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RESEARCH ARTICLE

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Canaleparolina darwiniensis, gen. nov., sp. nov., and other pillotinaceous spirochetes from insects

Summary We describe two new pillotinaceous spirochetes (*Canaleparolina darwiniensis*, *Diplocalyx cryptotermitidis*) and identify for the first time *Hollandina pterotermitidis* from both the subterranean termite *Cryptotermes cavifrons* and the wood-eating cockroach *Cryptocercus punctulatus* based on morphometric analysis of transmission electron micrographic thin sections. *C. darwiniensis*, gen. nov., sp. nov., limited to near Darwin, Australia, invariably is present on the surface of the treponeme-studded trichomonad *Mixotricha paradoxa*, a consistent inhabitant of the hindgut of healthy termite *Mastotermes darwiniensis*. The spirochete both attached to the surface of protists and free-swimming in the paunch (hindgut) lumen of the insect has 16 periplasmic flagella (16:32:16) and imbricated wall structures that resemble flattened crenulations of *Pillotina*. The flagella surround half the protoplasmic cylinder. *C. darwiniensis* is the largest (0.5 μm diameter × 25 μm length) of the three epibiotic bacteria (two spirochetes, one rod) that comprise the complex cortex of its host *Mixotricha paradoxa*. Several criteria distinguish *Diplocalyx cryptotermitidis* sp. nov. isolated from *Cryptotermes cavifrons* intestine: smaller diameter, fewer flagella, absence of inner and outer coats of the outer membrane, wider angle subtended by its flagella and, most notably, cytoplasmic tubule-associated centers, which are periodic electron dense spheres within the protoplasmic cylinder from which emanate cytoplasmic tubules up to 24 nm in diameter. This is also the first report of abundant populations of *Hollandina* in *Cryptotermes cavifrons* (those populations belong to the species *H. pterotermitidis)*. Morphometric analysis of the first thin sections of any spirochetes (published nearly 40 years ago by A.V. Grimstone) permits us to identify the large (0.9 μm diameter) free-swimming intestinal symbiont of *Cryptocercus punctulatus* also as *Hollandina pterotermitidis*.

Key words *Canaleparolina darwiniensis* · *Diplocalyx cryptotermitidis* · *Hollandina pterotermitidis* · *Mixotricha paradoxa* · Cytoplasmic tubule-associated center (CTAC)

Introduction

Pillotinaceous spirochetes (Gram-negative, helical, motile eubacteria greater than 0.4 μm in diameter that are symbionts of metazoa) are known only as normal intestinal microbiota of dictyopterid insects (i.e., xylophagous cockroaches and termites). Because their large diameters imply they contain more than 4–5 periplasmic flagella, observations of live hindgut contents suffice to classify them in the family Pillotinaceae. Further identification requires morphometric analysis of electron microscopic thin sections [14].

The tropical termite *Mastotermes darwiniensis* (Isoptera; Mastotermitidae), limited today to northern Australia, harbors several amitochondriate anaerobic protists (class Parabasalia) whose rRNA phylogeny is under current investigation [9]. Unlike other extant termites but typical of roaches, the female insect lays egg cases. The extrusion of such structures, comparable to oothecae, leads these modern termites to be classified in the family Mastotermiditidae along with their far more widely spread extinct Eocene, Oligocene and Miocene relatives. A unique wood-digesting microbiota in the hindgut of *Mastotermes darwiniensis* (the largest termites in Darwin, Australia) was reported by Sutherland [19]. She named *Mixotricha paradoxa* ("mixed-up hairs paradoxical") an abundant large uninucleate trichomonad protist that swims forward in a straight line. First suggested to be a ciliate, *Mixotricha* was claimed by Sutherland to be a unique cell that

simultaneously bears cilia and flagella. Further investigation revealed *Mixotricha* to be a hypertrophied trichomonad easily seen with the unaided eye (cells are 300–800 μm long). As do most trichomonads, the protist bears three forward directed and one trailing "cilium" at the anterior end. Such cell structures, composed of microtubules in the [9(2)+2] array are here called undulipodia, the generic name for eukaryotic flagella and cilia [2]. Ultrastructural examination showed that the motile protrusions that cover the cell in great profusion from its anterior portion to the rounded posterior region where wood particles are ingested were not cilia. They were shown to be two distinct morphotypes of attached symbiotic spirochetes by Cleveland and Grimstone [5]. The many smaller, far more abundant surface epibionts look just like a dense investment of cilia, but ultrastructure analysis shows they are treponemes with the standard 1:2:1 or 2:4:2 arrangement of flagella. The population size reaches more than 200,000 attached spirochetes per *Mixotricha* cell. Each treponeme has its own docking site on the cortex of the protist. The protist, studied by Prof. L.R. Cleveland (1892–1969) is seen live in his films [Cleveland LR (1956) 16 mm B&W films: Flagella of termites, part II, archived at the Smith College Library in the "non-print materials" section]. The larger, more casually associated spirochete was never identified.

Abundant large spirochetes in other dictyopterid insects, both the wood-eating cockroach (*Cryptocercus punctulatus*) and the dry wood-eating termite (*Cryptotermes cavifrons)* also have been depicted in film; however, the lack of electron microscopic study until now has precluded their identification. All work on the large spirochete found in *Cryptocercus punctulatus* is from unpublished micrographs kindly provided by A.V. Grimstone of Pembroke College, Cambridge University, Cambridge, England.

Materials and methods

Host insects A few individuals of *Mastotermes darwiniensis* were a gift from Prof. Betsey Dyer of Wheaton College, Norton, Massachusetts, who received them from the colony maintained by Thomas Hertel, under the direction of Prof. K. Hausmann of the University of Berlin, Germany. The large colony, originally from Darwin, Australia, is located in the materials processing laboratory in Berlin. We maintained samples of termites in plastic containers (<50 termites) for a few weeks on filter paper and native wood at 30°C in an incubator. High humidity was sustained by cotton wool saturation. Observations, videographs and still photographs were taken with a CDC-iris color video camera mounted on Nikon Diaphot (inverted), Optiphot (darkfield, phase-fluorescence), Microphot (Nomarski DIC-phase contrast) or Fluorophot (brightfield, phase contrast fluorescence) microscopes.

