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## A New Pathogen, *Microsporidium itiiti* n. sp. (Microsporidia), from the Argentine Stem Weevil, *Listronotus bonariensis* (Coleoptera, Curculionidae)<sup>1</sup>

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**ABSTRACT.** A new species of microsporidium (phylum Microspora) infecting the Argentine stem weevil, *Listronotus bonariensis* (Kuschel, 1955), is described on the basis of light and electron microscope observations. It has the following characteristics: nuclei always isolated; meronts spherical and sporonts ribbon-shaped, with variable numbers of nuclei; sporogony within a vacuole which is bounded by a thin membrane that usually breaks down before uninucleate spores mature; occasionally parts of the membrane remain so that clusters of variable numbers of spores may be seen in light microscopic preparations. Spores measure  $2.5 \times 1.4 \mu\text{m}$  (fresh) and development occurs mainly in the midgut, but also in the epidermis, fat body, muscle, and ovaries.

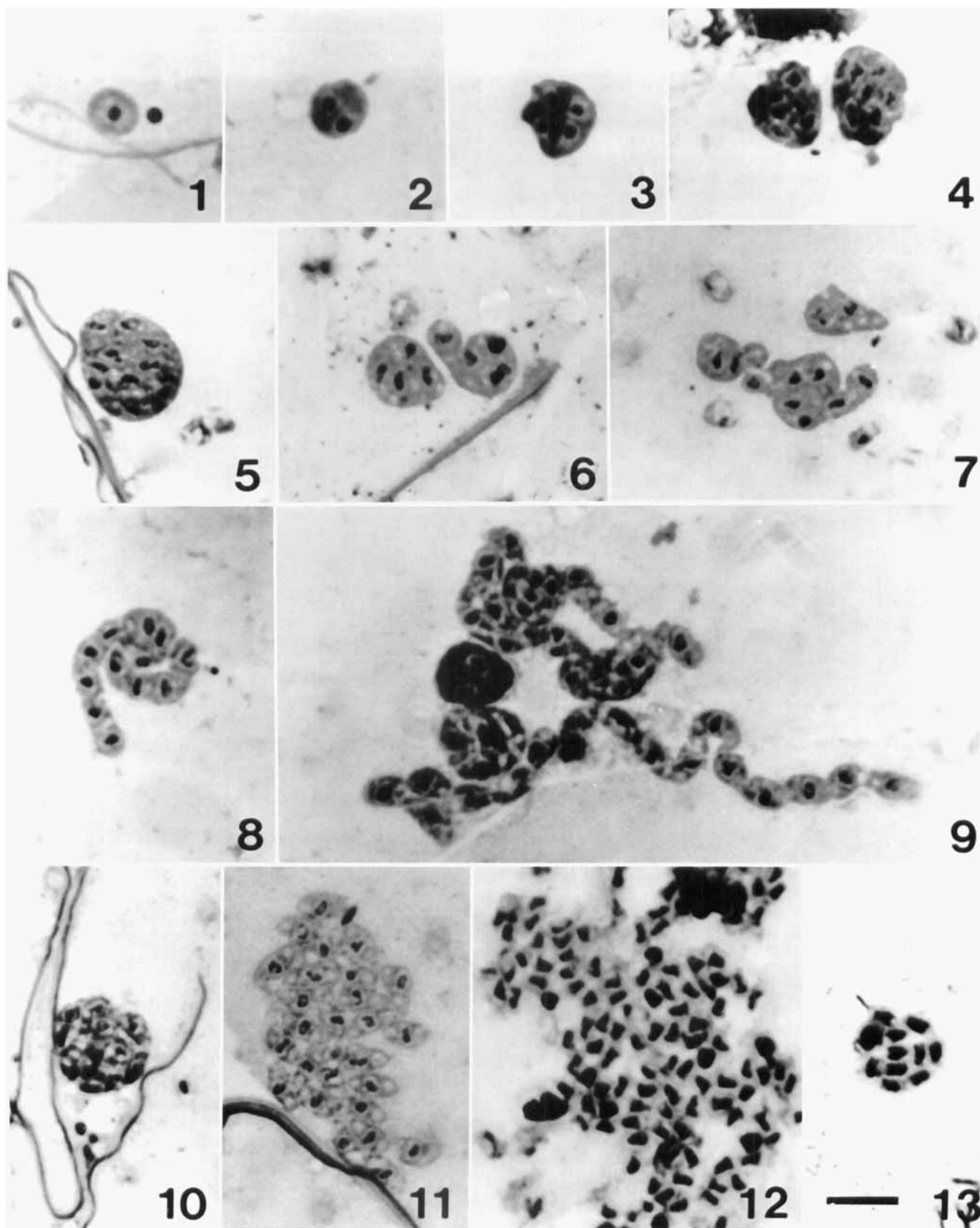
**D**URING a search for pathogens of the Argentine stem weevil, *Listronotus bonariensis* (Kuschel, 1955), a serious pest of New Zealand pastures, many adult weevils were found to be infected with a microsporidium with small spores (10). Microsporidia have been reported from the alfalfa weevil, *Hypera postica* (Gyllenhal) (3, 9, 19), the boll weevil, *Anthonomus grandis* Boheman (11), and the white pine weevil, *Pissodes strobi* (Peck) (15, 18) in the United States of America, and in *Oti-*

