.52
(distance vulva-Anus)	(59.64 - 166.26)	(59.64 - 140.36) 17	(65.06 - 122.29) 14	(63.25 - 166.26) 21	(69.28 - 122.29) 13	(76.50 - 122.29) 11	(63.85 - 119.28) 14
vulval body width	21.80 ± 4.38	20.67 ± 3.88	19.03 ± 2.61	23.69 ± 6.13	20.39 ± 2.94	25.62 ± 3.15	21.37 ± 2.90
	(11.45 - 31.93)	(12.05 - 26.51)	(13.25 - 23.49)	(11.45 - 31.93)	(13.25 - 27.71)	(18.67 - 30.12)	(14.46 - 26.51)
		19	14	26	14	12	14
a	17.86 ± 2.43	19.27 ± 2.32	19.77 ± 1.27	17.45 ± 3.19	18.04 ± 2.02	15.36 ± 0.99	17.27 ± 0.92
(L/max. body width)	(12.89- 30.68)	(16.68 -- 30.68)	(17.52 - 23.61)	(14.00 - 25.71)	(15.10 - 25.16)	(12.98 - 18.74)	(15.60 - 19.88)
		12	6	18	11	6	5
b	4.77 ± 0.55	4.95 ± 0.65	4.79 ± 0.54	4.87 ± 0.67	4.44 ± 0.42	4.91 ± 0.39	4.63 ± 0.41
(L/pharynx length)	(3.39 - 6.08)	(3.39 - 6.08	(3.61 - 5.72)	(3.51 - 5.78)	(3.62 -5.19)	(3.93 - 5.54)	(3.69 - 5.32)
		13	11	14	10	8	8
c	6.56 ± 0.77	7.07 ± 0.84	6.91 ± 0.55	6.30 ± 0.60	5.82 ± 0.58	6.89 ± 0.74	6.37 ± 0.45
(L/tail length)	(4.80 - 12.47)	(6.15 - 12.47)	(5.77 - 8.77)	(5.14 - 7.76)	(4.80 - 7.59)	(5.54 - 8.63)	(5.40 - 7.83)
		12	8	9	10	11	7
c'	5.12 ± 0.64	5.26 ± 0.52	5.20 ± 0.52	5.19 ± 0.57	5.65 ± 0.65	4.47 ± 0.48	4.94 ± 0.42
(tail/anal body	(3.41 - 7.43)	(3.44 - 6.69)	(3.59 - 6.50)	(4.05 - 6.71)	(4.14 - 7.43)	(3.41 - 5.63)	(3.83 - 5.88)
width)		10	10	11	11	11	9
ratio corpus/isthmus	2.54 ± 0.23	2.56 ± 0.28	2.63 ± 0.26	2.50 ± 0.22	2.50 ± 0.16	2.53 ± 0.22	2.55 ± 0.19
	(1.97 - 4.64)	(2.16 - 4.64)	(2.13 - 3.46)	(2.09 - 3.52)	(2.17 - 3.30	(1.97 - 3.35)	(2.05 - 3.15)
		11	10	9	6	9	7
gonad%	29.84 ± 5.68	29.08 ± 6.54	27.76 ± 3.03	29.96 ± 8.65	27.80 ± 4.40	32.57 ± 4.27	31.89 ± 3.22
(length reprod	(13.32 - 40.09)	(13.37 - 39.46)	(19.57 - 34.79)	(13.32 - 40.09)	(18.39 - 35.67)	(19.31 - 38.54)	(21.76 - 39.26)
system x 100 /L)		22	11	29	16	13	10
ratio ovary	0.64 ± 0.14	0.65 ± 0.18	0.70 ± 0.12	0.60 ± 0.13	0.64 ± 0.13	0.59 ± 0.15	0.63 ± 0.12
(post-v/pre-v)	(0.12 - 1.10)	(0.21 - 1.08)	(0.39 - 1.04)	(0.17 - 0.85)	(0.19 - 0.97)	(0.12 - -0.98)	(0.36 - 1.10)
		27	17	21	21	26	18
reflexed part ovary/	0.48 ± 0.19	0.38 ± 0.09	0.35 ± 0.07	0.56 ± 0.22	0.40 ± 0.11	0.64 ± 0.24	0.52 ± 0.17
ovary length	(0.11 - 1.07)	(0.14 - 0.66)	(0.16 - 0.49)	(0.11 - 0.91)	(0.14 - 0.62)	(0.20 - 1.07)	(0.17 - 0.779)
		25	19	40	27	37	33
reflexed part ovary/	1.30 ± 0.73	1.00 ± 0.24	0.87 ± 0.19	1.53 ± 0.69	1.08 ± 0.46	1.94 ± 1.20	1.35 ± 0.43
post-v	(0.33 - 7.33)	(0.45 - 1.93)	(0.34 - 1.56)	(0.47 - 4.58)	(0.33 - 3.995)	(0.51 - 7.33)	(0.56 - 2.08)
		24	22	45	43	62	32
V	58.96 ± 1.33	59.37 ± 1.51	59.33 ± 1.16	58.14 ± 1.07	58.14 ± 0.89	59.31 ± 1.29	59.50 ± 1.18
	(54.18 - 63.86)	(57.08 - 63.86)	(56.80 - 62.11)	(54.18 - 60.93)	(55.71 - 60.78)	(55.33 - 62.02)	(56.72 - 61.97)
		3	2	2	2	2	2
V'	69.74 ± 1.48	69.27 ± 1.53	69.44 ± 1.09	69.24 ± 2.06	70.34 ± 1.33	69.51 ± 1.00	70.64 ± 0.97
	(62.19 - 74.62)	(64.96 - 74.17)	(66.90 - 72.31)	(62.19 - 73.47)	(67.04 - 74.62)	(66.37 - 72.54)	(67.84 - 72.75)
		2	2	3	2	1	1
VA/tail length	1.69 ± 0.28	1.87 ± 0.31	1.81 ± 0.19	1.64 ± 0.28	1.44 ± 0.22	1.80 ± 0.24	1.58 ± 0.14
	(1.03 -- 4.02)	(1.45 - 4.02)	(1.49 - 2.41)	(1.15 - 2.56)	(1.03 - 2.11)	(1.29 - 2.42)	(1.28 - 2.09)
		16	10	17	15	13	9
(VA/2) / post-v	1.23 ± 0.56	1.35 ± 0.75	1.17 ± 0.20	1.36 ± 0.68	1.21 ± 0.43	1.24 ± 0.75	1.03 ± 0.19
	(0.71 - 6.50)	(0.77 - 4.71)	(0.91 - 2.27)	(0.71 - 4.18)	(0.83 - 4.18)	(0.79 - 6.50)	(0.74 - 1.77)
		56	17	50	35	60	19

INFLUENCE OF CULTURE CONDITIONS AND AGE GROUPS ON MORPHOMETRIC DATA

An analysis of variance (one-way ANOVA) was performed on all morphometric characteristics using the factors temperature, food availability and age group, respectively. Most morphometric characteristics are significantly ($P < 0.05$) influenced by the different factors, except stoma length, pharynx length, isthmus length, ratio corpus/isthmus, tail length and ratio V, which are not significantly influenced by a difference in food availability; total body length, which is not significantly influenced by temperature; and ratio ovary, which shows no significant differences between the age groups (ANOVA, $P > 0.05$; annotated as † in table 5.3). The *F*-values (table 5.3) indicate which characteristics are most influenced by the different factors (high *F*-values) and which characteristics are least influenced (low *F*-values). Note that comparison of *F*-values between characteristics should only be done within and not between factors. The results show that temperature, food availability and age group clearly have a dissimilar influence on the different morphometric characteristics. For example, whereas temperature has a strong effect on the pharyngeal measurements (corpus, isthmus and total pharynx length) and on ratio V, food availability and age group have little or no influence on these characteristics. Evidently, characteristics related to the reproductive system (post-v and the ratio (VA/2)/post-v) are highly influenced by the age of the adults (factor age group) and less by the factors temperature and food availability. Finally, stoma length and ratio corpus/isthmus, but also (VA/2)/post-v and ratio ovary, despite their huge overall variability (fig. 5.2), show no clear influence by either factor.

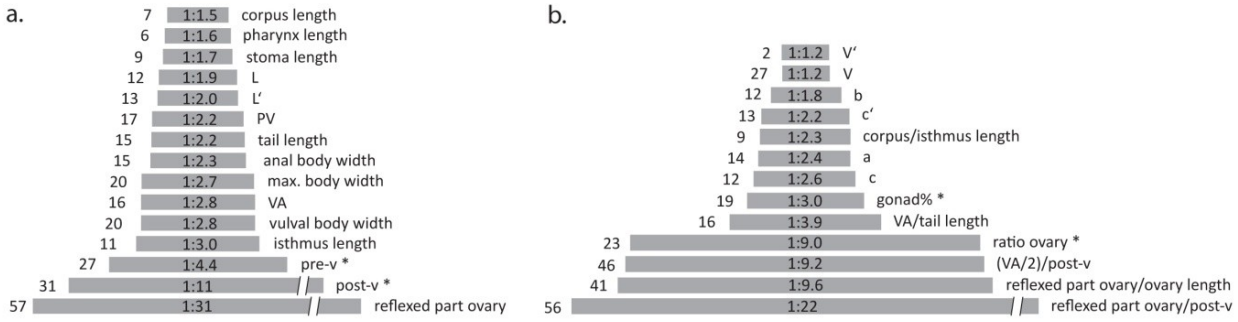

Figure 5.2. The grey bars represent the maximum variability of the measurements (**a**) and ratios (**b**), given as a proportion of 1 to the least common multiple, ranked from limited variation (top) to highly variable (bottom). The coefficient of variation (expressed as percentage) of each morphometric character is given left of the grey bar. *Abbreviations: pre-v = distance vulva to anterior ovarian flexure, post-v = distance vulva to posterior ovarian flexure, gonad% = (gonad length/L) x100, ratio ovary = post-v/pre-v.

Table 5.2. Coefficient of variation (CV) of specimens (n=30) originating from an *in situ* population from compost and specimens (n=30) originating from a population cultured under laboratory conditions (at 30°C with additional *E. coli* OP50).

origin character	*in situ* population	laboratory population
stoma length	7	8
pharynx length	8	5
PV	18	20
VA	19	21
tail length	17	9
L	13	14
L'	14	15
KV	12	13
V	3	2
V'	2	3
max. body width	12	26
a	7	18
b	11	14
c	14	9
c'	21	11
anal body width	12	16
pre-v	23	35
post-v	40	43
ratio ovary	31	21
reflexed	54	58
VA/tail length	18	21
(VA/2)/post-v	37	18
reflex/post-v	30	50
reflexed/ovary length	30	45
ratio corpus/isthmus	12	9

Table 5.3. Measurements and ratios with their respective *F*-values (ANOVA). The lowest *F*-values (printed in bold) represent the 10 characteristics least influenced by the factors temperature (15°C, 30°C, 38°C), food availability (with or without additional *E. coli* OP50) or age (young adults with a developed vulva but before the onset of oviposition, adults laying eggs, and old, post-reproductive adults near the end of their life cycle). Outlined bars represent the morphometrical characteristics that show a low influence by all three factors and have a limited maximal variability. Highlighted (grey bars) are the morphometrical characteristics that show a low influence by all three factors yet have a high maximal variability.

	temperature	food availability	age
Variable *	$F_{2,537}$	$F_{1,538}$	$F_{2,537}$
a	245	71	153
anal body width	175	79	302
b	23	133	524
c	120	62	**9**
c'	110	51	**7**
corpus length	47	**28**	**21**
ratio corpus/isthmus	**8**	**2.04**†	**4.8**
gonad%	93	31	443
isthmus length	41	**2.4**†	**10**
L	**1.6**†	108	552
L'	**3.5**	139	551
max. body width	216	388	819
pharynx length	57	**0.9**†	**23**
PV	**15**	138	618
post-v	**5.6**	**24**	299
pre-v	83	190	761
ratio ovary	**16**	**19**	**2.9**†
reflexed	284	356	858
reflexed/ovary length	244	168	478
reflexed/post-v	104	95	152
stoma length	**17**	**0.05**†	**4.6**†
tail length	108	**0.02**†	153
V	78	**0.27**†	**30**
V'	**20**	72	113
VA	**10**	167	477
(VA/2)/post-v	**5.5**	**20**	**46**
VA/tail length	99	88	**31**
vulval body width	202	422	810

* gonad%=(length reproductive system/L) x100; post-v=distance vulva to posterior ovarian flexure; pre-v=distance vulva to anterior ovarian flexure; ratio ovary=post-v/pre-v; reflexed=length dorsally reflexed part ovary. † ANOVA $P>0.05$, *i.e.*, character showing no significant influence of factor (temperature, food availability or age group).

Ordination by PCA of the most commonly used diagnostic morphometric variables did not show a clear pattern related to food, temperature or age (results not shown). Nevertheless, PCAs of selected data sets showed distinct patterns that illustrate clear differences between the subpopulations. For example, a PCA on 180 specimens of a subpopulation cultured at 15°C without food and a subpopulation cultured at 30°C with *E. coli* OP50 as food source showed a pattern distinctly related to culture conditions (fig. 5.3). This biplot represents 57.5% of the total variation in the morphometric data, the first principal component (PC1) axis explains 33.5% of the total variation and is mainly correlated with ratio c, ratio c' and VA/tail length, and the PC2 axis explains 23.92% of the total variation and is mainly correlated with ratio a, ratio V and ratio ovary.

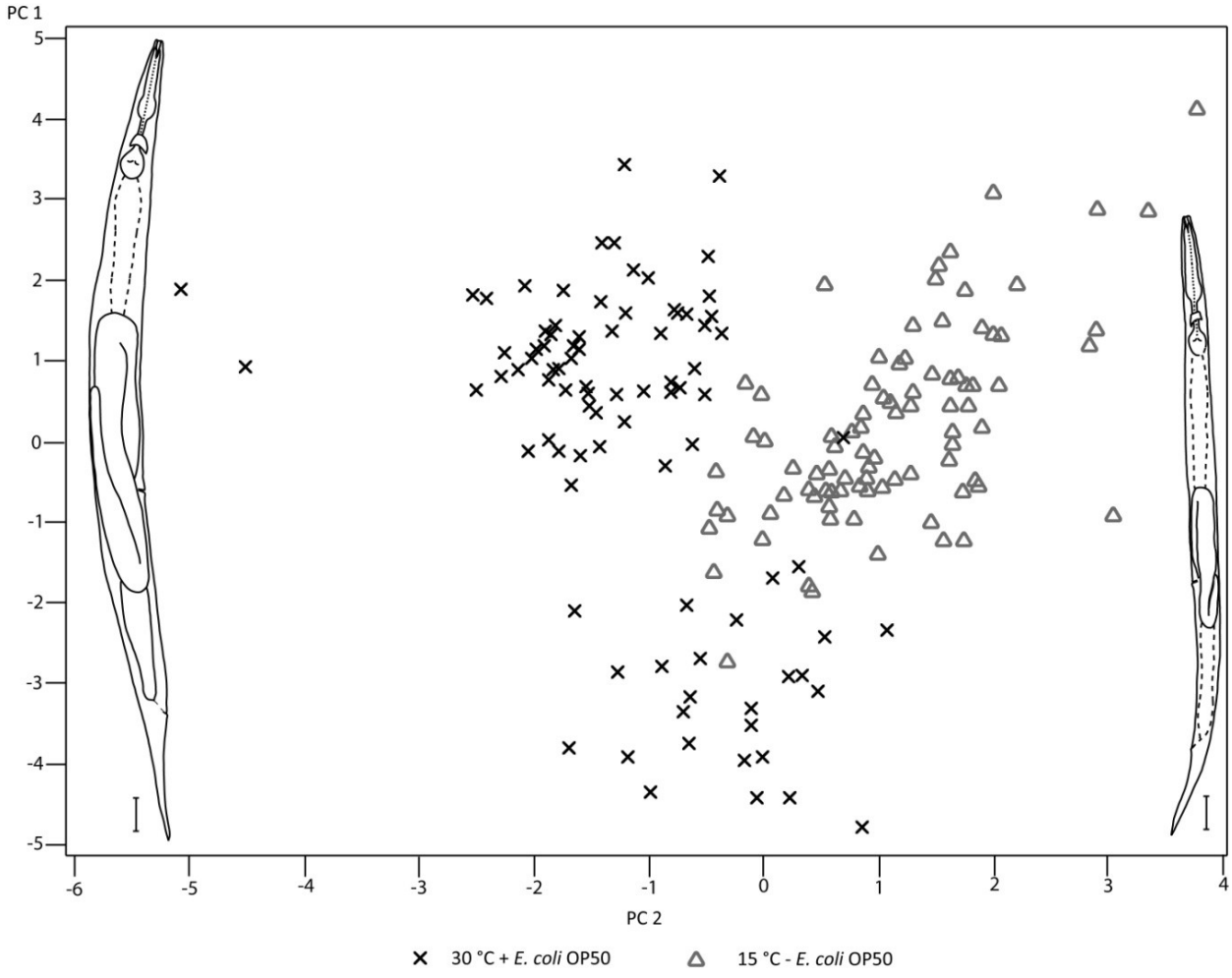

Figure 5.3. PCA plot of diagnostic characteristics of specimens cultured under two different culture conditions, 15°C without additional *Escherichia coli* OP50 and 30°C with additional *E. coli* OP50. A schematic representation of a specimen of each group is added left (30°C + *E. coli* OP50) and right (15°C - *E. coli* OP50) of the PCA plot. (Scale bar = 20 µm.)

Using the morphometric data of these two subpopulations in two independent 'species' identifications, has different outcomes. For example, following the key of (Geraert *et al.* 1988), which already takes the variability of some characteristics into account, the morphometrics of the subpopulation cultured at 15°C (c' = 3.5-5, VA/tail length > 1, ratio ovary < 0.5) correspond to *H. gingivalis*, whereas the morphometrics of the 30°C subpopulation (c' = 3.5-5, VA/tail length > 1, ratio ovary ± 0.66, tail tip straight) correspond to the species complex *H. palmaris*/*H. intermedius*.

Table 5.4. Pooled within canonical structure, *i.e.*, correlations between the canonical variables and the original variables, calculated by CDA analyses of the subpopulations induced by temperature (15, 30 and 38°C), food availability (with or without additional *E. coli* OP50) and age groups (young adult, adult and old adult). The amount of the variance of the data that each canonical variable accounts for is given between brackets. The highest correlations with each canonical variable are given in bold.

	temperature		food availability	age	
Variable *	Can1 (77.39%)	Can2 (22.61%)	Can1 (100%)	Can1 (89.14%)	Can2 (10.86%)
a	***0.4620***	-0.2678	-0.3192	-0.3372	0.1224
anal body width	***-0.3734***	0.0551	0.3284	***0.5081***	0.2860
c'	0.1672	***-0.6862***	-0.3577	-0.0838	-0.0639
ratio corpus/isthmus	0.1067	0.1357	-0.0900	-0.0920	-0.0219
L	0.0020	-0.0546	0.3616	***0.7815***	0.3425
post-v	-0.0113	0.1194	0.1947	***0.5798***	-0.3317
ratio ovary	0.1663	0.0090	-0.2608	0.0341	-0.1598
reflexed	***-0.3020***	0.1653	***0.4881***	***0.6490***	-0.0869
reflexed/post-v	***-0.3073***	0.2049	***0.4259***	0.3831	***0.2736***
stoma length	-0.1640	-0.0808	-0.0117	-0.0498	0.1840
tail length	-0.2082	***-0.4920***	-0.0058	0.3957	0.1880
V	0.1167	***0.6471***	-0.0283	-0.2000	0.1185
(VA/2)/ post-v	0.0435	-0.1423	0.2450	-0.2228	***0.3723***
VA/tail length	0.2829	***0.4282***	***0.4643***	0.1817	-0.0239

* post-v = distance vulva to posterior ovarian flexure; reflexed = length dorsally reflexed ovarian part

A canonical discriminant analysis was done including 14 of the 28 morphometric characteristics (listed in table 5.4) chosen based on their low mutual correlation (r < 0.80). While PCA analysis searches for the components that best explain the variability in the data, CDA appoints the variables that depict the differences between pre-defined groups, *i.e.*, the subpopulations. All analysed subpopulations are significantly different (Wilks' λ, P < 0.0001) and the morphometric characteristics that cause the overall largest separation between the groups are anal body diam., L, VA/tail length, length reflexed

part ovary and ratio reflexed part ovary/distance vulva to posterior ovarian flexure. The pooled within canonical structure, *i.e.*, the correlations between the canonical variables and the original variables, which illustrates how the subpopulations are grouped and separated, is listed in table 5.4.

Table 5.5. Summary of the results. The morphometric characteristics are categorized as potentially useful for species delineation (grey bars), *i.e.*, showing limited variation or low influence by age or external factors, or as not suitable for species delineation (X), *i.e.,* showing high variability or high influence by age or external factors. Maximum variability is given as a proportion of one to the least common multiple.

	max. variability	ANOVA/CDA		
Variable *		temp	food	age
a	1:2.4	X	X	X
anal body width	1:2.3	X	X	X
c'	1:2.2	X	X	
ratio corpus/isthmus	1:2.3			
L	1:1.9		X	X
post-v	1: > 3			X
ratio ovary	1: > 3			
reflexed	1: > 3	X	X	X
reflexed/post-v	1: > 3	X	X	X
stoma length	1:1.7			
tail length	1:2.2	X	X	X
V	1:1.2	X		X
(VA/2)/post-v	1: > 3			X
VA/tail length	1: > 3	X	X	

* post-v = distance vulva to posterior ovarian flexure; ratio ovary = post-v/distance vulva to anterior ovarian flexure; reflexed = length dorsally reflexed ovarian part

Finally, table 5.5 shows a summary of the results, which reveals that most morphometric characteristics used in *Halicephalobus* identification keys have a notable variability and/or can be highly influenced by environmental factors or age. Although ratio ovary and ratio corpus/isthmus are not clearly influenced by age and external conditions, they show a high intraspecific variability of 1 : 9 and 1 : 2.3, respectively. On the other hand, ratio V shows the lowest intraspecific variation (1 : 1.2), but is clearly influenced by differences in temperature and age. Stoma length is least influenced by

age and external factors (*i.e.*, temperature and food) and shows a relatively low maximal variability (1 : 1.7). Thus, only one morphometric character does not show distinct morphometric variability.

DISCUSSION

In the absence of other morphological features, measurements and ratios are often used to discriminate closely related species. However, when knowledge of the intraspecific variation of these characteristics is lacking, their taxonomic value is rather questionable. Therefore, when describing new species, the variability of each morphometric character should be carefully evaluated before utilising it for species delineation (Dodson and Lee 2006). However, this requirement is often not met, since many descriptions of new species are based on very few or even single specimens collected at one moment in time and from a single location. Here, by analysing the morphometric variability of the progeny of a single parthenogenetic female, the limits of the intraspecific variability of morphometric data are explored and implications for delineating species boundaries are discussed. Although several previous studies have shown a high variability in morphometric characteristics of nematodes, a variability of this magnitude has never been observed before.

The most obvious influence is that of food availability on the variation in body size and diameter. The influence of caloric restriction is well known for *Caenorhabditis elegans* where feeding-defective mutants are significantly smaller than wild type (N2) specimens (Morck and Pilon 2006), and in *C. briggsae,* which also shows a distinct decrease in body size and in gonad length correlated with a decrease in food (Schiemer 1982). Also, *Diplolaimelloides oschei* MEYL, 1954, *D. meyli* TIMM, 1961, and *Pellioditis marina* BASTIAN, 1865 show remarkably similar variations in body length and diameter due to variations in bacterial abundance (dos Santos *et al.* 2008). A general negative effect of caloric restriction on body size is not limited to invertebrates, but is also known for mice and rats (Weindruch 1996; Kristan and Hammond 2001). In the present study, the maximal variability in total body length, *i.e.*, 1 : 1.90, even exceeds that of *C. elegans* when comparing wild type specimens (N2) with long mutants, *i.e.*, 1 : 1.22 (Nyström *et*

al. 2002), or short mutants, *i.e*., 1 : 1.80 (Morita *et al.* 1999), thereby illustrating the huge variation observed in *H. gingivalis*.

Variation in size has a considerable effect on many other morphometric characteristics. However, variability may be reduced by using ratios instead of individual measurements when they are constant and both their entities show a high mutual correlation (Geraert 1968; Fortuner 1984; Roggen *et al.* 1986). In *Halicephalobus* species identification, especially ratio c' is frequently used. However, the present study reveals that ratio c' shows a relatively high intraspecific variability in *H*. *gingivalis* (1 : 2.2), exceeding the range of several other species of the genus (see also Geraert *et al.* 1988). Therefore, ratio c' can only be used to discriminate between a 'short-tailed' group consisting of *H. brevicauda* (MAVLJANOV, 1976) ANDRÁSSY, 1984, *H*. *intermedius* (POKROVSKAJA, 1964) ANDRÁSSY, 1984, *H. gingivalis*, *H. palmaris* (LORDELLO AND DE OLIVEIRA, 1963) ANDRÁSSY, 1974 and *H. limuli* TIMM, 1956, and a 'long-tailed' group encompassing *H. persicus* SHOKOOHI, ABOLAFIA AND ZAD, 2007, *H. parvus* (KÖRNER, 1954) ANDRÁSSY, 1974, *H. similigaster* (ANDRÁSSY, 1952) ANDRÁSSY, 1974*, H. minutus* (KÖRNER, 1954) ANDRÁSSY, 1974 and *H. mephisto* BORGONIE, GARCIA-MOYANO, LITTHAUER, BERT, BESTER, VAN HEERDEN, MOLLER, ERASMUS AND ONSTOTT, 2011. Ratio VA/T, also frequently used in *Halicephalobus* identification keys, exhibited a huge intraspecific variability (1 : 3.9) in our study and can be highly influenced by temperature and food availability. This corroborates Geraert *et al.* (1988), who found that the ratio VA/T values are overlapping in multiple species of the genus and are thus of limited diagnostic value.

When evaluating the boundaries of ratio variability, ratios V and V' have often been identified as very stable, thereby confirming the constancy of the position of the vulva and its importance in nematode taxonomy (Geraert 1979; Bert *et al.* 2010). In the current study, ratios V and V' show the least intraspecific variability, thus rendering them potential candidates for *Halicephalobus* species delineation. However, ratio V does appear to be influenced by temperature, which corroborates previous findings, *e.g*., in *A. avenae* (Monoson 1971). Hence, since a morphometric character with a low CV can still be modified by the environment (Tarte and Mai 1976; Lax *et al.* 2004), even the most stable characteristics, such as ratio V, must be used with caution.

