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A dry-fracture technique for the optimum preparation of microsporidia-infected tissues for scanning electron microscopy

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Summary

A technique for the optimum preparation of microsporidia for scanning electron microscopy is described. Microsporidian-infected tissues were subjected to a triple fixation procedure followed by slow dehydration and critical point drying. Fracturing of the tissue to reveal details of both host and parasite was delayed until immediately before coating of specimens with gold; the exposed parasites were therefore free of extraneous contamination, displayed minimum distortion and retained their spatial relationships with each other and with the host tissue. The value of the technique is illustrated by examination of microsporidia both with and without sporophorous vesicles, including species representing the genera *Tuzetia, Amblyospora, Pleistophora* and *Nosema*.

Key words: microsporidia, scanning electron microscopy, SEM technique, dry-fracture, *Tuzetia, Amblyospora, Pleistophora, Nosema*

Introduction

Microsporidia are among the smallest and yet structurally most intriguing of fungi (Vбvra and Lukeљ, 2013), with spores just a few micrometres long and extreme structural reduction associated with their obligate intracellular parasitic habit (Haag et al., 2014). The development and application of electron microscopic techniques has therefore contributed substantially to our understanding of microsporidia, not only with regard to their functional morphology, development and host parasite relationships (Liu and Liu, 1973; Cali and Takvorian, 1999; Terry et al., 1999), but also concerning their taxonomy (Hazard and Anthony, 1974; Hazard and Oldacre, 1975; Sprague, 1977; Canning and Vavra, 2000). The use of scanning electron microscopy (SEM) to reveal details of the surface structure of microsporidian spores and sporophorous vesicles is an important adjunct to transmission electron microscopy, and yet remains generally under-utilised. This may be due in part to the somewhat limited data that can be extracted from specimens prepared by common methods such as the layering of spore or sporophorous vesicle suspensions upon SEM stubs (Wang and Chen,

2007; Sharma et al., 2013). This paper describes a method for the preparation of microsporidian-infected tissue in which the intimacy of the host-parasite association is maintained, thereby providing support and protection during the preparatory stages and subsequently revealing in situ details of spores, sporophorous vesicles and host tissues.

Material and methods

Microsporidia-infected larval Simuliidae were collected from tributaries of the Taieri River in Otago, New Zealand and the Dreisam River in Breisgau, Germany. Infected tissues were dissected from the host into Clark's insect saline and sub-samples from the same host were then processed for both light and scanning electron microscopy. Saline smears were examined and imaged using Leitz Dialux and Zeiss Axiokop microscopes to complement the SEM imagery. For SEM, samples were transferred immediately from Clark's insect saline to 3% (v/v) glutaraldehyde in 0.1M phosphate buffer at pH 7.2. Fixation was for 2h at 4 °C. Post-fixation was in 1% (w/v) osmium tetroxide in 0.1M phosphate buffer pH 7.2 for 2h at 4 °C, followed by an additional 2h in saturated aqueous uranyl acetate. Dehydration was by means of an ethanol series to absolute ethanol, followed by transfer through an ethanol/acetone series to absolute acetone. Small pieces of infected tissue were then dried in a Polaron E5000 Critical Point Drying Apparatus.

At the point of mounting on SEM stubs each sample of infected host tissue was fractured with the use of fine-tipped tungsten needles produced by the electrolytic sharpening method of Brady (1965), a method that can produce very fine tips just a few micrometres across. Fracturing of specimens in this way thereby exposed the interior of the infected tissue for the first time. The fracturing procedure was conducted directly on the adhesive surface of SEM stubs under a stereo binocular microscope to facilitate deliberate and accurate manipulation of the specimens, which typically measured 0.5 to 3 millimetres across prior to fracturing. At the moment of fracturing, several fragments of the specimen would tumble randomly on to the prepared adhesive surface of the SEM stub thus presenting a wide variety of exposed facets for subsequent SEM examination. Following critical point drying, samples of infected tissue typically displayed a brittle quality and were easily fractured with gentle pressure of the needle tips applied to the external surface of the specimen. This step requires precise placement of the needle tips, but importantly avoids direct contact with, and potential damage to, the subsequently exposed interior of the specimen. Gold coating took place immediately after fracturing and specimens were then examined and imaged in Siemans Autoscan, Cambridge 150 or Jeol JSM Scanning Electron Microscopes.

