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Hibernacular behavior of spirochetes inside membrane-bounded vesicles of the termite protist *Staurojoenina assimilis*

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Abstract
Canaleparolina spirochetes have now been detected in parabasalid *Staurojoenina assimilis*, the dominant and largest wood-ingesting symbiont in the kalotermitid *Neotermes mona* (collected in Saint John, U.S. Virgin Islands, 1999). One to three 15–45 μm spherical vesicles per cell were detected in live preparations by differential interference contrast (DIC) microscopy and recorded on Sony U-matic videotape. The rotational activity inside the vesicles, reminiscent of snake movement in reptilian hibernacula ("snake pits"), suggested that the vesicles were inhabited by spirochetes. Subsequent collection of the same dry-wood-eating termite followed by transmission electron microscopic analysis confirmed that the vesicles were replete with several types of pillotinaceous and smaller spirochetes, many in synchronous movement. At least one morphotype, both inside the vesicle and free in the hindgut intestinal fluid, was identified on the basis of length, diameter and ultrastructure as *Canaleparolina* sp. This large spirochete characterized by a 16:32:16 periplasmic flagellar pattern heretofore had been reported only as the largest ectosymbiotic spirochete, *Canaleparolina darwiniensis*, on the hypertrophied xylophagous trichomonad, *Mixotricha paradoxa*, of Australia. *M. paradoxa* is found only in the intestine (hindgut or "paunch") of *Mastotermes darwiniensis*, the sole survivor, a South Pacific representative of the Miocene mastotermitids. In addition to *Canaleparolina darwiniensis* nearly half-a-million surface treponemes, surface spirochetes that act as motility organelles, are ectosymbiotic on *M. paradoxa*. None has ever been reported in association with *Staurojoenina* or anywhere else. Possible explanations for both the extremely disjunct distribution of the *Canaleparolina*–like spirochete and the "snake-pit" behavior are suggested.

Keywords: *Canaleparolina* sp., *Neotermes mona*, Oxymonadidae, pillotina spirochetes, "snake pits", treponemes, xylophagous dictyopterids

1. Introduction

Xylophagus termites, including the kalotermitid *Neotermes mona,* maintain a complex, unique symbiotic community dominated by wood-ingesting archaeprotists, such as *Staurojoenina assimilis* Kirby. Hundreds of distinctive wood-ingesting heterotrophic protists have been identified in these insects, none of which have been shown to contain standard mitochondria or to require molecular oxygen. All are subject to expulsion from the gut by vigorous peristaltic movement such that they are either highly motile or firmly attached to the chitin-lined intestine by specialized structures. Direct evidence from amber has verified that the termite-protist-bacterial wood-eating symbiosis is at least 20 million years old (Wier et al., 2002). Members of the genus *Mastotermes* have been described from fossil Cretaceous amber (Grimaldi, 1996; Thorne et al., 2000). This suggests not only that the *Mastotermes* sp. symbiosis may be far older (>65 million years old) but also that its fossil distribution was cosmopolitan in equatorial regions for over 40 million years. The xylophagous hindgut termite symbiosis involves both prokaryotic (eu- and archaebacterial microorganisms) and protists that form a metabolically active tissue. No member of the cellulolytic prokaryotic-protist community is found in the foregut, midgut or anywhere else inside the insect. Nor does the community exist beyond the termite in the wood, soil or nearby aquatic environments. Rather it is limited to the hindgut of kalotermitids.

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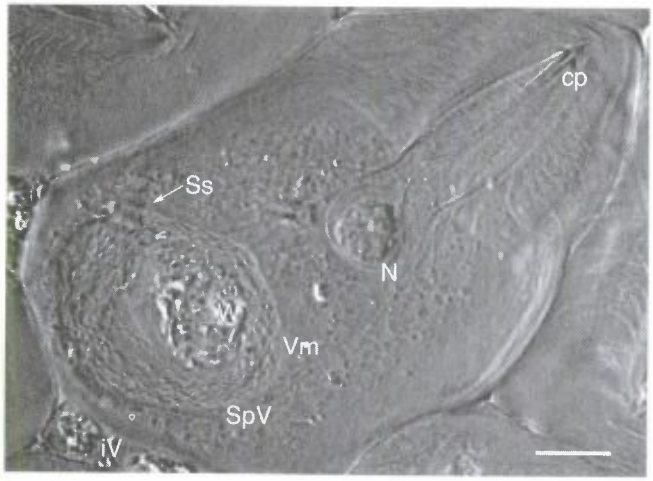

Figure 1. *Staurojoenina assimilis*, live whole cell with rotating posterior vesicle, Nomarski differential interference contrast microscopy. Bar = 20 μm. cp: "centrioles privilegés" (Hollande and Carruette-Valentin, 1971); W: wood; N: nucleus; Ss: synchronously moving spirochetes; SpV: spirochete vesicle; Vm: vesicular membrane; iV: isolated rotating vesicle.