Cryptotermes cavifrons, collected by Dr. Mark Deyrup (Archbold Biological Station, Lake Placid, Florida) and identified by Dr. Rudi Scheffrahn (Fort Lauderdale Research and Education Center, Florida), was maintained in the laboratory at room temperature in its original wood. Hindguts were extracted, punctured in 0.6% NaCl solution and their contents observed as previously described [7].

Cryptocercus punctulatus were collected in rotting timber in the wooded Appalachian mountain foothills around Mountain Lake Biological Station, Virginia, USA, by L.R. Cleveland.

Ultrastructure study 5–10 hindgut samples of *Cryptotermes cavifrons* were dissected into 1 ml of 2.0% glutaraldehyde in a 0.5 M Sorensen's phosphate buffer (pH 6.8). Animal tissues were removed and the suspension centrifuged for 10 seconds at 10,000 × *g*. The supernatant was mixed 1:1 with warm agar (45°) (1.5% in 0.05 M Sorensen's buffer) in a 2 ml Eppendorf tube for five minutes. The agar-specimens were stored in 2.0% glutaraldehyde overnight (4°C) and post-fixed in 1% OsO_4 for 1.5 h. Each specimen was rinsed in Sorensen's buffer, dehydrated in a graded ethanol series (5 min each: 10%, 25%, 50%, 75%, 95%, then 3 × 5 min in 100%). Specimens were infiltrated with Epon-Araldite-812 resin as follows: 2 h in 3:1 resin-acetone, 2 h in 1:1 resin-acetone and overnight in 3:1 resin-acetone (uncovered). Specimens were transferred to 100% resin for 1 h, vacuum desiccated for 24 h and polymerized at 55°C for 24 h. Ultra-thin sections were cut on a Reichert Ultramicrotome with a glass knife and collected on chloroform-cleaned grids (400 mesh copper). Sections were stained for 10–12 min with 2% uranyl acetate followed by 3–5 min with Reynold's lead citrate, allowed to dry at least 1 h and viewed on a JEOL JEM 100-S electron microscope at 80 kV. All sections were viewed within 12 h of cutting.

Spirochetes from *Cryptocercus punctulatus* were fixed for electron microscopy by A.V. Grimstone using techniques described by Grimstone and Gibbons [12]. Morphometric analysis involved the 15 characteristics described in [1]. They are expanded to include more measurements: granules, rosettes, composite structures in protoplasmic cylinders, and other traits [13, 16] presented in [15] and summarized here (Table 1).

Results

The *Mixotricha paradoxa* large spirochete On inspection of live termite gut material, *Mixotricha paradoxa* is immediately distinguishable from the other large protists of the *Mastotermes* gut (the hypermastigotes *Deltatrichonympha operculata* and *Koruga bonita)* by its straight swimming movement. The swimming motility of *M. paradoxa* is generated by surface spirochetes of two different sizes. *M. paradoxa* has a brown pigmented anterior with a triangular pointed shape, whereas the hypermastigotes have a conspicuous bulbous waving undulipodiated rostrum. At the anterior end of *M. paradoxa,* the smaller treponeme tends to the offbeat synchronous sort of movement pattern (called "metachronal waves" by protozoo-

Table 1 Morphometric criteria in spirochete analysis[1]

	Criteria (method)	Genus[2]	Reference
1	Diameter (LM, NS, TEM, VD)	AG	1
2	Number of flagella (one terminus) (TEM)	AG	1
3	Sillon (TEM)	*Pillotina*	1
4	Crenulations (TEM)	*Pillotina*	1
5	Ratio: OCOM/OM (TEM)	*Hollandina*	1
6	Ratio: ICOM/OM (TEM)	*Clevelandina*	1
7	Ratio: OCIM/IM (TEM)	*Diplocalyx*	1
8	Ratio: Diameter of protoplasmic cylinder/diameter (TEM)	*Spirosymplokos*	1
9	Angle protoplasmic cylinder subtended by flagella (TEM)	*Hollandina*	1
10	Flagellar bundle (TEM)	*Cristispira*	1
11	Length, µm (LM, NS, VD)	AG	1
12	Amplitude, µm (LM, NS, VD)	AG	1
13	Wavelength, µm (LM, NS, VD)	AG	1
14	Tubules (in protoplasmic cylinder) (TEM)	*Diplocalyx*	1
15	Polar organelle (TEM)	*Cristispira*	15
16	Rosettes (TEM)	*Cristispira*	15
17	Granulated cytoplasm (TEM)	*Spirosymplokos*	13
18	Composite (>1 protoplasmic cylinder/periplasm) (TEM)	*Spirosymplokos*	13
19	Cytoplasmic tubule-associated center (TEM)	*Diplocalyx*	This work

[1]See Fig. 5 for illustrations.
[2]Genus in which the feature is highly conspicuous or value is large. AG means the criterion is applicable to all genera. Cystic propagules reported for *Borrelia* [3] and *Spirosymplokos* [13].
Abbreviations: LM, light microscopy; NS, negative stain transmission electron microscopy; TEM, thin section transmission electron microscopy; VD, videomicroscopy; OCOM, outer coat of outer membrane; OM, outer membrane; ICOM, inner coat of outer membrane; IM, inner membrane; OCIM, outer coat of inner membrane.

logists, Fig. 1A, arrow). This form of movement is entirely different from the looser, less organized behavior of the larger spirochete (Fig. 1A–C). The larger spirochete extends all over the surface of the cell except at the ingestive smooth, rounded posterior (Fig. 1B). The large spirochetes are somewhat irregularly distributed at intervals of about 1 per 20–30 µm. The spirochetes are embedded in a conspicuous thick (5 µm) ribbed cortex, which, at ultrastructural resolution, is revealed to be an emergent structure (Fig. 1C). Protist filamentous material whose ultrastructure is reminiscent of typical actin fibers is present in a regular pattern in each raised portion of the protist's surface. The two kinds of embedded prokaryotic symbionts (the treponeme spirochete and the bacterial rod) adhere to the raised protist surface (refered to as the "bracket" by Cleveland and Grimstone [5]). Protist, rod bacteria, and spirochetes together form a ribbed regular surface cortex. The cortical pattern covers the anterior two-thirds of the *M. paradoxa* cell. The cortical pattern resembles the ciliate cortex. At the edges of the cortex, the large spirochetes extend as much as 30 µm out from the cell. They actively beat and twitch for longer than the underlying protist persists. Some move for 20 min or more after the total disintegration of the *M. paradoxa* cell. A large spirochete with identical features was videographed live on several occasions attached to the second conspicuous type of protist in the *Mastotermes darwiniensis* intestine: *Deltatrichonympha operculata* or *Koruga bonita.* These hypermastigote species were indistinguishable by us. The smaller *Deltatrichonympha nana* is obviously distinctive. Less frequently the large spirochetes were seen free-swimming in the gut.