In addition, other measurements and ratios related to the reproductive system are important in *Halicephalobus* identification keys (Andrássy 1984; Geraert *et al.* 1988;

Shokoohi *et al.* 2007). However, the gonad is the fastest growing organ in the adult nematode and is closely related to the post-embryonic increase in body size (Levsen and Berland 2002). It is therefore not surprising that maximal variability in the morphometric characteristics related to the reproductive system is mainly related to the factor age group. Conversely, ratio ovary, which plays an important discriminating role between some species of *Halicephalobus*, shows a large overall variability (1 : 9) but is not significantly influenced by age as is shown by the ANOVA results. Therefore, ratio ovary is also of limited discriminative use, even when comparing the same age groups.

Ratio corpus/isthmus and stoma length both show a low overall variability in *H. gingivalis* and the experimentally induced environmental factors have no or limited influence. The nematode stoma is a complex congregate of several cellular components, cuticular structures and tissues of both mesodermic and ectodermic origin (Bird and Bird 1991; De Ley *et al.* 1995). The low morphometric variability could be related to this complexity since, according to Soule's (Soule 1982) model of allometric variation, the variance of structures reduces as the number of independent developmental events that produce them increases. Mind that the small size of the stoma is no explanation for the low variability since all our morphometric characteristics were normalised for size prior to the statistical analysis. However, as Geraert *et al*. (Geraert *et al.* 1988) stated, the small size of the structure and the weakly developed posterior part makes previously observed interspecific variation less than convincing, rendering stoma length also not optimal for delineating *Halicephalobus* species.

In conclusion, by only adjusting the culturing conditions in subpopulations of the progeny of a single parthenogenetic female, intraspecific morphometric variation may surpass total interspecific variability, or measurements and ratios that have been used to appoint individuals to different 'species' of *Halicephalobus* according to the current keys for the genus. The present study also reveals that morphometric variability can be highly influenced by environmental conditions and that caution is necessary when using only morphometrics for delineating new species. At the same time, one might expect morphological variability within natural populations to be smaller than in single populations maintained at a given set of constant environmental conditions, since the lack of environmental variability in the laboratory cultures likely implies a lack of the selective pressures which act on natural populations. Such selective pressures would be

expected to result in loss of variability in natural populations. However, when comparing our data on a laboratory population with an *in situ* population obtained from compost, a comparable large intraspecific variability was found, including putative discriminating characteristics (Geraert *et al*., 1988). We therefore feel that the observed variability under the experimental conditions applied in our study is relevant to populations from natural habitats.

Additionally, given the presently found variability and its implications on species delimitation within *Halicephalobus*, a thorough revision of the genus is imperative. However, we are well aware that it is not feasible to describe species only after such an extensive assessment of variability, which can only be attained through an experimental culture approach, being only a substitute for natural environmental conditions. Finally, we also demonstrate, by showing that subpopulations of a single female can be statistically robustly separated, that delimitation of taxa based on multivariate analyses alone (Brown *et al.* 1997; Stock *et al.* 2000) should be avoided. Clearly, multivariate analysis of morphometric data have their merits to determine the most discriminating morphometric characteristics, especially when combined with independent molecular data (*e.g.*, Fonseca *et al.* 2008). The present paper is not a plea to discard morphometric data in (nematode) taxonomy altogether. With this experimental study we merely provide convincing elements to advocate an integrative taxonomic approach and to abandon the description of new species based only on morphometric differences.

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CHAPTER VI

EVOLUTION OF PARASITISM AND SPECIES DELINEATION IN THE PARTHENOGENETIC GENUS *HALICEPHALOBUS*: MORPHOMETRICAL DATA CORROBORATES A MULTIPLE GENE APPROACH IN A MORPHOLOGICALLY MINIMALISTIC GENUS

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ABSTRACT

Halicephalobus is an obligatory parthenogenetic, free-living genus with a remarkably wide environmental range, including facultative parasitism in different mammals. A multidisciplinary approach was carried out combining ecological, morphological, morphometrical and molecular data of 17 *H*. *gingivalis* isolates, of both free-living and parasitic origin, complemented with 4 isolates of other species of *Halicephalobus* with a distinctly different biology. A phylogeny based on two nuclear loci (18S and D2D3 expansion segment) and two mitochondrial loci (COI and ND4), and on both maximum likelihood and Bayesian inference, indicated a maximally supported sister relationship of *H. mephisto* with all other *Halicephalobus* isolates. Most other *Halicephalobus* isolates were also found both molecularly and morphometrically distinguishable from a distinct *H. gingivalis*-clade. Only for isolates within this *H. gingivalis*-clade, SSU sequence data revealed a high level of intra-genomic variability with associated alterations in the secondary structure model, which to the best of our knowledge has never been described for nematodes. Additionally, two morphological and morphometrical not discernible lineages were indicated reciprocal monophyletic by several species distinctiveness measures, consequently revealing cryptic speciation within the *H. gingivalis*-clade. Most remarkably, isolates originating from inside horses were concentrated in one of these lineages indicating that this lineage is evolving towards equine host specificity.

INTRODUCTION

Species of the genus *Halicephalobus* TIMM, 1954 (Panagrolaimidae) are known for their broad environmental range, *i.e.*, as inhabitants of compost, humus, soil, rotten wood, water enclosures in mines up to 1 km belowground, and in association with insects and chelicerates (table 1.1 Chapter 1). Males have never been described in any of the species, suggesting that they are all obligatory parthenogenetic. The genus is usually free-living and bacteriovorous, except for *H. gingivalis* (STEFAŃSKI, 1954) ANDRÁSSY, 1984 which is known as a facultative parasite. Approximately 75 equine infections have been described, all but two of which were fatal. Additionally, single cases have been described in a Grevy's zebra (Isaza *et al.* 2000) and a donkey (Schmitz and Chaffin 2004), and *H. gingivalis* resembling nematodes have been reported in the brain of a black Angus cow (Montgomery and O'Toole, 2006). Finally, four human cases have been reported, which were all fatal (Ondrejka *et al.* 2010). The distribution of reports on equine halicephalobiasis indicates that the species is cosmopolitan (see table 1.2, Chapter 1).

Little is known about the epidemiology of *H. gingivalis*. This opportunist appears able to colonize the host in several different ways, such as through open wounds, through the nasal and oral cavities, through the ingestion of contaminated food, via the respiratory tract (Spalding *et al*. 1990; Ruggles *et al*. 1993; Trostle *et al*. 1993; Bröjer *et al*. 2000), or facilitated by recumbency (Dunn *et al*. 1993). Insects may play an important role in *H. gingivalis* infections, which is especially true for infections through lesions in the skin or orbital infections (Anderson *et al*. 1988). Phoretic behaviour associated with insects and other invertebrates has been described for many nematodes (*e.g.*, Sudhaus and Kiontke 1996; Timper and Davies 1996), including the (endo)phoretic association of other species of the genus *Halicephalobus* with flies, beetles and ants (Köhler 2012), and termites (Kanzaki *et al.* 2012). However, the phoretic ability of *H. gingivalis* isolates as well as the possible role of phoresy in *H. gingivalis* infections and in the geographical distribution of the species in general, has never been investigated.

It is not yet clear whether free-living *H. gingivalis* are generally capable of infecting vertebrate hosts but only very occasionally do so, or whether a specific lineage of *H. gingivalis* is evolving towards vertebrate host specificity (Blunden *et al*. 1987, Nadler *et al*. 2003). The former hypothesis would be supported if free-living and

parasitic isolates do not exhibit consistent genetic differences, while the latter would be supported by reciprocal monophyly of isolates from vertebrate hosts versus those from soil (Blunden *et al*. 1987, Nadler *et al*. 2003). The phylogenetic relationship between several isolates of *H. gingivalis* based on their large subunit (LSU) rDNA sequences provided first evidence for the existence of distinct genetic lineages (Nadler *et al.* 2003), however, not reflecting evolution towards mammalian host specificity, thus pointing towards the first hypothesis, *i.e.*, that free-living isolates are generally capable of infections (Nadler *et al*. 2003). Furthermore, genetic lineages are not necessarily restricted in geographical distribution, and single geographic regions may contain a diverse pool of lineages capable of equine infection (Nadler *et al.* 2003). However, Nadler *et al.* (2003) only used one locus for the phylogenetic inference and DNA from pooled individuals was used. Hence, a multi-locus phylogenetic analysis based on single individuals is necessary to better investigate the delineation of the genetic lineages within the morphospecies *H. gingivalis* and the possible evolution of one lineage towards parasitism (Nadler *et al*. 2003).

Morphologically indiscernible *H. gingivalis* isolates previously used in other studies (Chapter 3) show an intraspecific sequence difference up to 6.3% in the SSU ribosomal DNA gene. Conversely, to illustrate the rDNA sequence differences in *H*. *gingivalis*, a SSU sequence difference of 0.2% between different isolates of the closely related *Panagrolaimus*, revealed two reproductively separated species (Abebe and Blaxter 2003). The genetic variability found in *H*. *gingivalis* may be a consequence of the clonal inheritance of rDNA due to its non-sexual reproduction resulting in a continuum of genetic differences between individuals with no gaps separating clades (Birky *et al.* 2005), or may indicate the presence of (multiple) cryptic species within the species complex *H*. *gingivalis*. An integrative approach combining data from multiple sources including molecular data has proven successful in disclosing cryptic biodiversity in sexually reproducing nematodes (*e.g*., Fonseca *et al.* 2008; De Oliveira *et al.* 2012). Because delineating species in non-sexual taxa is often difficult, especially when dealing with character-poor organisms of small size and few discriminating morphological traits, it is imperative to use an integrative approach when delineating parthenogenetic species (Heethoff *et al.* 2011).

The current study has the aim of revealing whether *H. gingivalis* is merely an opportunistic invader or, conversely, whether a lineage is evolving towards parasitism. Hence, a multidisciplinary approach was performed including 17 *H. gingivalis* isolates complemented with 4 isolates of other species of *Halicephalobus* which have a very different biology. Species boundaries were explored based on the evolutionary species concept (de Queiroz 2007 and references therein) and whereafter these results were compared with morphological and morphometrical data. The presence of permanent cultures ensures a sufficient number of specimens of several populations to screen genetic and morphological variability (Nadler 2002). Firstly, a thorough analysis of the genetic variability of the SSU rDNA gene revealed the presence of intraspecific and intra-genomic polymorphisms. Their occurrence and consequence for the SSU secondary structure model are described. Secondly, to reveal possible cryptic species within the morphospecies *H. gingivalis*, a multiple gene phylogenetic analysis based on single-specimen sequences of two ribosomal and two mitochondrial loci is performed. These molecular results are compared with morphological data, based on both light-microscopic and scanning electron microscopic (SEM) analyses, and morphometrical data.

MATERIAL AND METHODS

ISOLATION, IDENTIFICATION AND CULTURE OF NEMATODES

Molecular, morphometrical and scanning electron microscopic data were obtained from *Halicephalobus* spp. isolates WB0701-0705, WB0708, WB0709, WB0801, WB1101-1103, PF060103, PF060121, PF060144, PF190101, PF190106, JB043, SAN100, RGD838, RGD892 and *H. mephisto*. Isolates PF060103, 060144, 190106 and CaseReportBelgium were only included in the molecular analysis. The following isolates were already described in former studies, *i.e.*, WB0708 (Chapter 3), WB0801 (Chapters 3 and 5), JB043 (Nadler *et al.* 2003), JB128 and SAN100 (Chapter 3 and Nadler *et al.* 2003), *H. mephisto* (Borgonie *et al*. 2011). The origins of the isolates are listed in table 2.1 (Chapter 2), and include compost heaps, manure heaps, fresh horse dung, a clinical case, rotten plant material and in association with termites. WB1101 and WB1102 were obtained from rectal faecal

samples, representing the first direct evidence of the presence of *H*. *gingivalis* inside the equine digestive tract without causing clinical symptoms. Although there are no apparent characteristics to distinguish the new isolates light microscopically from other *H. gingivalis* isolates (*e.g*., JB128 and SAN100), considerable sequence differences in the D2D3 expansion region of LSU rDNA (28S) have been observed (Chapter 3).

All isolates were kept in culture on 1% bacteriological agar (Oxoid, Basinstoke, UK) enriched with cholesterol (Sigma-Aldrich, Belgium) at a final concentration of 1 µg ml^{-1} and with a bacterial lawn of *Escherichia coli* OP50 as a food source (Brenner, 1974).

MOLECULAR ANALYSIS

DNA extraction

All *Halicephalobus* species/isolates listed above and specimens collected from frozen horse tissue (Part II Chapter 2; further referred to as 'CaseReportBelgium') were used for the molecular analysis. *Procephalobus* sp. STEINER 1934 (strain JU169) is the most closely related genus (Borgonie *et al.* 2011) and was therefore selected as an outgroup. Single specimens of each nematode species/isolate were collected in 50µl of 90-95% acetone and kept at room temperature until processed (Fukatsu 1999). DNA was extracted of at least two specimens of each isolate.

The sample in acetone was dried under vacuum, whereafter 30 µl of lysis buffer (50 mM KCl, 10 mM Tris pH8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween2) was added. Subsequently, 30 µl of sterile water and 1 µl ProteinaseK were added, the tube was mixed and spun down. Samples were incubated for 1 hour at 65°C and subsequently for 10 min at 95°C to heat inactivate ProteinaseK. Finally, the DNA samples were centrifuged for 1 min at 14,000 rpm, and 1-5 µl of extracted genomic DNA was used as a template for double-stranded polymerase chain reactions (PCR).

Genetic markers

Because they are present in multiple copies, can easily be amplified as a whole fragment, and contain both variable and conserved regions, the ribosomal (r)DNA genes have been used extensively as markers for reconstructing nematode phylogenies. Especially the LSU (large ribosomal subunit) and SSU (small ribosomal subunit) regions

have been proven to be very useful because of its phylogenetic resolution at the genus and higher taxon level (De Ley *et al.* 2005). Conversely, mitochondrial (mt)DNA genes have been used successfully to unravel recent radiations (*e.g*., Blouin *et al.* 1998; Blouin 2002). The ND4 mtDNA gene has a high substitution rate which renders it excellent for identifying cryptic species, and resolving relationships among closely related congeners (Blouin *et al*. 1998), concurrently the COI mtDNA gene is used as a barcoding gene (*e.g*., Hebert *et al.* 2003). Therefore, since we are interested in both the relation between different species of the genus and in possible radiations within the *H*. *gingivalis* species complex, a portion of the mitochondrial gene coding for cytochrome oxidase *c* subunit 1 (COI) and the mitochondrial gene coding for NADH dehydrogenase subunit 4 (ND4) were complemented with the rDNA genes 18S (SSU) and the D2D3 expansion region of 28S (LSU) in a species phylogeny.

PCR amplification

The different molecular markers were amplified using the primers and PCR protocols listed in table 6.1. A PCR reaction volume (50 µl) contained 5 µl of 10X reaction buffer, 2.5 mM of $MgCl_2$, dNTP-mix at 0.2 mM each, 1 µM of each of the primers, and 0.02 U μl^{-1} of Taq polymerase (Goldstar, Eurogentec, Belgium). PCR amplifications were done on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplified PCR product was electrophoretically fractionated in 40 ml 0.5x TAE buffer in 1% agarose gel (Ultrapure Gibco BRL Life Technologies, UK) and visualized by staining with 0.003% ethidium bromide (0.02 µg ml^{-1}). A 1 Kbp DNA ladder (BRL Life Technologies, UK) was used as a size marker. Gels were viewed on a UV transilluminator.

Cloning

Initial sequence analysis revealed multiple ambiguous nucleotides present at the same sites in the SSU sequence of many *Halicephalobus* spp. isolates, indicating the possible presence of different copies of the SSU rDNA gene. Polymorphisms were indicated when both alternative nucleotide peaks were present in all sequence reactions representing both DNA strands and when the minor nucleotide peak represented at least 25% of the major peak (following Nadler *et al.* 2003). To define these polymorphisms, amplification

products were ligated in a vector using CloneJET PCR Cloning Kit (Fermentas Thermo Scientific) according to the manufacturer's protocol. Transformation of the vector in competent *E. coli* cells of strain DH5a was done by heat shock. After incubation at 37°C for 1 hour, the cells were concentrated by centrifugation in 100 µl and this suspension was spread on a Petri dish containing agar and ampicillin. Several (5 - 10) positive white colonies were selected for sequencing.

Table 6.1. Primers used for amplification of different genetic markers and respective PCR protocols

Primers & PCR protocol	Direction	Primer sequence 5'-3'	Usage	Reference
SSU				
G18S4	forward	GCT TGT CTC AAA GAT TAA GCC	PCR/Sequencing	Blaxter *et al*. 1998
4R	reverse	GTA TCT GAT CGC CKT CGA WC	PCR/Sequencing	Blaxter *et al*. 1998
18P	reverse	TGA TCC WMC RGC AGG TTC AC	PCR/Sequencing	Blaxter *et al*. 1998
PCR protocol	5 min at 94°C, 45 x(30 s 94°C, 30 s 54°C, 2 min 72°C), 5 min 72°C			
9FX	forward	AAG TCT GGT GCC AGC AGC CGC	Sequencing	Meldal *et al*. 2007
22F	forward	TCC AAG GAA GGC AGC AGG C	Sequencing	Blaxter *et al*. 1998
9R	reverse	AGC TGG AAT TAC CGC GGC TG	Sequencing	Blaxter *et al*. 1998
26R	reverse	CAT TCT TGG CAA ATG CTT TCG	Sequencing	Blaxter *et al*. 1998
2FX	forward	GGA AGG GCA CCA CCA GGA GTG G	Sequencing	Meldal *et al.* 2007
23F	forward	ATT CCG ATA ACG AGC GAG A	Sequencing	Blaxter *et al*. 1998
13R	reverse	GGG CAT CAC AGA CCT GTT A	Sequencing	Blaxter *et al*. 1998
23R	reverse	TCT CGC TCG TTA TCG GAA T	Sequencing	Blaxter *et al*. 1998
PCR protocol	5 min at 94°C, 45 x(30 s 94°C, 30 s 54°C, 2 min 72°C), 5 min 72°C			
D2D3 region				
D2A	forward	ACAAGTACCGTGAGGGAAAGTTG	PCR/Sequencing	De Ley *et al.* 1999
D3B	reverse	TCCTCGGAAGGAACCAGCTACTA	PCR/Sequencing	De Ley *et al*. 1999
PCR protocol	5 min at 94°C, 45 x (30 s at 94°C, 30 s at 54°C, 45 s at 72°C), 10 min 72°C			
CoxI				
CO1490F	forward	GGT CAA CAA ATC ATA AAG ATA TTG G	PCR/Sequencing	Folmer *et al.* 1994
CO2198R	reverse	TAA ACT TCA GGG TGA CCA AAA AAT CA	PCR/Sequencing	Folmer *et al*. 1994
PCR protocol	5 min at 94°C, 45 x (30 s at 94°C, 30 s at 50°C, 30 s at 72°C), 5 min at 72°C			
NDH4				
ND4f2	forward	GCT TAT TCT TCW GTM WSW CAT ATA GG	PCR/Sequencing	this study
ND4r2	reverse	GTW CCG ATG KTT TTA TGG TTA G	PCR/Sequencing	this study
PCR protocol	5 min at 94°C, 45 x (30 s 94°C, 30 s 50°C, 1 min 72°C), 5 min 72°C			

DNA sequencing

Sequencing was performed using an ABI 3130XL Genetic Analyser (Applied Biosystems, Foster City, California, USA). Excess primer and dNTP were removed with ExoSAP-IT (USB Corporation; Cleveland, Ohio, USA) for 15 min at 37°C, followed by 15 min at 80°C to inactivate the enzymes. This material was then used for cycle sequencing without any further purification, using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). The sequencing conditions were 30 s at 96°C, 15 s at 50°C and 1 min at 60°C for 27 cycles. Primers used for sequencing are listed in table 6.2. Cycle sequence products were precipitated by adding 25 µl of 95% ethanol and 1 µl of 3 M sodium acetate (pH 4.6) to each cycle sequencing reaction (10 µl). The samples were placed at room temperature for 15 min and centrifuged at 14,000 rpm for 15 min. The pellet was additionally washed with 125 µl of 70% ethanol and dried in a Speedvac concentrator, redissolved in formamide and run on 50 cm capillaries with POP7 polymer.

Before performing a multiple sequence alignment, consensus sequences were edited and assembled from contigs using SEQMAN 7.0 (DNASTAR Lasergene; Madison, WI, USA) and submitted to a BLAST search (Altschul *et al.* 1990) on the NCBI website (http://www.ncbi.nlm.nih.gov) to check for possible contaminations.

Sequence alignment and post-alignment editing

Multiple sequence alignment (MSA) was performed with MAFFT version 6.833 (Katoh and Toh 2008 a, b) at the freely available Bioportal server at Oslo University (www.bioportal.uio.no) (Whetzel *et al.* 2011). MAFFT has been indicated as one of the most accurate MSA programs presently available (Carroll *et al.* 2007). Both COI and ND4 datasets were aligned using the default algorithm, whereas both LSU and SSU rDNA sequences were aligned on the basis of their rRNA secondary structure information using the Q-INS-i algorithm in MAFFT (Katoh and Toh 2008a, b). Secondary structure models are used for aligning rDNA sequences because the conservation of secondary structures is thought to exceed that of nucleotides (Kjer 1995). Primer regions were removed from the alignment.

Since alignment errors significantly influence phylogenetic tree reconstruction (Ogden and Rosenberg 2006; Wong *et al.* 2008), random similarities[5], were eliminated using the parametric profiling method ALISCORE (Misof and Misof 2009, freely available from aliscore.zfmk.de) which selects randomness in multiple sequence alignments using Monte Carlo resampling within an adaptable sliding window. A window size of 4 base pairs was selected. To check for missing data in certain positions, gaps were treated as ambiguous characters. Additionally, multiple sequence alignments were also analyzed with the non-parametric alignment trimming program GBLOCKS (Castresana 2000) as implemented in SeaView v4.4.0 (Galtier *et al.* 1996; Gouy *et al.* 2010). GBLOCKS does not account for models of sequence evolution and excludes ambiguous sections based on an arbitrary chosen threshold and resulted in slightly different tree topologies. However, since different studies have consistently shown that GBLOCKS is outperformed by ALISCORE (Kück *et al.* 2010), the latter was chosen for post-alignment editing.

The alignment of each locus was tested for homoplasy using the substitution saturation test in DAMBE version 5.3.0 (Xia and Xie 2001), calculating the index of substitution saturation (I_{ss}). The proportion of invariable sites was determined for each locus, after which saturation tests were performed on all sites with gaps treated as missing data. Further, both COI and ND4 datasets were additionally screened for saturation at first, second and third codon positions. As a second control for substitution saturation, the overall transition/transversion ratio was calculated for each dataset in which a linear relationship between both distances indicates that no saturation has occurred. Final alignment lengths are listed in table 6.2.

Estimation of SSU rRNA secondary structure

The SSU rRNA secondary structure model of *H*. *gingivalis* was constructed to pin-point the regions with the polymorphisms in order to characterize the ribosomal diversity. Initially, the SSU rRNA sequence of isolate WB0705 was aligned against *Panagrolaimus rigidus* (Panagrolaimidae) within a MSA including secondary structure annotation obtained from Bert *et al.* (2008) and manually matched up with the RNA secondary

[5] According to Misof and Misof (2009) sequence similarity can be grouped into nonrandom similarity or nonrandom homoplasy, as a result of phylogenetic relatedness or selection, and random similarity caused by unrelatedness, high sequence divergence, and ambiguous alignment.

structure model of *P. rigidus*, visualized with RnaViz (De Rijk *et al.* 2003). Diverging regions were further refined by folding the sequences using the Mfold Webserver (www.mfold.rna.albany.edu) (Zuker 2003). Folding was done at a fixed temperature of 37°C and based on the default settings. Helices are numbered from 5'-3'terminus following the nomenclature proposed by Wuyts *et al.* (2001).

Choosing the models of DNA evolution and combining multiple genes

Multiple gene approaches generally reveal phylogenies with higher resolution and reliability (Nadler 2002). Moreover, combining the sometimes minor signals present in separate genes can yield resolution such that it becomes detectable above the background noise of homoplasy (Rokas *et al.* 2003). Therefore, next to single gene alignments, a concatenated alignment of all genes was generated using Geneious version R6 created by Biomatters and available from http://www.Geneious.com.

The model of DNA evolution was determined for each locus separately and for the concatenated dataset with jModeltest version 2.1.2 (Posada 2008) using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) (Posada and Buckley 2004). The different proposed models representing the lowest AIC and BIC scores, respectively, for the different loci and the concatenated alignment are listed in table 6.2.

Table 6.2. Alignment lengths and proposed evolutionary models following the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC).

	SSU	D2D3	COI	ND4	Concatenated dataset
Number of taxa	22	22	21	19	
Original alignment length	836	427	514	445	
Final alignment length	781	413	514	445	2148
AIC	GTR + Γ+ I	GTR + Γ	GTR + I	GTR + Γ	GTR + Γ+ I
BIC	HKY + Γ+ I	HKY + Γ	GTR + Γ	HKY + Γ	GTR + Γ+ I

Since the AIC tends to favour more complex, parameter-rich models whereas BIC penalizes complex models more, the models selected for the different markers were slightly different when based on the AIC as opposed to when based on the BIC. Most

importantly, evolutionary models were similar for all the analysed loci when either selected by the AIC or the BIC, which facilitates a concatenated analysis. Ultimately, the General time reversible model (GTR) that accounts for rate heterogeneity among sites (+ Γ) and a proportion of invariable sites (+ I) was favoured over the Hasegawa-Kishino-Yano (HKY, Hasegawa *et al.* 1985) by both AIC and BIC.

Phylogenetic inference

Maximum likelihood-based phylogenetic analysis was performed using RaxML version 7.2.8 (Stamatakis 2006) at the freely available Bioportal server (www.bioportal.uio.no) (Whetzel *et al.* 2011). Analyses were performed using the rapid hill climbing algorithm (-f d) and 1000 non-parametric bootstrap replicates (-N 1000) under the GTRGAMMAI model (Stamatakis 2006) with 4 distinct rate categories (-c 4). Gaps were treated as missing data. Bootstrap replicates were used to construct majority rule consensus trees and plot bootstrap proportions on best-scoring trees in TreeView as implemented in Geneious version R6.

Bayesian inference was performed with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Preliminary analyses on single loci were run on the BioPortal server, while the final concatenated analysis was done using the STEVIN Supercomputer Infrastructure at Ghent University. The analyses were done under the GTR model (nst=6) with rates varying following a gamma distribution and a proportion of invariable sites (rates=invgamma), using default prior settings. Final analysis on the concatenated alignment was performed under the same GTR model using a partitioned Bayesian strategy, with all parameters estimated separately for the individual genes. Each Bayesian analysis used four chains, one cold and three incrementally heated (default, temp=0.2) in two independent, simultaneous runs for several million generations (ngen=30,000,000 for monogenic analyses, ngen=50,000,000 for concatenated analysis), each started from different random trees and sampled every 1000th generation. Gaps were treated as missing data. To control whether the two different runs converged, average standard deviation of split frequencies and Potential Scale Reduction factors (PSRF) were assessed. Split frequencies approached zero and PSRF approached one at the end of the analysis providing proof for converged runs. The burnin was arbitrarily

chosen at 7,500,000 generations for monogenic analyses and at 12,500,000 for concatenated analysis (burnin 7,500 and 12,500, respectively) or 25% of the results. A generation/Log Likelihood scatter was used to evaluate the size of the burn in. Posterior probabilities were calculated using Metropolis-coupled Markov chain Monte Carlo samplers.