Although the cellulase enzymes apparently are of termite origin, the symbiotic protists, none of which contain mitochondria, ingest particles of wood whereas bacteria such as spirochetes digest sugars such as cellobiose to acetate. Fermentation products include hydrogen, methane and other gases (Breznak, 1984).

The complex ultrastructural morphology of the genus *Staurojoenina* was first described by André Hollande (1986) who confirmed Kirby's (1926) concept that the surface of the cell at its four lobes is covered with rows of symbiotic bacilli. The specific rod-shaped symbionts measure 1×4 μm as determined by scanning electron microscopy (Wier et al., 2004). In *Staurojoenina assimilis* Kirby from the Caribbean, these rods were misidentified as *Citrobacter* and later, by 16S rRNA *in situ* hybridization, the ectosymbionts were recognized as members of the anaerobic entero-eubacterial group: *Bacteroides* (Noda et al., 2006). Although resting cysts from the wood-feeding cockroaches are correlated with the insect molt, no cysts were known from protists of xylophagous termites before their discovery in this *Staurojoenina assimilis* in *Neotermes mona* (Dolan et al., 2004).

2. Methods

Neotermes mona was collected and identified in the field by Sean Werle (Department of Biology, University of Massachusetts-Amherst). The termites were taken on and in the vicinity of exposed red mangrove roots at the shore of Saint John in the United States Virgin Islands in early March, 1999. About forty termites were maintained in the laboratory in glass plates on the wood in which they lived to which were added small cotton-tipped vials with water to provide a steady controlled supply of moisture.

Samples of hindguts were removed with forceps and collected by placement into 0.6% sodium chloride solution. Those taken for electron microscopy were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 hour, and dehydrated in ethanol as previously described (Dolan et al., 2004). The whole guts were kept in unaccelerated Epon-Araldite, for at least 48 hours. Accelerated resin was changed 3 times over 24 hours and polymerized at 60°C overnight. Specimens were sectioned with a DDK diamond knife and examined at 60 kV on carbon coated copper grids with a Phillips CM120 TEM. Light microscopy used Nikon camera-mounted phase contrast, DIC optics and Sony-Umatic videomicrography with an Optiphot camera. The original footage was digitized with the Final Cut Pro program and recorded on a disk with DVD Studio Pro for analysis. Analysis of motion was made based on the digital moving images.

3. Results

The rotating spirochetes in vesicles were seen in about six *Staurojoenina assimilis* vesicles. The frequency, duration and conditions that lead to this "snake pit" phenomenon are unknown. The appearance of a typical live *S. assimilis* cell that contained rotating vesicles is shown in Fig. 1. The wood-filled vesicle is approximately 45 μm in diameter. All the vesicles we observed rotated as units, at speeds from two-to-three rotations per second. At least two different spirochete morphotypes, one larger and one smaller in diameter, were directly observable in the live cells of *Staurojoenina.* Probably at least three distinctive morphotypes participate in this activity, a treponema with a 1:2:1 flagella formula (T, Figs. 2D, 3C,D), a borrelia-like spirochete (B) with a 4:8:4 or 5:10:5 flagellar arrangement, and a thickened outer coat of the inner membrane (Fig. 3C), and the much larger *Canaleparolina* (Cp) morphotype with its 16:32:16 periplasmic flagella (Figs. 2D, 3C,D). The spirochete vesicle in Fig. 2C and at higher magnification in Fig. 2D is approximately three times the diameter of the nucleus (n) in which the chromatin (ch) is visible in the live cell (Fig. 2B). The vesicle is about 29 μm in diameter. The four cytoplasmic lobes in *Staurojoenina assimilis* are studded with ectosymbiotic rods. The lobes alternate with bundles of undulipodia. Spirochete "snake pit" activity is limited to vesicles in the lobes (Fig. 2C) or the posterior cytoplasm near the ingestive zone (Fig. 1, Fig. 2A,B). The four lobes, studded with aligned rod-shaped eubacteria (*Bacteroides* sp.) are unmistakable (Figs. 2C, 3A, 4B–D). Two spirochete membrane-bounded vesicles are clearly seen in Fig. 2C although it cannot be precluded that the thin section reveals a U-tube shaped single vesicle.