Transverse ultrastructural sections reveal an abundance of periplasmic flagella, at least 16 from each end that number as many as 32 where they overlap (Fig. 2). Probably because of incessant movement, the imbricated surface structures are only seen in favorable sections (Figs. 2B, C) where they resemble the crenulations of *Pillotina* that have collapsed. Inner and outer coats of the outer membrane, although small, are detectable. Their measurements are tabulated as criteria 5, 6 and 7 in Table 1. In some sections a small outer coat of the inner membrane can also be discerned. The presence of cytoplasmic tubules, suggested in Fig. 2C, needs further verification at higher power.

The presence of 16 periplasmic flagella, the lack of the conspicuous "calyx" (outer coat of inner membrane) and the imbricated crenulations confirm the uniqueness of the *M. darwiniensis* large spirochete as does comparison with other pillotinaceous spirochetes including *Diplocalyx*, its closest relative (Table 2).

The two *Cryptotermes cavifrons* spirochetes The two large spirochetes of *Cryptotermes cavifrons* belong to known genera. They were easily distinguished from each other but only by ultrastructural analysis. Their habitats, free-swimming in the gut, and diameters overlap; differences in their lengths are not discernible. Under the electron microscope their affinities to *Diplocalyx* (Fig. 3) and to *Hollandina* (Fig. 4) were clear. The data in Table 2 compare the *Cryptotermes cavifrons* large spirochetes with the type species from the European *Kalotermes (Calotermes) flavicollis*. *Diplocalyx* in the North American

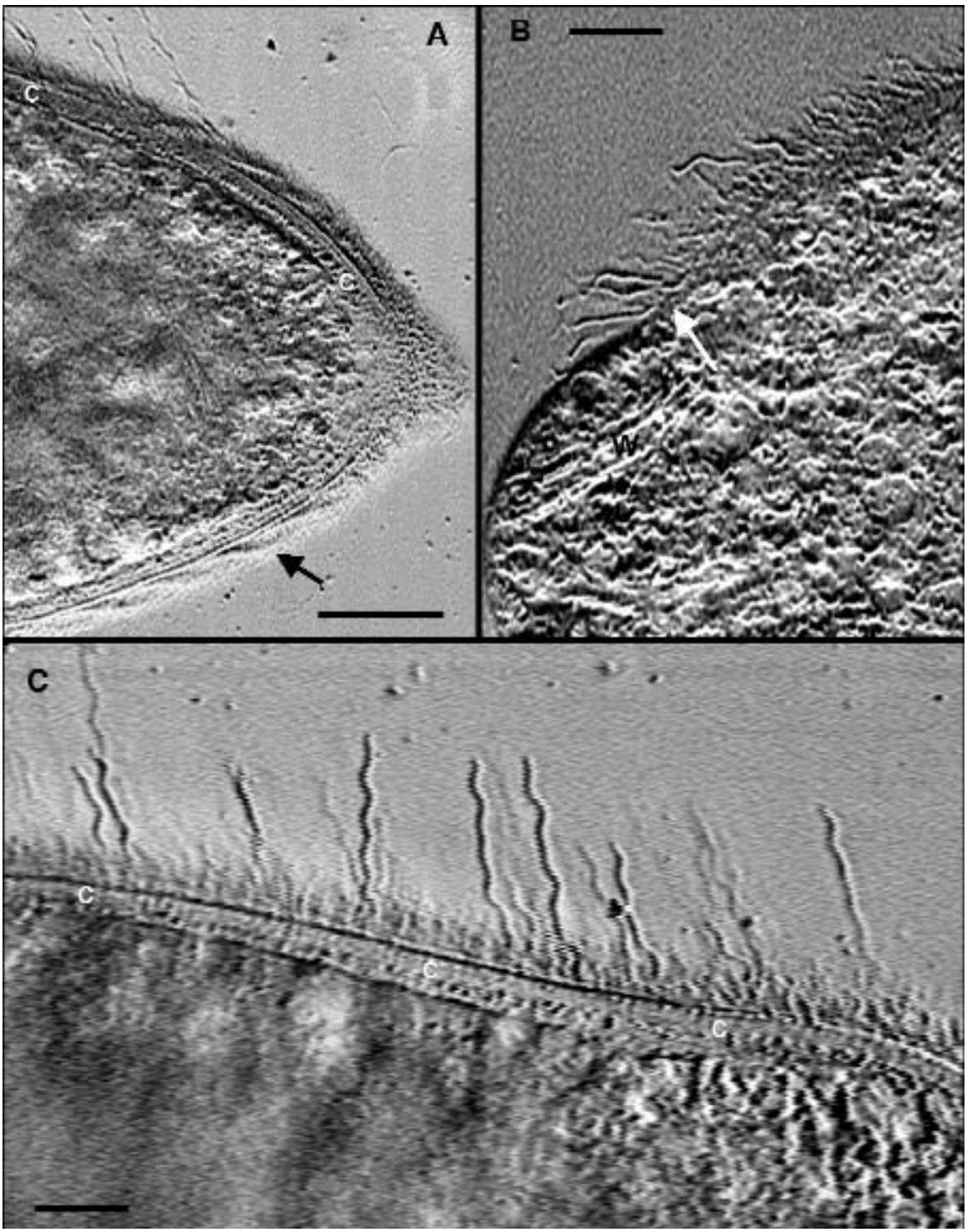

Fig. 1 *Canaleparolina darwiniensis* videographed live. (A) Anterior portion of *Mixotricha paradoxa*, the metachronal waves of the symbiotic treponemes at arrow. Cortex (c), emergent property of the three sorts of epibionts. At least six large spirochetes protrude at left. Bar 30 µm. (B) Large spirochete populations are most conspicuous at interface of cortex and posterior region marked by the arrow. Left posterior of *Mixotricha* from which at least seven large spirochetes protrude, wood (w) particle in posterior cytoplasm. Bar 20 µm. (C) More than twelve large spirochetes protrude from the *Mixotricha paradoxa* cortex (c). Bar 10 µm