Measures for species distinctiveness

Rosenberg's reciprocal monophyly or P(AB), and Rodrigo's P(RD) measures were calculated using the species delimitation plugin (Masters *et al.* 2011) in Geneious R6 (created by Biomatters, available from http://www.Geneious.com). Both calculations are based on a rooted genealogy containing user specified groups. The null hypothesis for Rosenberg's P(AB) (Rosenberg 2007) is that monophyly is a chance outcome of random branching, which can be rejected at $p < 10^{-5}$. Rodrigo's P(RD) (Rodrigo *et al.* 2008) is defined as the probability of an observed degree of distinctiveness and designates distinctive clades at $p < 0.05$. P(RD) ranges from 0 to 1 indicating complete distinctiveness and no distinctiveness, respectively.

Additionally, the genealogical sorting index (*gsi*, Cummings *et al.* 2008) statistic was calculated using the web interface freely available at www.genealogicalsorting.org. It is an R based algorithm, which quantifies the degree of exclusive ancestry of labelled groups on a rooted genealogy and is especially suitable for investigating distinctiveness among recent radiations. The *gsi* provides information on the extent of lineage divergence, which reaches a maximum value of 1 when the group is monophyletic, and is accompanied by a significance level (significant at $p < 0.05$). Bonferroni correction was used to assess the significance of the *gsi p*–values by dividing the *p*-value by the number of predefined groups.

Finally, differentiation between mtDNA clades/lineages identified by single gene phylogenies were analyzed according to the "4x rule" species criterion which is specifically designed for delimiting species in non-sexual and clonal organisms (Birky *et al.* 2010), which considers speciation to be complete when populations are separated by gaps too deep to be produced by random drift alone (Barraclough *et al.* 2003). Following this criterion, speciation has occurred when the mean sequence divergence (*K*) between

individuals from two clades or lineages is greater than 4 times θ, with $\theta = \pi/(1\text{-}4^* \pi/3)$, in which π is the mean sequence difference between individuals in a clade or lineage (Barraclough *et al.* 2003, Birky *et al*. 2010).

MORPHOLOGICAL ANALYSIS

Scanning electron microscopic (SEM) study

Nematodes were picked off culture plates and transferred to a drop of distilled water. Subsequently, 1 ml of freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, Sigma-Aldrich, Belgium), at 60°C was added. After 24 h, the specimens were dehydrated by passing them through a graded ethanol concentration series of 20, 50, 75, 95, 100% (20 min each), 100% (10 min) and 100% (10 min). To avoid collapsing the nematodes, they were critical-point dried with liquid CO_2 using a Balzers CPD 020. The dried nematodes were then removed individually, placed on a glass rod on a standard specimen stub, sputter-coated with gold (25 nm) using a 1200 JFC (JEOL, Tokyo, Japan), and observed using a JSM-840 (JEOL, Tokyo, Japan) at 12 kV. Images were taken on Kodak TMAX100 film.

Morphometric analysis

All cultured *Halicephalobus* spp. isolates where initially started from single individuals. Approximately 50 eggs of each isolate were placed on a Petri dish containing 1% bacteriological agar enriched with cholesterol at a final concentration of 1 µg ml^{-1}. To ensure that the observed morphological variability between *H. gingivalis* isolates is minimally influenced by environmental conditions, all developing eggs were incubated at a constant temperature of 30°C and contained a bacterial lawn of *E. coli* OP50 as a food source, obtained by adding 10 µl at a density of approximately 2.85×10^6 μl^{-1} to each plate and incubating them overnight at 37°C before the transference of the eggs. Three replicates of all *Halicephalobus* spp. isolates were included in the analysis.

After the first oviposition, the nematodes were removed from the plates using a stereomicroscope (Leica MZ95) and collected in 5 µl of water in an embryo dish. An excess (4-5 ml) of heated (70°C) formaldehyde (4%) with 1% glycerol was added to instantaneously kill and preserve the nematodes (Seinhorst 1966). The use of hot

formaldehyde has the advantage that nematodes are typically outstretched and not curled. The preserved nematodes were processed to anhydrous glycerin following the glycerin-ethanol method (Seinhorst 1966, as modified by De Grisse 1969), subsequently mounted on glass slides and covered with a cover slip. Finally, 10 specimens of each replicate of all isolates (17 isolates included in the morphometrical study, 30 specimens per isolate, 510 specimens in total) were measured digitally using the NIS-elements measuring software (Nikon Instruments Europe B.V., Brussels, Belgium). All 28 measurements and ratios are listed in appendix 1.

The resulting morphometrical data were analysed with Statistica 7.1 (StatSoft Europe GmbH, Hamburg, Germany). Residual plots indicated homogeneous variances and homoscedastic error distributions, however, formal testing using a Kolmogorov-Smirnov test and a Bartlett test often rejected the assumptions of normality and homogeneity of variances, respectively. However, when based on a large sample size ($n > 30$), deviations from normality are negligible because of the central limit theorem, according to which the sampling distribution of the mean approximates the normal distribution, regardless of the distribution of the variable in the population (StatSoft Electronic Statistics Textbook 2012, http://www.statsoft.com/textbook/).

A correlation matrix was generated to determine those characteristics that had no significant correlation with each other (significant correlation at $p < 0.05$, $r > 0.75$). This threshold was set based on the correlation between measurements that were expected to be correlated, such as body length and width, isthmus and pharynx length. Using these characteristics, a forward stepwise discriminant function analysis (DFA) was done to test whether molecular based clades or species were morphometrically significantly different. If significant differences were observed ($p < 0.05$), *a posteriori* canonical analysis was performed which determined an optimal combination of variables so that the first function (root 1) provided the largest overall discrimination between groups, which was visualized in a scatter plot (root 1 x root 2). Differences between molecularly based clades or species were determined by calculating *p*-values and squared Mahalanobis distances (D^2) for each pairwise comparison. Differences between clades or species were considered significant when $p < 0.01$.

Finally, analysis of variance (one-way ANOVA) with the factor species/isolate was used to pinpoint those morphometrical characters that can be used for differentiation between the morphometrically defined species/groups.

RESULTS

MOLECULAR ANALYSIS

Sequence analysis reveals polymorphisms

Sequence data obtained from non-cloned amplification product revealed the presence of ambiguities representing sequence polymorphisms in the SSU sequences of almost all isolates, with the exception of WB0701, RGD838, RGD892, *H. similigaster* and *H. mephisto.* Some polymorphic sites were randomly distributed single nucleotide polymorphisms (SNP), but most (40, *i.e.*, 78%) were concentrated in two distinctive regions. It is important to note that different specimens of one isolate did not always possess all polymorphisms, which could indicate that not all specimens had all polymorphisms or that sequence analysis is not always successful in indicating all polymorphisms in every specimen. Figure 6.1 represents the alignment of the two polymorphic regions for all *Halicephalobus* isolates. Isolate WB0705 comprised most polymorphisms, *i.e*., 51 (2.99%) in a total of 1705 sites.

Subsequent analysis of sequence data obtained from different clones of several specimens of SAN100, WB0702 and WB0705, revealed that polymorphic regions were not composed of completely random nucleotide sequences, but comprised two alternative sequence blocks of homogenous sequences (fig. 6.2, a vs. b). Interestingly, different homogenous sequences, or ribotypes, were not only identified intraspecific, *i.e.*, from different specimens of the same isolate (for WB0702 and WB0705), but also intra-genomic, *i.e*., from different clones of one specimen (for WB0705). For SAN100, only one homogenous sequence could be identified, however, only two clones of this isolate were analyzed.

To further characterize different ribotypes, the secondary structure model of the SSU rRNA of *H*. *gingivalis* isolate WB0705 was predicted (fig. 6.3). It revealed that the two polymorphic regions were located in three helices, *i.e.*, 10, 23/e1 and 23/e4. Both

alternative homogenous sequences, were not equally distributed. The predominant sequences (black box) were observed in 7 out of 9 clones of a single specimen for regions 23/e1 and 23/e4 and in 8 out of 9 clones of a single specimen for region 10. Alternative homogenous sequences resulted in different predictions of the secondary structure, for the subdominant alternative (red boxes) this resulted in a decreased size of the terminal loop in helix 10, a slightly different length of the stem in helix 23/e1, and an increased size of the internal loop in helix 23/e4.

	120																				130									140		
WB0701	C	A	T	A	A	A	T	G	A	C		T	A	T	A	T	A	G	T	T	-		-	A	T	G	C	T	A	T	A	T	A	G
WB0702	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
WB0703	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
WB0704	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
WB0705	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
WB0708	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
WB0709	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
WB0801	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
PF060103	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
PF060121	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
PF060144	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
PF190101	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
PF190106	.	.	A	W	.	.	W	.	.	Y		.	W	Y	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	R	.	R	R
SAN100	.	.	.	T	.	.	.	R	.	.		Y	.	.	.	.	.	A	.	.	-		-	W	Y	.	Y	.	.	.	.	Y	R	.
casus	.	.	.	T	.	.	.	R	.	.		C	.	.	.	.	.	R	.	.	-		-	.	.	.	T	.	.	.	.	Y	G	.
WB1101	.	.	A	W	.	.	W	.	.	Y		.	R	.	.	.	.	R	.	.	-		-	W	.	R	Y	.	.	.	.	.	R	R
WB1102	.	.	A	T	.	.	W	.	.	T		.	R	.	.	.	.	R	.	.	-		-	T	.	A	T	.	.	.	.	.	R	R
WB1103	.	.	A	T	.	.	.	.	G	T		.	R	.	.	.	.	A	.	.	-		-	.	.	.	T	.	.	.	.	.	G	A
H. mephisto	T	.	A	T	.	.	.	A	T	T		.	.	.	T	.	.	T	A	.	A		A	.	.	T	A	.	.	A	.	.	.	A
RGD838	.	.	.	.	.	.	.	.	.	.		.	G	.	.	.	.	.	.	.	-		-	.	.	.	.	.	.	.	.	.	.	.
RGD892	A	G	G	G	.	.	.	.	T	G		.	G	.	T	.	G	T	T	-	-		-	G	.	T	A	.	.	A	.	C	.	C

	590																				600									610		
WB0701	T	A	C	G	A	T	G	A	G	G		A	A	T	G	G	T	T	T	G	T		C	A	T	T	T	-	T	T	G	G
WB0702	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB0703	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB0704	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB0705	.	.	.	.	R	W	.	W	.	R		R	R	.	.	.	.	.	Y	R	W		.	-	.	.	A	-	.	.	.	W	
WB0708	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB0709	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB0801	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
PF060103	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
PF060121	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
PF060144	.	.	.	.	G	A	.	T	.	.		.	G	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	W	
PF190101	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
PF190106	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
SAN100	.	.	.	.	G	A	A	G	A	R		T	R	.	.	.	.	A	.	R	.		A	T	.	.	A	-	.	.	T	A	
casus	.	.	.	.	.	.	.	.	.	.		.	G	.	.	.	.	.	C	.	.		.	-	.	.	A	-	.	.	.	A	
WB1101	.	.	.	.	G	A	.	T	.	A		G	.	.	.	.	.	.	.	A	A		.	-	.	.	A	-	.	.	.	T	
WB1102	.	.	.	.	.	W	.	W	.	.		.	G	.	.	.	.	.	Y	R	W		.	-	.	.	A	-	.	.	.	W	
WB1103	.	.	.	.	G	A	.	.	.	A		G	G	.	.	.	.	.	C	A	A		.	-	.	.	A	-	.	.	.	T	
H. mephisto	.	.	.	A	T	A	A	.	A	T		T	.	.	.	.	.	.	.	.	.		T	-	.	.	.	-	.	.	.	A	
RGD838	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	C	A	.		.	-	.	.	A	-	.	.	.	A	
RGD892	.	G	A	.	.	.	A	.	T	.		T	G	.	.	.	.	.	.	.	.		T	.	.	A	A	C	.	.	A	A	