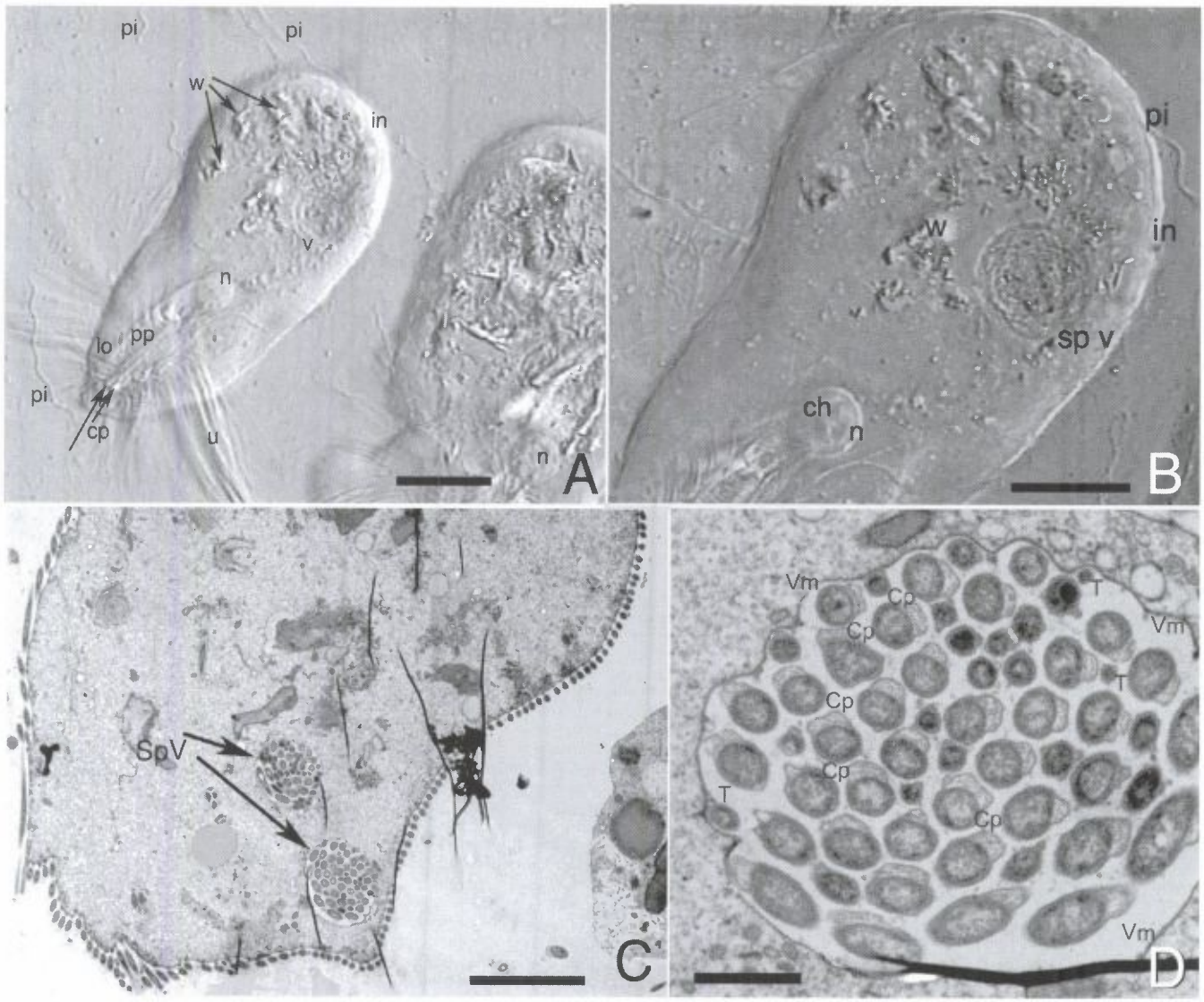

Figure 2. Spirochete-filled vesicles. A. *S. assimilis,* two live cells with wood and spirochete vesicles. A pillotina spirochete (pi) outside the cell is seen at left. Bar = 30 µm. B. The spirochete vesicle (SpV) makes three rotations per second. Bar = 30 µm. C. The bacterial-studded lobe harbors two spirochete vesicles. Bar = 5 µm. D. Two morphotypes, *Canaleparolina*-like (Cp) and treponemes (T) can be distinguished at higher magnification of the lower-most vesicle in C. Bar = 250 nm. (See Bermudes et al., 1988 and Margulis, 2000 for general morphometric spirochete analysis and Wier et al., 2000 for *Canaleparolina darwiniensis*). In: ingestive zone; V: vesicle; pp: parapodial plates; pi: pillotina spirochete; u: undulipodia; SpV: spirochete vesicle; n: nucleus; w: wood; ch: chromatin; Vm: vesicle membrane; lo: lobe; Cp: *Canaleparolina*; cp: "centrioles privilegés" (Hollande and Carruette-Valentin, 1971); T: treponeme.

At the greater magnification of Fig. 2D two spirochete morphotypes are visible at once, and the fact is confirmed that the membrane-bounded structure does not contain any other symbiotic bacteria such as the surface rods. The very rapid, incessant motility of the intracellular spirochetes, recorded without cessation for at least an hour, coupled with the electron micrographs, probably precludes the presence in the vesicles of other symbiotic bacteria.

The spirochete diameters vary by a factor of three. No vesicle had only a single spirochete morphotype. Close connections, probably adhesion structures like that seen in Fig. 3F, were observed. The rapid movement suggests that these adhesions are transient and dynamic.