Microsporidian taxa were identified in broad accordance with established morphological, developmental and ecological characteristics, mainly to genus level, as follows:

Tuzetia. Sporophorous vesicle encloses individual spore; numerous tubules between exospore and sporophorous vesicle membrane; unpaired nuclei throughout development (Larssen, 1983; Canning et al., 2002; Simakova et al., 2009; De Sousa et al., 2014).

Amblyospora. Octosporous sporophorous vesicles; smooth oval spores; diplokaryotic meronts; uninucleate spores (Hazard and Oldacre, 1975; De Sousa et al., 2014).

Pleistophora. Multisporous sporophorous vesicles with large and variable number of spores; diplokaryotic meronts; uninucleate spores (Canning and Hazard, 1982; De Sousa et al., 2014).

Nosema stricklandi. Sporophorous vesicle absent; diplokaryotic at all stages of development, parasite of Simuliidae (Jirovec, 1943; Sprague, 1977; Larssen, 1986; De Sousa et al., 2014).

Host simuliid larvae carrying patent microsporidian infections were identified to genus level only. Mature uninfected *Simulium* larvae from the same localities were identified as *Simulium reptans, Simulium ornatum* and *Simulium argyriatum*. The *Nosema stricklandi*, the *Pleistophora* species and the two species of *Tuzetia* could therefore have been infecting any one or a combination of these host species. The designation *Simulium* sp. used in this paper should therefore not necessarily be read as a single host species for these four microspordian infections.

Results

Microsporidian-infected insect tissues prepared for scanning electron microscopy according to the method described above reveal a great deal of information concerning not only the microsporidia themselves, but also their disposition within the host

