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Intestinal microsporidiosis in African skink *Mabuya perrotetii*

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Abstract. Intestinal microsporidiosis was documented by detecting abundant slightly curved spores (2.9 × 1.2 μm) in the faeces of five of twelve skinks *Mabuya perrotetii* Duméril et Bibron, 1839 that originated from Ghana. Clinically, the microsporidiosis was characterized by decreased appetite, diarrhea, and weight loss. Histopathological changes consisted of villous atrophy, blunting of mucosa and flattening of individual epithelial cells in the large intestine. The ultrastructure of microsporidian spores was consistent with an *Encephalitozoon* species. The PCR-RFLP assay and the heteroduplex mobility shift analyses were used to verify that the skink microsporidian is a species of the genus *Encephalitozoon* Levaditi, Nicolau et Schoen, 1923 and indicate that this microsporidian is not *E. hellem*, *E. intestinalis* or a strain of *E. cuniculi*. The microsporidia in African skink represent an *Encephalitozoon* species morphologically identical to *Encephalitozoon lacertae* Canning, 1981.

Microsporidia are ubiquitous, obligate intracellular protozoan parasites that infect many invertebrate and vertebrate hosts. Over 1,000 species of microsporidia have been described, and they can infect most invertebrates and vertebrates (Canning and Lom 1986). Among reptiles, there is only one microsporidian species that infect intestinal epithelium. Intestinal micro-sporidiosis was reported by Canning and Landau (1971) in single wall lizard *Podarcis muralis* and the causative agent was named *Encephalitozoon lacertae* by Canning (1981).

The purpose of this report is to present the diagnosis and pathology of naturally acquired intestinal micro-sporidiosis in skinks *Mabuya perrotetii* Duméril et Bibron, 1839 (Sauria: Scincidae) and characterization of this reptilian microsporidian species.

MATERIAL AND METHODS

Animals. A group of skinks *Mabuya perrotetii* originally captured in Ghana was imported by pet trade company into the Czech Republic. Fresh faecal samples from skinks were initially examined by wet-mount preparation in tap water. All samples were also screened routinely for parasites by flotation in modified Sheather's sugar solution (specific gravity 1.30). For detection of microsporidia, individual faecal smears were also screened by chemofluorescent optical brightening agent Calcofluor White 2MR (Sigma).

Light microscopy. Clinically affected and moribund lizards were euthanized and necropsied. At necropsy, tissue samples of the stomach, duodenum, small and large intestine, cloaca, heart, lung, liver, gall bladder and kidney were fixed in 10% buffered formalin. Fixed tissues were processed for light microscopy using standard methods. Paraffin sections were stained with hematoxylin and eosin (HE), Giemsa, Gram stain or with Calcofluor White 2MR.

Electron microscopy. Specimens for scanning electron microscopy (SEM) were fixed in 4% buffered para-formaldehyde at 4° C. Small portions of intestinal mucosa were rinsed several times in distilled water, dehydrated in alcohol series and desiccated by critical point drying in carbon dioxide. The samples were then gold-coated in spraying device and examined with a JEOL JMS 6300 scanning electron microscope. For transmission electron microscopy (TEM), intestinal specimens were fixed in 2.5% glutar-aldehyde in cacodylate buffer (0.1M, pH 7.4) at 4°C and post-fixed in 1% osmium tetroxide in the same buffer. Specimens were washed three times in the same buffer, dehydrated in graded alcohols and embedded in Durcupan. Semi-thin sections were stained with toluidine blue and examined with a light microscope. Thin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 1010 transmission electron microscope.

Isolation of microsporidian spores. Faecal material or intestinal content containing microsporidian spores were suspended in distilled water, strained through a series of sieves and concentrated by centrifugation. Spores were purified in a discontinuous gradient of Percoll (Jouvenaz 1981) and finally stored in distilled water with 0.1% gentamicin at 4°C until used for inoculation or cultivation. Before inoculation, spores were washed 3 times by centrifugation in sterile phosphate-buffered-saline (PBS, pH 7.2) and counted using hemocytometer.