*rhynchus* sp. in Czechoslovakia (16) and France (5) as well as in *Pissodes piceae* Ill. in the Soviet Union (7). None have been noted from *L. bonariensis* before. Unusual features, such as multinucleate sporonts in the form of long coiled ribbons and mature spores occurring either singly or in clusters of two to twelve spores, suggested that this microsporidium represented a new species and prompted the detailed light and electron microscope study reported here.

### MATERIALS AND METHODS

Infected larval and adult weevils were obtained from a field site at Ruakura Agricultural Research Centre, Hamilton, New Zealand.

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Figs. 1-13. Photomicrographs of the life cycle stages of *M. ititi* (Giemsa-stained smears). Bar = 5  $\mu$ m. 1. Uninucleate meront. 2. Binucleate meront. 3. Tetranucleate meront. 4. Meronts with six and seven nuclei. 5. Multinucleate meront. 6. Tetranucleate meronts, one undergoing a

TABLE I. *Microsporidia* from weevils.

Host	Microsporidium	Spore size ( $\mu\text{m}$ )	Reference
<i>Anthonomus grandis</i>	<i>Nosema gasti</i>	$4.3 \times 2.3$ (fresh)	(11)
<i>Hypera postica</i>	<i>Nosema</i> sp.	$4.3\text{--}5.7 \times 1.9\text{--}2.9$ (fresh)	(9)
	<i>Nosema</i> sp.	$6 \times 4.5$ (fresh)	(3)
	<i>Nosema hyperae</i>	$3.1 \times 1.7$ (fresh)	(14, 19)
<i>Otiorrhynchus fuscipes</i>	<i>Microsporidium longifilum</i>	$4\text{--}5 \times 3$ (stained)	(5, 14)
<i>Otiorrhynchus ligustici</i>	<i>Nosema otiorrhynchi</i>	$3.8\text{--}4 \times 1.8\text{--}2$ (stained)	(16)
<i>Pissodes piceae</i>	<i>Nosema</i> sp.	$3.6\text{--}5 \times 2\text{--}2.9$ (stained)	(7)
<i>Pissodes strobi</i>	<i>Nosema</i> sp.	$3.5\text{--}4.2 \times 1.8\text{--}2.2$	
		or	
		$5\text{--}6 \times 1.8\text{--}2.5$ (fresh)	(15)
	Unidentified sp.	$3.4 \times 1.6$ (fresh)	(18)

For light microscopy, air-dried smears of whole weevils were fixed in Carnoy's fluid and stained in 10% (v/v) Gurr's Improved R66 Giemsa stain in 0.02 M phosphate buffer at pH 6.9. Fresh spores were immobilized on slides coated with 1% (w/v) agar and photographed for measuring. In order to examine tissue specificity, whole infected larvae and adults were fixed in alcoholic Bouin's fluid, dehydrated in ethanol, and embedded in paraffin wax (56°C melting point). Sections (5–7  $\mu\text{m}$ ) were stained in 4% (v/v) Giemsa stain in distilled water overnight and differentiated in 0.07% glacial acetic acid for 2 min.