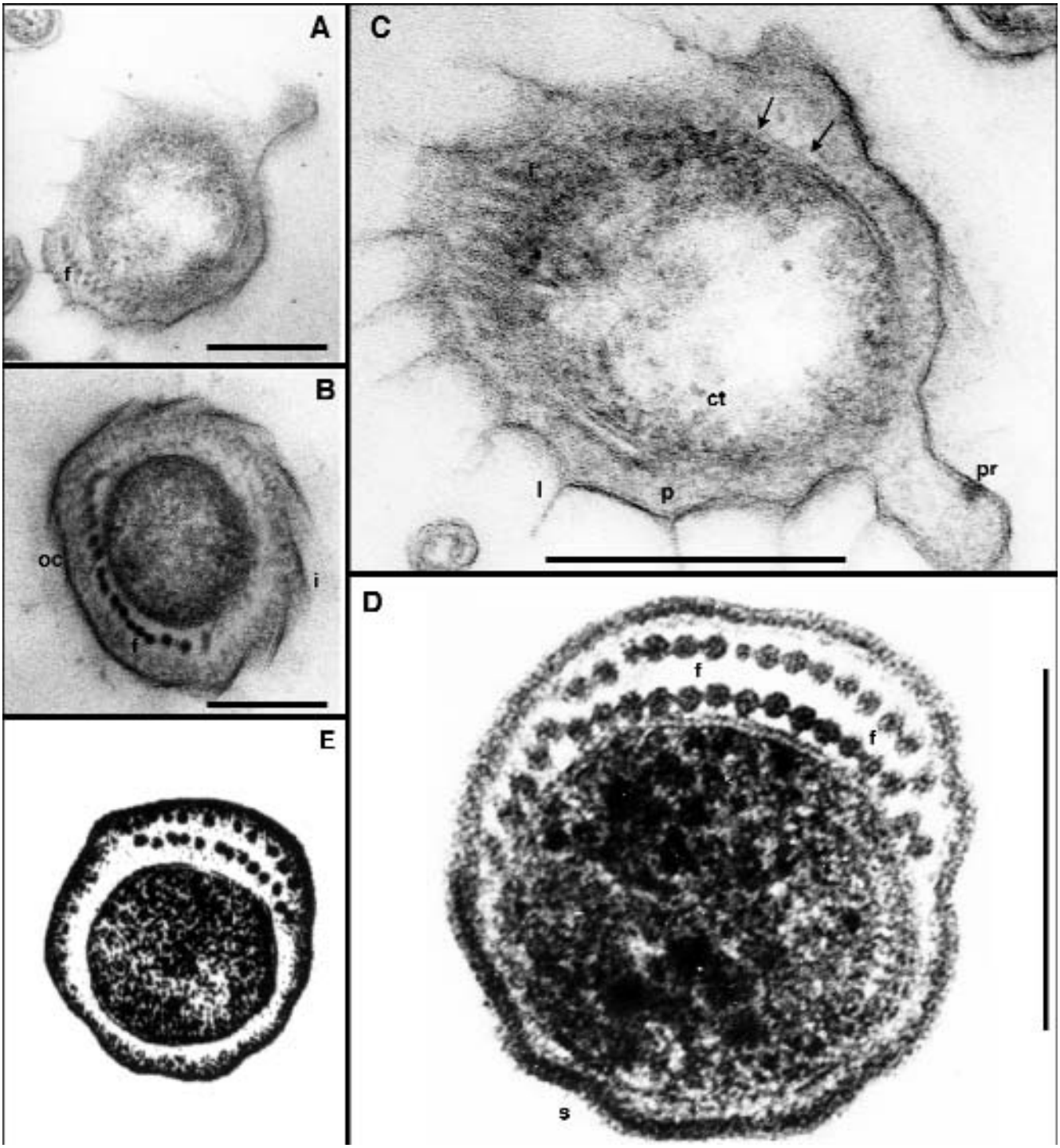

Fig. 2 (A–D) Large epibiotic spirochete, transverse sections *Canaleparolina darwiniensis*, (f) flagella, (p) periplasm, (ct) cytoplasmic tubule, protoplasmic cylinder (arrows), (pr) unidentified protrusion, (i) imbrications, (oc) outer coat of outer membrane. (D) Note overlapping flagella (f) and sillon (s). Bars 0.5 µm. (E) *C. darwiniensis* transverse section drawn from electron micrographs by Christie Lyons. Same as Fig. 5, lower right

Table 2 Morphometric comparisons of *Canaleparolina*, *Diplocalyx* and *Hollandina*

Criteria	*Canaleparolina* type sp. [this work]	*Diplocalyx* sp. [this work]	*Diplocalyx* type sp. [10]	*Hollandina* [11]	*Hollandina* sp. [this work]	*Hollandina* type sp. [16]
Host species	*Mastotermes darwiniensis*	*Cryptotermes cavifrons*	*Kalotermes* flavicollis*	*Cryptocercus punctulatus*	*Cryptotermes cavifrons*	*Pterotermes occidentis*
Location in host	Attached and unattached to *M. paradoxa* **	Free swimming in hindgut	Free swimming in hindgut	Free swimming in hindgut	Free swimming in hindgut	Free swimming in hindgut
Diameter (μm)	(0.5) 0.3–0.7	0.45–0.73	0.7–0.9	1.5–3	0.55–0.83	0.5–1.0
Flagella (ca.)	(16) 12–20	16	40–60	□ 80	□ 30	40–60
Sillon	–?	+	+	+	+	– (none or poorly developed)
Crenulations	+(modified with imbrications)	–	–	–	–	–
OCOM/OM[1]	0.318–0.360	ND	0.6–2.0	4–5?	1.5–2.5	2.4–8.0
ICOM/OM[2]	1.17–1.30	ND	0.9–2.1	0–0.2?	ND	0.0–1.3
OCIM/IM[3]	0.480–0.885	4.0–6.0	6.4–7.7	0	ND	1.0–2.5
PC/diameter[4]	0.5–0.6	0.53–0.68	0.48–0.53	0.5	0.57–0.70	0.6–0.8
Angle subtended by flagella (°)	170	65–105	50–70	120–180	85–150	105–330
Flagellar bundles	–	+	+	+	+	+/–
Length (μm)	22–26	ND	ND	ND***	ND	8–50
Amplitude (μm)	6.0–6.2	1.75	ND	ND***	1.8–2.0	1.8–2.2
Wavelength (μm)	(5) 4.5–5.5	12.0–12.5	ND	ND***	7.0–8.0	ND
Cytoplasmic tubules	+?	+	+	+	ND	+
Cytoplasmic tubule-associated center	–	+	?	–	–	–
Polar organelle	ND	–	+	+?	+	+
Rosettes	–?	–	–	–?	–	–
Granulated cytoplasm	–	–	–	–	–	–
Composite (>1 PC/periplasm)	–	–	–	–	–	–
Pili	ND	+	ND	ND	ND	ND
Length of pili (μm)	ND	0.3–0.5	ND	ND	ND	ND