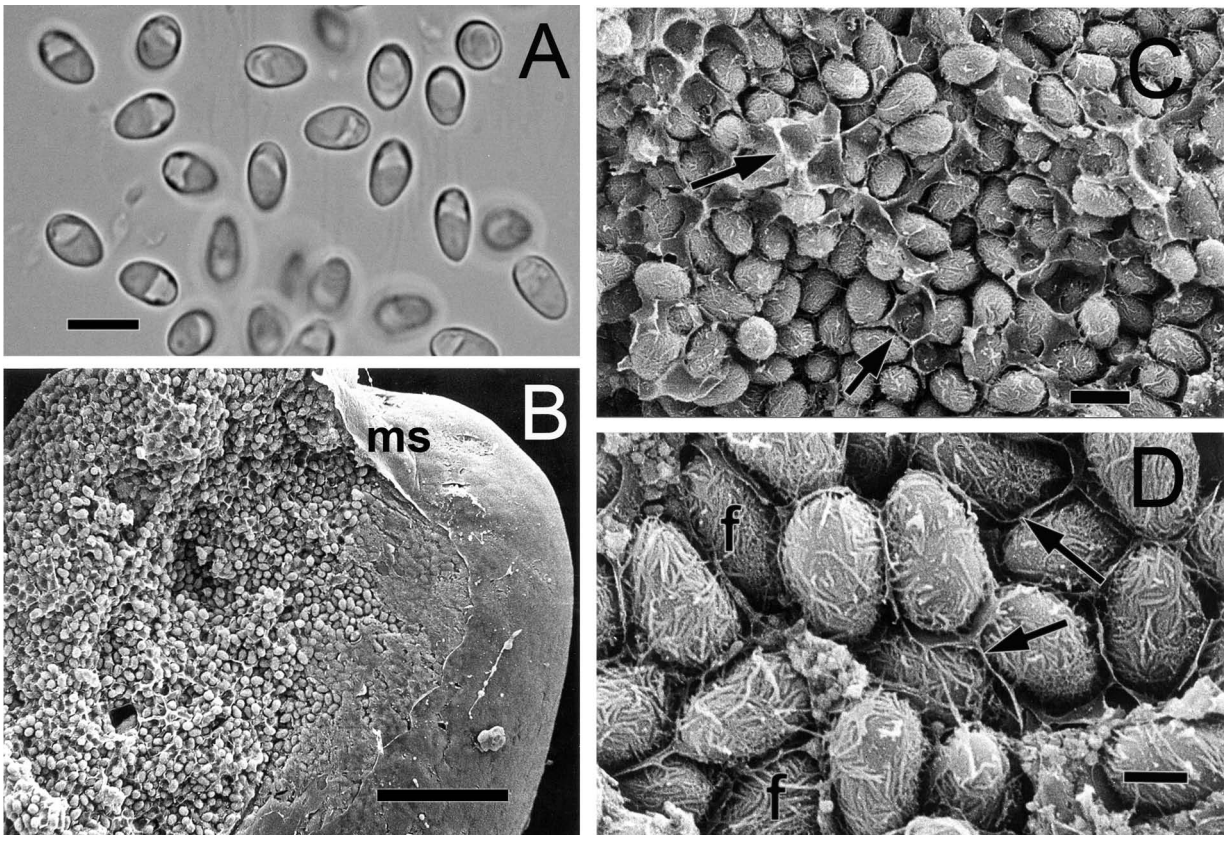

Fig. 1. Light micrograph of fresh saline smear (Fig. 1, A; brightfield) and SEM (Fig. 1, B, C, D) of dry-fractured *Simulium* sp. fat body lobe infected with *Tuzetia* sp.1. *Abbreviations*: f – spore fibrils, ms – membranous sheath of host fat body lobe, *arrows* indicate sporophorous vesicle membrane. Scale bars: A - 5 μm, B - 40 μm, C - 4 μm, D - 2 μm.

tissue. For comparison with the details revealed by SEM examination, each of the accompanying figures includes a light micrograph depicting a fresh saline mount of live spores or sporophorous vesicles of the microsporidian species concerned (Figs 1, A; 2, A; 3, A; 4, A; 5, A).

Figure 1, B-D, shows a fractured fat body lobe of *Simulium* sp. infected with *Tuzetia* sp.1. Low-power examination of such infected tissues graphically illustrates the full pathological extent of this invasive infection with tightly-packed spores extending throughout the fat body lobe (Fig. 1, B). A more detailed examination of the fractured face of the fat body lobe reveals a honeycomb-like appearance due to the location of each spore within the membrane-lined recess of individual sporophorous vesicles characteristic of the genus *Tuzetia* (Fig. 1, C). Where spores have remained attached to the removed complementary face of the fracture, the compartmentalisation is particularly conspicuous (Fig. 1, C). The arrangement of a membrane around each individual spore is most clearly seen at higher magnifications (Fig. 1, D), where ornamentation of the spore surface itself also becomes apparent; each spore bears an outer layer of coarse fibrillar appendages (Fig. 1, D).

A closely-related and morphologically very similar (by light microscopy) species of *Tuzetia*, sp. 2, was shown by dry-fracture SEM to be quite distinct from *Tuzetia* sp. 1 (Fig. 2). The arrangement of spores within the host tissue was very similar, with the same individual membranous compartments (Fig. 2, B). These were most conspicuous where spores had been removed in the dry-fracturing process to reveal niches lined with sporophorous vesicle membranes (Fig. 2, B). In this species the spore surface was adorned not with coarse fibres but with an anastomosing network of broad tubules (Fig. 2, C) beneath which occasional views of the spore surface revealed a pitted appearance (Fig. 2, C).