For electron microscopy, pieces of infected tissue were dissected into Karnovsky's fixative, left at room temperature for 10 min, then transferred to fresh fixative at 4°C for a further 1 h. Specimens were washed twice in 0.12 M sodium cacodylate buffer, pH 7.4, and then post-fixed in 2.5% (w/v)  $\text{OsO}_4$  in 0.1 M cacodylate buffer for 1 h at 4°C. After two washes in 0.1 M sodium acetate, specimens were passed through 50%, 70%, 90%, and 100% acetone at room temperature and embedded in Spurr's resin. To locate infected tissues, thick sections (1–2  $\mu\text{m}$ ) were cut with glass knives, mounted on slides and stained briefly in hot 1% (w/v) toluidine blue in 1% (w/v) aqueous sodium tetraborate for light microscopic examination. Ultrathin sections were cut with glass knives, mounted on uncoated 400-mesh grids, and post-stained in a saturated solution of uranyl acetate in 70% ethanol followed by Venable's lead citrate; a JEOL JEM 100B electron microscope was used at an accelerating voltage of 60 kV.

## RESULTS

**Light microscopy.** The earliest meronts are spherical cells containing one or two isolated nuclei (Figs. 1, 2). Later meronts are larger and have variable numbers of nuclei, up to about twelve per cell (Figs. 3–5). Meronts containing odd numbers of nuclei are quite common and probably result from asynchronous nuclear division. Some meronts have bizarre shapes (Figs. 6, 7) and these may represent a transition phase between the spherical meronts and the early sporonts, which are shaped like ribbons, with nuclei evenly spaced along their lengths (Fig. 8). Ribbon-like stages may have from four to twelve nuclei and the longer ones often appear coiled or folded back on themselves. During sporogony, the cytoplasm constricts around each nucleus, producing chain-like late sporonts (Fig. 9), which sometimes remain

coiled in a spherical mass (Fig. 10). The chains eventually break up to form uninucleate, ovoid or fusiform sporoblasts (Fig. 11). Mature spores are mostly free in the cytoplasm (Fig. 12), but some occur in clusters containing from two to twelve spores (Fig. 13). Diplokaryotic nuclei were not observed in any stage. Fresh spores had dimensions (mean  $\pm$  standard error of the mean) of  $2.5 \pm 0.04 \mu\text{m}$  by  $1.4 \pm 0.03 \mu\text{m}$ , with a range of 2.1–3.0  $\mu\text{m}$  by 0.9–2.1  $\mu\text{m}$  ( $n = 50$ ). Giemsa-stained spores had dimensions of  $2.7 \pm 0.04 \mu\text{m}$  by  $1.7 \pm 0.03 \mu\text{m}$ , with a range of 2.0–3.5  $\mu\text{m}$  by 1.2–2.7  $\mu\text{m}$  ( $n = 100$ ). Although spores varied considerably in size, there was no indication of dimorphic development in larvae or adults.

There are no obvious external symptoms of infection in larvae or adults. In adult weevils, parasite development occurs principally in the midgut epithelial cells. A great number of spores are produced and many are shed into the gut lumen and passed out with the feces. Spores also occur in lesser numbers in the epidermis, the fat body, and in small pockets within some muscle fibers. Misshapen ovaries full of microsporidian spores were noted upon dissection of two adult female weevils. The midgut epithelium and midgut ceca are the main sites of parasite growth in the larvae although a few spores occur in the fat body. The microsporidium is sometimes transmitted from one generation of laboratory-reared weevils to the next, but it is not certain whether the route of infection is transovarial or transovum.