* *Kalotermes* in the English and German literature is equivalent to *Calotermes* in French literature.
** Also adheres to other large mastigotes: *Deltatrichonympha* and *Koruga.*
*** Estimated from LR Cleveland video (c.1946) *Sex and reproduction in flagellates of the wood-digesting cockroach*.
ND, not detected. + and – mean presence and absence, respectively.
[1]Ratio of thickness of outer coat of outer membrane to outer membrane thickness.
[2]Ratio of thickness of inner coat of outer membrane to outer membrane thickness.
[3]Ratio of thickness of outer coat of inner membrane to inner membrane thickness.
[4]Ratio of diameter of protoplasmic cylinder to cell diameter.

termite is smaller than previously reported in members of the genus but in other ways entirely like it. We extend the observations of *Diplocalyx* by noting the presence of spherical structures, approximately 0.2 μm in diameter. With these are associated cytoplasmic tubules, from smaller to a maximum of 24 nm in diameter. Some are over to 75 nm in length in thin sections. That *Diplocalyx* spirochetes contain one such structure per cell length equivalent is clear at low power. One of the spheres from which the cytoplasmic tubules emanate is shown in the inset (Fig. 3B). *Hollandina*, on the other hand, has a reduced outer coat of the outer membrane. A smaller angle of the protoplasmic cylinder is subtended by flagella. A polar organelle (Table 1, criterion 15) of the *Hollandina* is also seen (Fig. 4B).

The wood-eating cockroach large *Hollandina* spirochete The transverse section of the one large pillotinaceous spirochete from the wood-eating cockroach (Fig. 4B) permits us to identify it in Grimstone's micrograph as a *Hollandina* (Fig. 4A). The highly conspicuous outer coat of the outer membrane, here of a value from 4–7, is typical of most hollandinas as depicted in the references [1, 16]. The thin filaments that connect the protoplasmic cylinder to the uncoated inner surface of the outer membrane remain unidentified (Fig. 4A).

Genus identification of pillotinaceous spirochetes from *Cryptotermes cavifrons* Ultrastructural comparison to type species *Diplocalyx calotermitidis* and *Hollandina pterotermitidis* allows the identification of the spirochetes of *Cryptotermes cavifrons* as *Diplocalyx* sp. and *Hollandina* sp. (Table 2; [1, 16]). In both cases, ultrastructure unique to the genus in question is clearly demonstrated (see Fig. 2).

Species identification of *Hollandina pterotermitidis* from *C. cavifrons* Comparison of the morphometric data from *Hollandina* sp. found in *C. cavifrons* and the type species