Figure 6.1. Alignment of two regions of SSU sequence that contain polymorphisms in all *Halicephalobus* spp. isolates except *H. mephisto*, RGD892, RGD838, WB0701 and *H. similigaster*. Dots represent bases that match the first sequence, '-' represent gaps. Sequence polymorphisms are indicated with W = A or T, R= A or G, Y= C or T.

```
                                                  620                                                  630                                                  640
WB0701          C A A T A C T A T A     A C T C T C G T - -     T A A G T A T G T A
WB0702          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB0703          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB0704          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB0705          Y R . . . . . . . .     W W . .     M Y . . . - -     . . .     R R R K T W .
WB0708          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB0709          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB0801          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
PF060103        T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
PF060121        T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
PF060144        Y . . . . . . . . .     T . . .     A C . . . - -     . . .     A G G . T A .
PF190101        T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
PF190106        T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
SAN100          C . T . . . . . . .     . . T C     A C . . . - -     . . . .     G G . T G .
casus           C G . . . . . . . .     . . . .     C . . . . - -     . . .     A G R G T . .
WB1101          T . . . . . . . . .     T . . .     A C . . . - -     . . . .     A G . T A .
WB1102          Y R . . . . . . . .     W W . .     M Y . . . - -     . . .     R R R K T W .
WB1103          T G . . . . . . . .     T T . .     A C . . . - -     . G . .     G G G T G .
H. mephisto     . . . . . . . . . .     . A T .     T G T . - - -     . . .     T A . A A . .
RGD838          T G . . . . . . . .     . A . .     . . . . . - -     . . . . . . . . . .
RGD892          . . . . . . . . . .     . C A .     A . . T C A A     A . . .     A G A T A .
                                                  650                                                  660                                                  670
WB0701          T A T - G T A G A A     A T A C A - - - T A     T C A T A C A G T T
WB0702          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB0703          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB0704          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB0705          . . . G K . K A . W     R M C . - - - - W W     W W M Y Y Y . . . .
WB0708          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB0709          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB0801          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
PF060103        . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
PF060121        . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
PF060144        . . . G T . G A . T     . C C . . - - - . .     . A M C Y Y . . . .
PF190101        . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
PF190106        . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
SAN100          . . . G . . T A . T     . C C . . - - - . .     . A - C Y . . . . .
casus           . . . - . . G . . .     . G . - . - - - . .     . A C . C T . . . .
WB1101          . . . G . . T A . T     . C C . . - - - . .     . A . C T . . . . .
WB1102          . . . G . . T A . W     . C C . . - - - . .     . A . C T . . . . .
WB1103          . . . A . . G A . T     . A C T . - - - . .     C A C . C A . . . .
H. mephisto     . G . A . . T T . T     T . . T . A A T . .     C A T A T T . . . .
RGD838          . . . - . . . . . .     . . . . . . . - - -     . . . . . . . . . .
RGD892          . G . T T . T A . C     . - - - - - - - - .     . A T C T . . . . .
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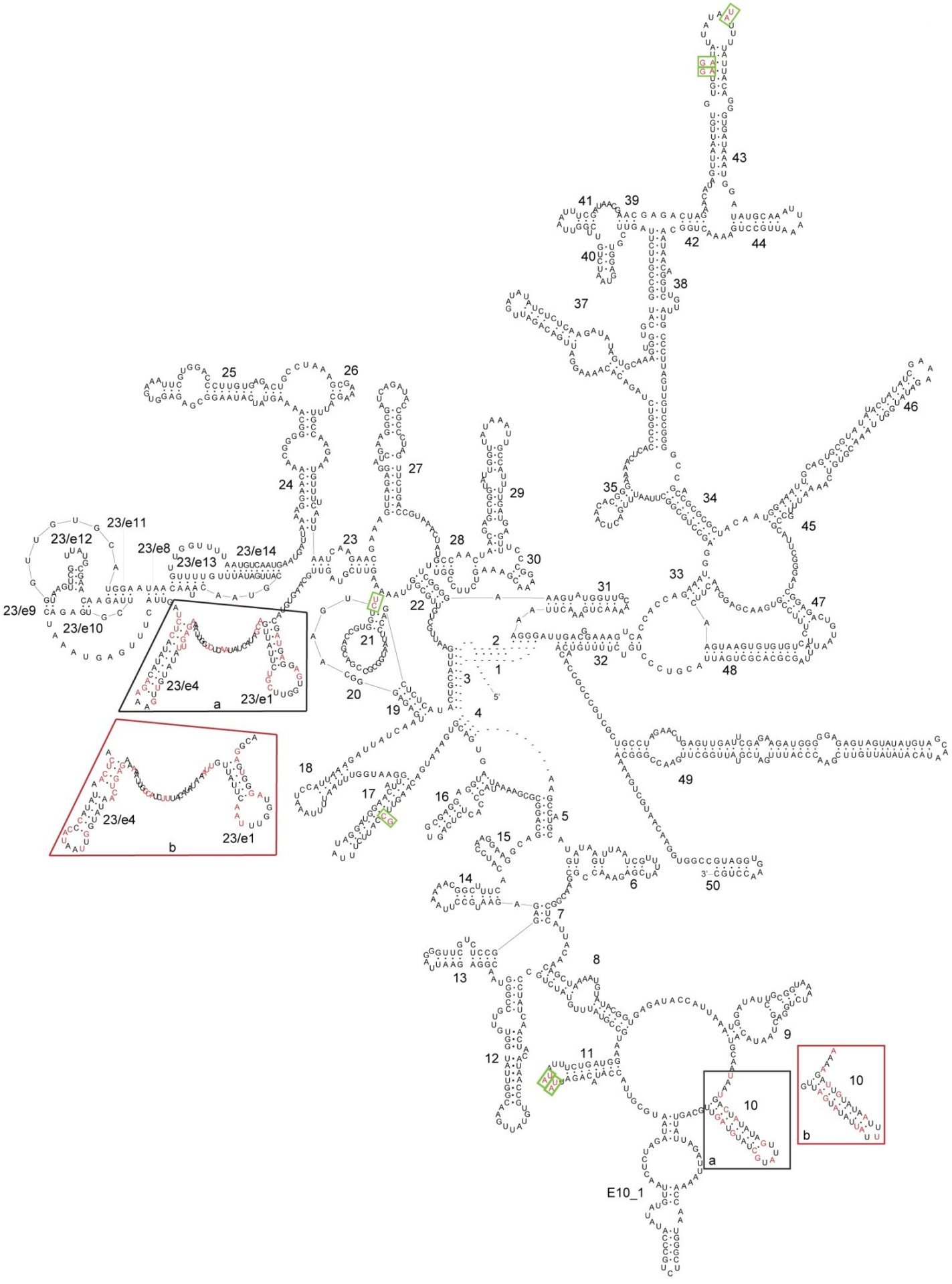
Figure 6.1 continued. Alignment of two regions of SSU sequence that contain polymorphisms in all *Halicephalobus* spp. isolates except *H. mephisto*, RGD892, RGD838, WB0701 and *H. similigaster*. Dots represent bases that match the first sequence, '-' represent gaps. Sequence polymorphisms are indicated with W = A or T, R= A or G, Y= C or T.

Figure 6.2. Secondary structure model of the partial SSU rRNA sequence of *H. gingivalis* WB0705. Numbers correspond to the helices next to them, following the nomenclature proposed by Wuyts *et al*. (2001). Variable positions of the rRNA molecule are indicated in red inside coloured boxes: green boxes represent single nucleotide polymorphisms (SNP), whereas the two polymorphic regions are shown in black boxes, representing the predominant homogenous sequence, with the alternative sequence in red boxes.


23/e11
23/e12
23/e8
23/e13
23/e14
23/e9
23/e10
23/e4
23/e1
a
b
E10_1
5'
3'
1
2
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To further characterize different ribotypes, the secondary structure model of the SSU rRNA of *H. gingivalis* isolate WB0705 was predicted (fig. 6.3). It revealed that the two polymorphic regions were located in three helices, *i.e.*, 10, 23/e1 and 23/e4. Both alternative homogenous sequences, were not equally distributed. The predominant sequences (black box) were observed in 7 out of 9 clones of a single specimen for regions 23/e1 and 23/e4 and in 8 out of 9 clones of a single specimen for region 10. Alternative homogenous sequences resulted in different predictions of the secondary structure, for the subdominant alternative (red boxes) this resulted in a decreased size of the terminal loop in helix 10, a slightly different length of the stem in helix 23/e1, and an increased size of the internal loop in helix 23/e4.

Theoretically, as two sequences were possible for the first region (corresponding to helix 10) and two for the second region (corresponding to helices 23/e1 and 23/e4, in short helix23), there were four putative combinations of polymorphic regions, and therefore four conceivable ribotypes, which were, however, not all found in the present dataset. Within all available clones of WB0705 (9 clones from one specimen complemented with two clones from other specimens) revealed the presence of only three ribotypes, *i.e.*, combinations 10a and (23/e1,e4)a, 10a and (23/e1,e4)b, and 10b and (23/e1,e4)b in 8, 2 and 1 out of 11 clones, respectively.

Phylogenetic inference

Based on ALISCORE, in the SSU and D2D3 alignment, respectively 12 and 14 positions were indicated as ambiguous and therefore omitted from the alignment. Additionally, 43 polymorphic positions were omitted from the SSU alignment since their presence or absence can depend on the chosen individual (see higher) and this could erroneously be observed as fixed differences between the isolates when based on one sequence. The COI and ND4 alignments were retained without modification. Substitution saturation tests on the resulting alignments, including screening of all codon positions for COI and ND4, did not reveal homoplasy, as the index of substitution saturation (I_{ss}) were significantly ($p < 0.05$) smaller than the critical index of substitution saturation ($I_{ss.c}$) for symmetrical and asymmetrical topologies. Saturation plots, *i.e.*, transitions and

transversions over divergence, revealed near linear relationships for all loci, confirming no saturation had occurred. Final alignment lengths are listed in table 6.2.

Both maximum likelihood (ML) and Bayesian (BI) methods of phylogenetic inference yielded trees with a limited resolution, however, tree topologies for each locus were not contradictory (fig. 6.3). *Procephalobus* JU169 was used as outgroup for the rDNA phylogenies. Based on SSU, *H. mephisto* had a maximally supported sister relationship with all other *Halicephalobus* isolates; termite isolate RGD892 was sister to the remaining *Halicephalobus* isolates (100 BS, 0.96 PP); and RGD838 and WB0701 formed a well supported clade. Phylogenies based on the D2D3 expansion segment showed maximal support for the relationship of SAN100 and CaseReportBelgium, the only two clinical isolates in this study. Since sequence analysis of *Procephalobus* JU169 was not successful for COI and ND4, *H. mephisto* was chosen as outgroup for the mtDNA phylogenies based on its highly supported sister relationship with all other *Halicephalobus* isolates in the phylogeny based on SSU. Both mitochondrial genes provided maximal support for a *H. gingivalis*-clade, with two distinct lineages: lineage I encompassing WB0708, 0801, 1101, 1102, SAN100, CaseReportBelgium, PF060103 and PF060144, and a second lineage (II) encompassing isolates WB0702, 0703, 0704, 0705, 0709, PF060121, 190101 and 190106.

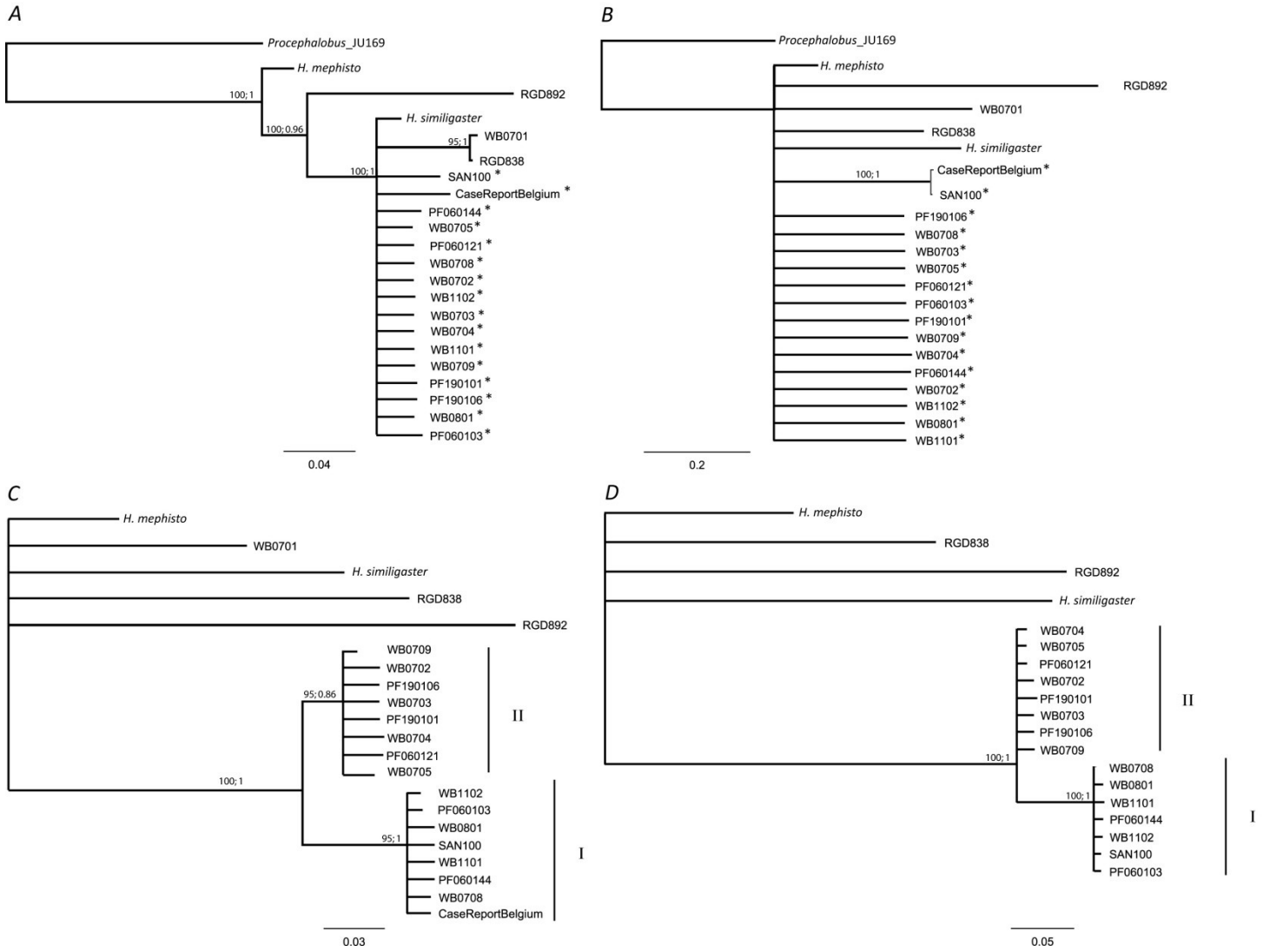

Figure 6.3. Monogenic phylogenies of SSU (*A*), D2D3 expansion segment (*B*), COI (*C*) and ND4 (*D*). Values above branches represent RAxML bootstrap support and Bayesian posterior probabilities, respectively. Branches were collapsed if bootstrap or posterior probabilities values were less than 85% and 0.95, respectively. SSU sequences originally revealing polymorphic positions, are indicated with an asterisk (*). Scale bar denotes nucleotide substitutions per site.

Table 6.3. Range of divergences of SSU (above diagonal) and D2D3 (below diagonal) sequences expressed as percentages. Isolates assigned to lineage I and II are based on mtDNA phylogenies

	Procephalobus JU169	*H. mephisto*	RGD892	RGD838	WB0701	*H. similigaster*	*H. gingivalis* lineage I	*H. gingivalis* lineage II
Procephalobus JU169		18.9	22.9	21.3	21.1	21.4	21.5 - 23.6	21.5 - 22
H. mephisto	25.9		12.3	8.3	8.4	7.8	8.0 - 10.3	8.0 - 8.6
RGD892	30.5	24.2		11.9	12.0	12.0	11.8 - 14.1	11.9 - 12.3
RGD838	33.2	17.9	24.7		0.4	3.8	2.7 - 6.0	2.8 - 3.2
WB0701	32.9	22.0	25.7	16.7		4.1	3.1 - 6.3	8.2 - 8.6
H. similigaster	32.4	19.6	23.2	12.8	15.5		2.3 - 5.8	2.4 - 2.8
H. gingivalis lineage I	31.5 - 32.4	17.2 - 18.4	23.5 - 25.2	9.7 - 11.4	14.5 - 17.7	9.7 - 12.3		0 - 4.1
H. gingivalis lineage II	31.5 - 32.2	17.2 - 17.9	22.8 - 23.5	10.4 - 10.7	15.3 - 15.5	9.7 - 10.2	0 - 5.8	

Table 6.4. Range of divergences of COI (above diagonal) and ND4 (below diagonal) sequences expressed as percentages. Divergences within lineages I and II are indicated in grey boxes, above and below diagonal line for COI and ND4, respectively.

	H. mephisto	RGD892	RGD838	WB0701	*H. similigaster*	*H. gingivalis* lineage I	*H. gingivalis* lineage II
H. mephisto		10.1	8.2	7.6	8.8	8.6 - 8.9	7.2 - 7.6
RGD892	15.1		7.2	8.9	9.3	7.2 - 7.4	8.6 - 9.1
RGD838	14.6	14.2		8.0	7.4	8.0 - 8.2	7.0 - 7.6
WB0701	n.a.	n.a.	n.a.		6.8	7.0	6.6 - 7.0
H. similigaster	16.6	13.0	13.4	n.a.		7.8	6.4 - 7.2
H. gingivalis lineage I	15.9 - 16.2	14.8 - 15.1	12.1 - 12.4	n.a.	11.2 - 11.5	0.2 / 0.9	4.3 - 4.8
H. gingivalis lineage II	16.2 - 16.6	14.4 - 15.1	11.5 - 11.9	n.a.	10.8 - 11.5	4.0 - 4.5	0.9 / 0.6

SSU, D2D3, COI and ND4 sequence divergences expressed as percentages are listed in tables 6.3 and 6.4. The SSU divergence between *H*. *mephisto vs*. *Procephalobus* (18.9%) as opposed to that of *H. mephisto vs*. all other taxa (7.8 – 12.3%) lends support to the position of *H. mephisto* within the genus *Halicephalobus*. The COI dataset revealed a maximal divergence of 0.6% and 0.9% within lineage I and II, respectively,

whereas the divergence between the lineages ranged from 4.3-4.8%. Sequence divergences between these two lineages and all other taxa ranged from 6.4-10.1%. ND4 sequence differences revealed a similar pattern, *i.e*., a maximal sequence divergence of 0.2% and 0.9% within lineages I and II, respectively, a divergence of 4-4.5% between these two lineages, and a divergence of 10.8-16.6% between these two lineages and all other isolates.

A monogene species phylogeny based on *Halicephalobus* isolates used in the present study complemented with D2D3-only isolates from GenBank sequences was constructed to investigate the possible relationship of clinical isolates. GenBank sequences included both free-living, *i.e.*, AY294178 and AY294181, and clinical isolates of *H. gingivalis* obtained from equine infections, *i.e.*, AY294179, 80, 82 (Nadler *et al*. 2003), AB288935 (Akagami *et al*. 2007), and JX194163 (Rodriguez *et al*. 2013). Both ML and BI analyses rendered similar trees with a limited resolution, however, showing a highly supported relationship for most clinical isolates with the exception of the Japanese isolates AB288935 and AY294179. However, this cluster of clinical isolates also includes one free-living isolate obtained from compost (JB128 = AY294181).

Finally, a concatenate analysis of the four genetic markers, and based on ML and BI, rendered highly concordant, well resolved trees with high branching support (fig. 6.6). For both phylogenetic inference methods, *H. mephisto* had a maximally supported sister relationship with all other *Halicephalobus* isolates, whereas the termite isolate RGD892 was the second most early diverging taxon (89 BS, 1 PP). The other termite isolate had a well supported relationship with WB0701, an isolate originating from a manure heap at a horse stable (97 BS, 1 PP). This isolate was the only horse associated isolate that did not fall within the *H*. gingivalis-clade. *H. similigaster* had a well supported sister relationship with the *H*. *gingivalis*-clade. This clade was further separated into two highly supported distinct lineages (100 BS, 1 PP), referred to as '*H*. g*ingivalis* lineage I', *i.e*., encompassing WB0708, 0801, 1101, 1102, PF060103, 060144, SAN100 and CaseReportBelgium with a maximally supported relationship (100 BS, 1 PP) for the latter two isolates, and '*H*. *gingivalis* lineage II' (100 BS, 1 PP), *i.e.*, encompassing WB0702, 0703, 0704, 0705, 0709, PF060121, 190101 and 190106. The two *H. gingivalis* lineages did not correspond with geographical origin and only partly with habitat: lineage I

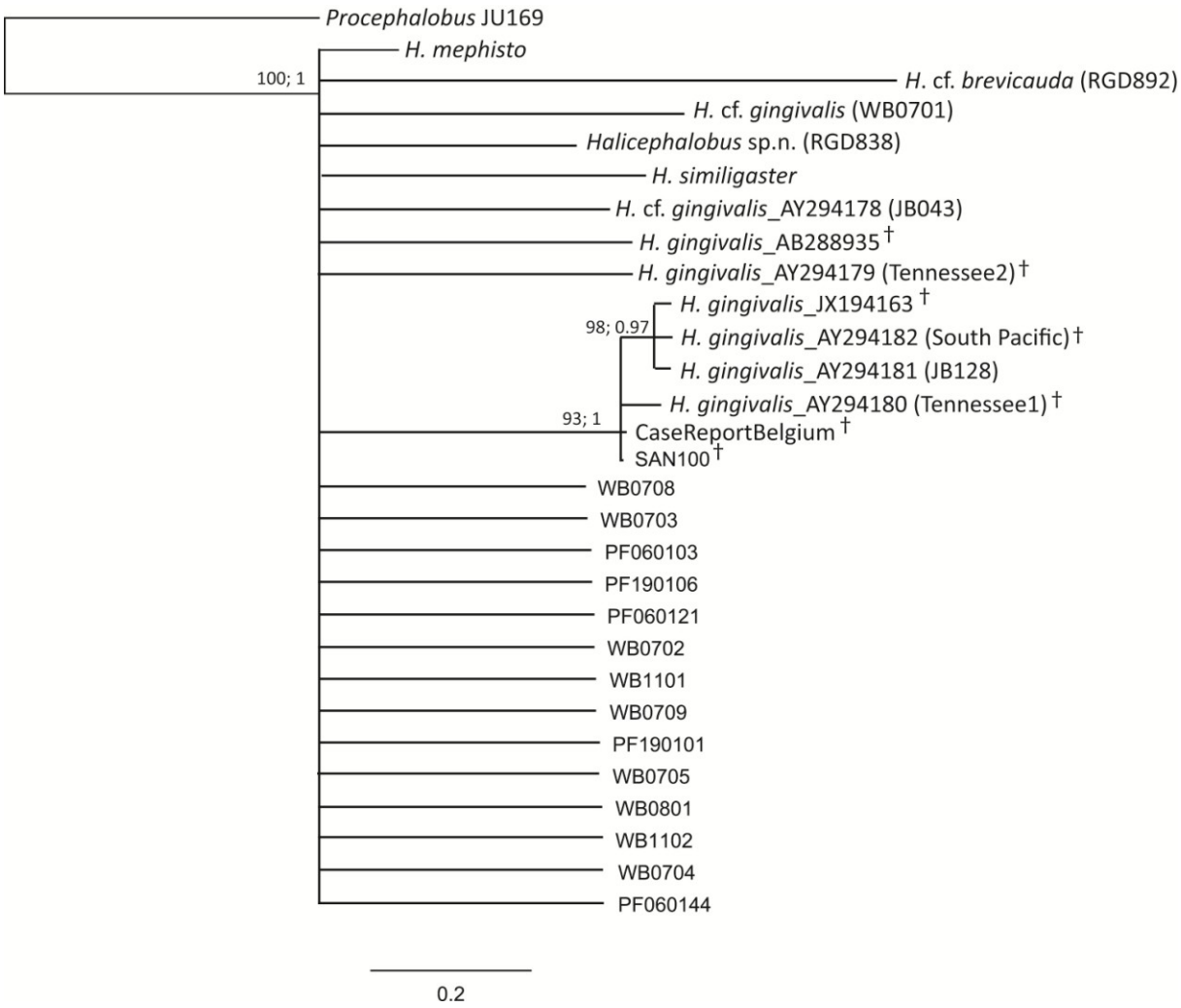

Figure 6.4. Monogene species phylogeny of D2D3 expansion segment including *Halicephalobus* isolates of the present study complemented with GenBank sequences of both free-living (JB043 and JB128) and clinical isolates (AY294179, 80, 82, JX194163, and AB288935) of *H. gingivalis*. Values above branches represent RAxML bootstrap support and Bayesian posterior probabilities, respectively. Branches were collapsed if bootstrap support or posterior probability values were less than 85% and 0.95, respectively. All clinical isolates are indicated (†). Scale bar denotes nucleotide substitutions per site.

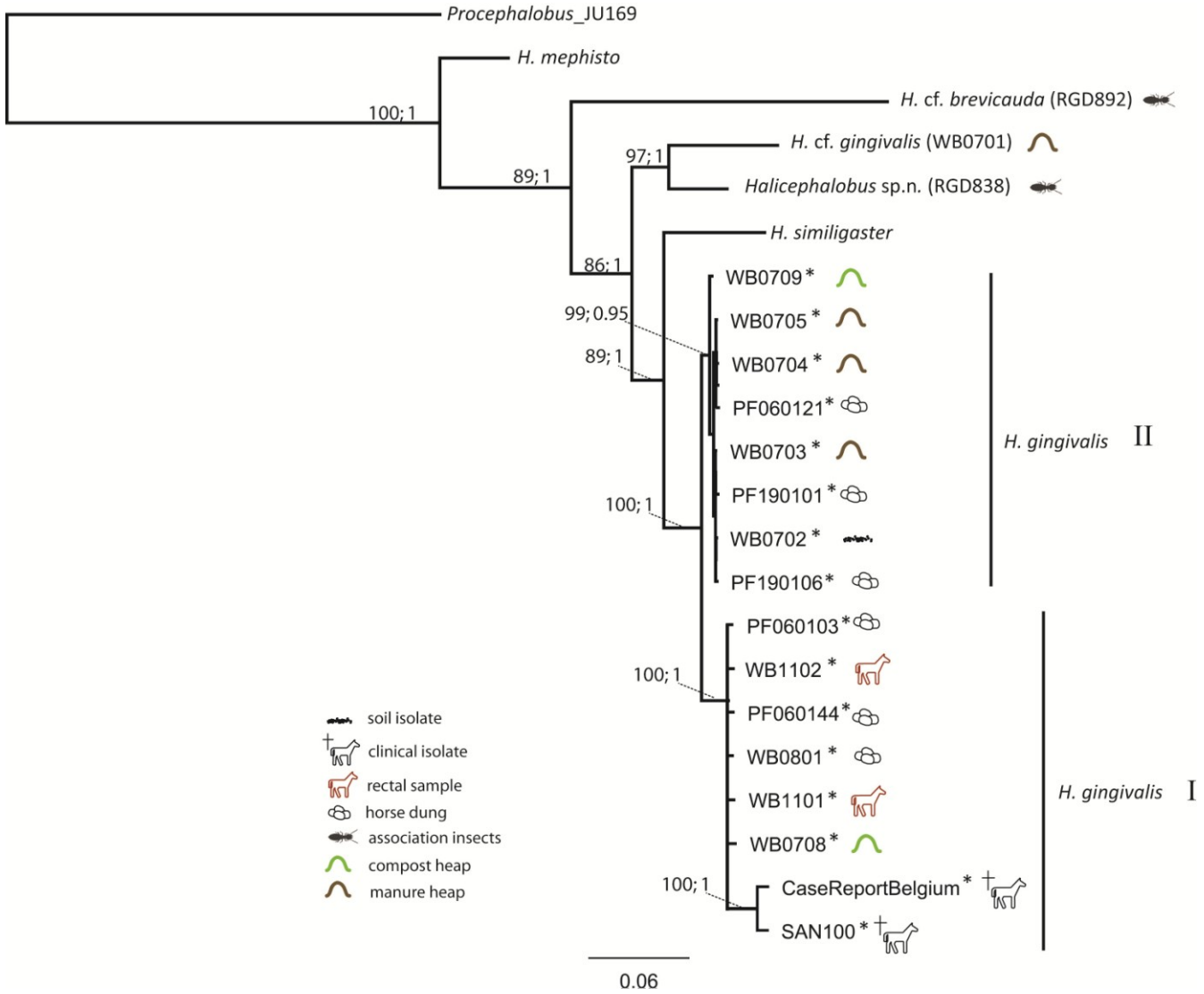

Figure 6.6. Phylogeny of concatenated data based on ML and partitioned Bayesian inference using the GTR + Γ+ I model of evolution. Values above branches or indicated with a dashed line represent RAxML bootstrap support and Bayesian posterior probabilities, respectively. Branches were collapsed if bootstrap support or posterior probability values were less than 85% and 0.95, respectively. The origin of each isolate is schematically represented. Isolates originally containing polymorphic positions in their SSU sequences, are indicated with an asterisk (*). Isolates RGD892, RGD838 and WB0701 are named based on morphological data. Scale bar denotes nucleotide substitutions per site.

comprised all clinical isolates and rectal samples but also included compost isolates (fig. 6.4, annotation on tree), while lineage II comprised both compost isolates and manure isolates, but no clinical isolates. Remarkably, all *H. gingivalis* isolates have polymorphic positions in their SSU DNA sequences, but this apomorphy is absent in all other *Halicephalobus* species.

Measures for species distinctiveness

The two *H. gingivalis* lineages indicated by concatenated analysis contained multiple haplotypes and could therefore be tested using the different species distinctiveness measures (table 6.5). Reciprocal monophyly was indicated for both lineages by Rosenberg's P(AB) ($p < 10^{-5}$). This was partially corroborated by Rodrigo's P(RD) which indicated complete taxonomic distinctiveness for lineage II ($p < 0.05$) but not for lineage I ($p = 0.95$). The extent of lineage divergence as indicated by the *gsi* was maximal (= 1) for lineage II, revealing monophyly, and high (0.77) for lineage I. Both *gsi*-values were significant after Bonferroni correction ($p < 0.05/2 = 0.025$). In conclusion, all measures indicated species distinctiveness for lineage II, whereas for lineage I, only P(RD) was not significant and the *gsi*-value indicated incomplete lineage divergence.

Table 6.5. Measures for species distinctiveness: Rosenberg's P(AB), *i.e.*, the degree of reciprocal monophyly, Rodrigo's P(RD), *i.e.*, the probability that a clade has the observed degree of distinctiveness, and the genealogical sorting index (*gsi*) and its associated *p*-value (significant at $p < 0.025$ after Bonferroni correction). Clade support: bootstrap (BS) and posterior probability (PP). Shaded values indicate species distinctiveness.

	H. gingivalis	
	lineage I	lineage II
Rosenberg's P(AB)	$1.0\ e^{-5}$	$1.0\ e^{-5}$
Rodrigo P(RD)	0.95	< 0.05
gsi	0.77	1
gsi p-value	< 0.0001	< 0.0006
clade support (BS, PP)	100, 1	0.99, 0.95

Finally, calculations of speciation in accordance with the 4x rule, specifically designed for delimiting species in non-sexual organisms, for both COI and ND4 are presented in table 6.6 and reveal that *K* is well over 4 times θ (*K*/θ > 4) for both *H. gingivalis*-lineages based on both COI and ND4.

Table 6.6. Calculations of speciation between *H. gingivalis* lineages I and II in accordance with the 4x rule for species delimitation in non-sexual organisms.

	H. gingivalis	Nucleotide diversity (π)	θ [=π/(1-4*π /3]	Sequence divergence between lineages (*K*)	*K* /θ
COI				0.04645	
	lineage I	0.002501	0.00251		18.5
	lineage II	0.004655	0.00468		9.9
ND4				0.043018	
	lineage I	0.000642	0.000643		66.9
	lineage II	0.004414	0.004440		9.7

MORPHOLOGICAL ANALYSIS

Scanning electron microscopy

Except for *H. mephisto,* scanning electron microscopy (SEM) did not reveal any substantial differences between the studied isolates (fig. 6.7). Cuticle with fine annulations, approximately 1 µm wide at mid body. Lateral field with two outer lines. Head continuous with body and lip region continuous with neck. Oral aperture hexagonal and strengthened by a thin ridge. Six lips not clearly separated and orientated along the sides of the hexagon. One circle of six inner labial papillae, with one papilla on each lip, and one circle of four cephalic papillae, with one on each subventral and subdorsal lip. Amphid aperture round to oval and situated on both lateral lips. Slightly protruding vulva. Anus well developed, slit-like. Phasmids posterior to anus. Tail elongated, conical, slightly curved ventrally, tapering to pointed terminus with a terminal mucro.

H. mephisto differs from all other *Halicephalobus* spp. isolates by the presence of a triangular oral aperture (*vs*. a hexagonal oral aperture) and the absence of a mucro at the tail tip (fig. 6.8).

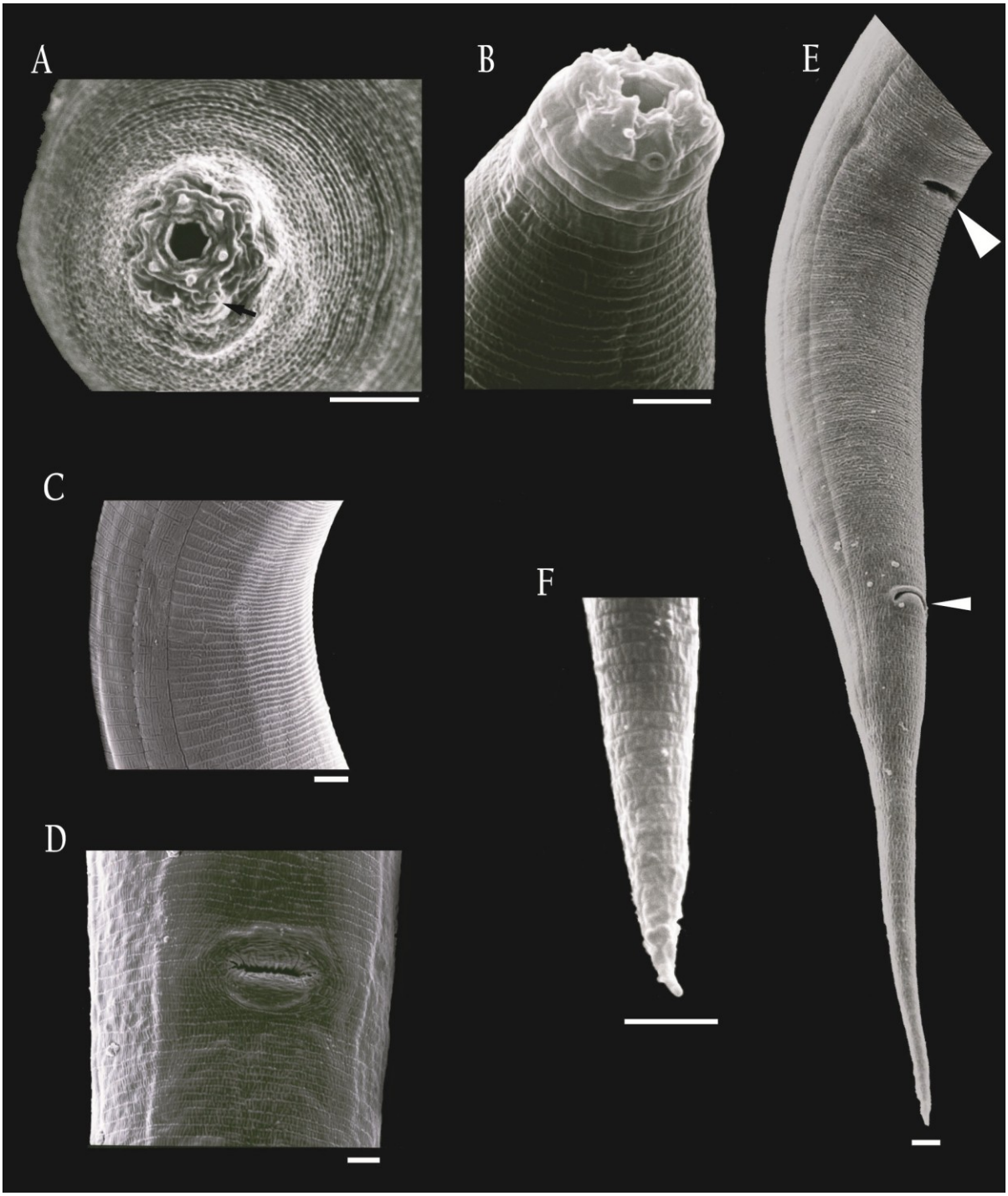

Figure 6.7. SEM photographs of *Halicephalobus gingivalis*. A: En face view of head region showing hexagonal oral aperture lined with thin ridge, six fused lips with labial papillae, and cephalic papillae (arrow); B: Lateral view head region, showing amphid aperture on lateral lip; C: lateral field at mid body; D: ventral view vulva; E: Lateral view posterior body region, with vulva (large arrowhead) and anus (small arrowhead); F: tail tip with terminal mucro. Scale bars = 2 µm.

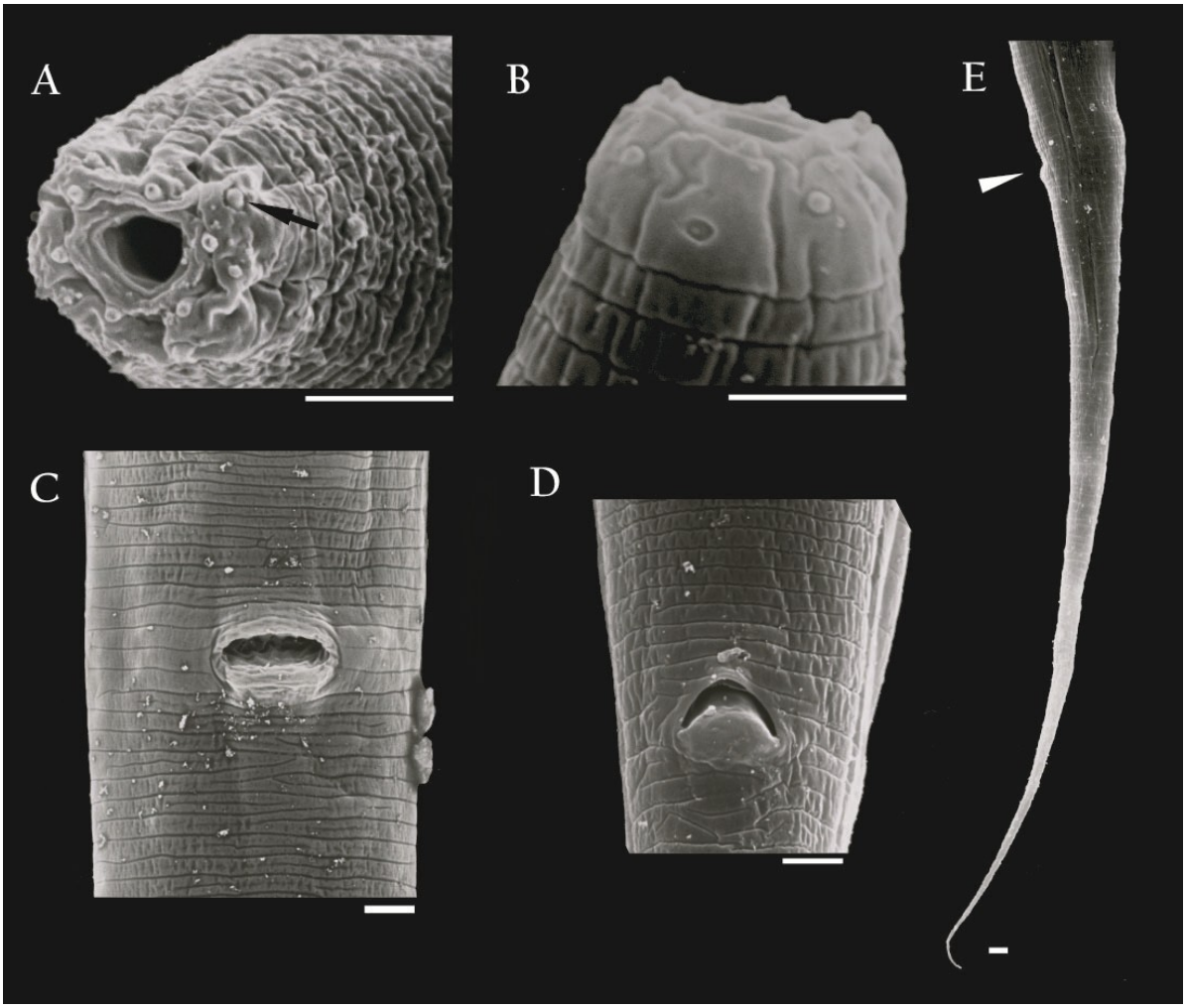