**Electron microscopy.** In ultrathin sections, the meronts appear as simple cells with isolated nuclei and cytoplasm containing scattered ribosomes and a few strands of rough endoplasmic reticulum (Figs. 14, 15). The mitotic spindle plaques surmounted by polar vesicles, noted in some, indicate that these are actively dividing cells (Fig. 15). The meronts are separated from the host cytoplasm by two membranous layers. The outer layer is generally studded with ribosomes on the surface facing the host cytoplasm (Fig. 16). The sporonts are elongate and, as their plasmalemmas become thickened by the deposition of a layer of electron-dense material, they appear to pull away from the host cytoplasm (Fig. 17) and eventually lie within a vacuole (Fig. 18) bounded by a membrane, still studded on one surface with ribosomes, as in the meronts (Fig. 19). Each vacuole appears to contain a number of sporonts (Fig. 18), but these may in fact be sections of a single folded ribbon-like stage. The sporoblasts develop within these vacuoles (Fig. 20) although the

shape change. 7. Two binucleate meronts and meront, apparently with seven nuclei, undergoing a shape change. 8. Coiled, ribbon-like sporont with 12 nuclei. 9. Long, folded chains of sporoblasts. 10. Sporoblasts grouped in a spherical mass. 11. Group of uninucleate sporoblasts, some separate. 12. Free mature spores. 13. Eleven mature spores in a cluster.