Experimental inoculation. Two adult skinks *Mabuya perrotetii* were used for experimental inoculation. Skinks were

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kept in separate cages (~27° C; 14L/10D photoperiod) and fed laboratory reared crickets with mineral and vitamin supplements. Prior to the experimental inoculation, individual faecal samples of skinks were monitored repeatedly (four to five times) for microsporidia four to six weeks before the inoculation. Only skinks that were microsporidia-free animals were used for experimental peroral inoculation with 10^3-10^4 three-week- old purified microsporidia spores.

Two female ten-week-old severe combined immunodeficient (SCID) mice (AnLab, Czech Republic) were perorally inoculated with 10^5 four-week-old purified spores. Two other SCID mice were given 10^5 spores by intraperitoneal injection. All mice were monitored daily for clinical signs of disease. On day 48 post inoculation (DPI), all inoculated SCID mice were euthanized and necropsied. At necropsy, tissue samples of the stomach, duodenum, small and large intestine, heart, lung, liver, gall bladder, kidney, urinary bladder, diaphragm and brain were fixed in 10% buffered formalin and processed for light microscopy. Peritoneal macrophages, urine samples and faecal material from all necropsied SCID mice were examined for presence microsporidia by Calcofluor White 2MR.

Cultivation. Purified spores were suspended in RPMI 1640 medium (Sigma) supplemented with 5% heat-inactivated fetal calf serum and overlaid on partially confluent E6 cells (Vero green monkey kidney cells) The ratio of host cells to spores was approximately 5 : 1. Cultures were incubated at 28°C or 37°C. Media were replaced every 3 to 4 days and tissue-culture supernatants were concentrated by centrifugation and screened for microsporidia by Calcofluor White 2MR.

Microsporidian isolates. Microsporidia were grown in RK-13 cells using procedures as previously described (Didier et al. 1996). *Encephalitozoon cuniculi* strains I, II, and III were isolated originally from a rabbit, mouse, and a dog, respectively, as previously described (Didier et al. 1995). *Encephalitozoon hellem* was isolated from an AIDS patients with corneal/conjunctival keratitis (Didier et al. 1991), and *E. intestinalis* was cultured from an isolate of a bronchoalveolar lavage specimen of an HIV-infected individual as described (Didier et al. 1996). Microsporidia from the African skinks were collected from faecal material and intestinal content as described above.

DNA Isolation. Microsporidia were washed with sterile distilled water, pelleted in 1.5 ml microfuge tubes by centrifugation at 15,000 *g* for 1 min and resuspended in 200 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 0.1 g of 0.5 mm sterile glass beads. The tubes were vortexed for 45 seconds followed by addition of 200 µl lysis buffer (0.2 M NaOH, 2% SDS, 0.5 mg/ml proteinase K). After overnight incubation at 56°C, 200 µl neutralization buffer (2.55 M potassium acetate, pH 4.8) was added to each tube. The tubes were centrifuged at 15,000 *g* for 5 min and the DNA was purified using Magic Minipreps (Promega Corporation, Madison, WI) as described by the manufacturer.