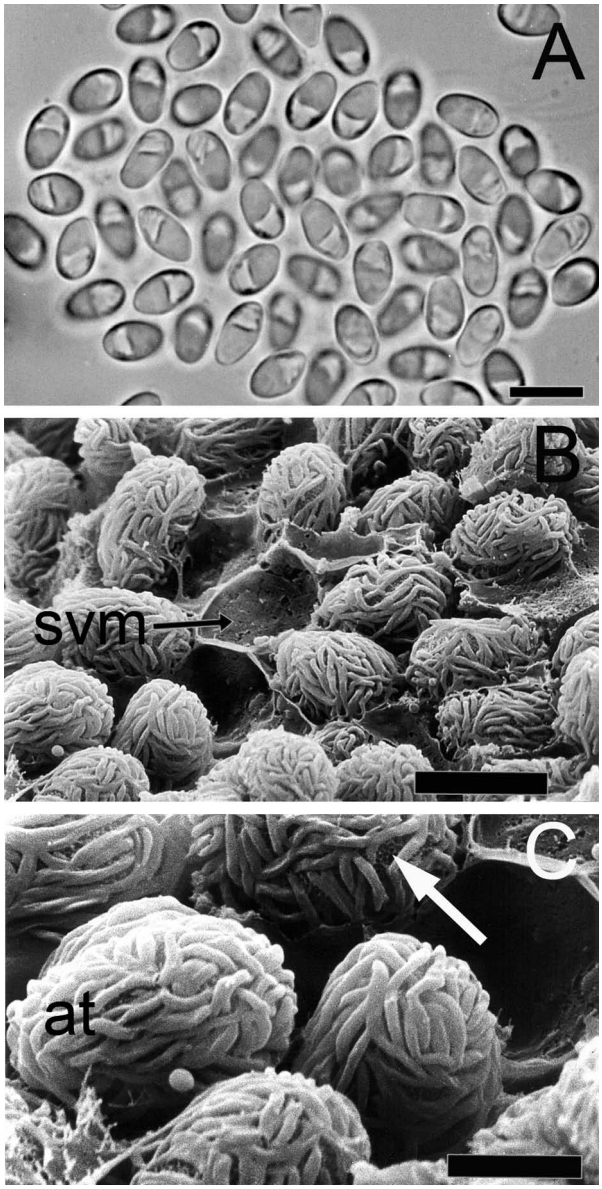

Fig. 2. Light micrograph of fresh saline smear (Fig. 2, A; brightfield) and SEM (Fig. 2, B, C) of dry-fractured *Simulium* sp. fat body lobe infected with *Tuzetia* sp.2. *Abbreviations*: at – anastomosing tubules, svm – sporophorous vesicle membrane, *arrow* indicates pitted appearance of spore surface. Scale bars: A - 5 μm, B - 4 μm, C - 2 μm.

Amblyospora sp. from *Austrosimulium* sp. possessed a persistent sporophorous vesicle (Fig. 3, A) so that even after dry-fracturing most of the vesicles retained their integrity (Fig. 3, B). Only sparse remnants of host cell components were apparent in mature infections, most commonly residual mitochondria which persisted in close association with the parasites long after other host cell constituents had degenerated and disappeared (Fig. 3, C). The sporophorous vesicle membrane was usually constricted around the contents of the vesicle, revealing the outline of the enclosed spores (Fig. 3, C). Sometimes the sporophorous vesicle membrane was partially removed during the dry-fracturing process, providing a clear view of the smooth unornamented surface of the mature spores of that species (Fig. 3, D).

In other sporophorous-vesicle-producing microsporidia the fracture plane created by the dry-fracturing process rarely leaves the sporophorous vesicle intact. Figure 4B shows a cluster of entire sporophorous vesicles and host cell debris on the exposed surface of a dry-fractured *Simulium* sp. fat body lobe infected with *Pleistophora* sp. However, more typical views are shown in Figs 4C and 4D where the sporophorous vesicles have themselves been cleaved by the fracture plane creating a honeycomb-like pattern and exposing the numerous spores within each sporophorous vesicle. The infection has almost completely replaced the host tissue (Fig.4, C), with only diminutive islands of host cell residue visible between the sporophorous vesicle membranes of adjacent vesicles (Fig.4, D). The broadly oval spores of *Pleistophora* sp. were smooth and unornamented but numerous small granules produced during sporogenesis were retained in the mature sporophorous vesicle (Fig 4, D).

SEM of dry-fractured specimens confirms the absence of a sporophorous vesicle membrane in *Nosema* stricklandi from *Simulium* sp. Figure 5, B shows the pathological impact of this invasive infection with free spores packing the fat body lobe from immediately below its outer membranous sheath. The spores of *Nosema stricklandi* exhibit distinct surface-sculpting; the mature spores have a wrinkled appearance with irregular ridges running over most of the surface except at one end of the spore where a distinct circumpolar annulus is always present (Fig. 5, C). Living mature spores of this species have an uneven profile when examined by light microscopy (Fig. 5, A) and the wrinkled appearance seen in scanning electron microscopy is therefore not an artefact.