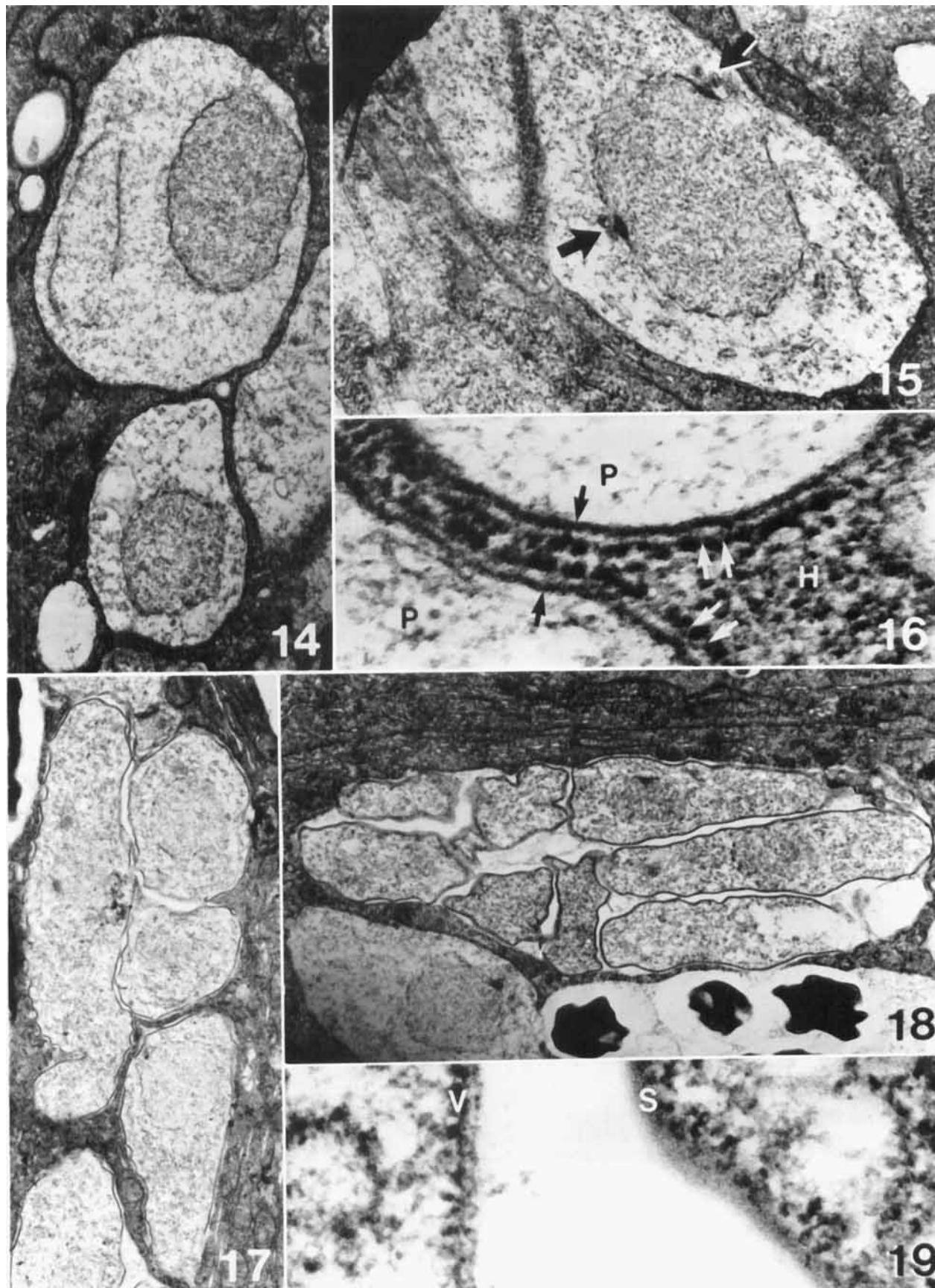


TABLE II. *Microsporidia with ribbon-shaped stages.*

Microsporidium	Hosts	Spore size ( $\mu\text{m}$ )	Reference
<i>Orthosoma operophterae</i>	<i>Operophtera brumata</i>	2.5–6 $\times$ 1–1.5	(2)
<i>Pleistophora fidelis</i>	<i>Polygramma undecimlineata</i> , <i>Leptinotarsa decemlineata</i>	2–2.5 $\times$ 1–1.2	(6)
<i>Pleistophora neustriiae</i>	<i>Malacosoma neustriiae</i> , <i>Lymantria dispar</i> , <i>Hyponomeuta malinella</i>	3 $\times$ 1.8	(4, 14)
<i>Pleistophora schubergi schubergi</i>	<i>Euproctis chrysorrhoea</i> , <i>Lymantria dispar</i> , <i>Malacosoma neustria</i> (and other Lepidoptera)	2.5 $\times$ 1.5	(14, 20)
<i>Pleistophora schubergi neustriiae</i>	<i>Malacosoma neustria</i>	2.5–3 $\times$ 1.5–1.8	(13)

membrane surrounding them appears to break down somewhat in the later stages of sporoblast formation (Figs. 20, 21). Mature spores are uninucleate, with eight to ten coils of the polar tube and they mostly occur singly and free in the cytoplasm (Fig. 22). Some spores, however, do remain grouped within the remnants of the vacuole, which accounts for the clusters of spores seen in light microscope preparations. Synaptonemal complexes, indicating meiotic division, were not observed at any stage.

### DISCUSSION

As diplokarya were never observed in the microsporidium from the Argentine stem weevil, it is clearly not a *Nosema* species and thus may be distinguished from all but two of the microsporidian species recorded from weevils (Table I). Details of the vegetative stages of *Microsporidium longifilum* (Hesse, 1905) are not available (5, 17), but its spores are large enough to be beyond the size range of those of the stem weevil microsporidium. The small unidentified microsporidium from the white pine weevil, *Pissodes strobi* (Peck) (18), bears a strong resemblance to the stem weevil microsporidium; both have uninucleate spores containing about nine coils of the polar tube and both have multinucleate meronts which infect mainly mid-gut tissue. They differ, however, in that ribbon-like stages have not been recorded from the parasite of *P. strobi*. Only a direct comparison could establish identity between these two microsporidia.

Distinctly ribbon-shaped vegetative stages, i.e. elongate, with approximately parallel sides and containing more than four, evenly spaced, isolated nuclei, have been recorded from only a few microsporidian species (Table II). Each species has spores similar in size to those of the stem weevil microsporidium.

The stem weevil microsporidium was at first tentatively identified as an *Orthosoma* species (10) because its stages seemed morphologically similar to those of *Orthosoma operophterae* (Canning, 1960) (2); however, the genus *Orthosoma* is characterized by apansporoblastic sporogony and the stem weevil microsporidium has a membrane-bounded vacuole.

As the sporonts of the microsporidium from the Argentine stem weevil do not develop in a well-defined sporophorous vesicle, this species cannot belong to the genus *Pleistophora* or the genus *Vavraia* (1). Neither does it have the diplokaryotic meronts that characterize the genus *Polydispyrenia* (1). There are, however, a number of species described as belonging to the