Membrane fragments in the vesicles (Fig. 3B–D) and coated spirochete outer membranes (Figs. 3D,F) suggest metabolic interactions between the spirochetes in contact within the same vesicle. Vesicular exudates are seen leaving and/or entering *Staurojoenina*'s outer membrane (Fig. 4C). Strong metabolic interaction between the symbiotic surface bacteria (in longitudinal and transverse section (Figs. 4A,C) and the protist is manifest from the morphological observation that each regularly spaced

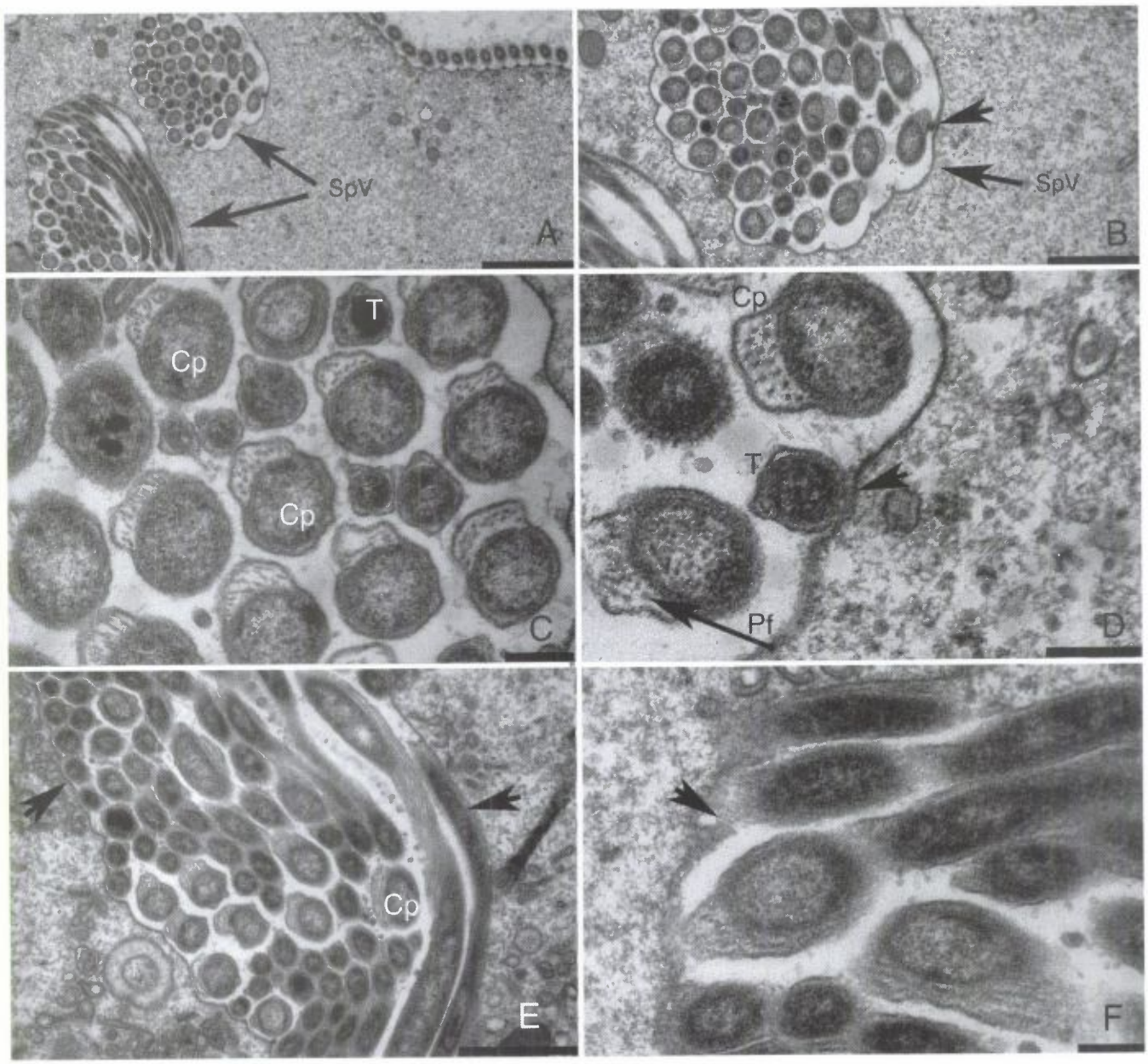

Figure 3. Spirochete-vesicle composite (TEMs = transmission electron micrographs). A. Motility in the videos correlates with the longitudinal spirochete sections at the left most vesicle. Bar = 2 μm. B. Contact between the outer membrane of the spirochetes and the vesicle membrane implies transfer of substances. Bar = 1 μm. C. At least three spirochete morphotypes but no other bacterial forms confirms observations of live cells: the vesicles contain mixtures of spirochete populations but not other bacteria. Bar = 200 nm. D. Tight contact over at least one third of the diameter of this treponeme between its outer membrane and the vesicle membrane and the abundant fuzz and membrane fragments suggest substance transfer, probably in two directions (to and from the bacteria to the protist). Bar = 200 nm. E. Vesicle membrane contact along the longitudinally aligned axis and at least 12 transversely aligned spirochete outer membranes reinforces the possibility that the nature of the relationship of the spirochetes with *S. assimilis* is metabolic exchange for cellulose and/or lignin degradation. In the reticulitermitids that presumably evolved later, the role of intracellular motility for "mixing of the brew" may have been taken over by axostylar protists (Order