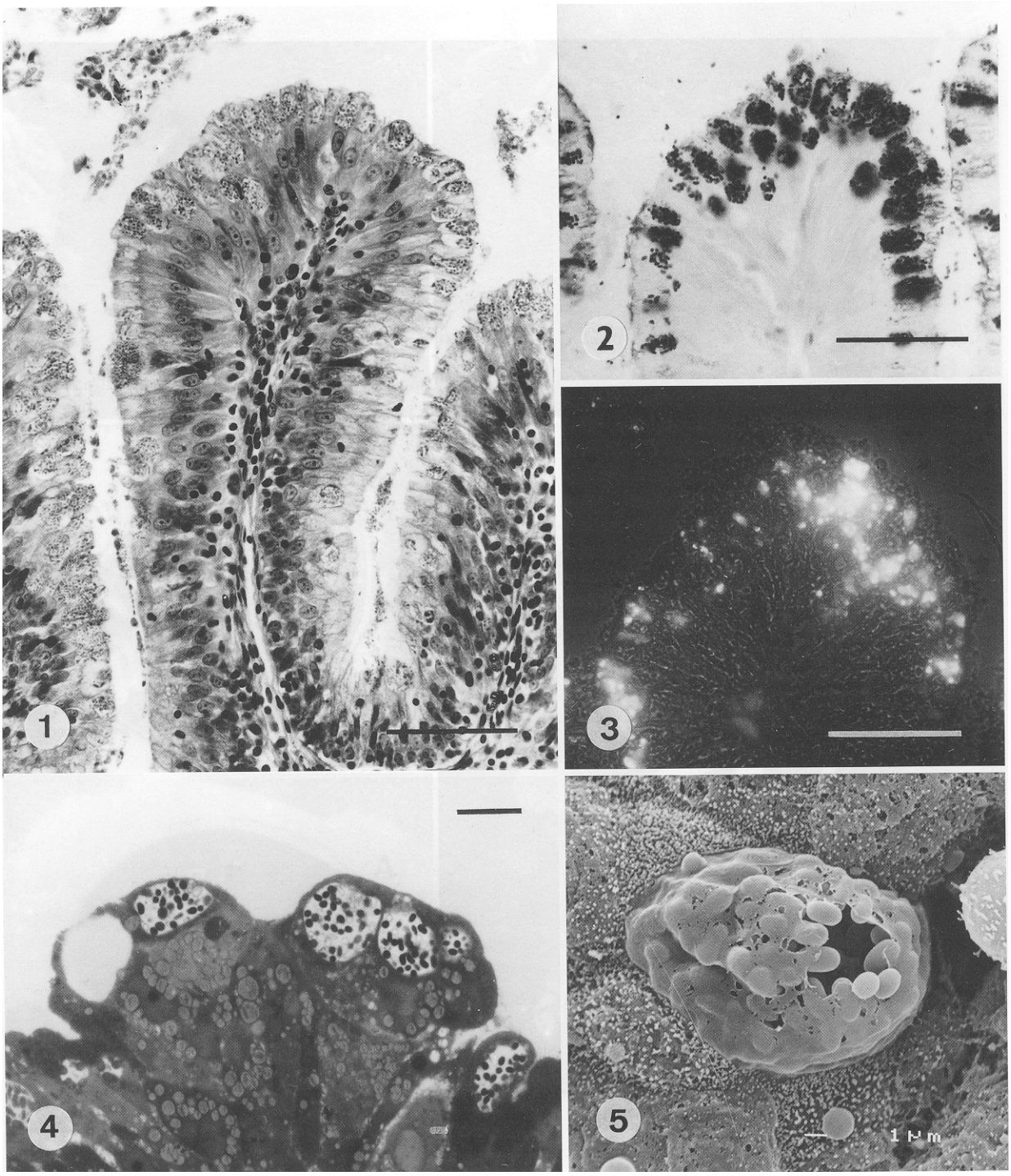
Polymerase chain reaction (PCR). To amplify the ribosomal RNA genes of the microsporidia, PCR was employed using a pair of *Encephalitozoon* genus-specific primers (Didier et al. 1995, 1996). These primers amplify products of approximately 1,000 base pairs which include a large portion of the small subunit ribosomal RNA gene, the entire intergenic region and a short segment of the large subunit ribosomal RNA gene. These primers do not amplify rDNA from tissue culture-derived *Vittaforma corneae* or *Enterocytozoon bieneusi*, and the sequences are (int530f) 5'-TGCAGTTAAAATGTCCGTAGT-3' and (int580r) 5'-TTTCACTCGCCGCTACTCAG-3'. Gene amplification by PCR was performed in 100 µl reactions containing 0.05 µM of each primer, 0.2 mM of each dNTP (Promega Corp., Madison, WI), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 2.5 units *Taq* DNA polymerase (Promega Corporation, Madison, WI), approximately 100 ng of genomic DNA, and 100 µl of overlayed mineral oil. The PCRs were performed using a Perkin-Elmer thermocycler (Norwalk, CT) with a profile of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min for 35 cycles. The PCR products were purified using Magic PCR Prep mini-columns as described by the manufacturer (Promega Corp., Inc., Madison, WI), and aliquots were examined by electrophoresis in 1.5% agarose gels. The remaining portions of the PCR products were stored at 4°C until used.