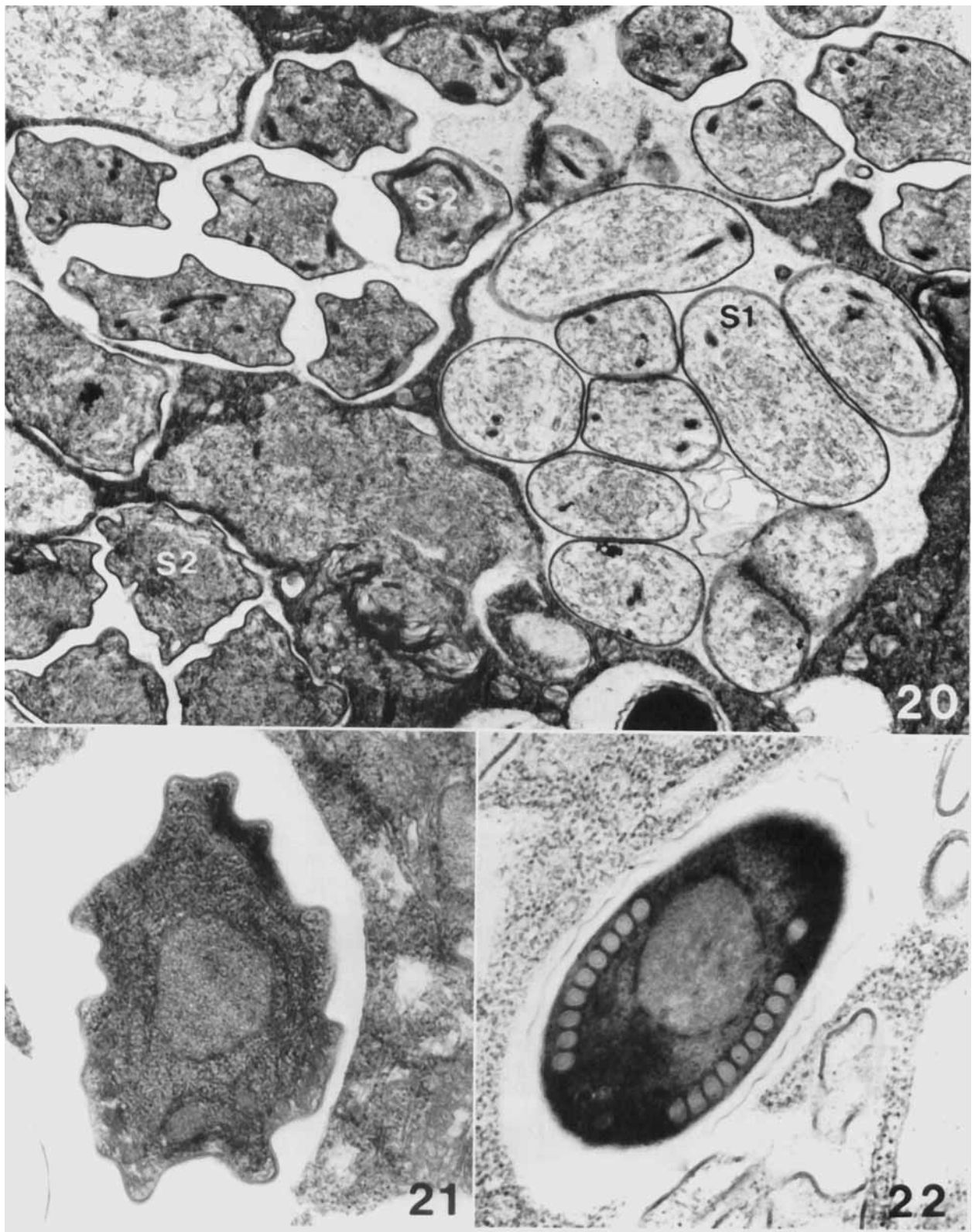
genus *Pleistophora* that have vegetative stages very similar to those of the stem weevil microsporidium (Table II). Only the arrangement of the spores is different; spores of the stem weevil microsporidium are predominantly free whereas those of the *Pleistophora* species are always in membrane-bounded groups. Of the *Pleistophora* species with ribbon-like stages, only *Pleistophora schubergi neustriiae* Purri, 1982 has been examined using electron microscopy (13), and ultrastructural details of the meronts and sporonts are not recorded. The others are described on the basis of light microscopic observations only. *Pleistophora fidelis* Hostounsky & Weiser, 1975 and *Pleistophora neustriiae* (Gunther, 1958) are morphologically indistinguishable from each other and from *P. schubergi schubergi* (4, 6, 14). These three species have been separated on the basis of differing host ranges only. *Pleistophora schubergi neustriiae* is separated from the other subspecies only because a detailed investigation revealed two generations of schizonts (meronts) (13). The susceptibility of insect species other than *Malacosoma neustria* to *P. schubergi neustriiae* was not recorded in this study (13) and no comparison was made with *P. neustriiae*, which also infects this insect. The stem weevil microsporidium may be closely related to each of the *Pleistophora* species listed in Table II, and perhaps also to the other subspecies of *P. schubergi*, but ultrastructural studies of all stages of these microsporidia are needed before proper comparisons can be made.