Oxymonadida especially the family Pyrsonymphidae). Bar = 1 μm. F. The membranes of the spirochetes and the membrane vesicle of the protist appear fused at higher magnification. Bar = 200 nm. SpV: spirochete vesicle; Pf: periplasmic flagella; Cp: *Canaleparolina*; cp: "centrioles privilegés" (Hollande and Carruette-Valentin, 1971); T: treponeme.

ectosymbiont is underlain by its single microtubule that we presume developed in *Staurojoenina* itself (Wier et al., 2004 and Fig. 4C here).

From the temporarily apposed membranes, both spirochete-vesicular and spirochete-spirochete (Figs. 2D, 3B–D and F) we infer that both metabolic exudates,

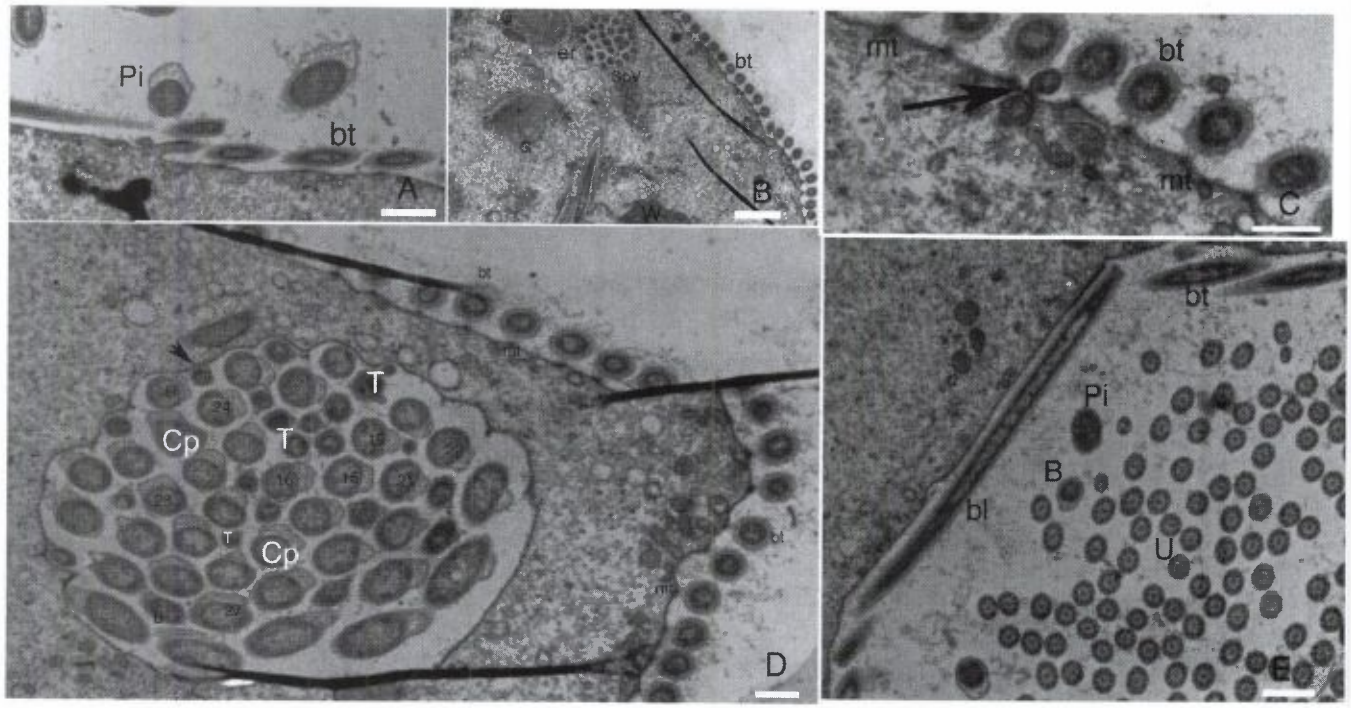

Figure 4. A. Rod bacteria that rib the lobes in transverse section (bt in A–E) verify the observations in live cells (and in E) that pillotina spirochetes (Pi) are found in and among the undulipodia (U). Bar = 500 nm. B. Between the spirochete vesicle and one of the Golgi (G) bodies is a fragment of endoplasmic reticulum (er). Golgi = parabasal bodies in these members of the Archaeprotist phylum "Parabasalia". Bar = 3 μm. C. Material either entering or leaving *Staurojoenina* through its bacterial-studded lobe surface. Microtubules (mt) that subtend each attached bacterium. See Wier et al. (2004) for higher magnification micrographs where the identification of the cortical microtubules is indisputable. Bar = 500 nm. D. "Hibernacular" vesicle contents with approximate periplasmic flagellar counts. The spirochete n:2n:n flagella count of *Canaleparolina* displays a 16-32-16 pattern, 16 per row in two rows, in ideal transverse sections. T = treponeme. Bar = 500 nm. See Margulis (2000). bl: bacteria in longitudinal section; bt: bacteria in transverse section; er: endoplasmic reticulum; G: Golgi; mt: microtubules.