Double-stranded DNA heteroduplex mobility assay. PCR-amplified products were assayed for the generation of heteroduplexes by a slightly modified version of the method of Soto and Sukumar (1992) as previously described (Didier et al. 1995, 1996). Briefly, 10 µl aliquots of PCR products from two microsporidian isolates were mixed together, heated to 95°C and cooled to room temperature to allow random and complete duplex formation. Heteroduplexes and homoduplexes were visualized after electrophoresis of the products in PCR Purity Plus gels (J.T. Baker, Inc., Phillipsburg, NJ). If complete sequence identity exists between complementary strands, the homoduplexes will migrate to the same position as the original PCR-amplified DNA product. If even one mismatch exists between the complementary strands, heteroduplexes form which migrate much more slowly than the homoduplexes due to conformational changes.

Restriction fragment length polymorphism (RFLP). PCR products were digested overnight with the restriction endonuclease *Fok I* (New England Biolabs, Beverly, MA) as described (Sambrook et al. 1989). The restriction digests were electrophoresed through 2% Seakem GTG agarose (FMC Bioproducts, Rockland, ME), stained with ethidium bromide, and photographed under ultraviolet illumination.

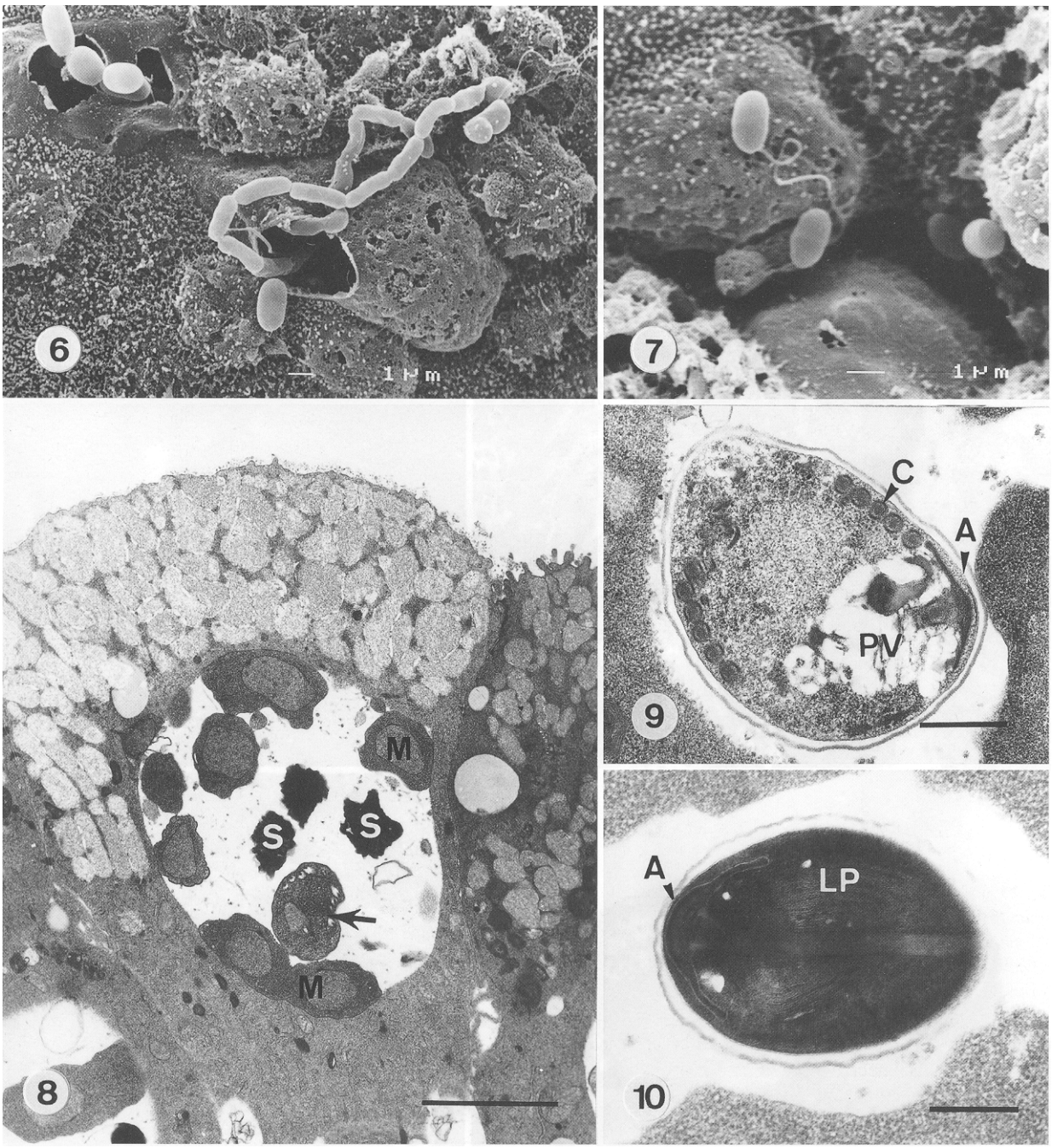
RESULTS

Microsporidia were detected in a group of twelve skinks *Mabuya perrotetii* originally captured in Ghana. Approximately two months after import, some of skinks exhibited decreased appetite and diarrhea followed by progressive weight loss, and two skinks died. Histopathology of the large intestine of the both skinks showed heavy infection of intestinal epithelium with microsporidia. The microsporidia were identified using Gram and Giemsa stains, and applying Calcofluor White 2MR directly on paraffin sections. Subsequently, examination of faecal samples collected from remaining ten skinks revealed that three animals passed micro-