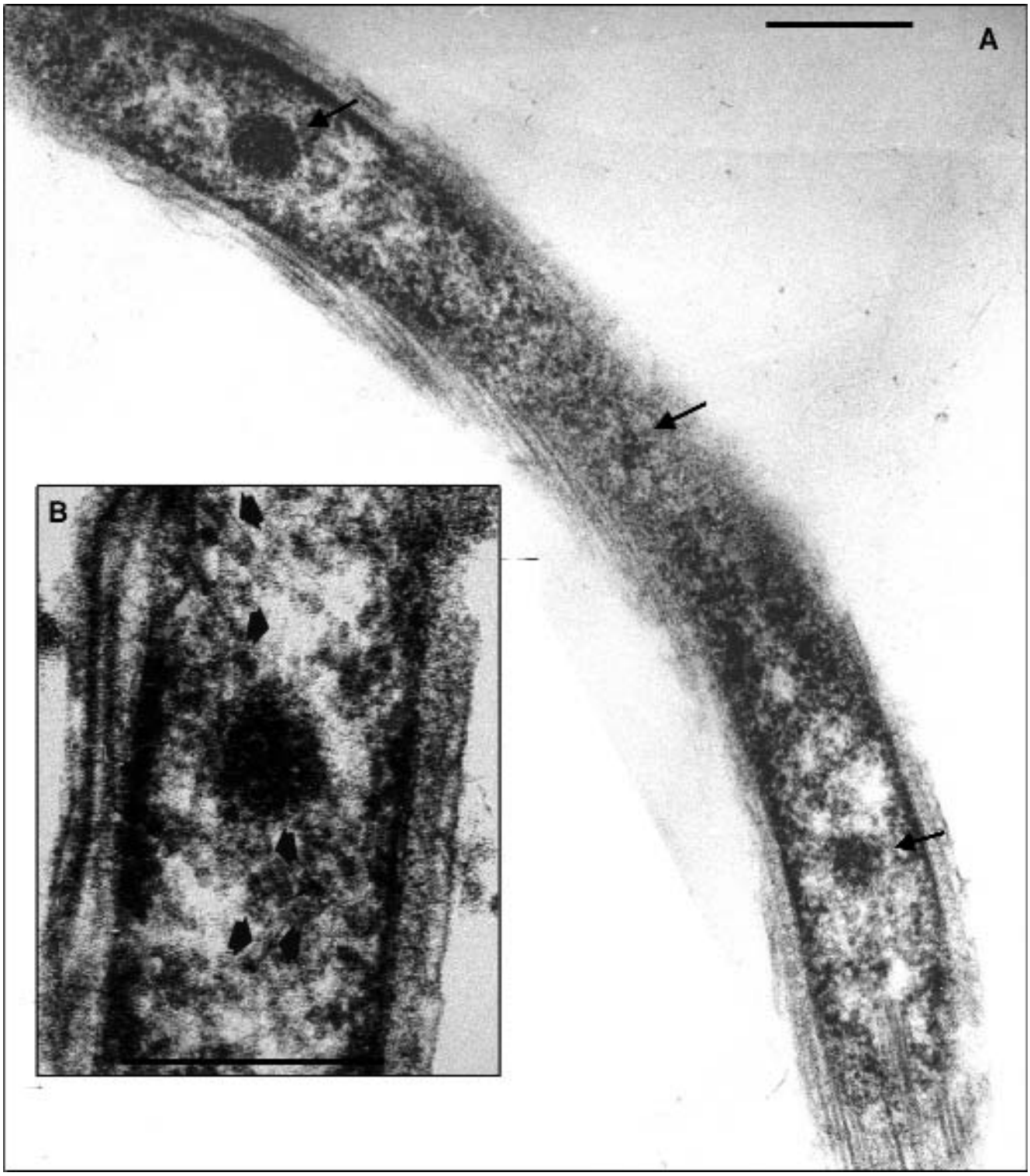

Fig. 3 *Diplocalyx cryptotermitidis* (A) Three cytoplasmic tubule-associated centers (CTACs) at arrows. Bar 0.5 µm. (B) CTAC, cytoplasmic tubules at arrows. Bars 0.3 µm

H. pterotermitidis from *Pterotermes occidentis* [1] reveals no significant differences between the two (Table 2). Values for *Hollandina* sp. fall within, or slightly below, the ranges reported for *H. pterotermitidis*. As *Hollandina* sp. from *C. cavifrons* conforms to the type species, this study proposes its formal identification as *Hollandina pterotermitidis*.

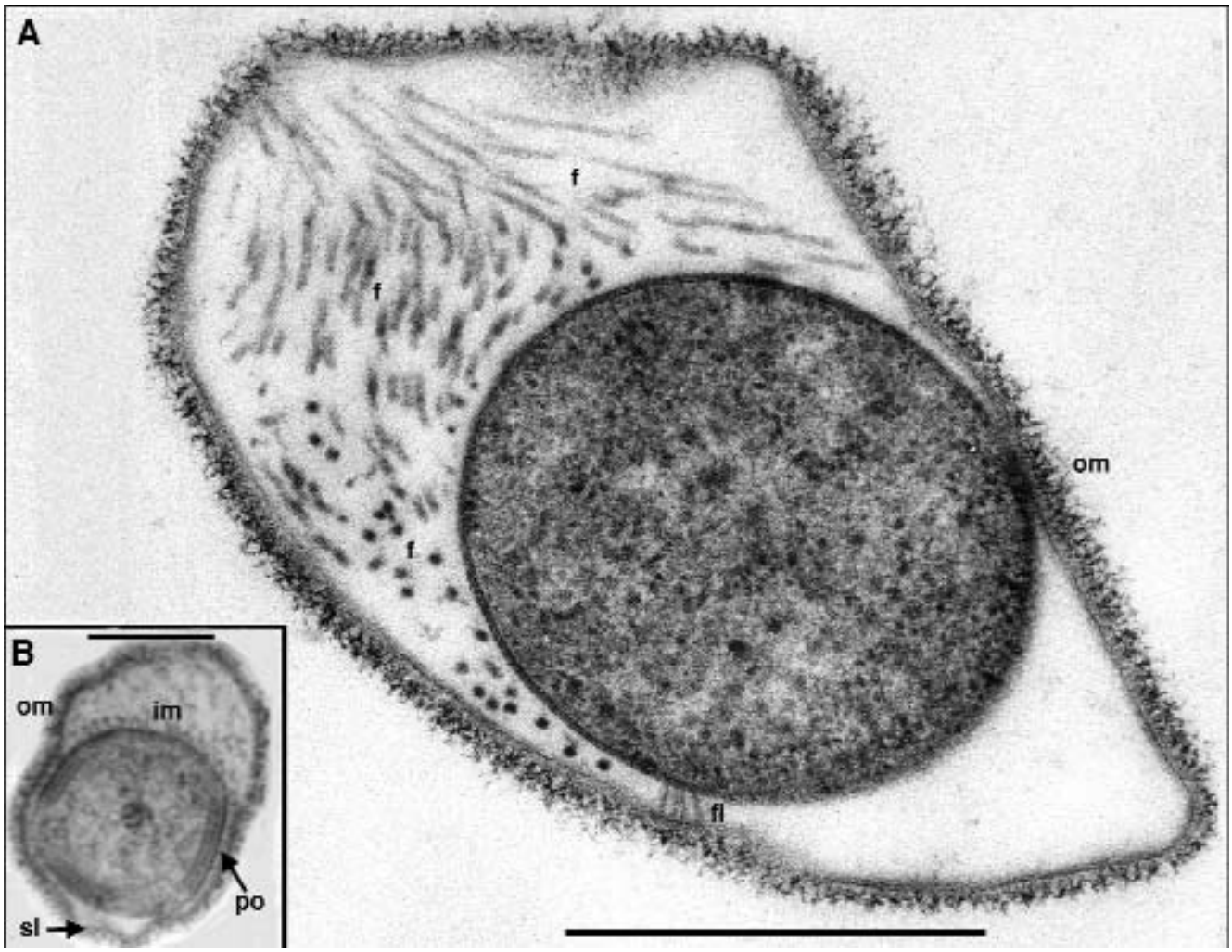

Fig. 4 (A) *Hollandina pterotermitidis* from the wood-eating cockroach *Cryptocercus punctulatus.* Note the flagella (f) in both transverse and longitudinal section, unidentified filaments (fi) that link the protoplasmic cylinder to the outer membrane in the periplasm, and the outer coat of the outer membrane (om). Thin section by A.V. Grimstone. Bar 1 µm. (B) The smaller, thinner *Hollandina* from *Cryptotermes cavifrons*. Polar organelle (po), (si) sillon, outer coat of outer membrane (om), outer coat of inner membrane (im). Bar 0.5 µm