mechanostimulation and its response occur incessantly inside the vesicular "snake pits". Such an inference is justified by the synchronously beating spirochetes (Fig. 1 at Ss arrow) that extended beyond the membrane of the vesicle for a length of about 20 μm. The synchrony, recorded as short horizontal parallel lines, over 40 of which can be counted in Fig. 1, lasted for more than the 10 minutes during which it was videographed. The lines represent two wavelengths of the spirochetes that beat in synchrony according to standardized spirochete morphometrics (Fig. 5).

Spirochetes in this *Neotermes mona* are not limited to the "snake pit" intracellular vesicles. *Canaleparolina*-like multiflagellated spirochetes are outside the protist just beyond the investment of rod bacteria on the lobes and among the undulipodia of the bundles (Figs. 4A,E). The electron micrograph images of Figs. 2 and 4 that reveal spirochetes in both intra- and extracellular positions correlate entirely with observations and video recordings of live cells.

4. Discussion

Comparison of the live spirochetes, the transverse thin section electron micrographic composite plates in which periplasmic flagella number may be estimated (Figs. 3C,D and Figs. 4B,D) with the morphometric guide (Fig. 5) permits the classification of the large diameter "snake pit" spirochetes unambigously as pillotinas. The lack of crenulations, the diameter and length, and the probable number of periplasmic flagella, suggest that the largest of the snake pit spirochetes should be assigned to the genus *Canaleparolina*. However, because the diameter of *Canaleparolina darwiniensis* from the Australian termite *Mastotermes darwiniensis* is larger, it has the periplasmic flagella in one or two rows and the flagellar bundle subtends at least half of the diameter, it is likely that the *Canaleparolina*-like spirochete from *Neotermes mona* will need assignment to a second species. The *Canaleparolina*-like snake pit spirochete lacks the neat flagellar rows, the imbricated external plates seem to be absent and the flagella

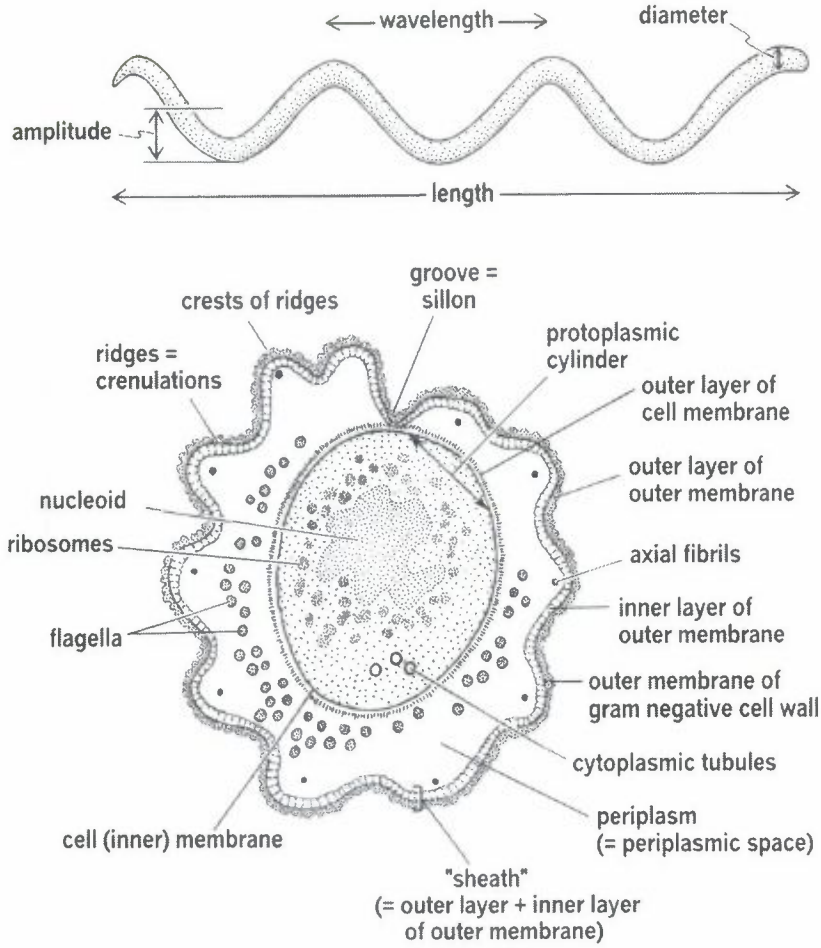

Figure 5. General features that permit identification of pillotina spirochetes. Although the crenulated *Pillotina* sp. was not seen in *S. assimilis* membrane vesicles application of this set of criteria leads to conclusion based on morphometric analysis that the dominant pillotinaceous spirochete in the vesicles most resembles published features of *Canaleparolina darwiniensis* (Wier et al., 2000). (See details in Bermudes et al., 1988).

subtend as little as 90° of the diameter relative to the 180° seen in the *Mixotricha* epibiont. The species assignment will probably require further morphogenetic correlated with molecular biological analysis.