The occurrence of both free and grouped spores in light microscopic preparations of the stem weevil microsporidium does not appear to be the result of true dimorphic development as observed in *Vairimorpha* species (12) and *Burenella* species (8). In these genera, two different classes of spores are produced, each the result of a different developmental pathway. The stem weevil microsporidium, however, has a single type of spore development and some of the spores are clustered simply because the membrane surrounding the sporonts fails, in some cases, to break down completely before the spores mature. The observation that the clusters contain variable numbers of spores seems to support this idea.

The stem weevil microsporidium is most similar to microsporidian species of uncertain generic status, i.e. *Pleistophora fidelis*, *P. neustriiae*, *P. schubergi schubergi*, and *P. schubergi neustriiae*, and it is not identical to any of them. Further information may show that they are taxonomically related but for now it is proposed that this new species be placed in the col-

Figs. 14–19. Electron micrographs of *M. itiiti*. 14. Two spherical uninucleate meronts.  $\times 17,700$ . 15. Uninucleate meront undergoing nuclear division. Spindle plaques are marked with arrows.  $\times 28,000$ . 16. Detail of membranes surrounding two meronts. Parasite cytoplasm, P, is bounded by an inner layer (single arrow) and an outer layer (double arrow) studded with ribosomes on the surface facing the host cytoplasm, H.  $\times 172,300$ . 17. Elongate sporonts with thickening plasmalemmas.  $\times 12,850$ . 18. Sporonts with completely thickened plasmalemmas, lying within a vacuole.  $\times 13,600$ . 19. Detail of the membranes surrounding both sporont, S, and vacuole, V.  $\times 150,000$ .





Figs. 20–22. Electron micrographs of *M. ititii*. 20. Early, S1, and late, S2, sporoblasts within vacuoles.  $\times 17,000$ . 21. Late, uninucleate sporoblast.  $\times 38,950$ . 22. Mature, uninucleate spore with nine coils of polar tube.  $\times 53,500$ .

lective group, *Microsporidium* Balbiani, 1884, and named *Microsporidium itiiti* after the Maori word, *itiiti*, meaning "smaller than the eye can see."

*Microsporidium itiiti* n. sp.

**Diagnosis.** Isolated nuclei throughout the life cycle; meronts spherical with variable numbers of nuclei, later changing shape to form long, ribbon-like sporonts, which are often coiled or folded; sporogony and some sporoblast development within a vacuole bounded by a thin membrane; in most cases, this breaks down later, releasing the free, uninucleate spores; occasionally parts of the membrane remain so that clusters of two to twelve spores are formed; no evidence of meiosis; spores ovoid, when fresh, averaging  $2.5 \pm 0.04 \mu\text{m}$  by  $1.4 \pm 0.03 \mu\text{m}$  in size, and  $2.7 \pm 0.04 \mu\text{m}$  by  $1.7 \pm 0.03 \mu\text{m}$  after Giemsa-staining; eight to ten coils of polar tube.

**Host.** Larvae and adults of the Argentine stem weevil, *Listronotus bonariensis* (Kuschel, 1955). Ruakura Agricultural Research Centre, Hamilton, New Zealand.

**Infection sites.** Mainly midgut epithelium of both larvae and adults; also in ceca and fat body of larvae; fat body, epidermis, muscle, and ovaries of adults.

**Type material.** Type slides deposited in collection of the Insect Pathology Section, Department of Scientific and Industrial Research, Entomology Division, Auckland, New Zealand. Holotype slide number 597 56. Paratype slide number 597 66.

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