Figs. 1-5. Intestinal microsporidiosis in African skink *Mabuya perrotetii*. **Fig. 1.** Histological section from large intestine demonstrating several cells with microsporidia. HE. **Fig. 2.** Gram-positive microsporidia densely aggregated in cytoplasma of cells from tip of intestinal villus. Gram stain. **Fig. 3.** Calcofluor White 2MR staining showing microsporidian spores in epithelial cells from tip of intestinal villus. **Fig. 4.** Semi-thin plastic section showing prominent swelling of epithelial cell containing microsporidia. Scale bars 50 µm (Figs. 1-3) and 10 µm (Fig. 4) **Fig. 5.** Sloughed cell containing microporidian spores. SEM.

sporidian spores. Elongate ellipsoid and slightly curved spores measured 2.9 × 1.2 (2.5-3.5 × 1.0-1.5) µm in wet-mount preparation from fresh faecal material. Examination of faeces by flotation in Sheather's sugar solution revealed that two of twelve skinks also passed oocysts of coccidia and oxyurid ova.



Figs 6-10. Intestinal microsporidiosis in African skink *Mabuya perrotetii*. **Fig. 6.** Ruptured epithelial cells containing microsporidian developmental stages in chains. SEM. **Fig. 7.** Spore with extruded polar tube on mucosal surface of large intestine. SEM. **Fig. 8.** Goblet cell with parasitophorous vacuole containing meronts (M), early sporoblast (arrow) and immature spores (S). TEM. **Fig. 9.** Immature spore showing posterior vacuole (PV), polar tube coils (C) and anchoring disc (A). TEM. **Fig. 10.** Mature spore depicting anchoring disc (A) and lamellar polaroplast (LP). TEM. Scale bars 5 µm (Fig. 8) and 200 nm (Figs. 9, 10).