Figure 6.8. SEM photographs of *Halicephalobus mephisto*. A: En face view of head region showing trigonal oral aperture lined with thin ridge, six fused lips with labial papillae, and cephalic papillae (arrow); B: Lateral view head region, showing amphid aperture on lateral lip; C: Ventral view vulva; D: Ventral view anus; E: Tail region, arrowhead indicates position of anus. Scale bars = 2 µm.

Morphometrical analysis

The 28 measurements and ratios (Appendix to Chapter 6) are based on ten specimens for each *Halicephalobus* spp. isolate, with three replicates (3 X 10 individuals). A correlation matrix including all morphometrical characters selected 7 morphometrics (listed in table 6.7) that are not significantly related with other characters ($p > 0.05$, $r < 0.75$). Based on these 7 characters, a forward stepwise DFA identified the variables that best discriminate between *a priori* defined groups, *i.e*., phylogenetically defined taxa or lineages (see tree, fig. 6.6): *H. mephisto*, *H. similigaster*, RGD892, RGD838, WB0701 and

H. *gingivalis* lineages I (including isolates WB1101, 1102, 0707, 0708, 0801, SAN100) and II (including WB0702, 0703, 0705, PF190101, PF060121).

Table 6.7. Morphometrics with low correlation of *Halicephalobus* spp. isolates (n = 30). Measurements are given in μm and are represented as mean ± standard deviation with (ranges) and coefficient of variation expressed as percentage.

	WB0701	WB0702	WB0703	WB0704	WB0705	WB0708	WB0709	WB0801
stoma length	7,1 ± 0,4 (6,2 - 8,2) 6	7,7 ± 0,3 (7,1 - 8,3) 4	7,8 ± 0,4 (7,0 - 8,6) 6	7,0 ± 0,3 (6,1 - 7,6) 5	7,8 ± 0,5 (7,1 - 9,1) 6	7,9 ± 0,4 (6,8 - 8,6) 5	8,2 ± 0,5 (7,3 - 9,1) 6	7,4 ± 0,4 (6,1 - 8,0) 5
a	15,5 ± 0,8 (13,8 - 17,5) 5	16,5 ± 1,2 (12,0 - 18,1) 7	15,2 ± 0,6 (14,3 - 16,8) 4	15,6 ± 0,9 (14,1 - 17,7) 6	16,8 ± 1,1 (15,2 - 19,8) 7	15,2 ± 1,0 (12,9 - 17,2) 7	16,0 ± 0,5 (15,2 - 17,6) 3	16,7 ± 0,8 (15,0 - 18,6) 5
c'	4,8 ± 0,4 (4,1 - 5,6) 8	4,9 ± 0,4 (4,0 - 6,0) 9	4,3 ± 0,3 (3,8 - 5,0) 6	4,7 ± 0,4 (4,0 - 5,5) 8	4,1 ± 0,4 (3,3 - 4,8) 9	4,0 ± 0,4 (2,8 - 4,6) 9	4,3 ± 0,2 (3,4 - 4,7) 6	4,8 ± 0,4 (4,1 - 5,5) 7
ratio corpus/isthmus	2,4 ± 0,2 (1,9 - 2,8) 8	2,7 ± 0,2 (2,3 - 2,9) 6	2,8 ± 0,2 (2,4 - 3,2) 7	2,4 ± 0,2 (2,2 - 2,8) 6	2,7 ± 0,2 (2,3 - 3,2) 8	2,7 ± 0,2 (2,4 - 3,2) 8	2,6 ± 0,2 (2,2 - 3,3) 9	2,5 ± 0,1 (2,1 - 2,8) 6
ratio ovary (post-v/pre-v)	0,7 ± 0,1 (0,6 - 0,8) 8	0,7 ± 0,1 (0,5 - 0,8) 9	0,7 ± 0,1 (0,6 - 0,8) 7	0,7 ± 0,1 (0,5 - 0,8) 8	0,8 ± 0,1 (0,6 - 1,3) 19	0,6 ± 0,1 (0,5 - 0,8) 11	0,6 ± 0,1 (0,5 - 1,0) 14	0,6 ± 0,1 (0,5 - 0,8) 11
V'	70,8 ± 1,4 (65,9 - 73,6) 2	68,1 ± 0,7 (66,2 - 69,1) 1	69,4 ± 1,4 (65,6 - 72,3) 2	70,1 ± 0,8 (69,1 - 72,4) 1	69,9 ± 1,0 (68,1 - 71,9) 1	69,7 ± 1,4 (67,9 - 73,1) 2	69,1 ± 1,0 (67,9 - 71,5) 1	69,5 ± 0,9) (68,1 - 71,0) 1
(VA/2) / post-v	1,0 ± 0,1 (0,9 - 1,1) 7	1,0 ± 0,1 (0,9 - 1,2) 7	0,9 ± 0,1 (0,8 - 1,1) 7	1,0 ± 0,1 (0,9 - 1,2) 7	0,9 ± 0,1 (0,7 - 1,1) 8	1,0 ± 0,1 (0,8 - 1,1) 8	1,0 ± 0,1 (0,7 - 1,3) 11	1,0 ± 0,1 (0,9 - 1,1) 7

	PF060121	PF190101	SAN100	WB1101	WB1102	*H. similigaster*	*H. mephisto*	RGD838	RGD892
stoma length	8,0 ± 0,5 (7,2 - 8,9) 6	7,8 ± 0,6 (6,6 - 9,0) 7	7,4 ± 0,5 (6,4 - 8,5) 7	8,3 ± 0,8 (6,9 - 9,6) 10	8,6 ± 0,8 (7,0 - 10,2) 10	8.8 ± 0.8 (7.2 - 9.8) 8	9.2 ± 0.9 (7.2 - 10.4) 9	7,2 ± 0,8 (5,6 - 8,6) 11	7.7 ± 0.6 (6,13 - 8,71) 8
a	18,6 ± 0,8 (17,0 - 20,2) 4	18,4 ± 1,7 (11,2 - 21,1) 9	16,1 ± 0,9 (14,3 - 18,5) 6	17,7 ± 1,1 (15,9 - 20,3) 6	19,5 ± 1 (16,9 - 21,5) 5	19.6 ± 2.5 (16.5 - 30) 13	19. ± 2.8 (14.3 - 26.2) 15	17,3 ± 1,1 (15,4 - 19,5) 6	14,9 ± 0,8 (13,4 - 16,7) 5
c'	4,7 ± 0,3 (4,1 - 5,7) 7	4,7 ± 0,4 (4,0 - 5,5) 8	4,6 ± 0,4 (3,3 - 5,3) 9	4,8 ± 0,7 (3,9 - 6,9) 14	5,4 ± 0,3 (4,8 - 5,8) 6	7.4 ± 0.8 (6.1 - 9.6) 11	8.1 ± 1.2 (6 - 11.3) 14	6,3 ± 0,5 (5,5 - 7,2) 8	3,4 ± 0,2 (2,9 - 3,9) 6
ratio corpus/isthmus	3,0 ± 0,3 (2,5 - 3,7) 9	2,9 ± 0,2 (2,4 - 3,2) 7	2,9 ± 0,2 (2,3 - 3,4) 8	3,0 ± 0,2 (2,6 - 3,6) 7	3,1 ± 0,3 (2,7 - 3,6) 9	2.1 ± 0.1 (1.9 - 2.4) 7	2.6 ± 0.3 (2.2 - 3.3) 11	2,1 ± 0,2 (1,9 - 2,5) 8	2,9 ± 0,2 (2,4 - 3,3) 6
ratio ovary (post-v/pre-v)	0,7 ± 0,1 (0,6 - 0,9) 11	0,7 ± 0,1 (0,4 - 0,8) 14	0,6 ± 0,1 (0,3 - 0,9) 17	0,7 ± 0,1 (0,5 - 1,1) 16	0,7 ± 0,1 (0,6 - 0,9) 10	0.7 ± 0.1 (0.4 - 1) 20	0.9 ± 0.4 (0.5 - 2.3) 41	0,5 ± 0,1 (0,3 - 0,8) 22	0,5 ± 0,07 (0,3 - 0,7) 15
V'	70,1 ± 0,8 (67,85 - 71,6) 1	69,3 ± 1,6 (67,6 - 76,0) 2	69,7 ± 1,0 (67,0 - 71,2) 1	69,1 ± 1,2 (66,1 - 70,8) 2	67,7 ± 0,9 (66,1 - 39,4) 1	70.1 ± 1.2 (68.7 - 73.7) 2	67.6 ± 2,2 (58.6 - 70.2) 3	70,1 ± 0,9 (68,4 - 71,8) 1	70,7 ±1,3 (65,7 - 72,9) 2
(VA/2) / post-v	0,9 ± 0,1 (0,8 - 1,1) 6	1,0 ± 0,2 (0,5 - 1,7) 18	1,0 ± 0,2 (0,8 - 1,8) 18	1,0 ± 0,1 (0,8 - 1,3) 11	1,0 ± 0,1 (0,8 - 1,1) 8	1.21 ± 0.3 (0.8 - 2.8) 28	1.1 ± 0.4 (0.53 - 1.9) 33	1,4 ± 0,3 (1,0 - 2,78) 24	1,4 ± 0,2 (0,9 - 2,0) 14

All pairwise comparisons showed significant morphometrical differences, except between the two *H*. *gingivalis* lineages ($p > 0.01$, table 6.8). The *H*. *gingivalis* lineages are most divergent from *H. mephisto* and *H*. *similigaster* (D^2= 56 and 42, respectively). The highest divergence overall was observed between RGD892 *vs. H. mephisto* and *H. similigaster* (D^2= 81 and 65, respectively) and the least, but significant, divergence was found between WB0701 and both *H*. *gingivalis* lineages. The canonical analysis visualized that both *H*. *gingivalis* lineages could not be separated based on morphometrical data, and that WB0701 and RGD892 were also plotted close to the *H*. *gingivalis* lineages (fig. 6.9).

Table 6.8. Squared Mahalanobis distances (D^2) (upper right, in bold) and *P*-values (lower left) between pre-defined groups based on the molecular analysis.

	H. mephisto	*H. similigaster*	RGD892	RGD838	WB0701	*H. gingivalis* I	*H. gingivalis* II
H. mephisto		**9,4**	**81**	**28**	**49**	**56**	**56**
H. similigaster	0,00		**65**	**12,1**	**32**	**42**	**42**
RGD892	0,00	0,00		**35**	**16,0**	**9,4**	**9,4**
RGD838	0,00	0,00	0,00		**15,1**	**25**	**24**
WB0701	0,00	0,00	0,00	0,00		**6,0**	**4,6**
H. gingivalis I	0,00	0,00	0,00	0,00	0,00		**0,2**
H. gingivalis II	0,00	0,00	0,00	0,00	0,00	0,03	

In a forward stepwise DFA based on the individual isolates of both *H. gingivalis* lineages and complemented with WB0701 and RGD892, the termite associated isolate RGD892 was found significantly ($p < 0.05$) different from all other isolates, whereas isolate WB0701 was morphometrically not discernible ($p > 0.05$) from WB0704, which was nested within *H*. *gingivalis* lineage II. Analysis further revealed significant morphometrical differences ($p < 0.01$) (data not shown) between most isolates within either *H. gingivalis* lineage, whereas some isolates from different lineages (WB0703 and WB0708) were morphometrically not discernible ($p > 0.01$). This high morphometrical variability within *H*. *gingivalis* did not correspond to the molecular based groupings, *i.e*., the two lineages.

Table 6.9. Comparison of morphometrical characters (ranges, in µm) of *H. brevicauda* Mavljanov 1976, derived from the original description, and RGD892, measured in the present study. Measurements based on drawings of one specimen (*), and on an unknown number of specimens (§) are indicated.

	H. brevicauda	RGD892
n	8	30
L	0.24 - 0.33	0.22 - 0.30
a	13 - 17.4	13.4 - 16.7
b	3.9 - 4.1	3.9 - 4.7
c	11.4 - 14.4	5.9 - 9
c'	2*	2.9 - 3.9
V	56 - 60.4	56.2 - 64.8
stoma	16	6.3 - 8.7

Subsequently, analysis of variance (one-way ANOVA) identified those morphometric characters that could significantly ($p < 0.05$) differentiate between *H. mephisto*, *H. similigaster*, RGD892, RGD838 and the *H. gingivalis*-clade. *H. mephisto* had a straight ovary *vs*. a dorsally reflected ovary in all other isolates, and a higher c' value (8.1 ± 1.2, mean ± standard deviation) due to its long tail with filiform terminus. Both *H. similigaster* and the termite isolate RGD838 had a lower ratio corpus/isthmus length (2.1 ± 0.1 and 2.1 ± 0.2, respectively) and a higher c' ratio than the *H. gingivalis*-clade, and differed from one another by their c' ratio (8.1 ± 1.2 and 6.3 ± 0.5, respectively). Since RGD838 does not resemble any species of the genus, it is proposed as *Halicephalobus* sp. n. The other termite isolate RGD892 could be characterized by its shorter total body length (266 ± 19) and low c' ratio (3.4 ± 0.2), resembling *H. brevicauda* MAVLJANOV 1976 and is therefore referred to as *H.* cf. *brevicauda*.

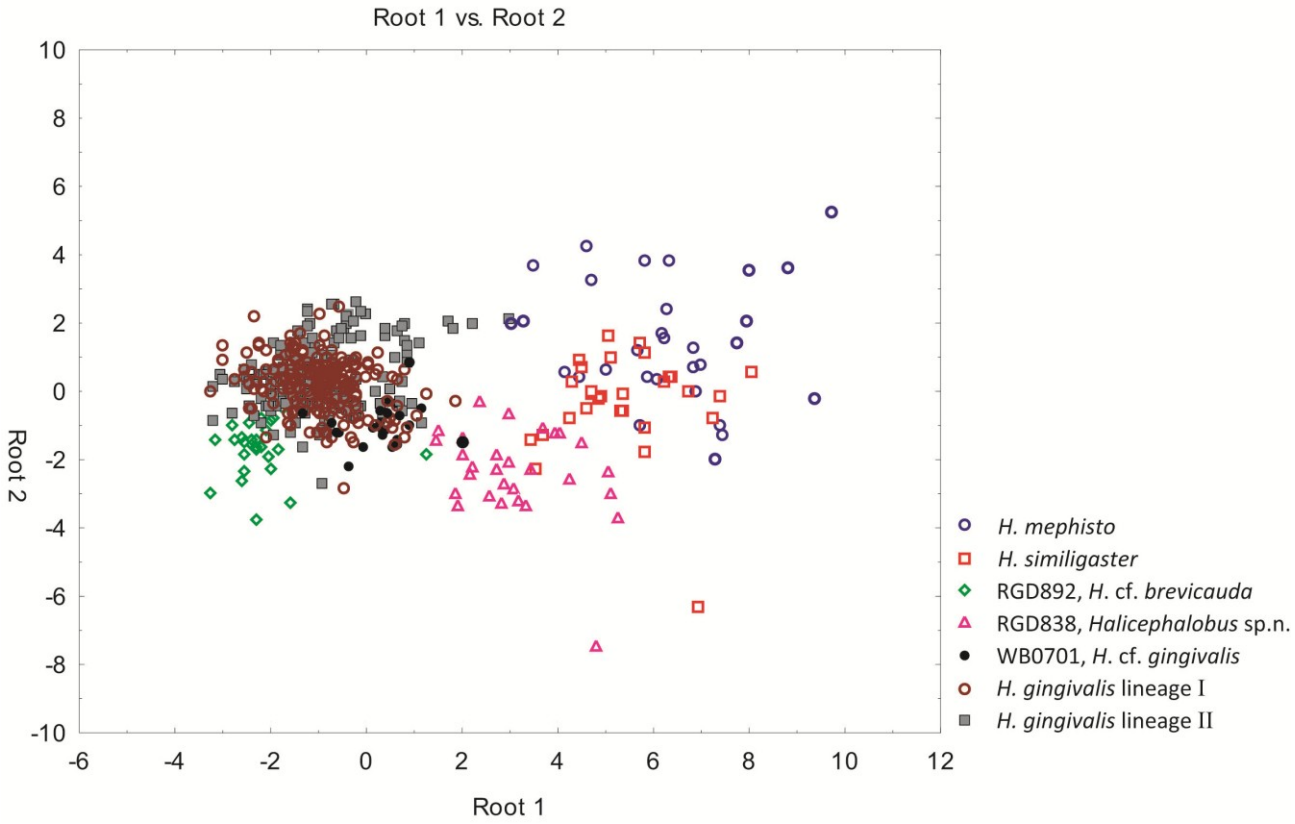

Figure 6.9. Scatterplot of the first two canonical scores obtained by forward stepwise DFA on the morphometrics retained based on their low mutual correlation.

DISCUSSION

ORIGIN AND POSSIBLE FUNCTION OF POLYMORPHISM IN RDNA

The ribosomal DNA genes have been used extensively as markers for inferring phylogenies since they can easily be amplified as a whole fragment and contain both variable and conserved regions. Although the eukaryotic ribosomal DNA (rDNA) array consists of several hundred tandem repeated copies, these appear nearly identical within a given organism, which has been attributed to concerted evolution (Arnheim 1983). Hence, intraspecific variation of the rDNA genes is expected to be minimal (Dover and Tautz 1986). However, more and more exceptions to this rule have emerged as intraspecific and intra-genomic rDNA variability have been reported in various organisms. For example, the flatworm *Dugesia* (*Schmidtea*) *mediterranea* contains two different types of SSU rDNA within single organisms with an overall sequence divergence

of 8% between the two, and of which only one seems to be transcribed to RNA (Carranza *et al.* 1996). Pillet *et al.* (2012) found both single nucleotide polymorphisms (SNP's) and polymorphisms concentrated in certain regions (defined as expansion segment polymorphisms or ESP's) in the SSU sequence of the foraminifer *Elphidium macellum*. Heterogeneity in the rDNA array, mostly found in the ITS region and then generally consisting of SNP's, has also been reported in different nematode taxa, *e.g.*, in *Meloidogyne* (Zijlstra *et al.* 1995; Blok *et al.* 1997; Hugall *et al.* 1999), *Heterodera* (Szalanski *et al.* 1997), *Belonolaimus* (Cherry *et al.* 1997), *Haemonchus* (Gasser *et al.* 1998; Heise *et al.* 1999), *Globodera* (Subbotin *et al.* 2000), *Nematodirus* (Heise *et al.* 1999; Nadler *et al.* 2000), and *Bursaphelenchus* (Cardoso *et al.* 2012).

Next to SNP's, the present study revealed 2 polymorphic regions (similar to the ESP's found in *E. macellum* (Pillet *et al.* 2012)) in the SSU sequence of isolates of the *H. gingivalis*-clade. This is a unique trait for the *H. gingivalis*-clade, as SNP's and polymorphic regions were not found in any of the other *Halicephalobus* isolates. Although intraspecific variation of the rDNA genes is expected to be, the transient existence of polymorphism among copies of the rDNA gene is considered intrinsic to the concerted pattern of evolution (Porazinska *et al*. 2009). However, since the traditional approach using PCR and direct sequencing results in a single (consensus) sequence from individual nematodes, such aberrant copies will most likely not be detected when present in low numbers. Using new high-throughput DNA sequencing technologies[6], different copies of the rDNA gene have been identified in nematodes before (Porazinska *et al*. 2009), albeit not to the extent that it is found here. Ribosomal DNA polymorphisms in *H. gingivalis* had previously been suspected in the LSU sequence of *H. gingivalis* (Nadler *et al.* 2003), however, the use of pooled individuals in that study precluded the possibility to determine whether they represented intra-individual heterogeneity or variation between individuals. The occurrence of polymorphisms in both sequenced strands of single individuals of *H. gingivalis*, reflects genuine sequence variants and not artefacts resulting from amplification, cloning or sequence procedures. Secondary structure modelling further revealed that the polymorphic regions directly affected the secondary structure of the expansion segment in which they occurred. Because both

[6] also referred to as next generation sequencing, in which single DNA molecules are sequenced in a massively parallel fashion in a flow-cel (Mardis 2008)

polymorphic regions had two possible sequence alternatives, 4 different ribotypes are theoretically possible. However, based on 20 clones of three *H. gingivalis* isolates only three ribotypes were identified in current study with a strong predominance towards the homogenous sequence combination 10a – (23/e1, e4)b. Theoretically, the analyses of additional clones of each isolate could reveal additional ribotypes and determine in which proportion they are present. Further, to assess whether all sequence variants are functional, as in *Plasmodium* (Gunderson *et al.* 1987; Rogers *et al.* 1996), it may be useful to directly sequence the ribosomal RNA (Sonnenberg *et al.* 2007).

Several hypotheses can be forwarded to explain the origin and function of the observed SSU heterogeneity in *H. gingivalis*. An important consideration in this discussion is the presumed non-sexual reproduction in *Halicephalobus*, as males have never been reported for any of its species (Stefański 1954; Andrássy 1984; Akagami *et al*. 2007). Current study corroborates non-sexual reproduction, since males have never been observed in over 3000 light microscopically observed specimens of 55 *Halicephalobus* isolates of different species, both from culture under different environmental conditions and from direct field samples. Generally, in mitotic non-sexual lineages in the absence of recombination, two alleles at any given locus begin to accumulate mutations independently from each other and subsequently diverge in their nucleotide sequence, which is called the Meselson-effect (Welch and Meselson 2000). Although this effect has been reported in bdelloid rotifers (Welch and Meselson 2000) and in *Meloidogyne* species (Lunt 2008), it is not common for all non-sexual species. Moreover, it is unlikely that the Meselson-effect is the cause of SSU rDNA heterogeneity as concerted evolution could still be effective in non-sexual lineages through mitotic recombination and gene conversion, thereby maintaining the homogeneity of rDNA (*e.g*., Birky 1996). However, several circumstances have been described in which the rDNA repeats can escape concerted evolution, thereby resulting in rDNA heterogeneity. An important assumption for evolution under the concerted evolution model is the organization of genes in tandem arrays (Pillet *et al*. 2012). Recombination and gene conversion occur less frequently between sequences on heterologous chromosomes than on homologous chromosomes (Ironside 2013), therefore, in the rare case that the repeats are not strictly organized in tandem arrays and are dispersed throughout the genome, concerted evolution acts less strongly, as was suggested for the 5.8S rDNA

heterogeneity in the amphibian *Xenopus laevis* (*e.g.*, Peterson *et al.* 1980) and the loach fish *Misgurnus fossilis* (Mashkova *et al.* 1981). DNA heterogeneity can also be caused by polyploidization, which has on several occasions been described in nematodes, *e.g.*, in *Meloidogyne* (Triantaphyllou and Hirschmann 1997). In case of polyploidization concerted evolution is restricted to each set of chromosomes that are encoding for a different ribotype, as was suggested for the microsporidian *Nosema bombi* (O'Mahony *et al.* 2007). Other proposed explanations for rDNA heterogeneity are inter-specific hybridisation events, as suggested for *Meloidogyne* (Lunt 2008; Fargette *et al.* 2010) and the foraminifer *E. macellum* (Pillet *et al*. 2012). However, the chromosome state within *Halicephalobus* has not been established and unless non-sexual reproduction has evolved independently several times within the genus *Halicephalobus*, it seems unlikely that inter-specific hybridisation forms the basis for the presence of the polymorphic regions in the *H. gingivalis*-clade.

Although the origin of the SSU heterogeneity in *Halicephalobus* is uncertain, it is remarkable that only the isolates of the *H. gingivalis*-clade, including facultative parasites, show these polymorphic regions in their SSU rDNA. Given that in *Plasmodium* the expression of two distinct types of SSU rDNA has been shown to be developmentally regulated (*i.e.*, correlated to discrete stages of sporozoite development), linked to different parasitic life stages, *i.e.*, one predominantly expressed in the mosquito host and the other in the mammalian host (Gunderson *et al.* 1987; Li *et al.* 1994; Rogers *et al.* 1996), and possibly related to functional differences between ribosome types or mechanisms of transcriptional control, it is possible that the presence of alternating life histories is the key to understand the presence of the SSU heterogeneity. Hence, this trait could facilitate the survival of a single population of *H. gingivalis* in extremely different habitats and consequently enables it to be opportunistically parasitic.

USING BOTH MOLECULAR AND MORPHOLOGICAL DATA TO INFER TAXONOMIC STATUS

Regardless of the used method of phylogenetic inference, the concatenated analysis of 2 rDNA genes and 2 mtDNA genes rendered a highly concordant topology with high support (>85% RAxML bootstrap values and >0.95 Bayesian posterior probability) along most branches, suggesting that a sufficient number of genes are concatenated to

elucidate the poorly supported single gene signals (Rokas *et al.* 2003). In non-sexuals, the evolutionary divergence of mtDNA and nuclear DNA is expected to be similar since they are both inherited clonally (Barraclough *et al.* 2003). However, results show that on average, mtDNA divergences are consistently lower than rDNA divergences in *Halicephalobus* species. A similar low, but strongly structured, mtDNA diversity against a higher nuclear diversity has been suggested for some polyploid parthenogenetic lineages of *Meloidogyne* and has been attributed to multiple, possibly hybrid, origins from closely related females (Hugall *et al.* 1999). However, since the ploidy of the different species of *Halicephalobus* has not been established, no such conclusions can be drawn in the present study.

All phylogenetically supported taxa, with the exception of WB0701, were morphometrically discernible from each other mainly based on the c' ratio and the ratio corpus/isthmus length. Termite isolate RGD838 can be distinguished mainly based on its c' ratio and ratio corpus/isthmus length (2.1 ± 0.2 and 6.3 ± 0.5, respectively), thereby differing from all other *Halicephalobus* taxa. Based on its phylogenetic position, which is corroborated by its distinct morphometry, isolate RGD838 is considered a new species. The termite isolate RGD892 resembles *H. brevicauda* based on its short body length, although its c' ratio and stoma length are larger. However, the c' ratio of *H. brevicauda* (c' = 2), which is often used in *Halicephalobus* species identification keys, is based on a single drawing from the original description and should therefore only be considered as an approximation, whereas the stoma is a weak developed and difficult characteristic (Geraert *et al.* 1988). The isolate WB0701, here appointed as *H.* cf. *gingivalis,* was morphologically not discernible from the *H. gingivalis*-clade but formed a clearly separated evolutionary line indicating cryptic speciation. Cryptic speciation, *i.e.*, morphologically alike but genetically distinct speciation, is found in species throughout the tree of life (Bickford *et al.* 2007), in non-sexual taxa (Schön *et al.* 2012) and in nematodes (*e.g.*, Derycke *et al.* 2005; Sudhaus and Kiontke 2007; De Oliveira *et al.* 2012), including parasitic species (*e.g.*, Ferri *et al.* 2009; Pérez-Ponce de Leon and Nadler 2010). Finally, only *H. mephisto* can be separated from other *Halicephalobus* isolates by other (non-morphometrically) distinct morphological features, including SEM, *i.e.*, by the absence of a dorsally reflexed ovary and a trigonal oral aperture. Its deviant morphology corroborates its phylogenetic sister relationship to the other *Halicephalobus* isolates,