Two mysteries present themselves immediately. What is the biological significance of the intracellular vesicles full of writhing spirochetes in *Staurojoenina assimilis*? The activity is so conspicuously comparable to reptilian "snake pit" behaviour that the image conjured by this name is appropriate. Furthermore this is a case of hypersymbiosis: The spirochetes are endosymbiotic in healthy parabasalid hypermastigotes, themselves intestinal symbionts in wood-feeding termites.

Secondly, what might be the relationship between *Canaleparolina darwiniensis* on the Australian *Mixotricha paradoxa* and the similar large spirochete in *Staurojoenina assimilis* vesicles in the intestine of *Neotermes mona*? These cellulolytic isopterans, presumably directly descended from cryptocercid wood-ingesting social cockroaches, are placed at the base of the dictyopterid (cockroaches and termites) lineage. The *M. darwiniensis* termite, and therefore the spirochetes, are limited to dry hot habitat in the northern Australian tropics i.e., to Kakadu National Park and contiguous regions near Darwin. On each normal *Mixotricha paradoxa* cell approximately 200 long distinctive *Canaleparolina darwiniensis* spirochetes are attached to the cortex (Wier et al., 2000). The length of these spirochetes is 25–30 μm. As a scattered presence they are limited to the cortical surface of this anomalous giant trichomonad; none is present on the posterior ingestive zone.

The Australian and the Caribbean "*Canaleparolina*-like" pillotina spirochetes today are separated by half the world (approximately 12,000 miles of ocean) and disjunct

in isopterans whose ancestors are thought to have diverged in the late Paleozoic or at least, no more recently than the late Cretaceous Period of the Mesozoic Era.

Of course we can not answer these questions, but we offer some suggestions that warrant further study. The answers may be related to the refractory nature of the cellulose and lignin polymers of wood, especially in anoxic environments, and the peculiar ecological conditions required for the wood-feeders to complete digestion of these polymers to carbon dioxide and water.

Why, as temperatures warm and become permissive, do pits in the ground full of writhing snakes, of different sizes, ages and most often members of different species, appear? These serpent "hibernacula", according to Professor Alan Richmond (Dept. of Biology, University of Massachusetts-Amherst) provide safe and comfortable habitat for frenetic matings. Chemical communication where specific pheromones are elicited and responded to has been observed such that fertile intraspecific mating patterns are the rule. Perhaps, in only an analogous and far smaller way, the necessity of intense microbial motility is dictated by a flux, not of pheromones (there is no evidence of cell-to-cell conjugation in any spirochete) but of enzymes required for cellulose and lignin degradation through sugar to acetate. The two-carbon intermediate, acetate, was identified as the carbon source that travels from the hindgut to the chitin layer of the termite intestine. The direct search for specific cellulases and glycolytic enzymes might be instructive.

It may be relevant that rhinotermitids (subterranean termites, Family Rhinotermitidae) tend to harbor pyrsonymphids: members of the protist order Oxymonadida (e.g., *Notila, Oxymonas, Pyrsonympha, Saccinobacculus*), many of which agitate the cellulolytic environment by axostylar-mediated microtubular intracellular motility. Except for nonmotile *Oxymonas* sp., pyrsonymphids are unknown, or scarce, in Kalotermitidae. Might it be that the role of "mixing the brew" accomplished in part by the "snake-pit" phenomenon here was taken over by the evolution of the pyrsonymphids in the "higher" wood-ingesting termites? Experiments to measure the actual metabolites in the "snake-pit" vesicles and the reticulitermitid intestine with and without pyrsonymphids, can, in principle, resolve or refute this hypothesis.