Discussion

Three dimensional images of the surface of microsporidian spores were first obtained using shadowed replicas of the spores of *Glugea weis-*

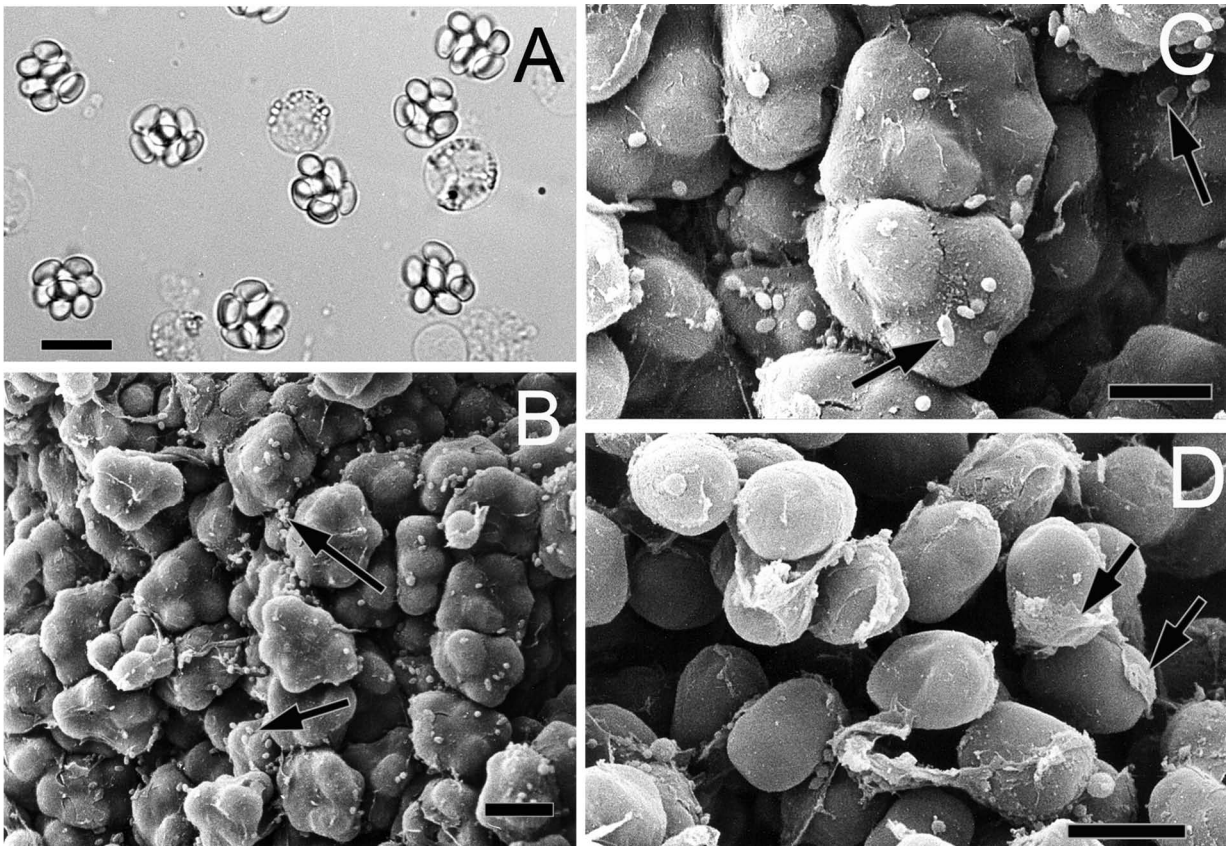

Fig. 3. Light micrograph of fresh saline smear (Fig. 3, A; brightfield) and SEM (Fig. 3, B, C, D) of dry-fractured *Amblyospora* sp. in the adipose tissue of *Austrosimulium* sp. *Arrows* indicate residual host cell debris, including probable mitochondria (B and C) and fragments of remaining sporophorous vesicle membrane (D). Scale bars: A - 10 μm, B - 10 μm, C and D - 4 μm.