however its presence within an unresolved LSU based *Halicephalobus* clade (fig. 6.3B; fig. 6.4) and the SSU divergence between *H. mephisto vs*. *Procephalobus* (18.9%) as opposed to that of *H. mephisto vs*. all other taxa (7.8 – 12.3%), supported the position of *H. mephisto* within the genus *Halicephalobus*.

Concatenated analysis and single gene analysis of COI and ND4 revealed two molecular lineages in the *H. gingivalis*-clade, which were morphometrically and morphologically not distinguishable. The two lineages were supported by species distinctiveness measures except Rodrigo's P(RD) that indicated incomplete reciprocal monophyly for lineage I. The divergence into two distinct molecular lineages is also supported by both single mtDNA phylogenies. Except in Bivalvia (Breton *et al*. 2009 and references therein), mtDNA is generally only inherited maternally, in which case its divergence is not influenced by reproductive strategy (Schön *et al.* 1998). However, heteroplasmy, *i.e*., the presence of different copies of the mitochondrial genome in one individual, can fail to distinguish species delimitation from phylogenetic relationships (Magnacca and Brown 2010 and references therein). However, although only one mtDNA sequence of each isolate was included and therefore multiple copies of the mitochondrial genome cannot be excluded, heteroplasmy seems unlikely in the *H. gingivalis*-clade since both COI and ND4 data identically divide the *H. gingivalis* isolates into two lineages. Moreover, the number of fixed differences between the lineages, *i.e*., the number of base positions at which all sequences of one lineage differ from all sequences of the other lineage, is 18 and 19 for COI and ND4, respectively, which corroborates the existence of distinction. The number (18) of fixed differences of COI between the lineages of *H. gingivalis* approach those found for COI of cryptic species (22 - 29) within *Litoditis marina* (*Rhabditis* (*Pellioditis*) *marina*) (Derycke *et al.* 2005; Derycke *et al.* 2008) and the number of fixed differences for ND4 between both lineages is comparable (19). Application of the 4x rule for speciation, specifically designed for non-sexual organisms (Birky *et al.* 2010), indicated that the two lineages are sufficiently distinct to infer species status under the evolutionary genetic species concept (Barraclough *et al*. 2003; Birky *et al*. 2010). Conversely, the rDNA phylogenies do not support the existence of two lineages in the *H. gingivalis*-clade. The SSU rDNA gene in nematodes has often been found uninformative for defining closely related groups such as species within a genus (*e.g*., Nadler *et al.* 2005), however, on other occasions

unequivocally distinguishes between morphologically indiscernible species (*e.g*., Abebe and Blaxter 2003). Theoretically, there is no outcrossing and recombination in non-sexual organisms, therefore the phylogeny of one gene should be the phylogeny of all other genes within the organism (Birky and Barraclough 2009). However, since the origin of rDNA variability within the *H. gingivalis* clade is unclear, we deem it too speculative to draw conclusions based on the information at hand.

Geographical distribution of the isolates in current study does not explain the two lineages, because isolates originating from one horse riding-school (WB0701, 0801, PF060103, 21 44, and PF190101, 06) are found scattered over the two lineages. These results corroborate the findings of Nadler *et al.* (2003) on the distribution of different *H. gingivalis* isolates obtained from equine clinical cases, *i.e.*, genetically different isolates were found together in single geographical regions, whereas genetically homogeneous isolates were found in different regions. Since coexistence of seemingly similar species is possible and, although not necessarily, might be facilitated by some distinct difference in their biology (Zhang *et al.* 2004; Leibold and McPeek 2006), it is possible that several cryptic species of *H. gingivalis* coexist at one location due to an unknown difference in their behaviour, physiology or morphology, which might be identified by further research. However, at this stage, in the absence of any known observable difference, we deem it not pragmatic to describe new species within the *H. gingivalis*-clade.

In conclusion, molecular characterization and analyses on different markers indicate molecular distinct lineages in the non-sexual genus *Halicephalobus* which are considered species based on the evolutionary species concept (de Queiroz 2007 and references therein). The phylogenetic supported taxa were corroborated by morphological and morphometrical data, except for the lineages found in *H. gingivalis*. The presence of two cryptic lineages within the morphospecies *H. gingivalis*, which cannot be separated based on geographical origin, is supported by different species distinctiveness measures. Although free-living and parasitic isolates do not exhibit consistent genetic differences, remarkably, all isolates from inside horses belong to one lineage, *i.e*., *H. gingivalis* lineage I. This supports the hypothesis that only one lineage is capable of equine host parasitism. However, this should be confirmed by a more extensive phylogeny including more clinical isolates.

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APPENDIX TO CHAPTER 6

Table with all measurements and ratios of *Halicephalobus* spp. isolates (n = 30), represented as mean ± standard deviation with (ranges) and coefficient of variation expressed as percentage. Measurements are given in μm.

	WB0701	WB0702	WB0703	WB0704	WB0705	WB0708	WB0709	WB0801
anal body width	9.0 ± 0.5 (8.1 - 10.7) 6	12.5 ± 1.0 (9.8 - 14.2) 8	12.0 ± 0.8 (10.1 - 13.6) 7	9.1 ± 0.5 (8.3 - 10.0) 6	12.5 ± 0.8 (11.2 - 14.3) 6	12.5 ± 0.9 (10.9 - 15.0) 7	12.2 ± 0.6 (11.2 - 13.6) 5	12.1 ± 0.8 10.5 - 13.5) 6
bulbus length	12.0 ± 0.9 (10.3 - 14.1) 7	15.2 ± 1.1 (13.1 - 17.7) 7	15.0 ± 1.1 (12.8 - 18.1) 7	12.1 ± 0.9 (10.5 - 15.0) 8	15.7 ± 0.7 (14.4 - 17.5) 4	15.0 ± 1.2 (13.0 - 17.7) 8	15.2 ± 0.8 (13.2 - 16.6) 5	14.6 ± 0.9 (12.3 - 16.3) 6
corpus length	36.7 ± 1.5 (34.3 - 39.5) 4	47.1 ± 2.1 (43.0 - 52.2) 4	45.5 ± 2.0 (40.9 - 48.3) 4	36.7 ± 1.6 (33.5 - 39.6) 4	49.5 ± 3.1 (43.0 - 54.6) 6	44.7 ± 2.8 (39.7 - 53.6) 6	45.9 ± 1.8 (42.5 - 49.6) 4	45.6 ± 1.8 (42.3 - 49.6) 4
dorsally reflexed part ovary	31.6 ± 6.1 (17.8 - 41.4) 4	75.8 ± 17.4 (48.2 - 108) 23	78.3 ± 16.8 (52.1 - 114) 22	33.1 ± 4.8 (22.6 - 44.0) 15	64.4 ± 15.2 (41.2 - 95.1) 23	66.8 ± 16.9 (31.3 - 95.0) 25	67.4 ± 12.9 (46.3 - 88.1) 19	72.8 ± 14.3 (40.4 - 99.8) 20
isthmus length	15.5 ± 1.1 (13.3 - 17.9) 7	17.8 ± 1.1 (15.4 - 19.8) 6	16.6 ± 1.3 (13.8 - 19.0) 8	15.1 ± 1.2 (12.6 - 17.1) 8	18.3 ± 1.4 (14.8 - 21.9) 8	16.7 ± 1.4 (13.8 - 19.4) 8	17.5 ± 1.5 (13.8 - 21.1) 9	18.5 ± 1.3 (15.5 - 20.8) 7
L	265 ± 18 (223 - 298) 7	398 ± 30 (340 - 470) 7	367 ± 37 (301 - 443) 10	271 ± 22 (236 - 316) 8	376 ± 25 (331 - 430) 8	362 ± 33 (302 - 436) 9	378 ± 24 (338 - 428) 6	383 ± 26 (326 - 432) 7
L'	222 ± 17 (186 - 249) 8	338 ± 27 (282 - 404) 8	315 ± 35 (251 - 388) 11	229 ± 18 (198 - 264) 8	325 ± 23 (287 - 380) 7	312 ± 31 (257 - 380) 10	326 ± 23 (288 - 374) 7	325 ± 24 (275 - 375) 8
max. body width	17.1 ± 1.1 (14.6 - 19.6) 6	24.3 ± 2.5 (20.4 - 32.7) 10	24.1 ± 2.4 (21.0 - 29.0) 10	17.4 ± 1.3 (15.5 - 20.0) 8	22.5 ± 2.0 (18.7 - 27.5) 9	23.9 ± 2.6 (17.8 - 28.2) 11	23.7 ± 1.7 (19.7 - 26.8) 7	22.9 ± 1..7 (19.9 - 25.7) 7
pharynx length	64.3 ± 2.6 (58.8 - 67.6) 4	80.1 ± 3.0 (73.0 - 85.7) 4	77.0 ± 3.6 (69.2 - 82.4) 5	63.9 ± 2.9 (58.3 - 69.1) 5	83.4 ± 4.0 (74.0 - 90.1) 5	76.3 ± 4.1 (68.9 - 87.9) 5	78.6 ± 2.8 (73.7 - 84.3) 4	78.8 ± 3.0 (72.6 - 84.7) 4
post-v (vulva to posterior ovarian flexure)	34.1 ± 4.27 (22.4 - 40.6) 12	56.0 ± 5.8 (45.4 - 66.6) 10	52.6 ± 6.1 (38.1 - 61.37) 12	34.0 ± 3.6 (28.1 - 42.5) 10	52.8 ± 6.7 (41.3 - 73.6) 13	48.9 ± 6.2 (36.3 - 64.4) 13	49.0 ± 6.7 (38.2 - 70.6) 14	49.9 ± 5.0 (38.9 - 58.2) 10
pre-v (vulva to anterior ovarian flexure)	48.0 ± 5.7 (33.1 - 58.1) 12	83.1 ± 8.9 (68.5 - 109) 11	77.2 ± 9.3 (59.9 - 94.7) 12	50.8 ± 5.1 (42.7 - 60.1) 10	70.5 ± 10.1 (53.0 - 89.6) 14	76.2 ± 10.7 (51.4 - 94.1) 14	77.4 ± 6.8 (61.4 - 90.6) 9	78.9 ± 8.6 (59.6 - 94.0) 11
PV	93.2 ± 11.3 (61.8 - 122) 12	150 ± 17 (119 - 192) 11	142 ± 20 (107 - 173) 14	97.9 ± 9.8 (80 - 118) 10	143 ± 14 (122 - 173) 10	140 ± 18 (100 - 182) 13	147 ± 15 (118 - 181) 10	147 ± 16 (116 - 178) 11
stoma length	7.1 ± 0.4 (6.2 - 8.2) 6	7.7 ± 0.3 (7.1 - 8.3) 4	7.8 ± 0.4 (7.0 - 8.6) 6	7.0 ± 0.3 (6.1 - 7.6) 5	7.8 ± 0.5 (7.1 - 9.1) 6	7.9 ± 0.4 (6.8 - 8.6) 5	8.2 ± 0.5 (7.3 - 9.1) 6	7.4 ± 0.4 (6.1 - 8.0) 5
tail length	43.3 ± 3.2 (36.5 - 49.5) 8	60.8 ± 3.6 (53.4 - 68.2) 56	51.9 ± 2.9 (46.1 - 57.4) 6	42.3 ± 4.4 (35.8 - 52.5) 11	51.5 ± 4.4 (39.2 - 59.6) 9	50.2 ± 4.1 (33.5 - 56.1) 8	51.8 ± 2.9 (42.0 - 58.6) 6	58.0 ± 3.8 (49.7 - 65.9) 6
VA	64.9 ± 5.8 (50.6 - 74.4) 10	108 ± 9 (88 - 126) 9	97 ± 14 (70 - 134) 14	66.8 ± 6.0 (55.7 - 76.5) 9	97.9 ± 8.7 (81.3 - 119) 9	95.0 ± 12.6 (73.6 - 120) 13	101 ± 8.5 (85 - 117) 8	99.4 ± 7.9 (81.5 - 116) 8
vulval body width	16.8 ± 1.1 (14.6 - 19.6) 7	23.6 ± 2.5 (19.8 - 31.6) 10	23.5 ± 2.54 (19.9 - 28.4) 10	16.9 ± 1.2 (15.4 - 19.7) 7	22.2 ± 1.69 (18.7 - 26.4) 9	23.2 ± 2.4 (17.7 - 27.2) 11	23.2 ± 1.8 (19.4 - 26.6) 8	22.3 ± 1.7 (19.1 - 24.8) 7
a	15.5 ± 0.8 (13.8 - 17.5) 5	16.5 ± 1.2 (12.0 - 18.1) 7	15.2 ± 0.6 (14.3 - 16.8) 4	15.6 ± 0.9 (14.1 - 17.7) 6	16.8 ± 1.1 (15.2 - 19.8) 7	15.2 ± 1.0 (12.9 - 17.2) 7	16.0 ± 0.5 (15.2 - 17.6) 3	16.7 ± 0.8 (15.0 - 18.6) 5
b	4.1 ± 0.3 (3.6 - 4.6) 6	5.0 ± 0.3 (4.4 - 5.5) 5	4.8 ± 0.4 (4.0 - 5.4) 8	4.2 ± 0.2 (3.7 - 4.6) 5	4.5 ± 0.3 (3.9 - 5.3) 7	4.8 ± 0.4 (3.8 - 5.5) 9	4.8 ± 0.3 (4.2 - 5.6) 6	4.9 ± 0.3 (4.1 - 5.3) 5
c	6.2 ± 0.4 (5.3 - 7.3) 7	6.6 ± 0.4 (5.9 - 7.5) 6	7.1 ± 0.5 (6.1 - 8.0) 7	6.4 ± 0.3 (5.9 - 7.0) 5	7.3 ± 0.5 (6.7 - 9.1) 7	7.2 ± 0.7 (6.3 - 9.2) 9	7.3 ± 0.5 (6.3 - 8.4) 7	6.6 ± 0.4 (5.9 - 7.5) 6

	PF060121	PF190101	SAN100	WB1101	WB1102	*H. similigaster*	*H. mephisto*	RGD838	RGD892
anal body width	12,9 ± 0,8 (11,2 - 14,7) 6	11,4 ± 0,6 (9,7 - 12,6) 6	12,7 ± 0,9 11,0 - 15,4) 7	12,5 ± 1,0 (10,7 - 14,3) 8	12,6 ± 0,69 (10,8 - 14,3) 7	10 ± 1.9 (6.4 - 12.7) 19	13.4 ± 3.0 (9.1 - 21.3) 22	7,7 ± 1,1 (6,1 - 9,4) 14	9,5 ± 0,9 (6,8 - 10,6) 9
bulbus length	14,9 ± 0,9 (12,0 - 16,6) 6	13,7 ± 1,0 (11,8 - 15,9) 7	14,7 ± 1,2 (12,7 - 17,5) 8	15,4 ± 1,8 (11,4 - 19,1) 11	16,8 ± 1,2 (13,5 - 18,7) 7	13.6 ± 1.6 (10.7 - 16.6) 11	16.3 ± 2.4 (12.5 - 22.2) 15	11,5 ± 1,3 (9,2 - 13,2) 11	12,1 ± 1,1 (9,2 - 14,3) 9
corpus length	51,4 ± 2,9 (45,7 - 57,0) 6	45,5 ± 1,9 (41,4 - 49,0) 4	47,6 ± 2,0 (43,3 - 52,0) 4	50,2 ± 3,5 (43,3 - 56,1) 7	52,8 ± 3,4 (46,8 - 60,0) 6	44.5 ± 4.6 (34.3 - 52.8) 10	59.8 ± 3.3 (52 - 67.2) 5	38,0 ± 4,6 (30,3 - 43,9) 12	35,5 ± 2,1 (32,1 - 39,8) 6
dorsally reflexed part ovary	72,5 ± 15,8 (41,5 - 94,8) 22	52,9 ± 8,4 (37,5 - 72,7) 16	68,0 ± 13,5 (31,4 - 90,7) 20	62,3 ± 10,6 (39,8 - 83,5) 17	74,6 ± 15,9 (43,9 - 107) 21	30.6 ± 10.3 (8.2 - 59.3) 34	n.a.	16,4 ± 4,5 (8,7 - 26,7) 27	27,6 ± 5,1 (15,8 - 39,5) 18
isthmus length	17,2 ± 1,3 (14,9 - 19,7) 7	15,6 ± 1,2 (13,6 - 18,2) 7	16,4 ± 1,5 (14,0 - 20,2) 9	17,0 ± 1,9 (13,7 - 21,2) 11	17,0 ± 2,2 (13,2 - 20,5) 13	21.1 ± 3 (14.5 - 26.1) 14	23 ± 2.9 (16.3 - 27.2) 12	18,5 ± 3,4 (13,8 - 22,2) 18	12,5 ± 1 (10,3 - 14) 8
L	411 ± 25 (358 - 453) 6	355 ± 29 (244 - 397) 8	405 ± 16 (359 - 427) 4	384 ± 29 (336 - 434) 8	419 ± 25 (357 - 456) 6	364 ± 50 (285 - 441) 14	483 ± 82 (398 - 670) 17	279 ± 34 (230 - 330) 12	266 ± 19 (223 - 301) 7
L'	351 ± 25 (299 - 388) 7	302 ± 27 (195 - 342) 9	347 ± 14 (305 - 364) 4	384 ± 29 (228 - 371) 8	352 ± 22 (296 - 382) 6	291 ± 42 (224 - 356) 14	376 ± 27 (305 - 530) 18	231 ± 28 (189 - 268) 12	233 ± 19 (191 - 264) 8
max. body width	22,1 ± 1,5 (18,4 - 24,2) 7	19,3 ± 1,5 (15,9 - 22,3) 8	25,2 ± 1,6 (21,2 - 28,1) 6	21,7 ± 1,2 (19,2 - 23,9) 6	21,6 ± 1,9 (17,4 - 25,9) 9	19 ± 3.8 (9.9 - 25.5) 20	25.5 ± 5.9 (15.8 - 39.1) 23	16,1 ± 1,5 (13,6 - 18,8) 9	17,9 ± 1,3 (13,7 - 19,9) 7
pharynx length	83,4 ± 3,6 (75,6 - 89,0) 4	74,8 ± 3,1 (68,9 - 80,5) 4	(78,6 ± 3,5) (71,8 - 85,9) 4	82,7 ± 6,4 (70,8 - 94,8) 8	86,6 ± 6,1 74,4 - 97,1) 7	79.2 ± 8.9 (59.5 - 93.8) 11	99.2 ± 7.2 (82.1 - 111.4) 7	68,0 ± 9,1 (54,5 - 78,7) 13	60,1 ± 3,4 (54,8 - 65,4) 6
post-v (vulva to posterior ovarian flexure)	55,6 ± 5,5 (41,0 - 64,2) 4	45,7 ± 6,6 (23,9 - 55,0) 14	53,1 ± 7,9 (30,1 - 65,9) 15	50,6 ± 5,6 (37,8 - 61,9) 11	59,4 ± 5,9 (46,6 - 72,4) 10	38.2 ± 10.6 (12.4 - 63.1) 28	65 ± 38 (27.1 - 162) 58	25,6 ± 5,9 (12,5 - 39,8) 23	25,4 ± 3,8 (14,6 - 30,9) 15
pre-v (vulva to anterior ovarian flexure)	82,3 ± 9,7 (65,9 - 100) 12	70,0 ± 6,6 (55,1 - 84,1) 9	84,2 ± 7,3 (69,3 - 96,7) 9	72,9 ± 11,1 (37,8 - 61,9) 15	83,0 ± 10,9 (62,2 - 106) 13	51.3 ± 8.3 (28.6 - 67.4) 16	69.5 ± 22.9 (41.9 - 132) 33	49,2 ± 6,3 (38,9 - 60,7) 13	57,2 ± 5,7 (43,5 - 66,3) 10
PV	163 ± 15 (133 - 187) 9	135 ± 16 (72,4 - 155) 12	164 ± 10 (142 - 186) 6	142 ± 16 (109 - 173) 11	152 ± 13 (127 - 172) 9	124 ± 21 (91 - 159) 17	155 ± 38 (118 - 235) 25	93,6 ± 11,2 (76,7 - 108,6) 12	104,4 ± 11,6 (73,9 - 120) 11
stoma length	8,0 ± 0,5 (7,2 - 8,9) 6	7,8 ± 0,6 (6,6 - 9,0) 7	7,4 ± 0,5 (6,4 - 8,5) 7	8,3 ± 0,8 (6,9 - 9,6) 10	8,6 ± 0,8 (7,0 - 10,2) 10	8.8 ± 0.8 (7.2 - 9.8) 8	9.2 ± 0.9 (7.2 - 10.4) 9	7,2 ± 0,8 (5,6 - 8,6) 11	7.7 ± 0.6 (6,13 - 8,71) 8
tail length	60,3 ± 3,2 (52,3 - 67,6) 5	52,9 ± 3,9 (44,3 - 59,5) 7	57,8 ± 4,6 (45,5 - 65,2) 8	60,0 ± 6,6 (52,7 - 76,9) 11	67,3 ± 4,4 (59,2 - 75,8) 6	73.4 ± 9,0 (55.3 - 87.8) 12	106 ± 15 (88 - 144) 14	48,8 ± 6,8 (39,7 - 62,4) 14	33,7 ± 2,5 (26,8 - 39,1) 7
VA	105 ± 8,3 (85 - 118) 8	92,9 ± 11 (46,9 - 110) 12	105 ± 5 (88 - 114) 5	100 ± 9 (82 - 122) 10	114 ± 8 (94 - 128) 7	87.3 ± 14.3 (63.4 - 111) 16	122 ± 27 (97 - 191) 22	68,9 ± 8,2 (55,6 - 81,4) 12	68,2 ± 6,0 (55,7 - 78,7) 9
vulval body width	21,5 ± 1,5 (18,4 - 24,1) 7	18,2 ± 1,2 (15,0 - 20,9) 6	24,1 ± 1,5 (20,9 - 26,7) 6	21,2 ± 1,2 (18,5 - 23,3) 6	21,3 ± 1,8 (17,3 - 24,7) 9	18.9 ± 3.6 (9.9 - 24.8) 19	24.3 ± 5.6 (15.7 - 36.2) 23	15,4 ± 1,6 (12,8 - 18,0) 11	17,3 ± 1,3 (13,6 - 19,6) 8
a	18,6 ± 0,8 (17,0 - 20,2) 4	18,4 ± 1,7 (11,2 - 21,1) 9	16,1 ± 0,9 (14,3 - 18,5) 6	17,7 ± 1,1 (15,9 - 20,3) 6	19,5 ± 1 (16,9 - 21,5) 5	19.6 ± 2.5 (16.5 - 30) 13	19. ± 2.8 (14.3 - 26.2) 15	17,3 ± 1,1 (15,4 - 19,5) 6	14,9 ± 0,8 (13,4 - 16,7) 5
b	4,9 ± 0,3 (4,4 - 5,4) 5	4,8 ± 0,4 (3,2 - 5,2) 8	5,2 ± 0,2 (4,8 - 5,8) 5	4,7 ± 0,3 (3,9 - 5,3) 7	4,9 ± 0,4 (4,2 - 5,8) 7	4.6 ± 0.3 (3.9 - 5.2) 7	4.9 ± 0.6 (4.2 - 6.4) 13	4,1 ± 0,1 (3,9 - 4,3) 3	4,4 ± 0,2 (3,9 - 4,7) 5
c	6,8 ± 0,5 (5,8 - 8,0) 7	6,7 ± 0,5 (5,0 - 7,7) 8	7,0 ± 0,5 (6,2 - 8,6) 6	6,5 ± 0,6 (5,5 - 7,8) 10	6,2 ± 0,3 (5,8 - 6,9) 4	5 ± 0.3 (4.4 - 5.7) 6	4.5 ± 0.3 (4 - 5.2) 6	5,7 ± 0,3 (5,3 - 6,3) 5	7,9 ± 0,7 (5,9 - 9) 8

	WB0701	WB0702	WB0703	WB0704	WB0705	WB0708	WB0709	WB0801
c'	4,8 ± 0,4	4,9 ± 0,4	4,3 ± 0,3	4,7 ± 0,4	4,1 ± 0,4	4,0 ± 0,4	4,3 ± 0,2	4,8 ± 0,4
	(4,1 - 5,6)	(4,0 - 6,0)	(3,8 - 5,0)	(4,0 - 5,5)	(3,3 - 4,8)	(2,8 - 4,6)	(3,4 - 4,7)	(4,1 - 5,5)
	8	9	6	8	9	9	6	7
ratio corpus/isthmus	2,4 ± 0,2	2,7 ± 0,2	2,8 ± 0,2	2,4 ± 0,2	2,7 ± 0,2	2,7 ± 0,2	2,6 ± 0,2	2,5 ± 0,1
	(1,9 - 2,8)	(2,3 - 2,9)	(2,4 - 3,2)	(2,2 - 2,8)	(2,3 - 3,2)	(2,4 - 3,2)	(2,2 - 3,3)	(2,1 - 2,8)
	8	6	7	6	8	8	9	6
ratio ovary	0,7 ± 0,1	0,7 ± 0,1	0,7 ± 0,1	0,7 ± 0,1	0,8 ± 0,1	0,6 ± 0,1	0,6 ± 0,1	0,6 ± 0,1
(post-v/pre-v)	(0,6 - 0,8)	(0,5 - 0,8)	(0,6 - 0,8)	(0,5 - 0,8)	(0,6 - 1,3)	(0,5 - 0,8)	(0,5 - 1,0)	(0,5 - 0,8)
	8	9	7	8	19	11	14	11
V	59,3 ± 1,5	57,7 ± 0,9	59,5 ± 1,1	59,8 ± 0,9	60,3 ± 0,9	60,0 ± 1,2	59,6 ± 0,9	58,9 ± 1,2
	(53,4 - 61,8)	(55,9 - 59,2)	(57,4 - 61,8)	(58,1 - 61,2)	(58,0 - 62,1)	(58,4 - 65,1)	(58,4 - 62,6)	(56,7 - 60,7)
	2	2	2	1	2	2	2	2
V'	70,8 ± 1,4	68,1 ± 0,7	69,4 ± 1,4	70,1 ± 0,8	69,9 ± 1,0	69,7 ± 1,4	69,1 ± 1,0	69,5 ± 0,9)
	(65,9 - 73,6)	(66,2 - 69,1)	(65,6 - 72,3)	(69,1 - 72,4)	(68,1 - 71,9)	(67,9 - 73,1)	(67,9 - 71,5)	(68,1 - 71,0)
	2	1	2	1	1	2	1	1
VA/tail length	1,5 ± 0,1	1,8 ± 0,1	1,9 ± 0,2	1,6 ± 0,1	1,9 ± 0,2	1,9 ± 0,2	1,9 ± 0,2	1,7 ± 0,1
	(1,3 - 1,9)	(1,5 - 2,1)	(1,4 - 2,4)	(1,4 - 1,8)	(1,7 - 2,5)	(1,5 - 2,3)	(1,6 - 2,3)	(1,5 - 2,0)
	10	7	11	6	10	12	8	7
(VA/2) / post-v	1,0 ± 0,1	1,0 ± 0,1	0,9 ± 0,1	1,0 ± 0,1	0,9 ± 0,1	1,0 ± 0,1	1,0 ± 0,1	1,0 ± 0,1
	(0,9 - 1,1)	(0,9 - 1,2)	(0,8 - 1,1)	(0,9 - 1,2)	(0,7 - 1,1)	(0,8 - 1,1)	(0,7 - 1,3)	(0,9 - 1,1)
	7	7	7	7	8	8	11	7

	PF060121	PF190101	SAN100	WB1101	WB1102	*H. similigaster*	*H. mephisto*	RGD838	RGD892
c'	4,7 ± 0,3	4,7 ± 0,4	4,6 ± 0,4	4,8 ± 0,7	5,4 ± 0,3	7.4 ± 0.8	8.1 ± 1.2	6,3 ± 0,5	3,4 ± 0,2
	(4,1 - 5,7)	(4,0 - 5,5)	(3,3 - 5,3)	(3,9 - 6,9)	(4,8 - 5,8)	(6.1 - 9.6)	(6 - 11.3)	(5,5 - 7,2)	(2,9 - 3,9)
	7	8	9	14	6	11	14	8	6
ratio corpus/isthmus	3,0 ± 0,3	2,9 ± 0,2	2,9 ± 0,2	3,0 ± 0,2	3,1 ± 0,3	2.12 ± 0.14	2.6 ± 0.3	2,1 ± 0,2	2,9 ± 0,2
	(2,5 - 3,7)	(2,4 - 3,2)	(2,3 - 3,4)	(2,6 - 3,6)	(2,7 - 3,6)	(1.9 - 2.4)	(2.2 - 3.3)	(1,9 - 2,5)	(2,4 - 3,3)
	9	7	8	7	9	7	11	8	6
ratio ovary	0,7 ± 0,1	0,7 ± 0,1	0,6 ± 0,1	0,7 ± 0,1	0,7 ± 0,1	0.7 ± 0.1	0.9 ± 0.4	0,5 ± 0,1	0,5 ± 0,07
(post-v/pre-v)	(0,6 - 0,9)	(0,4 - 0,8)	(0,3 - 0,9)	(0,5 - 1,1)	(0,6 - 0,9)	(0.4 - 1)	(0.5 - 2.3)	(0,3 - 0,8)	(0,3 - 0,7)
	11	14	17	16	10	20	41	22	15
V	59,8 ± 1,0	59,0 ± 1,2	59,8 ± 0,9	58,3 ± 1,5	56,9 ± 1,0	55.9 ± 1.3	52.6 ± 1.8	57,9 ± 0,8	61,7± 1,6
	(57,4 - 61,7)	(57,0 - 61,2)	(57,5 - 61,6)	(54,0 - 61,2)	(55,1 - 59,3)	(53.5 - 60.2)	(44.9 - 55.3)	(56,2 - 59,3)	(56,2 - 64,8)
	2	2	2	3	2	2	3	1	3
V'	70,1 ± 0,8	69,3 ± 1,6	69,7 ± 1,0	69,1 ± 1,2	67,7 ± 0,9	70.1 ± 1.2	67.6 ± 2,2	70,1 ± 0,9	70,7 ±1,3
	(67,85 - 71,6)	(67,6 - 76,0)	(67,0 - 71,2)	(66,1 - 70,8)	(66,1 - 39,4)	(68.7 - 73.7)	(58.6 - 70.2)	(68,4 - 71,8)	(65,7 - 72,9)
	1	2	1	2	1	2	3	1	2
VA/tail length	1,7 ± 0,2	1,8 ± 0,2	1,8 ± 0,2	1,7 ± 0,2	1,7 ± 0,1	1.2 ± 0.1	1.1 ± 0.1	1,4 ± 0,1	2,0 ± 0,2
	(1,4 - 2,1)	(1,0 - 2,0)	(1,6 - 2,3)	(1,3 - 2,0)	(1,5 - 1,9)	(0.97 - 1.46)	(0.93 - 1.37)	(1,2 - 1,6)	(1,4 - 2,3)
	9	11	9	12	5	8	11	7	9
(VA/2) / post-v	0,9 ± 0,1	1,0 ± 0,2	1,0 ± 0,2	1,0 ± 0,1	1,0 ± 0,1	1.21 ± 0.3	1.1 ± 0.4	1,4 ± 0,3	1,4 ± 0,2
	(0,8 - 1,1)	(0,5 - 1,7)	(0,8 - 1,8)	(0,8 - 1,3)	(0,8 - 1,1)	(0.8 - 2.8)	(0.53 - 1.9)	(1,0 - 2,78)	(0,9 - 2,0)
	6	18	18	11	8	28	33	24	14

CHAPTER VII

GENERAL DISCUSSION

Within the framework of this PhD research, several aspects of the general biology and evolutionary history of *Halicephalobus gingivalis*, *i.e*., one of the most deadly facultative parasitic nematodes of mammals, was investigated. The study revealed that the species is more widely distributed than previously assumed, has an unseen high tolerance for ivermectin and thiabendazole, has dichotomously branched intestinal microvilli most likely associated with parasitism, shows an intraspecific morphometrical variability which surpasses interspecific variability, has a high level of intra-genomic rDNA variability, and that cryptic speciation in ongoing.

In this closing chapter, speciation and species delineation in the genus *Halicephalobus* is further discussed. The incidence of infections in the light of our results on the distribution of *H. gingivalis* is evaluated, including possible precautionary measures. The discussion on the evolution of *H. gingivalis*, especially regarding parasitism in mammals, is further elaborated. Finally, future research prospects are proposed.

SPECIATION AND SPECIES DELINEATION IN PARTHENOGENETIC ORGANISMS

The existence of species in parthenogenetic lineages remains controversial. In a traditional biological view, species arise in sexual clades because interbreeding maintains cohesion within species whereas reproductive isolation causes divergence between them (Maynard Smith and Szathmary 1995 in Fontaneto *et al*. 2007). Following this rational, non-sexually reproducing organisms could not diversify into species. However, if other processes were at least equally important, *e.g*., specialization into distinct niches or geographical separation, than organisms diversify into species independently from their reproductive mechanism (Barraclough *et al*. 2003).