Two of three skinks passing microsporidian spores were necropsied and processed for light and electron microscopy. At necropsy, gross findings included dilated large intestine and cloaca and accumulation of ascites fluid in the abdominal cavity. Histopathological lesions consisted of villus blunting, mucosal thickening and infection of epithelial cells in the large intestine and cloaca with microsporidia (Fig. 1). Both the Gram and Calcofluor White 2MR stains greatly enhanced detection of microsporidia in paraffin-embedded sections (Figs. 2 and 3). The most heavily infected epithelial cells were those at the distended tips and on

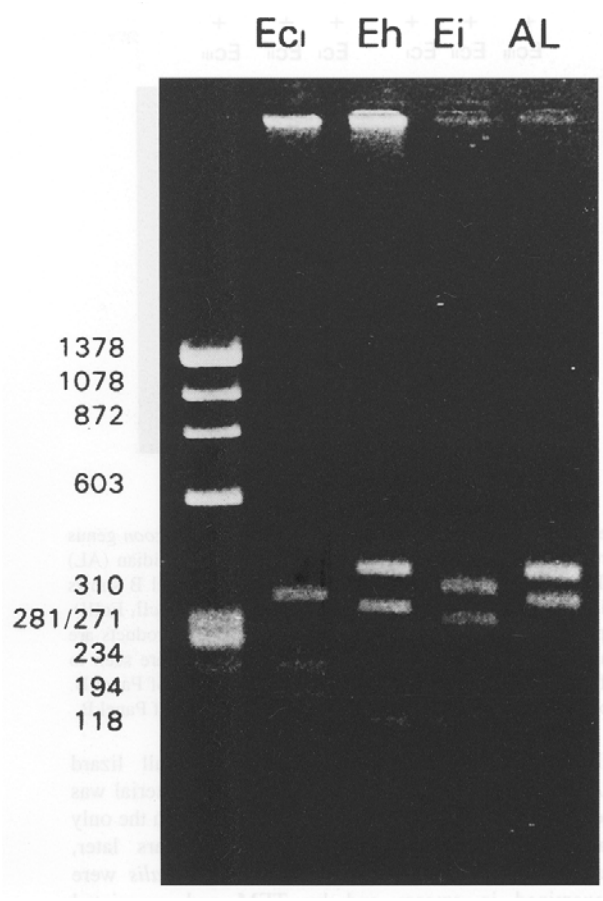


Fig. 11. PCR-RFLP analysis of rDNA from *Encephalitozoon cuniculi* strain I (EcI), *E. hellem* (Eh), *E. intestinalis* (Ei), and the skink isolate (AL). *Fok I* was used to generate the restriction digest and DNA markers from *Hae III*-digested fX174 are shown to the left.

the sides of the intestinal villi, but microsporidia were also present in enterocytes of the intestinal crypts (Fig. 1). The lamina propria and submucosa were edematous and infiltrated by inflammatory cells consisting mainly of heterophils and lymphocytes. In semi-thin plastic section, microsporidia were dark blue and were located in the supranuclear area of the epithelial cells. Semi-thin plastic sections also showed prominent swelling of the epithelial cell containing microsporidia (Fig. 4).

Electron microscopy. SEM of a mucosal surface of the large intestine showed severe microsporidian infections at the distended tips of intestinal villi and confirmed prominent swelling of the epithelial cell containing microsporidia (Fig. 5). Many epithelial cells containing microsporidia were ruptured and elongate developmental stages in chains and spores were seen on the mucosal surface (Fig. 6). Occasionally, spores after extrusion of their polar tube were identified (Fig. 7). Ultrastructurally, microsporidia were located in parasitophorous vacuoles lying between the nucleus and the mucus goblet (Fig. 8). Host cell mitochondria were closely attached to the vacuolar membrane. The earliest developmental stages were spherical uninucleate meronts. These meronts were 1.5-2.5 µm in diameter and developed into elongate binucleated cells or chains within the parasitophorous vacuole adjacent to the vacuolar membrane. Elongate sporonts measured 3.0-4.5 × 1.5-2.0 µm and were separated from vacuolar membrane. Nuclear division was followed by constriction of sporonts and resulting two uninucleated sporoblasts displayed a rudimentary polar tube, vesicular inclusions, and rough endoplasmatic reticulum. In immature and mature spores, the polar tubes in cross section has an electron-lucent center bound by thin electron-dense layer and thin outer transparent halo (Fig. 9). Spores contained a single nucleus, a polar tube with five to seven coils (Fig. 9), an anterior polar tube anchoring complex, lamellar polaroplast and both exospore and endospore layers (Fig. 10). Ultrastructural features of the organism found in large intestine and cloaca of skinks *M. perrotetii* were characteristic for microsporidia of the genus *Encephalitozoon* Levaditi, Nicolau et Schoen, 1923 (Canning and Lom 1986).