senbergi (Vernick, 1969). The early application of conventional scanning electron microscopy to microsporidia used preparatory techniques which relied upon the air-drying of spore suspensions upon metal stubs (Frost and Nolan, 1972; Lom and Weiser, 1972; Fowler and Reeves, 1975; Vavra, 1976). The specimens had therefore been subjected to significant physical forces that result from the scalar effects of surface tension during dehydration, and they consequently displayed variously wrinkled or collapsed appearances.

Other early explorations employed the fracturing of hard blocks of epon-embedded tissue (Humphreys et al., 1973) to expose the surface of *Nosema* and *Thelohania* spores for scanning electron microscopy (Liu and Liu, 1973; Liu and Liu, 1974; Liu and McEwan, 1977; Liu, 1983). Unfortunately the penetration of resin throughout the tissue drastically reduced the detail that could be visualised by scanning electron microscopy.

In major revisions of microsporidia within the family Thelohanidae, Hazard and Anthony (1974) and Hazard and Oldacre (1975) were the first to use data from scanning electron microscopy as a diagnostic tool (in conjunction with light and electron microscopic information) in studying the systematics of this group of Protozoa, as they were then classified. Spore suspensions were subjected to an air-drying technique which has continued to be used through to recent times (Larsson, 1980; Larsson, 1983; Larsson, 1985; Simakova et al., 2005; Wang and Chen, 2007; Bhat et al., 2012; Chakrabarty et al., 2012; Kwak et al., 2013; Sharma et al., 2013). A variety of other SEM preparatory techniques have been utilised for microsporidia (Liu, 1983; Del Aguila et al., 1998; Schottelius et al., 2000) but the exposed surfaces have also inevitably been subjected to the physical trauma of handling and processing. A recent study has utilised a liquid nitrogen pathway in the preparation of spore suspensions for SEM (Hyliš et al., 2013).