Species identification of *Diplocalyx cryptotermitidis* sp. nov., from *C. cavifrons* Despite the apparent similarities between *Diplocalyx* sp. from *C. cavifrons*, and the type species, *Diplocalyx calotermitidis* from *Kalotermes flavicollis*, differences in the morphometric details of the *C. cavifrons* spirochete are not consistent with its classification as *D. calotermitidis*. Comparison of *Diplocalyx* sp. with the type species reveals differences in values for most primary morphometric criteria (Table 2). *Diplocalyx* sp. has a smaller range of cell diameter, fewer flagella, and lacks both inner and outer coats of the outer membrane. The range of the angle subtended by its flagella is wider and it has a smaller ratio of the thickness of the outer coat of the inner membrane (calyx) to inner membrane thickness (OCIM/IM). The primary criteria which are common between *Diplocalyx* sp. (*C. cavifrons*) and the type species are the presence of a sillon (opposite the flagellar bundle) which bisects the calyx (a defining character of the genus) and the presence of the flagellar bundle. The flagellar bundle, however, is arranged differently than in *Diplocalyx calotermitidis*, with 2–3 distinct rows of flagella closely appressed to the protoplasmic cylinder. In auxiliary criteria, *Diplocalyx* sp. (*C. cavifrons*) is distinguished by the presence of pili, best seen in negative stain preparations, and the apparent absence of polar organelles (seen only in TEM). As negative stain of the type species had not been done, the presence or absence of pili in *Diplocalyx* from other species and thus the taxonomic significance of this character to the genus cannot yet be assessed. Since no polar organelle was seen in *Diplocalyx* from *C. cavifrons* in 10 longitudinal and more than 50 transverse sections, the tentative conclusion is that it is absent. The cytoplasmic tubules often found in transverse and longitudinal sections of *Diplocalyx* from

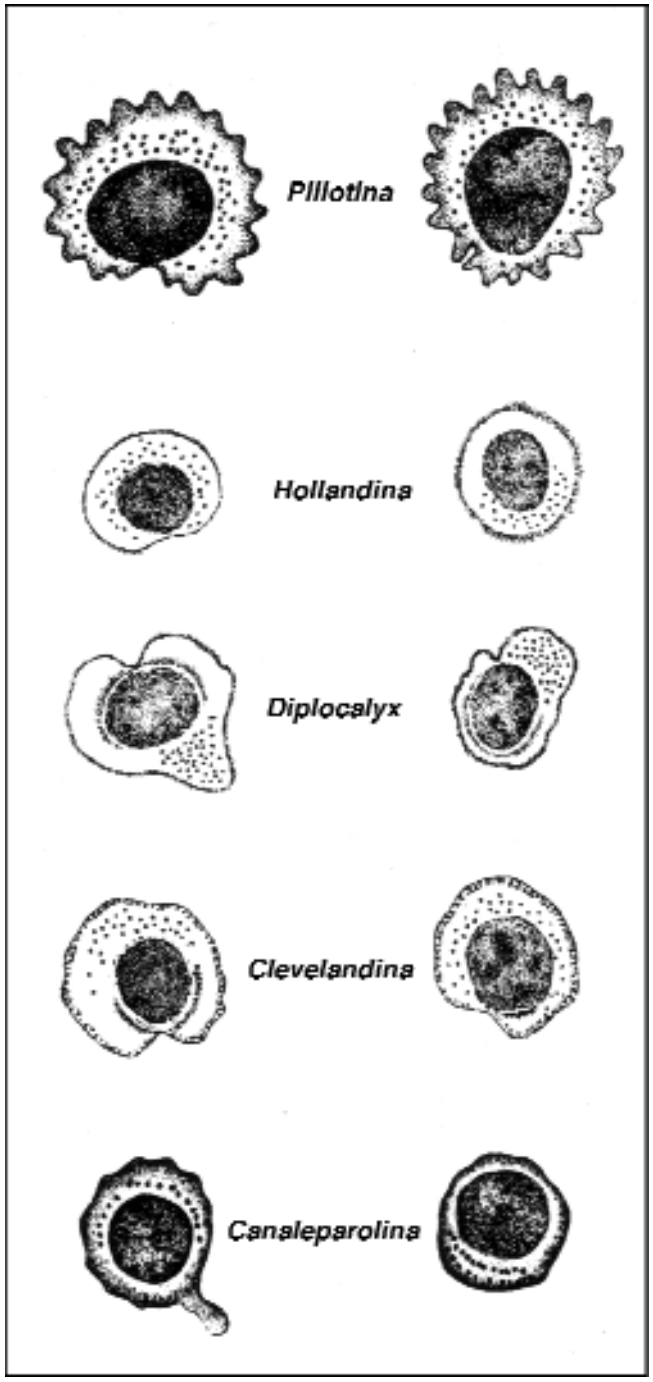

Fig. 5 The five genera of pillotinaceous spirochetes of termites and wood-eating cockroaches distinguished morphometrically. Two examples of members in each genus in transverse section drawn from electron micrographs by Christie Lyons. Table 1 lists morphometric criteria depicted here. At least one of the arthropod genera in the intestine of which these spirochetes are found is presented in Table 2

C. cavifrons (see Fig. 3) are clearly visible in several micrographs. Their appearance and abundance per cell was enhanced when tannic acid was included in the fixative. Since tannic acid causes artificial inflation of cytoplasmic tubules, however, both the smallest and largest measured values obtained in this study of the *Diplocalyx* tubules were obtained from fixed preparations which lacked tannic acid [22]. Although the cytoplasmic tubules ranged in size from 15 to 26 nm, their average size (n = 70) was 23.5 nm which falls very close to the 24 nm diameter size reported for most eukaryotic microtubules [8].

Nine out of ten *Diplocalyx* sp. cells analyzed in longitudinal section contained cytoplasmic tubules, a greater frequency than reported for any other pillotinaceous spirochete [22, 16]. Some axonemal microtubule doublets from an unidentified parabasalid protoctist were compared in the same thin sections to the cytoplasmic tubules of *Diplocalyx*. They measured from 26 to 30 nm in diameter. The similar ultrastructural appearance of the prokaryotic and eukaryotic tubules was notable. The spirochete tubules apparently emanate from the electron dense spherical structures (approximately 0.2 μm in diameter) seen in Fig. 3. The similarity of the *Diplocalyx* cytoplasmic tubules to certain non-centriolar microtubule-organizing centers (MTOCs) in eukaryotes such as protoctists (the ciliate *Nassula* [23]) or fungi like *Taphrina* is striking. Both *Diplocalyx* and these eukaryotes display dark staining regions of amorphous granulofibrosal material from which the tubules are seen to radiate.

We propose, in accordance with the morphometric criteria of Bermudes [1], that *Diplocalyx* from *Cryptotermes cavifrons* be considered a new species in the family of Pillotinaceae spirochetes: *Diplocalyx cryptotermitidis*. The proposed binomial reflects its symbiotic habitat in the *C. cavifrons* termite.