According to Birky *et al.* (2005), the most common model of parthenogenetic reproduction is when the number of offspring is equal for all individuals, which differ from their ancestor and each other by mutations. This results in a continuum of genetic differences between individuals without gaps separating the clades (Figure 7.1A). However, reproduction is more likely asynchronous with a varying number of offspring and the occurrence of extinction (zero offspring), causing random genetic drift (Figure 7.1B). Yet, the produced clusters of similar organisms are transient and can therefore

not be considered species. Conversely, divergent adaptation to different ecological niches gives rise to long-lasting, independently evolving clades in parthenogenetic species (Figure 7.1C).

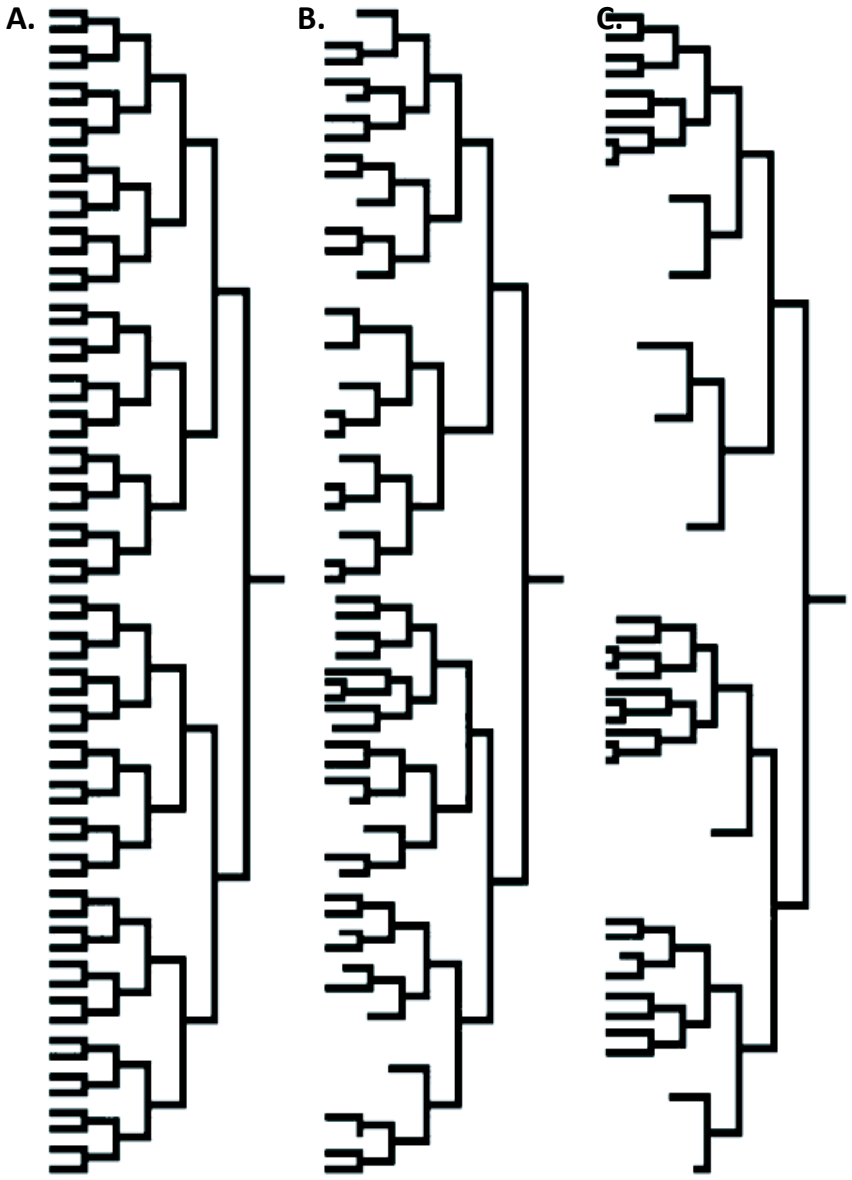

Figure 7.1. Graphical representation of parthenogenesis that is **(A)** synchronous with an equal amount of offspring for all individuals, **(B)** asynchronous with a variable number of offspring and extinction (zero offspring), and **(C)** random genetic drift and divergent adaptation to different ecological niches give rise to independently evolving clades (Figure taken from Birky *et al.* 2005).

Several species concepts, including the evolutionary, ecological and phylogenetic concept, consider the existence of parthenogenetic species (Heethoff *et al*. 2011 and references therein). However, their description in a biological meaningful context

remains difficult. Purely molecular based approaches have been criticized mainly because no general definition is available for the amount of genetic distance that indicates separate species in a lineage (*e.g.*, Heethoff *et al*. 2011). Therefore, it is imperative to use an integrative approach when delineating parthenogenetic species, especially when they have few discriminating morphological traits (Heethoff *et al*. 2011). Such an integrative approach, in which species boundaries were studied from molecular and morphological perspectives, has been proven useful when demonstrating speciation in parthenogenetic lineages such as the bdelloid rotifers (Fontaneto *et al.* 2007) and the oribatid mite genera *Tectocepheus* (Lauman *et al.* 2007) and *Trhypochthonius* (Heethoff *et al*. 2011).

Within the suspected obligate parthenogenetic genus *Halicephalobus* current study revealed the presence of several well supported molecular clades (Chapter 6), which could be considered species based on the evolutionary species concept (de Queiroz 2007 and references therein). Moreover, all phylogenetic supported taxa, except WB0701, were confirmed by morphological and/or morphometrical data (Chapter 6). Divergent adaptation can explain at least some of these species. *H. mephisto*, is found in a distinctly different niche than the other included isolates, *i.e.*, in a water enclosure in a mine up to 3 km belowground (Borgonie *et al*. 2011). *H. laticauda*, although not included in this study, is also found in mines (Geraert *et al.* 1988) but in a different habitat and a distinctly different morphology confirms that it represents a different molecular lineage. *H*. cf. *brevicauda* (RGD892) and *Halicephalobus* sp. n. were both found in association with termites. The co-occurrence of representatives of both *H. gingivalis* lineages on one equestrian facility (Chapter 2 and Chapter 6) could be explained by inhabitation of different micro-environments.

In conclusion, most of the *Halicephalobus* species identified in the present research are based on well supported molecular clades, are also supported by morphological and/or morphometrical data, and also on differential ecology.

SPECIES IDENTIFICATION IN *HALICEPHALOBUS*

As described in Chapter 5, *H. gingivalis* has an unseen high degree of intraspecific morphometric variation to the extent that different species of *Halicephalobus* can be

identified within the progeny of a single female. Based on these results, only ratio corpus/isthmus length remains potentially useful as a morphometric discriminating factor. However, a morphometric analysis by means of multivariate analyzing techniques revealed that the interspecific variation of some characteristics is larger than their intraspecific variation. Consequently, phylogenetically supported taxa, with the exception of WB0701, were discernible from each other mainly based on ratio c' and ratio corpus/isthmus length.

Table 7.1. Morphometrics of different *Halicephalobus* species, both from original species descriptions and from the present study. Measurements are given in µm, except total body length (mm).

	L	a	b	c	c'	V	stoma length	corpus/ isthmus	VA/tail length	
H. mephisto	0.52-0.56	25-29	5.5-6.6	4.3-4.7	9-10	48-51	7-9.5	1.9-2	1.1-1.4	Borgonie *et al*. 2011
H. laticauda	0.25-0.35	24-29	3.3-4.2	4.5-7.3	4-6	53-64	8-11	1-2†	1.1-1.9	Geraert *et al*. 1988
H. brevicauda	0.24-0.33	13-17	3.9-4.1	11.4-14.4	2.4‡	56-60	16°		4°	Mavljanov 1976
H. cf. *brevicauda*	0.22-0.30	13-17	3.9-4.7	5.9-9	3-4	56-65	6-9	2.4-3.3	1.4-2.3	this study
H. similigaster	0.23-0.39	17-21	3.7-4.3	4.0-4.6	8*	54-56	6-8*	3*	1*	Andrássy 1954
H. similigaster	0.28-0.44	16-30	3.9-5.2	4.4-5.7	7-10	54-60	7-10	1.9-2.4	1.0-1.4	this study
H. minutus	0.26-0.28	22-25	3.9-4.3	5.2-6.5	8*	57-63	7*	2.1‡	1.2‡	Körner 1954
H. parvus	0.25-0.41	17-24	4.0-5.2	3.8-4.5	10*	53-56	9*	2.5‡	0.8‡	Körner 1954
H. persicus	0.20-0.25	21-26	3.7-4.2	4.4-5.2	7-12	54-61	6-8	2.2-2.6	0.9-1.1	Shokoohi *et al*. 2007
Halicephalobus sp. n.	0.23-0.33	15-20	3.9-4.3	5.3-6.3	5.5-7	56-59	6-9	1.9-2.5	1.2-1.6	this study
H. deletrix[7]	0.25	15-20	2.9-3.6	4.4-6.3	5.3‡	56-63				Anderson, Bemrick 1965
H. gingivalis	0.25-0.43	15-23	2.9-3.8	4.4-7.0	5-6	56-65	4-10		1.0-1.3	Andrássy 1984
H. gingivalis	0.24-0.47	11-22	3.2-5.8	5.0-8.6	3-7	56-65	6-10	2.1-3.6	1.0-2.5	this study
H. cf. *gingivalis*	0.22-0.29	14-18	3.6-4.6	5.3-7.3	4-6	53-62	6-8	1.9-2.8	1.3-1.9	this study
H. intermedius	0.23-0.32	13-23	3.2-4.5	5.0-6.8	4-5*	58-66			1.0-1.3*	Pokrovskaja 1964
H. palmaris	0.36-0.41	17-19	3.8-4.5	6.9-7.5	4.5-6†	56-60	7-8	0.6*	1.6-1.8*	Lordello, De Oliveira 1963
H. limuli	0.42-0.46	20-21	4.0-4.8	6.7-7.0	4.5-5†	59-61	11-12		1.6-1.8	Tim 1956

† calculated from original descriptions; ‡ measurements from original illustration; ° based on one specimen in the original description; * according to Andrássy 1984. For the denotation of the used ratios, we refer to Chapter 5.

Table 7.1 lists the morphometrics of different *Halicephalobus* species based on the original descriptions together with the results of the present study. The morphometrics of cultured specimens of *H. similigaster* are in accordance with those measured from an *in situ* population from the same beech tree (Köhler 2011). This confirms that the observed morphometric variability under experimental conditions as applied in this study are relevant to populations from natural habitats, as was also observed for *H. gingivalis* (Chapter 5). *H. brevicauda* and *H.* cf. *brevicauda* identified in

[7] *H. deletrix* is a synonym of *H. gingivalis* (see General Introduction), but is often still used en therefore included in this table.

this study showed some minor morphometrical differences (table 7.1). However, since both stoma length and ratio VA/T were based on a single individual (Mavljanov 1976) and c' was based on a single illustration, we appoint this isolate *H*. cf. *brevicauda*.

Stoma length and the ratios VA/T, V, and c' are predominantly used in current identification keys. The present study revealed that ratio V showed the least intraspecific variability within a fixed environment, but was unable to discriminate between the different *Halicephalobus* spp. used in the present study and by extension in the entire genus (table 7.1). Also ratio VA/tail length has a large intraspecific variability which overlaps between the different species (Geraert *et al*. 1988 and table 7.1). The stoma it is a small sized structure with a weakly developed posterior part which makes observed differences less convincing (Geraert *et al*. 1988). Although c' had a relatively high intraspecific variability (Chapter 5), the difference between the phylogenetically delimited clades (Chapter 6) is larger and this corroborates with Geraert *et al.* (1988) that c' is a useful character to discriminate certain species. We demonstrated that c' can be used to delimitate the following groups: c'<4 (*H*. cf. *brevicauda*), c'=4-7 (*H. gingivalis* and *Halicephalobus* sp. n.), and c'>7 (*H. similigaster* and *H. mephisto*). Ratio corpus/isthmus length was able to distinguish *H. similigaster* and *Halicephalobus* sp. n. (isolate RGD838) from all other isolates based on a discriminant function analysis (Chapter 6). However, the range of the characteristic is slightly overlapping and it is therefore omitted as a discriminating characteristic. Isolate WB0701 is not morphologically or physiologically discernible from the *H. gingivalis*-clade but forms a clearly separated evolutionary lineage indicating cryptic speciation, and was appointed *H*. cf. *gingivalis* (Chapter 6).

Current study corroborates Köhler (2011), who proposed *H. parvus* and *H. minutus* as junior synonyms of *H. similigaster* because the morphometrical ranges of the latter encompass those of the first two. Further, with the exception of its smaller total body length, most morphometrics of *H. persicus* overlap with the morphometrical ranges of *H. similigaster* measured in the present study. Moreover, the minute mucro at the tail tip in *H. persicus* (Shokoohi *et al*. 2007), proposed as unique in the differential diagnosis, has also been found in *H*. cf. *brevicauda*, *Halicephalobus* sp. n., *H. similigaster*, *H*. cf. *gingivalis* and *H. gingivalis* in the present study. Therefore, at this point, there are

no reliable characteristics to further distinguish *H. similigaster* (including *H. parvus* and *H. minutus*) *and H. persicus.*

Geraert *et al*. (1988) already recognized that *H. intermedius* and *H. palmaris* are two difficult-to-distinguish species. Moreover, since ratio VA/T and ratio ovary showed a high intraspecific morphometrical variability that was highly influenced by environmental conditions (Chapter 5), both morphometrics were proven not useful for *Halicephalobus* species identification. Consequently, *H. gingivalis* and *H. intermedius*, *H. palmaris* and *H. limuli* are no longer morphometrically distinguishable. *H. limuli* was always considered having a different biology since it was isolated from a marine environment. However, since specimens of *H. limuli* were also able to survive in tap water (Timm 1956) and given the wide environmental range of *Halicephalobus* spp., the origin of isolation should not be used to distinguish between the species.

Despite the difficulty to characterise the majority of the species within *Halicephalobus*, two species can be easily characterised. *H. mephisto* is discernible from all other taxa by a straight ovary *vs*. a dorsally reflected ovary, whereas *H. laticauda* can be identified based on its thick tail with a sclerotized tip.

Although a more thorough revision of the genus is imperative, we here propose a preliminary simplified key to the genus enabling identification of some species or species groups. Since there are few discriminating characters between the species, we feel an integrative approach based on multivariate analysis of morphometric data and molecular data is required for accurate species identification.

Key to species

1	ovary straight	*H. mephisto*
	ovary dorsally reflexed	2
2	tail with sclerotized tip	*H. laticauda*
	tail without sclerotized tip	3
3	tail conical, c’ < 4	*H. brevicauda*
	c’≥ 4	4
4	c’= 4 – 7	*H. gingivalis* (= *H. deletrix*), *H. palmaris, H. limuli, H. intermedius, Halicephalobus* sp. n.
	c’ > 7 . . .	*H. similigaster, H. parvus, H. minutus, H. persicus*

DISTRIBUTION OF *H. GINGIVALIS* AND THE INCIDENCE OF HALICEPHALOBIASIS

Because of its wide geographical distribution and its ability to tolerate a wide variety of environments, *H. gingivalis* can be classified as an eurytopic species. Current study demonstrated that populations of free-living *H. gingivalis* are more abundantly present than previously thought. Although never reported in Belgium at the beginning of the PhD, a sampling of manure heaps at 73 different equestrian facilities yielded five free-living isolates and a more focused analysis at one facility revealed the occurrence of *H. gingivalis* in approximately 55% of the sampled horse stables (Chapter 2). A parallel study further demonstrated that *H. gingivalis* is numerously present in compost heaps (see figure 2.1 and Steel *et al*. 2010). However, since the sample sites were restricted to horse associated environments and compost heaps, the possible distribution of *H. gingivalis* outside these habitats was not investigated. As infections with this species have been reported, albeit rarely, in other Equinidae and in cattle, it would be interesting to map the presence of *H. gingivalis* populations in the vicinity of these animals as well.

The present study also established phoresy as a possible transport mechanism for *H. gingivalis* (see Appendix), which could facilitate the dispersal of this species between distant habitats such as different compost heaps, manure heaps and dung patches. However, other mechanisms, which are summarized in figure 7.2, may also play a role in the distribution of this facultative parasite. Treading of contaminated dung patches could explain the presence of *H. gingivalis* in individual stables and in a paddock (isolate WB0702). Whereas in a fly rich environment such as in the vicinity of horses, phoresy might also be an important dispersal mechanism and may even play a role in equine infections, especially when these occur in the orbital region or through lesions in the skin. The retrieval of *H. gingivalis* from fresh dung suggested its presence in the horse's intestine, which has been confirmed by two positive rectal faecal samples and consequently represents a possible dispersal mechanism through defecation. As seen in some plant-parasitic nematodes, intensive trade can facilitate the cosmopolitan distribution of local species (Jasmer *et al*. 2003). Equally, transportation of horses due to trading or participation in equestrian sports could facilitate the distribution *H. gingivalis* globally.

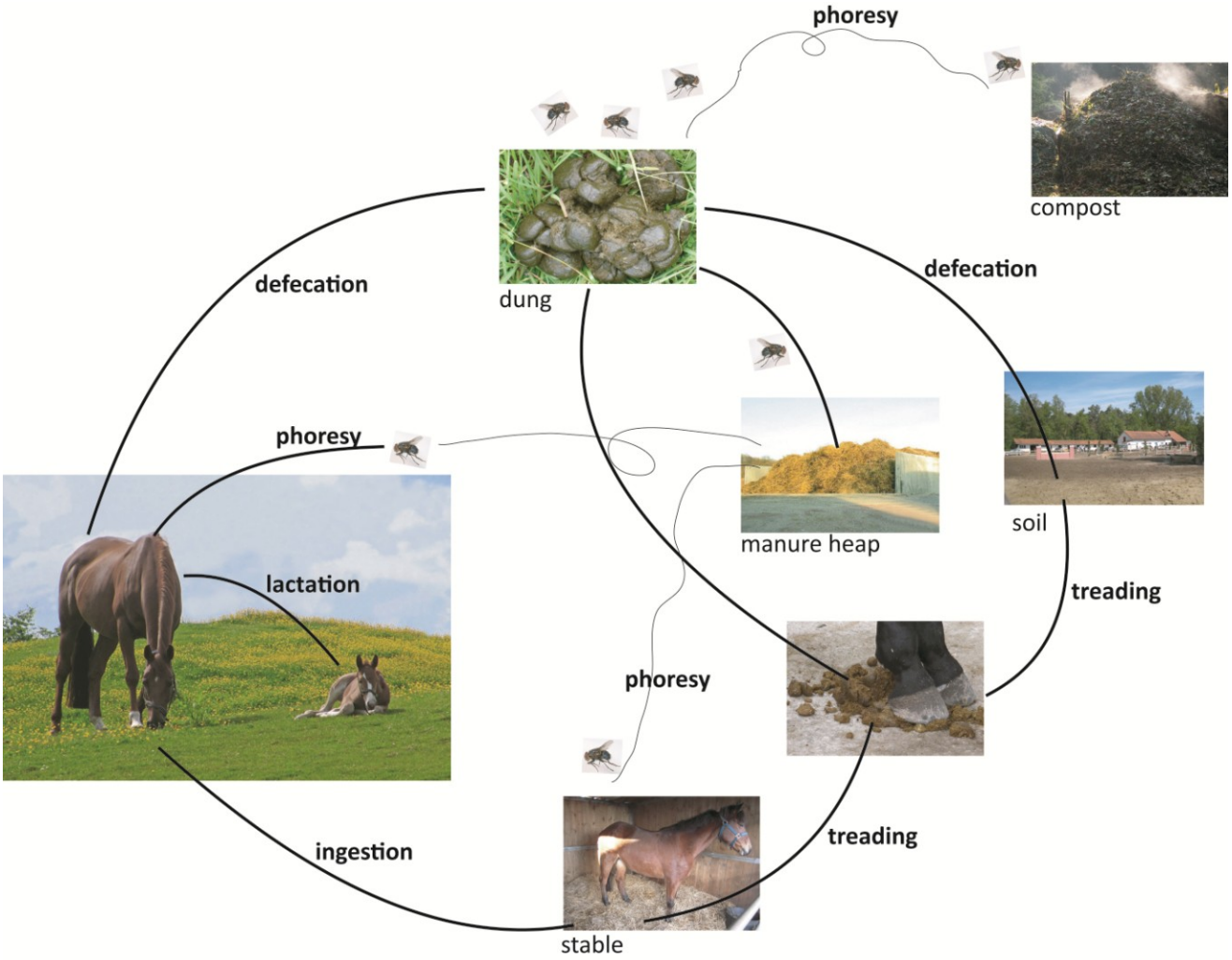

Figure 7.2. An overview of possible mechanisms in the dispersal of *H. gingivalis*. Infections can occur both through phoresy and by direct contact. **Phoresy** enables *H. gingivalis* to disperse between dung patches, compost heaps, manure heaps, stables and can play a role in infections. **Treading** of contaminated patches can distribute *H. gingivalis* in more locally arranged habitats. **Ingestion** of *H. gingivalis* could facilitate its dispersal through **defecation**. A mare with a *H. gingivalis* infection in the mammary glands can transmit the infection to her foal by **lactation** (*e.g.*, Wilkins *et al.* 2001).

Presuming that free-living populations of *H. gingivalis* are also abundantly present in or near equestrian facilities in other countries, the number of reports of halicephalobiasis is surprisingly low. However, equine infections could be overlooked because of the difficult diagnosis resulting in an underestimation of the incidence of halicephalobiasis (see General Introduction). For example, during a study on causes of equine encephalitis in Egypt, histological examination of the brain revealed the presence of *H. gingivalis* in 2 out of 28 animals, of which just one was previously diagnosed with halicephalobiasis (Ferris *et al*. 1972). Although the incidence of equine halicephalobiasis

is possibly underestimated, human infections have less likely been overlooked, since a post-mortem examination is generally performed in case of an inexplicable death.

Most likely not all *H. gingivalis* infections are, or at least not immediately, deadly in horses. This is supported by the two horses carrying *H. gingivalis* in their intestine (Chapter 2) without showing clinical symptoms which could be related to halicephalobiasis. It is conceivable that this facultative parasite can reside in the intestine for longer periods of time, merely thriving in an alternative food rich environment. The discrepancy between the high abundance of *H. gingivalis* in horse rich environments and the low incidence of halicephalobiasis could also be explained by the fact that not all free-living forms are able to become parasitic. Both molecular lineages in the *H. gingivalis*-clade (Chapter 6) contain free-living isolates, *i.e.*, from compost or manure/fresh dung, but only lineage I contains isolates obtained from inside horses, *i.e.*, clinical or from rectal faecal sample. This supports the hypothesis that only one lineage represents equine host specificity.

TREATMENT AND PRECAUTIONARY MEASURES

Considering the high anthelmintic tolerance of *H. gingivalis*, it is unlikely that current *in vivo* drug treatments are sufficient in controlling infections of this species (Chapter 3). Therefore, surgical debulking of the granulomatous lesions in an early stage, before dissemination of nematodes to vital organs, can be forwarded as the best treatment. The subsequent admission of a high dosage of ivermectin, as was proposed by Pearce *et al*. (2001), seems redundant in the light of our results. The prognosis is unfavourable once the infection has spread throughout the body. At this moment no treatment can be proposed in case of a disseminated infection. However, given its suitability as a model organism (Chapter 3), the effects of other anthelmintic drugs on the life cycle of *H. gingivalis* should be tested in order to explore other drug treatment options.

Present study revealed that cross contamination of *H. gingivalis* between different horses may be possible, because one of the horses carrying *H. gingivalis* in its intestine had been stabled with a lethally infected Connemara mare (Case Report in Chapter 2) while the other originated from the same French farm as the infected mare. Phylogenetic analysis further showed that all three isolates belonged to same molecular

lineage, *i.e.*, lineage I (Chapter 6). Therefore, some precautionary measures can be forwarded to prevent the further distribution of *H. gingivalis*. In case halicephalobiasis has been suspected in a mare, she should no longer be allowed to carry or suckle foals to prevent *in utero* or transmammary transmission of *H. gingivalis*. In case *H. gingivalis* has been known to occur at an equestrian facility, it would be advisable to closely follow up on the healing of open wounds. Further, since the distribution of *H. gingivalis* through urine or dung is possible, horses with a (suspected) history of halicephalobiasis should be stabled separately and preferably transported to other locations as little as possible. Finally, stored semen of stud horses diagnosed with halicephalobiasis should be destroyed since the presence of *H. gingivalis* has been proven in the semen of infected stallions (*e.g.*, Kinde *et al*. 2000) and several nematode species, including Panagrolaimids, are able to revive after cryopreservation for longer periods of time (*e.g.*, Hwang 1970, Gill and Redwin 1995).

IS *H. GINGIVALIS* EVOLVING TOWARDS ANIMAL PARASITISM?

Parasitism is usually described as an association in which the parasitic partner harms but not kills its host, whereas parasites that kill their hosts are often called 'poorly adapted' (Blaxter 2003). Nematodes living in saprobiontic ephemeral habitats, as shown here for *Halicephalobus gingivalis*, have acquired several adaptations, *e.g.*, enduring low oxygen levels, changing osmotic pressures, and higher temperatures, which are essential for survival and ultimately parasitism inside a vertebrate host (Sudhaus 2010). The biological mechanisms that subsequently enable parasites to thrive in their host environment, probably resulted from gradual adaptations to a changing environment after nematodes entered the host body (Sangster and Dobson 2002). The current study revealed several adaptations in *H. gingivalis* which could facilitate its facultative parasitic life style and the presence of two distinct phylogenetic lineages in the *H. gingivalis*-clade of which one includes all parasitic isolates.