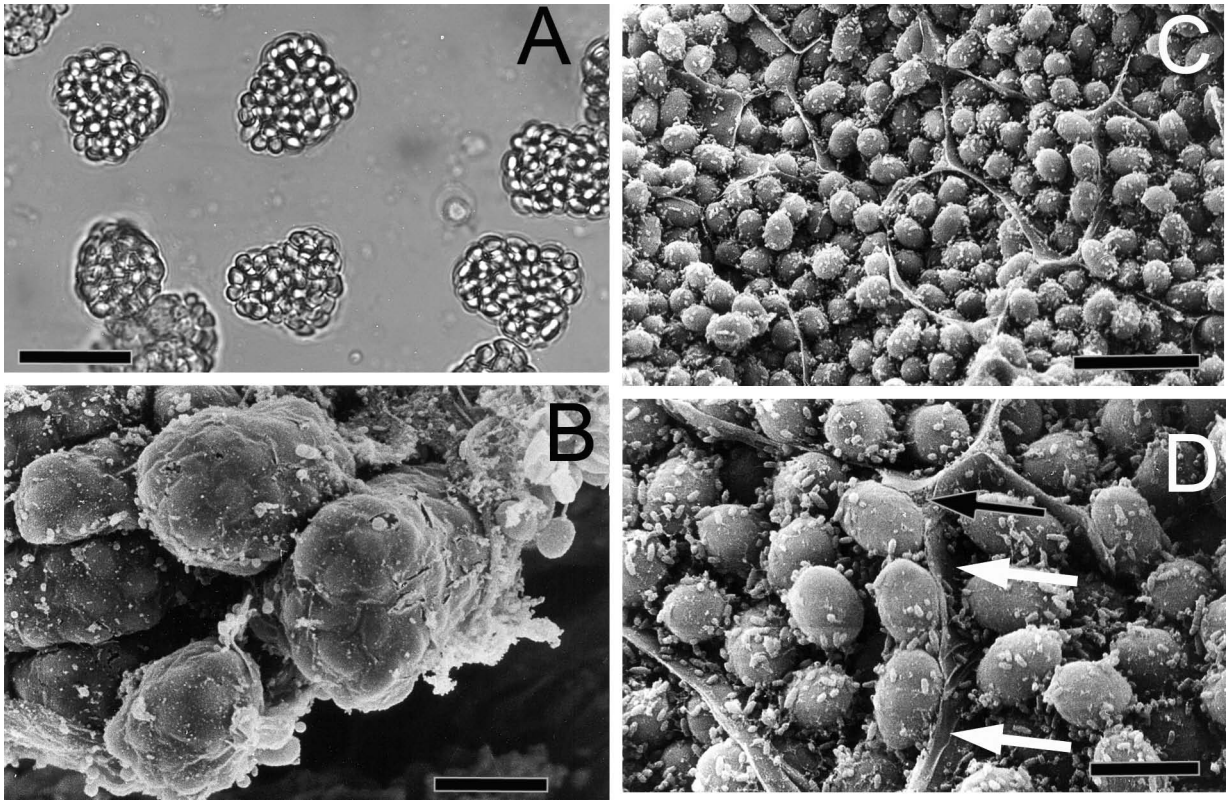

Fig. 4. Light micrograph of fresh saline smear (Fig. 4, A; brightfield) and SEM (Fig. 4, B, C, D) of dry-fractured *Pleistophora* sp. in the adipose tissue of *Simulium* sp. Black *arrow* indicates residual host tissue confined to the diminutive space between abutting sporophorous vesicle membranes (white *arrows*). Scale bars: A - 20 μm, B and C - 10 μm, D - 4 μm.

Critical point drying was initially developed in response to the need for a preparatory technique incurring the minimum distortion of biological materials being prepared for SEM (Anderson, 1966), and was subsequently applied to microsporidians (Vavra and Barker, 1980; Vavra et al., 1981). However, critical-point drying has been applied almost exclusively to spore or sporophorous vesicle suspensions layered upon metal stubs, or microscope slide smears of infected tissue, in which the spatial relationships of the parasites to each other and to the host tissue have inevitably been lost or seriously disrupted (Hashimoto and Takinami, 1976; Larsson, 1983; Ovcharenko and Wita, 2001). The dry-fracture technique described here provides several significant benefits. First, the fixation procedure is identical to that employed for transmission electron microscopy, so that specimens selected for scanning electron microscopy need not be handled separately until after dehydration; this permits the examination by both transmission and scanning electron microscopy of infected tissues from a single host prepared in essentially the same way. Second, the fracturing process relies upon the transmission of cleavage forces from the surface of the specimen, where the needle-tips are applied, through the depth of the sample without direct physical contact with the surfaces thereby exposed; this removes the potential for damage or distortion produced by blades slicing or cutting the specimen at an earlier hydrated stage of processing. Third, the fractured surface of infected host tissue is not exposed until immediately before coating with gold prior to examination in the scanning electron microscope; the possibility of surface contamination is therefore minimised or eliminated. Fourth, during all preparatory stages from initial fixation to final critical point drying the infected tissue sample retains its integrity and the production of fixation- or dehydration-induced artefacts is thereby minimised. Fifth, the spatial relationships of the parasites to each other and to the host tissue are generally undisturbed by the

technique, so that features such as the presence and detailed configuration of any parasite-associated membranes are readily visualised. Finally, the fracture plane is rarely uniform; for example, in some parts of the dry-fractured specimen sporophorous vesicles, where present, remain intact while in other regions these structures are fractured to expose the contents; thus a great variety of clean undistorted images of the parasites are available for documentation and analysis.

Description of microsporidian taxa has traditionally relied upon morphological characterisation together with host specificity and life-cycle data. The development of cost-effective genome analysis technology, especially since the turn of the millennium, has added a new dimension to taxonomic descriptions within this group and to the quest to unravel the phylogenetic and evolutionary relationships within microsporidia and between microsporidia and other eukaryotes (Corradi and Keeling, 2009; Scazzocchio, 2014). This approach has supported a fungal affiliation for the microsporidia rather than its traditional protozoan domicile, a re-alignment that began as a result of protein and cell division studies during the 1990s (Flegel and Pasharawipas, 1995; Fast et al.,1999). Perhaps ironically, the first description of a microsporidian, *Nosema bombycis* in the middle of the nineteenth century, placed it in the fungi (Nageli, 1857). And yet recently, as a further indication of flux in this area, a major new classification of all living things has returned the microsporidia, along with another taxonomically labile group, the rozellids, to the Protozoa (Ruggiero et al., 2015).