Comparative transverse sections of the intestinal symbiotic spirochete genera assigned to the family Pillotineaceae, including the new *Canaleparolina* (see description in the next section), are depicted in Fig. 5.

Discussion

Name of the new large epibiotic spirochete The n:2n:n flagella arrangement where n = 16, the flattened crenulations and other features listed in Table 2 for the *Mixotricha* spirochete warrant both a new generic and specific name for this symbiont. This new bacterial epibiont we name in the tradition of other genera in the family Pillotinaceae (*Hollandina, Clevelandina* and *Pillotina*). The names of those genera honor researchers André Hollande (France), Lemuel Roscoe Cleveland (USA) and Maurice Pillot (France), respectively. The generic epithet *Canaleparolina* is derived from the surname of Prof. Ercole Canale-Parola (see below). The specific, *darwiniensis*, indicates the only known geographical location of the spirochete in the region of northern Australia in and near the city of Darwin. Although not found anywhere else in the world, *Mastotermes darwiniensis* termites abound at Darwin. They are considered formidable pests. The newly named long spirochete is attached to the surface of all the large amitochondriate archaeprotists in the *Mastotermes darwiniensis* termite. The anomalous intestinal

trichomonad, *Mixotricha paradoxa* consistently bears the *Canaleparolina* spirochete. *Canaleparolina darwiniensis* is also sporadically attached to the accompanying hypermastigotes that are similar to *M. darwiniensis* in size (*Deltatrichonympha operculata*/*Koruga bonita*).

Genus *Canaleparolina* Large periplasmically multiflagellated (n = 12–20; 16:32:16 array) spirochete attached to the cortex of *Mixotricha paradoxa* limited to the intestine of *Mastotermes darwiniensis*. Named for Professor Emeritus Ercole Canale-Parola, Department of Microbiology, University of Massachusetts at Amherst, Massachusetts, USA, who trained a generation of microbiology researchers, especially experts in spirochete biology.

Species *darwiniensis* From the only known location Darwin, Australia, of the only living mastotermitid termite. Epibiotic on amitochondriate protists. Limited to the hindgut of *Mastotermes darwiniensis*, northern tropical Australia, the spirochete is abundantly attached to the entire anterior region of *Mixotricha paradoxa*. Especially dense populations are present at the junction of the cortex and the ingestive zone. However, the spirochete also attaches to the posterior region of the two other large hypermastigotes, *Koruga bonita* and *Deltatrichonympha operculata.* It may also adhere to the smaller *Deltatrichonympha nana* and/or swim freely in the gut fluid.

***Cryptotermes* spirochetes** The habitat of the new *Hollandina*, is the hindgut of *Cryptotermes cavifrons* and therefore the spirochete is widely distributed in the southern United States. *C. cavifrons* is well-known because it also harbors *Caduceia versatilis*, "Rubberneckia", the devescovinid protist with a eukaryotic cell wheel. The single-direction clockwise rotation of the anterior portion of the unique *Caduceia* demonstrated the fluid model of cell membranes [20]. Recently identified as *C. versatilis* [6], this cell and its immediate surroundings also serves as habitat for the new *Hollandina* and *Diplocalyx* morphotypes described here.

The regularly spaced cytoplasmic spheres in *Diplocalyx* whose measurements we report here can be seen in the micrographs. We suggest that they be referred to as cytoplasmic-tubule associated centers or CTACs. The function of CTACs is not known but either analogy or homology to microtubule-organizing centers of eukaryotes (MTOCs) deserve investigation. The spherical entity, the CTAC, provides another criterion to distinguish between spirochetes that we add to the list of morphological traits in Table 1. The CTAC cytoplasmic feature along with other differences suffice to raise the organism to species status. We call it *D. cryptotermitidis* after its host termite.

An expansion of the type species of *Hollandina* (*H. pterotermitidis*) on the other hand suffices to accommodate the two hollandinas (that from the termite *Cryptotermes* and from the cockroach *C. punctulatus*) without construction of any new taxa. The presence of cytoplasmic tubules in the *Diplocalyx* from *Cryptotermes cavifrons* was previously reported in a review on microtubules [2] but not described.

Canaleparolina darwiniensis spirochetes persist on dead protists. They maintain morphology for minutes and sometimes up to an hour after the protists are destroyed by extraction from the gut, starvation in situ, oxygen increase, or water loss or other unfavorable environmental conditions on glass slide preparations. Cells of *Mixotricha darwiniensis, Deltatrichonympha operculata* and *Koruga bonita* seem moribund as their motion slows and the cells stop. The latter two genera are indistinguishable by us. All the protists twitch, inflate, distort, twist and cease all swimming movement within 1–2 h after removal from the gut in even the most favorably sealed and watered preparations. The *Canaleparolina* spirochetes on *Mixotricha* and the other large protists may have some oxygen tolerance as they are among the last gut organisms to die. Although bending precedes the small amount of rounding up that can be seen, unlike free-living spirochetes, i.e., *Spirosymplokos* [13] and *Borrelia burgdorferi* in culture [3], no real "cystic forms" are produced. Rather, the spirochetal forms persist. With impending death the outer membranes loosen, the coils become flattened, the termini swell, but only the first step in the tendency to form round bodies ("cystic forms") is noted. The process does not continue because all movement ceases first. Whether *Canaleparolina* and other pillotinaceous spirochetes ever form viable cystic forms is yet to be determined. In any case, the strong impetus typical of *Spirosymplokos* [18] and for *Borrelia* [3] grown in vitro for all swimmers to round up and form cysts was not observed in termite intestines.

Preliminary observations of both optical and electron microscopical museum quality specimens of amber-embedded *Mastotermes electrodominicus* from Miocene samples from the Dominican Republic were kindly provided by Dr. Jorge Wagensberg (Director, Barcelona Museum of Science) and by Dr. David Grimaldi (Curator of Entomology, American Museum of Natural History, New York City). All samples were in amber of the leguminous tree, *Hymenea* sp. Work in progress has convinced us that the size and preservation potential of both *Mixotricha* and its attached *Canaleparolina* spirochetes is high. The distinctive distribution of the bacteria and protists in the hindgut of the large, gas bubble-producing wood-eating dictyopterids of the genus *Mastotermes* augurs well for the eventual detection and identification of the fossil counterparts of both microbial genera (that of *Mixotricha* and of its new spirochete, *Canaleparolina darwiniensis*).

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