Morphological adaptations

Pharyngeal glands, the stoma and gut, and the cuticular surface of parasitic nematodes are known to have adaptations related to host-parasite interactions (Jasmer *et al.* 2003).

Cuticular structures can facilitate attachment to host surfaces or function in the evasion of host defence systems and the suppression of immune responses (*e.g.*, Meri *et al.* 2002), whereas pharyngeal glands can secrete proteins that interact with the host (*e.g.*, Harrop *et al.* 1995). None of these adaptations related to parasitism can be identified in *H. gingivalis*. However, a TEM study (Chapter 4) revealed thread-like and enlarged globular vesicles in the intestinal lumen of *H. gingivalis*, also found in the entomoparasitic species *Hexatylus viviparus* (Shepherd and Clark 1976) and in the vertebrate parasite *Metastrongylus* sp. (Jenkins and Erasmus, 1969). Although no function in relation towards an adaptation to parasitism is known, these aberrant types of secretion were to our knowledge only observed in parasitic nematode species and the apical surface of the parasite intestine is an interface for host-parasite interactions (Jasmer *et al*. 2003). Deviations from usual finger-like intestinal microvilli have arisen independently several times within the phylum, their occurrence always being in parasitic or in parasitism-related nematodes such as *Ascaris suum* (Kessel *et al*. 1961) and *H. viviparus* (Shepherd and Clark 1976). In *H. gingivalis,* both dichotomous and trichotomous branched intestinal microvilli were observed adjacent to regular cylindrical microvilli representing an increase of the surface area of the intestinal lumen needed to optimise the intake of nutrients when in the vertebrate parasitic phase.

Biological adaptations

H. gingivalis has a stunning temperature range of 4°C – 40°C and can be cultured in a wide variety of conditions (Discussion Chapter 3), including in human blood plasma (unpublished results). This temperature range also enables the species to cope with temperature differences between its free-living and parasitic stage. *H. gingivalis* has a predilection for warm environments as shown by its optimal culture temperature of 30°C and its natural environment, which includes compost (Steel *et al.* 2010) and manure heaps (current study). When cultured at 38°C, which approximates the body temperature of horses and other mammals, its fecundity is lower compared to 30°C (table 7.2); however, the life cycle is faster (table 7.2) enabling rapid proliferation after entering the hosts body. Nematodes inhabiting decaying organic matter are usually able to withstand oxygen deprivation and can accommodate changing osmotic pressure in their environment, which are also adaptations necessary for surviving inside a host. *H.*

mephisto has been described from an oxygen poor environment (Borgonie *et al*. 2011) and *H. similigaster* has been found in decaying organic matter (Köhler 2011), thereby suggesting that species of the genus have these adaptations.

Table 7.2. Comparison of life cycle of *H. gingivalis* isolate WB0801 when incubated at different temperatures. Values represent mean (n = 8) ± standard deviation.

	30°C	38°C
hatching (hrs)	13 ± 1	11 ± 1
oviposition (hrs)	47 ± 1	38 ± 1
fecundity	337 ± 116	147 ± 70,4
reproductive faze (days)	12,7 ± 5,2	7,5 ± 1,4
postreproductive faze (days)	1,2 ± 0,2	1,3 ± 0,5

As described in Chapter 3, a study on both free-living and facultative parasitic isolates of *H. gingivalis* revealed an unseen high tolerance for both thiabendazole and ivermectin and showed that it is very unlikely that *in vivo* anthelmintic treatments are effective for infections with this facultative parasite. Further, the results showed a remarkable increase of tolerance from fully free-living towards horse associated isolates. However, these biological differences are not unequivocally related to their evolutionary history. The studied isolates with a range of anthelminthic tolerance belong to only one molecular lineage, *i.e*., *H. gingivalis* lineage I.

Different animal parasitic nematodes live in symbiosis with intracellular bacteria, which have a possitive effect on their biology, and antibiotic treatment can cause inhibition of development, blocked embryogenesis and fertility, and reduced viability (Hoerauf *et al.* 2001; Foster *et al.* 2005). To investigate if intracellular bacteria facilitated the high anthelmintic tolerance of *H. gingivalis*, the MALDT-test described in Chapter 3 was repeated with different concentrations of IVM or TBZ in combination with tetracycline at different concentrations. This resulted in no visible increase in the susceptibility of the *Halicephalobus* isolates, thus rejecting the presence of intracellular bacteria as a possible explanation of the extraordinary anthelmintic drug tolerance of *H. gingivalis.*

Parthenogenesis

Parthenogenesis is very common among parasites and can be obligatory or cyclical (Price 1980). It allows an increased reproductive capacity necessary to compensate the difficulty in finding suitable hosts (Sudhaus 2010). Its parthenogenetic reproduction enables *H. gingivalis* to rapidly invade and colonize the hosts' tissue, but also results in a high mortality rate as it passes multiple generations inside its host, thereby suggesting that *H. gingivalis* is poorly adapted to a parasitic life style.

Phylogenetic relationships

A multiple gene phylogenetic analysis revealed the presence of two distinct evolutionary lineages within the *H. gingivalis*-clade. Only one of these lineages (*i.e.*, lineage I) contains isolates from inside horses. Although, the presence of free-living isolates, *i.e.*, a compost isolate and isolates from fresh dung suggests that this clade is not exclusively parasitic, it is not unlikely that horse associated *H. gingivalis* strains can reside in compost heaps as well due to their predilection for warm environments.

Endoparasitic nematodes can be divided in gut-dwelling and tissue-dwelling parasites (Blaxter 2003). Gut-dwelling parasites can be seen as bacteriovores exploiting a rich food source in a warm environment, whereas tissue-dwelling parasites require further adaptations as they actively invade the hosts' body and take up reproductive residence in organs and tissue (Blaxter 2003). Within *Halicephalobus* lineage I, the clinical isolates (SAN100 and CaseReportBelgium) are molecularly distinct from free-living isolates and isolates obtained from rectal dung samples (WB1101 and WB1102) based on their D2D3 differences, *i.e.*, a 5.8% sequence difference between the clinical isolates and free-living or rectal isolates, as opposed to an approximately 1% sequence difference between all free-living and rectal isolates (results not shown). This phylogenetic support of both clinical isolates may suggest the presence of a distinct subclade within lineage I which is able to invade the hosts' tissue.

CONCLUSION

The present study based on a multidisciplinary approach demonstrates that the facultative parasitic nematode *H. gingivalis* has several characteristics that enable it to opportunistically invade and colonize vertebrate hosts, *i.e*., a remarkable temperature range and parthenogenetic reproduction, and adaptations that are function of a parasitic life style, *i.e*., ultrastructural adaptations at the intestinal level and an unseen tolerance for commonly used anthelmintic drugs. Moreover, a phylogeny based on multiple genes revealed two distinct evolutionary lineages within *H. gingivalis* of which only one includes isolates obtained from inside a host and thus appears to be evolving towards vertebrate specific parasitism.

SUGGESTIONS FOR FUTURE RESEARCH

1. In retrospect, there are some indications for biological differences between the different isolates. For example, *H*. cf. *gingivalis* (WB0701) cultures appeared more difficult to optimize for experimental use by incubating at 30°C. This might be caused by a different optimal temperature for its life cycle in comparison to the optimal temperature *of H. gingivalis* isolates. Also, cultures of the termite isolates (RGD838 and 892) appeared to thrive better when fungi were present on the culture plates. A more thorough investigation of isolates belonging to different evolutionary lineages might reveal biological differences, such as differences in optimal temperature, generation time, and feeding-type.

2. Using laboratory animals such as mice to test different routes of infection for different isolates (intestine dwelling, tissue dwelling and free-living of both lineages) under controlled conditions and their subsequent ability for parasitism would determine if free-living and rectal dung isolates are also able to persist inside tissue of a vertebrate host or if the clinical isolates are unique in their ability to thrive inside host tissue.

3. The present study revealed a high number of polymorphisms in the SSU rDNA gene. To fully understand the mechanisms underlying these polymorphisms, it is imperative to know the chromosomal status of *H. gingivalis*.

4. Since *Diploscapter coronatus*, which is also known as a facultative parasite (for an introduction to the species see Chapter 1), has often been found in the samples together with *H. gingivalis* throughout the present study, several isolates of *D. coronatus* from various origins (*e.g.*, from manure, fresh dung, rectal dung samples, rotten wood, and a water well) were brought into culture. As opposed to *H. gingivalis*, *D. coronatus* has to date not been known to invade the tissue of its host. To investigate the biology of *D. coronatus* a comparable study could be performed to determine its general biology.

APPENDIX

PHORESY IN DIFFERENT ISOLATES OF *HALICEPHALOBUS GINGIVALIS*

Results concerning compost isolate of *H. gingivalis* partially published in: Steel H., Verdoodt F., Čerevková A., Couvreur M., **Fonderie P**., Moens T. and Bert W. (2013). Survival and colonization of nematodes in a compost process. *Invertebrate Biology* 132, 108-119.

INTRODUCTION

The possible transference of *H. gingivalis* by flies has on occasion been forwarded as a possible infection route (refs). Although phoresy is known for other species of the genus, i.e., *H. similigaster* associated with ants, beetles and flies (Köhler 2011), *H. limuli* associated with chelicerates (Timm 1956), the possible role of phoresy in the distribution (both geographically and in case of infection) of *H. gingivalis* has never been investigated. This appendix reports on the phoretic ability of two isolates (from compost and horse dung) of *H. gingivalis*, which was tested under laboratory conditions with the blue bottle fly as possible vector.

MATERIAL AND METHODS

Calliphora vomitoria (blue bottle fly) was used as a possible vector to investigate the phoretic ability of *H. gingivalis*. The flies were obtained from mature maggots, which pupated into imagos after a few days. Two isolates of *H*. *gingivalis* from different habitats were included in the experiment, *i.e.*, compost isolate WB0707 and isolate WB0801 from fresh horse dung.

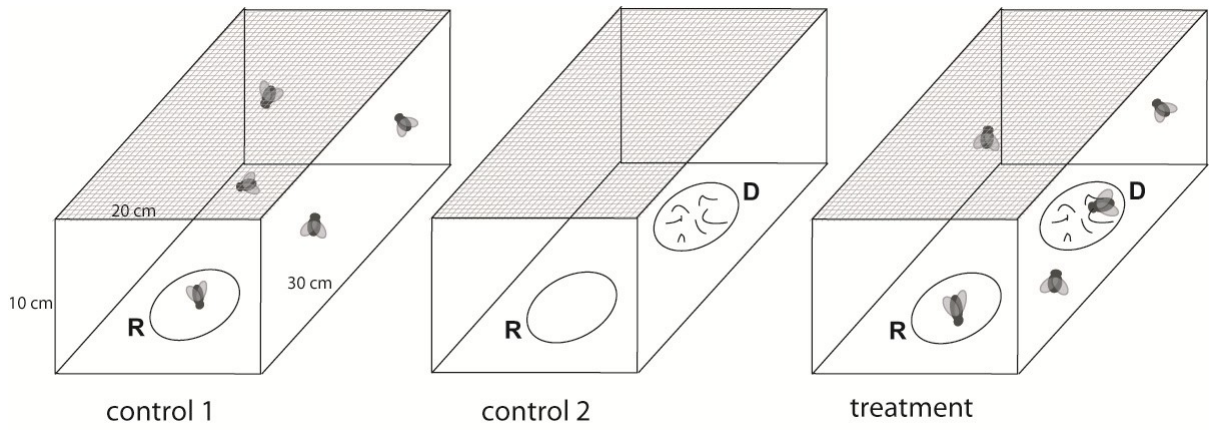

Figure A1. Experimental design for testing possible phoresy in *Halicephalobus gingivalis* by the vector *Calliphora vomitoria.* D=donor plate maintaining a culture of nematodes, R=nematode-free receptor plate. Dimensions of container are indicated in control 1.

Experiments were carried out simultaneously in several identical plastic containers, which were covered with an insect net and placed in a closed culture room with constant environmental conditions (22°C and 60% humidity). Each treatment contained 20 adult flies, a donor plate maintaining a monoxenic culture of nematodes, and a nematode-free receptor plate (fig. A1). Both donor and receptor plate contained 2% bacteriological agar enriched with cholesterol at a final concentration of 1 µg ml^{-1} and *E. coli* OP50 as a food source. The receptor plates were checked and replaced by a new plate every 48 hours for six days. Since at 20°C *H. gingivalis* has a generation time of approximately 8 days (results not shown), this allowed us to disentangle the actual life stage that was transferred by the vector, and avoided reproduction obscuring the results. Two controls were included in the experiment. The first served to verify the absence of nematodes associated with the flies prior to their introduction in the experiment, and contained two nematode-free receptor plates and 20 flies (fig. A1, control 1). A second control included a donor plate and a receptor plate without flies to test for non-fly-dependent transfer of nematodes (*e.g.*, through any circulation of air inside the containers) (fig. A1, control 2). Since the aim of the experiment was merely to investigate to what extent different isolates of *H. gingivalis* are capable of phoresy and, if so, which life stages would be transferred by the vector, the experiment was only once replicated in time.

RESULTS

All donor plates contained a thriving culture with different life stages at a mean density of approximately 300 nematodes cm^{-2}. Figure A2 represents the mean number of nematodes of both isolates of *H. gingivalis, i.e.*, WB0708 from compost and WB0801 from horse dung, that were transferred from the donor plate to the receptor plate, at each consecutive observation time. All controls remained empty for the entire period of the experiment.

Several nematodes of both isolates had already been transferred to the receptor plate after 48h of incubation. Similar numbers of nematodes were found on the receptor

plate after 96h and 144h of incubation for both isolates, indicating that the capacity to use a fly as a vector was similar for both isolates.

Mostly juvenile nematodes of different stages were transferred by the flies. Only a limited number of adults and very few eggs were found on the receptor plate. The eggs and immobile dauer[8] stages were found only in the fly faeces, indicating the possibility that they were sucked up by the flies and subsequently excreted undigested, therefore suggesting that at least part of the phoresy was internal. No nictation (*i.e.*, waving behaviour of nematodes) has been observed in any of the donor plates at any given observation moment during the treatment.

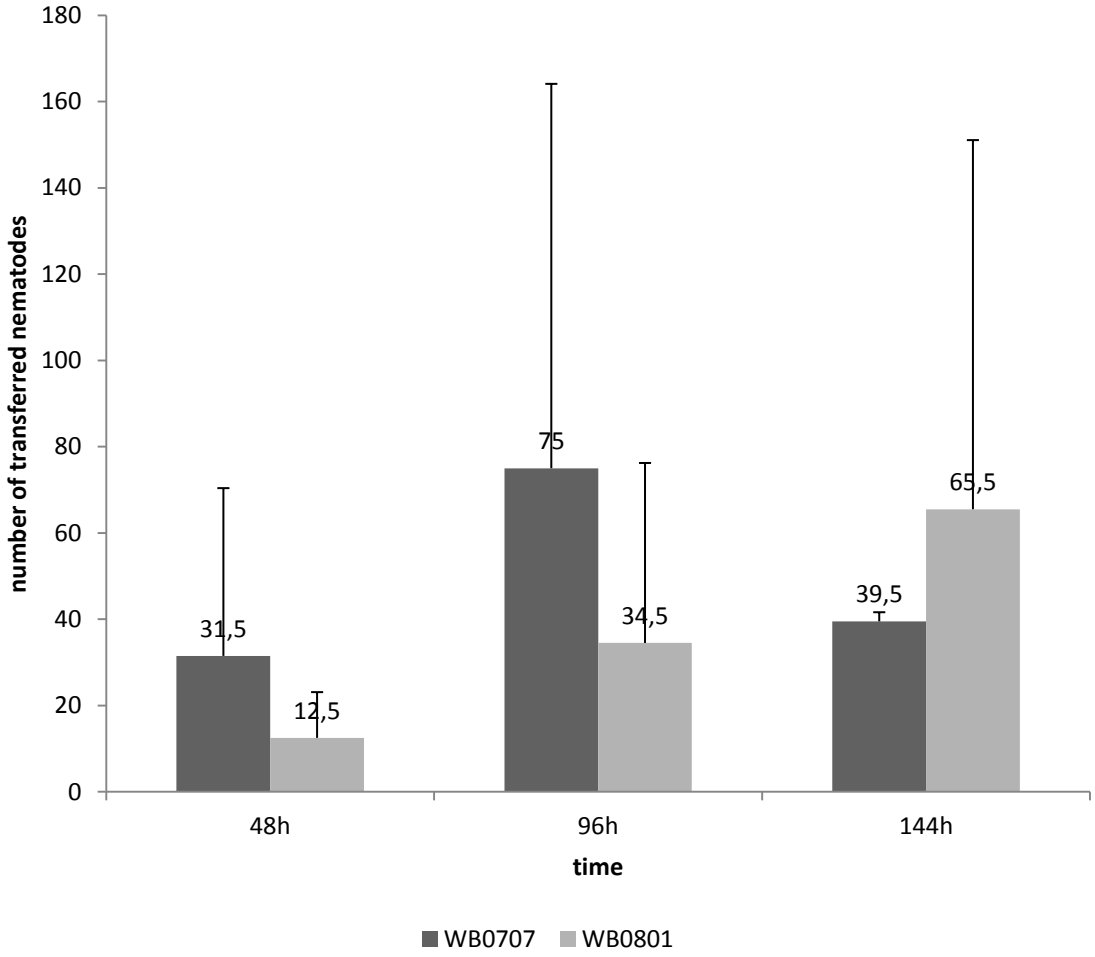

Figure A2. Mean (of two replicates) number of nematodes transferred by *Calliphora vomitoria* after 48 h, 96 h and 144 h incubation for two isolates of *H*. *gingivalis* of different origin, *i.e.*, compost isolate WB0707 and horse dung isolate WB0801. Error bars represent standard deviation.

[8] The dauer stage is the alternative L3 stage in which the larvae go into a kind of stasis enabling them to survive harsh conditions

DISCUSSION

Due to their small size and inability to move long distances, terrestrial nematodes inhabiting ephemeral habitats are challenged to reach new habitats when these are no longer suitable due to crowding or habitat deterioration (*e.g.*, Kruitbos *et al.* 2009). Therefore, larger and more mobile organisms, in most cases arthropods and other invertebrates, which share the same habitat or food preference, are used as vehicles for dispersal or migration (*e.g*., Timper and Davies 2004). Transport of the nematodes can occur internally or externally, passive or active, can be facultative or obligatory (Timper and Davies 2004), and very often are highly species specific (*e.g.*, Krishnan *et al.* 2010). Phoresy is often considered a stepping stone to insect parasitism, such that parasites evolve from non-parasitic insect associates (*e.g*., Anderson 1984; Sudhaus 2010). Additionally, there appears to be an association between invertebrate and vertebrate parasitism since invertebrate-pathogenic and –parasitic clades are all placed sister to vertebrate-parasitic clades (Dorris *et al*. 1999). Phoresy is widespread among bacteriovorous nematodes which exploit ephemeral habitats such as dung (Kiontke 1996; Kühne 1996). The formation of dauer juveniles has long been thought a prerequisite for phoresy (*e.g*., Timper and Davies 2004). Another adaptation towards phoresy displayed by dauer juveniles of some parasitic and phoretic nematodes is nictation, *i.e*., waving behaviour in which they 'stand' on their tails and wave their anterior ends above the substrate in an attempt to make contact with a potential carrier (*e.g*., Timper and Davies 2004).

Chapter 6 revealed that two isolates of *H. gingivalis* originating from different habitats, *i.e*., from compost (WB0707) and from horse dung (WB0801), showed an equally pronounced capacity for phoresy under experimental conditions using *Calliphora vomitoria* (blue bottle fly) as a vector. This study also revealed that phoresy is not restricted to dauer juveniles as both juvenile and adult stages were transferred by the flies, hereby corroborating a study on other compost inhabiting nematodes (Steel *et al.* 2013). And, comparable to Kruitbos *et al*. (2009), the absence of nictation in *H. gingivalis* juveniles demonstrated that this waving behaviour is not a prerequisite for phoresy. The most remarkable observation in *H. gingivalis* was that transferred dauer juveniles and eggs were found in the fly faeces, suggesting that flies sucked up these stages and

passed them without digestion, thus representing some form of passive, internal transport. Phylogenetic analyses further revealed that the used isolates were each placed in one of the distinct molecular *H. gingivalis* lineages, thereby suggesting that phoresy is a general trait for the entire *H. gingivalis*-clade. Other *Halicephalobus* species are also known from their association with insects: *H. limuli* has originally been described from an association with the chelicerate *Limulus* (Timm 1956), *H. similigaster* has been found in a phoretic association with ants, beetles and flies (Köhler 2012), and two other *Halicephalobus* isolates (RGD892 and RGD838) used in the current study originate from an association with termites (Giblin-Davis, personal communication; also see Kanzaki *et al.* 2012). However, information on the phoretic abilities of the other isolates is missing, including *H. mephisto* which is placed sister to all other *Halicephalobus* isolates.

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- **Fonderie P**., Steel H., Moens T., Bert W. (2013) Experimental induction of intraspecific morphometric variability in a single population of *Halicephalobus gingivalis* may surpass total interspecific variability. *Nematology* published on-line, DOI:10.1163/15685411-00002699

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ACTIVE CONTRIBUTION TO INTERNATIONAL CONFERENCES

- 31st International Symposium of the European Society of Nematologists – Adana, Turkey. (2012) Oral presentation:
  "The fascinating biology of *Halicephalobus gingivalis*, a free-living bacterivore with a deadly streak." Fonderie P., Moens T., Steel H., Bert W.

- 17th Benelux Congress of Zoology. Ghent, Belgium. (2010) Oral presentation:
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- 30th International Symposium of the European Society of Nematologists – Vienna, Austria. (2010) Oral presentation:
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- 29th International Symposium of the European Society of Nematologists – Blagoevgrad, Bulgaria. (2006) Poster:
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