Experimental inoculation. Both skinks experimentally inoculated with purified spores developed infection. Spores were first detected in faecal material of inoculated skinks 12 days post inuculation (DPI). Both skinks excreted spores without clinical signs for two months, were then necropsied, and intestinal tissues were used for cultivation. Oral and intraperitoneal inoculations of SCID mice did not result in infection. Also, stained smears and histological sections did not confirm infection with microsporidia.

Cultivation. No continuous propagation of microsporidia was observed in E6 cells maintained at two different temperatures and decreased numbers of spores were only detected in tissue-culture supernatants.

PCR-RFLP analysis. The ultrastructural features of the microsporidian observed in the skink were similar to those of the *Encephalitozoon* species, so PCR was performed using a set of genus-specific primers, and a PCR product was successfully generated. PCR-RFLP analysis was performed using *Fok I* to compare the rDNA PCR products of the skink isolate with those of the three species of *Encephalitozoon*. The results indicate that the RFLP pattern of the African skink microsporidian rDNA is similar but distinct from the RFLP patterns generated by *E. cuniculi* strain I, *E. hellem*, and *E. intestinalis* (Fig. 11).

Double-stranded DNA heteroduplex mobility shift analysis. PCR rDNA products from the African skink microsporidian isolate were compared with the three *Encephalitozoon* species (Fig. 12 - panel A) as well as with the three *E. cuniculi* strains species (Fig. 12 - panel B) by double-stranded DNA heteroduplex mobility shift analyses. Only homoduplexes of DNA were seen in