It seems unlikely to us that pillotinaceous spirochetes, since they originated, have ever left the anoxic confines of the insect intestines. The protist symbionts tend to die within minutes of removal from the gut. The very few protists that were grown anoxically on cellulase in culture by Yamin (1979) were difficult to sustain *in vitro*. Neither *Pillotina*, nor any other pillotinaceous (large, morphologically complex spirochetes in wood-eating coackroach and termite handguts) have ever been grown in culture nor seen elsewhere. The wet, salty, and gas-regulated intestinal communities that predictably harbor unique spirochetes, amitochondriate mastigotes and other bacteria are apparently profoundly stable and conservative. The termites are photophobic voracious eaters of the wood in which they nest. Their diets are primarily wood and water. Five sources of nitrogen are known, each by itself inadequate: (1) fixation of dinitrogen gas in the intestine by competent bacteria including N_2 fixation by specialized spirochetes and other capable eubacterial microbes, (2) cannibalism of infirm and damaged nest-mates, (3) ingestion of fungal hyphae and spores brought in with wood, (4) digestion of plant wood nitrogenous compounds and (5) limited ingestion of the intestinal protists and bacterial bodies. This conservative microbial regime most likely has persisted for millions of years in hot woody habitats. If so, the disjunct distribution of *Canaleparolina* spirochetes has followed the speciation patterns of the tropical trees and wood-feeding termites, and the movement of continental plates. If true, the xylophagous spirochete-termite-protist symbiosis may be traced back 200 million years to the contiguous land mass of Pangaea before it separated into Gondwana, that gave rise to Antarctica and the Australian subcontinent. The original cryptocercid termite ancestors must have ranged, in at least the last 100 million years since the early Cretaceous, in tropical woody habitats that maintained connections with the mastotermitid ancestors of the kalotermitidae, including *Neotermes,* in landmasses of what became Central America because *Mastotermes electrodominicus* and *Mastotermes mexicanus* are known from the Miocene fossil record. The colonization of the island of Saint John by the modern population of *Neotermes mona* was, by this reckoning, an extremely recent event.

Spirochetes that range in diameter from 0.1 to 0.5 μm abound in the xylophagous insect gut. They are found free in the lumen, attached casually or precisely fixed to wood-ingesting protists. They have entered with no loss of motility or viability the insides of various anaerobic protists. Such spirochetes are especially common on devescovinids, calonymphids and other parabasalids. The significance of this report of thriving balls of spirochetes inside *Staurojoenina assimilis* vesicles relates to our model of the origins of the nucleus and its cytoskeletal system as recombinant from an archaebacterial-eubacterial (*Thermoplasma-Spirocheta*-like) fusion.

We envision the earliest nucleus (Margulis et al., 2006) to have co-evolved with intracellular motility (including mitotic) systems. The first nucleus, on our model, began as a nucleoid (like those of the free-living prokaryote *Gemmata obscuriglobus*; Margulis et al., 2005). The nucleus evolved from eu- and archaebacterial recombination that formed DNA-RNA-protein membrane-bounded structures. From the beginning it was attached to a protein connector (the "rhizoplast" in old protozoological literature). Comparable to extant symbionts of *Cryptotermes cavifrons* and other kalotermitids, the spirochetes contained 24 nm cytoplasmic tubules as do

today's *Hollandina*, *Diplocalyx* and other pillotinas. They became the undulipodia (cilia, eukaryotic flagella, etc). The spirochete attachment sites evolved into the kinetosome-centrioles that underlie the axonemes of the undulipodia of protoctists, of animal cells including sperm as well as plant sperm. Amitochondriate swimming protists (wood-feeding roach mastigotes) require this microtubule-based cytoskeleton (e.g., "missing piece" of Dolan, 2005) for cell division and meiotic sexuality, as do all sexual eukaryotes. Unlike other eukaryotic cells these protists lack all vestiges of mitochondria at all stages in their life histories yet they show well-developed microtubule systems with standard undulipodia. They tend, like *Staurojoenina*, to have profound variations on the mitosis pattern typical of animals and plants (Hollande and Valentin, 1968). Thus the original nuclei were tethered; by our reckoning, in other words, they were part of the karyomastigont organellar system (Margulis et al., 2006).

The mitotic and meiotic processes (present in archaeprotists such as *Barbulanympha* and *Notila*) must have evolved prior to the symbiotic acquisition of mitochondria since over 200 species, all in anoxic environments (e.g., trichomonads, trichonymphids, monocercomitids, calonymphids, hypermastigotes, etc.) have mitotic cytoskeletal systems but lack evidence (morphological, physiological and biochemical) of mitochondria. Both gamma tubulin and scleroderma fluorescent antibodies specifically label the rotary shear zone of the karyomastigont of *Caduceia versatilis*, an amitochondriate devescovinid (Melnitsky and Margulis, 2004). The most parsimonious explanation of the antibody results is that these conserved antigens and the attached and released nuclei of karyomastigonts of parabasalid protists (i.e., archaeprotists) represent the base of the eukaryotic lineage. The symbiotic spirochetes, once-independent, are modern legacies of Proterozoic-eon muddy bacterial communities whose living components genetically integrated to form the first nucleated cells. The spirochete behavior, biochemistry, structure and community interactions in anoxic and "dysaerobic" marine near-shore habitats, rich in cellobiose, cellulose and sulfide from cyanobacterial, algal and later plant decomposition, were internalized by ancestors of wood-feeding roaches and termites. The spectacular Darwinian "imperfections and oddities" of the bacteria and protists now in xylophagous insect intestines have not substantially changed in over a thousand million years. They offer a glimpse of the evolutionary sequence that led to eukaryotic cells at the base of the lineage from which we, and all other animals, descended (Margulis et al., 2006; Chapman and Alliegro, 2007 in this volume).

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