Caution has been advised in the over-zealous adoption of genomic studies as the sole basis of new phylogenies (Larsson, 2005), especially given the relatively narrow set of lineages that have so far been explored (Corradi and Keeling, 2014). The rapid evolutionary changes in these parasites has for example highlighted the tendency towards genomic contraction and reduction (Corradi and Keeling, 2009). Increasingly, new microsporidian taxa are being described based upon the traditional morphological characteristics revealed by light and electron microscopy, together with molecular attributes (Rao et al., 2007; Andreadis et al., 2013; Ovcharenko et al., 2013). Within this emerging blended approach the merits of effective scanning electron microscopy are significant, providing a three dimensional morphological perspective that is beyond the resolution of light microscopy and not easily revealed by transmission electron microscopy. The dry-fracture technique described here has the additional benefits of supporting and protecting both host and parasite cells during preparation thereby optimising the final visualisation once fracturing has been implemented, plus providing clear *in situ* 3-D imagery.

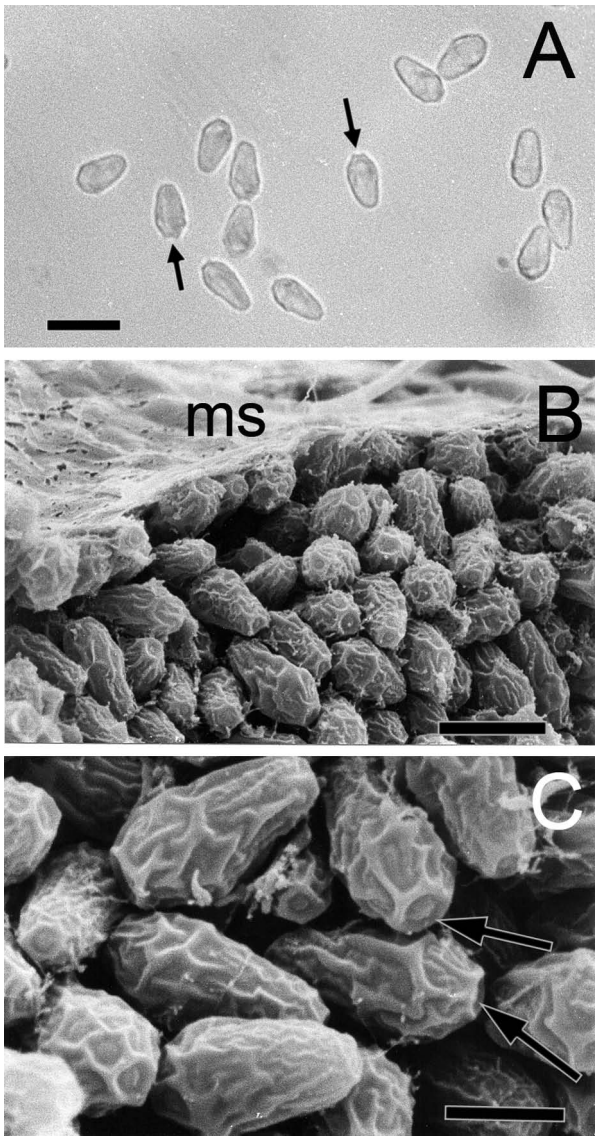

Fig. 5. Light micrograph of fresh saline smear (Fig. 5, A; brightfield) and SEM (Fig. 5, B, C) of dry-fractured *Nosema stricklandi* in the adipose tissue of *Simulium* sp. *Abbreviations*: ms – membranous sheath of host fat body lobe, *arrows* indicate distinctive polar annulus of spores. Scale bars: A - 5 μm, B - 4 μm, C - 2 μm.

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