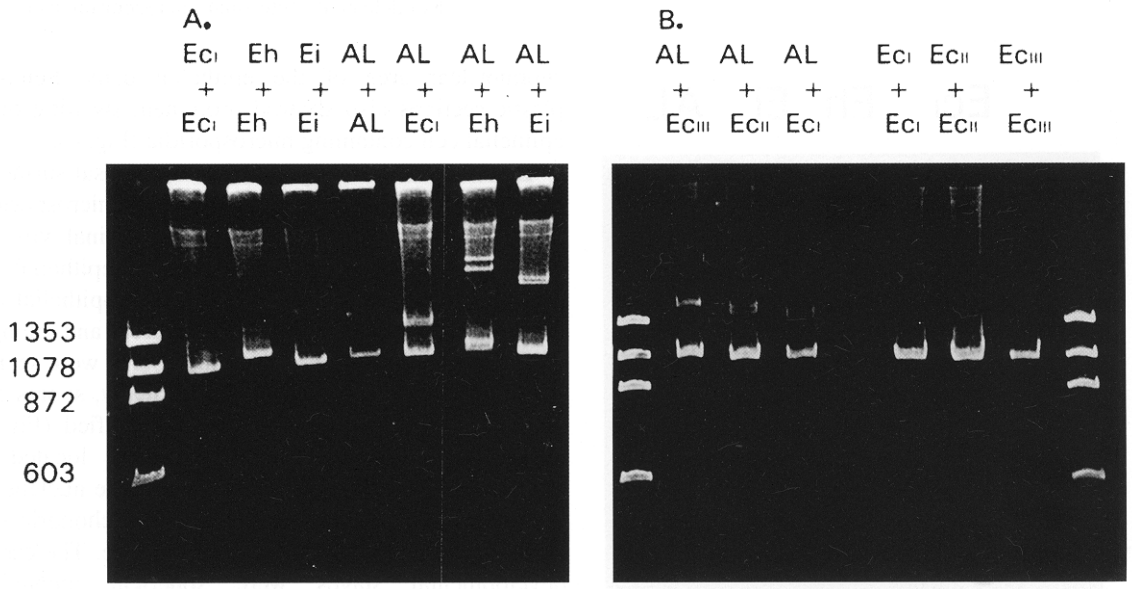


Fig 12. Double-stranded DNA heteroduplex mobility shift analyses. PCR products were generated using *Encephalitozoon* genus primers and assayed for heteroduplex formation. Panel A compares the rDNA PCR products of the skink microsporidian (AL) with those of the *Encephalitozoon* species: *E. cuniculi* strain I (EcI), *E. hellem* (Eh), and *E. intestinalis* (Ei). Panel B shows comparisons of the rDNA PCR products of the skink microsporidian (AL) with the three strains of *E. cuniculi* (EcI, EcII, EcIII). Controls to demonstrate fidelity of reannealling to form homoduplexes in mixtures containing two identical PCR products are shown in the second through fifth lanes of Panel A and fifth through seventh lanes of Panel B. Heteroduplexes are seen to migrate more slowly than homoduplexes in the last three lanes of Panel A, and in the second through fourth lanes of Panel B. DNA markers generated from *Hae III*-digested fX174 are shown to the left of Panel A, and on the left and right lanes of Panel B.

mixtures containing two like PCR products which controls for the fidelity of the reannealing process. Heteroduplexes (as well as homoduplexes) were generated when the rDNA PCR product from the skink was mixed with rDNA PCR products from any of the *Encephalitozoon* species (Fig. 12 - panel A) or with those from any of the three strains of *E. cuniculi* (Fig. 12 - panel B). Results of the PCR-RFLP assay, and the heteroduplex mobility shift analyses indicate that the skink microsporidian is a species of the genus of *Encephalitozoon.*

DISCUSSION

The first confirmed case of intestinal microsporidiosis in reptiles was reported by Canning and Landau (1971) and involved a single wall lizard *Podarcis muralis* collected in France. The material was examined only in histological sections, in which the only recognizable stages were spores. Ten years later, additional specimens of wall lizards *P. muralis* were examined in smears and by TEM and associated microsporidian species was named *Encephalitozoon lacertae* by Canning (1981).

In the present study, intestinal microsporidia in African skink *Mabuya perrotetii* were detected using routine histopathologic methods and were confirmed through examination by electron microscopy. The microsporidia appeared to be a species of the genus *Encephalitozoon* based on the size of the mature spores and growth within a parasitophorous vacuole. Also, the absence of diplokarya at any stage and coil number in the polar tube was characteristic for a species of the genus *Encephalitozoon*. Moreover, the results of the PCR-RFLP assay, and the heteroduplex mobility shift analyses indicate that the skink microsporidian is a species of the genus of *Encephalitozoon.* Our results also showed that the RFLP pattern of the African skink microsporidian rDNA is similar but distinct from the RFLP patterns generated by other *Encephalitozoon* species (*E. cuniculi* strain I, *E. hellem*, and *E. intestinalis*).

The microsporidian parasite found in skinks *M. perrotetii* was identical to the previously described *E. lacertae* Canning 1981 in development within intestinal goblet cells and all ultrastructural features (Canning 1981). This author described similar sizes of spores, spherical meronts and elongate sporonts, proliferation by binary fission, chains of meronts and sporonts and disporoblastic sporogony.

While we are not able to distinguish between microsporidia found in different species of lizards (*L. muralis* and *M. perrotetii*), we suggest that microsporidian parasites from skinks *M. perrotetii* are conspecific with *E. lacertae.*

In this study, spores were found in the faeces and microsporidia developed within non-phagocytic epithelial cells. It is probable that spores which are

released into the environment via faeces are sources of infection and sporoplasms are inoculated directly into intestinal epithelial cell cytoplasm through the polar tube. Result of experimental inoculation, in which no intermediate host or vector was found necessary, and our SEM findings of spores after extrusion of their polar tube on the intestinal surface confirmed this support of direct horizontal transmission of microsporidia among lizards.

Microsporidian species of the genus *Encephalitozoon* can usually be propagated in cell culture system (Weber et al. 1994) and SCID mice provides a useful animal model for *E. cuniculi* microsporidiosis (Koudela et al. 1993). Our preliminary data on cultivation of presumptive *E. lacertae*, however, showed that a cell culture system for *Encephalitozoon* spp. and SCID mice as an animal model could not be used for this microsporidian species infecting reptiles.

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