J. Eukaryot. Microbiol., 51(2), 2004 pp. 207–213

Transfer of *Nosema locustae* (Microsporidia) to *Antonospora locustae* n. comb. Based on Molecular and Ultrastructural Data[1]

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ABSTRACT. *Nosema locustae* is a microsporidian parasite of grasshopper pests that is used as a biological control agent, and is one of the emerging model systems for microsporidia. Due largely to its diplokaryotic nuclei, *N. locustae* has been classified in the genus *Nosema*, a large genus with members that infect a wide variety of insects. However, some molecular studies have cast doubt on the validity of certain *Nosema* species, and on the taxonomic position of *N. locustae*. To clarify the affinities of this important insect parasite we sequenced part of the rRNA operon of *N. locustae* and conducted a phylogenetic analysis using the complete small subunit rRNA gene. *Nosema locustae* is only distantly related to the nominotypic *N. bombycis*, and is instead closely related to *Antonospora scoticae*, a recently described parasite of bees. We examined the ultrastructure of mature *N. locustae* spores, and found the spore wall to differ from true *Nosema* species in having a multi-layered exospore resembling that of *Antonospora* (one of the distinguishing features of that genus). Based on both molecular and morphological evidence, therefore, we propose transferring *N. locustae* to the genus *Antonospora*, as *Antonospora locustae* n. comb.

Key Words. Microsporidia, parasite, phylogeny, rRNA, taxonomy.

MICROSPORIDIA are obligate intracellular parasites that were traditionally considered to be a unique eukaryotic phylum (Microspora Sprague, 1977), but are now recognized as highly derived fungi (Hirt et al. 1999; Katinka et al. 2001; Keeling 2003; Keeling et al. 2000; Van de Peer et al. 2000). Although members of this diverse group of eukaryotes infect a broad array of animal phyla, microsporidian infections are best known from those animals with medical or economic importance, such as insects, fish, and humans (Wittner and Weiss 1999).

The genus *Nosema* was established by Nägeli in 1857 for the first microsporidian species to be described, *N. bombycis*, which caused important losses to the silk industry (Becnel and Andreadis 1999). The original diagnosis of the genus was very broad, and it inevitably gathered a large number of species possessing oval diplokaryotic spores (Larsson 1999). A major problem with the taxonomy of the *Nosema* is the convergent nature of their main taxonomic feature: being diplokaryotic throughout their life cycle. Indeed, Baker et al. (1994) analysed a short fragment of the large subunit (LSU) rRNA of eight *Nosema* species, and found two distinct groups. Although the sampling of other microsporidia was not sufficient at that time to tell how closely related these two groups were to one another, they considered the true *Nosema* species to be those that infected Lepidoptera and were closely related to the nominotypic *N. bombycis* [molecular studies have since showed that this group includes species infecting Lepidoptera and Hymenoptera (Bell et al. 2001; Muller et al. 2000)]. Not surprisingly, as more refined techniques became available, many *Nosema* species were proven to be distant from *N. bombycis* in ultrastructure, physiology, and molecular phylogeny (Canning et al. 2002; Lowman et al. 2000; Silveira and Canning 1995).

Nosema locustae was first described from the grasshopper *Locusta migratoria* (Canning 1953), but it is now known that its potential host range involves at least 102 orthopteran species (Sokolova and Lange 2002). As a pathogen of orthopterans, *N. locustae* has been widely used as a microbial insecticide for grasshopper control in several countries (Johnson 1997; Lange and Cigliano 1999; Lockwood et al. 1999; Lomer et al. 2001). *Nosema locustae* is also emerging as an important model species for microsporidian biology: it has been the subject of extensive molecular investigations into metabolism, evolution, and phylogenetics (Fast and Keeling 2001; Fast et al. 1998, 1999, 2003; Germot et al. 1997; Keeling 2003; Keeling et al. 2000), and its complete genome is currently being sequenced (http://jbpc.mbl.edu/Nosema). Despite the practical relevance of this species and its important place in microsporidian molecular biology, the SSU rRNA of *N. locustae* has surprisingly not been sequenced and details on the ultrastructure of intracellular stages were unknown until recently (Sokolova and Lange 2002). Moreover, the taxonomic status of *N. locustae* has not been seriously disputed, but doubts have been raised. In the early analysis using LSU rRNA, *N. locustae* branched among the group that did not include *N. bombycis* (Baker et al. 1994), and a phylogenetic analysis of RNA polymerase including *N. locustae* and *N. tyriae* showed that these two species were not closely related (Cheney et al. 2001). However, the sampling available in these analyses was restricted and they did not show an affinity of *N. locustae* to any other microsporidian group.

With the aims of resolving the phylogenetic affinities of *N. locustae* and clarifying its taxonomic status, we have examined spore wall ultrastructure, characterized the rRNA operon of *N. locustae*, and conducted a phylogenetic analysis of microsporidia using the complete sequence of the SSU rRNA. Our results clearly show that *N. locustae* does not belong to the genus *Nosema* as previously suggested by Baker et al. (1994), and consistently support a relationship between *N. locustae* and the newly described *Antonospora scoticae*. We accordingly propose the new combination, *Antonospora locustae*.

MATERIALS AND METHODS

Sequence and analysis of the SSU rRNA. The *N. locustae* rRNA operon was identified in an ongoing genomic survey (Fast and Keeling 2001), and the complete sequence of a genomic fragment (4319 bp) containing part of the rRNA operon, including the entire SSU rRNA, was determined. The sequence was deposited in Genbank under accession number AY376351. The spores were provided by M&R Durango Inc. (Bayfield, CO), with the original source being ATCC 30860, supplied by J. E. Henry (Bozeman, MT). This identity is supported by other sequences from our survey, which include a 900-bp sequence 100% identical to the *N. locustae* mitochondrial HSP70 reported by Germot et al. (1997) and also, the alpha and beta tubulin and actin from an EST survey based on the same material (unpubl. data). The SSU rRNA sequence was aligned with all known homologues from other microsporidians available in public databases (accession numbers are given in Supplemen-

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[1] New DNA sequence included: AY376351

tary Data Table I, see Appendix). Sequences were aligned using ClustalX (Thompson 1997) and the alignment edited manually. Poorly aligned regions were excluded, resulting in a data set of 850 characters from 88 taxa. Phylogenetic analyses were conducted using neighbor-joining with Log-Det distances and maximum likelihood (ML) using PAUP* 4.0 b10 (Swofford 2003). For ML trees, a reduced set of 45 sequences was used due to computational limitations. ML analyses followed the GTR model with site-to-site rate variation modeled on a discrete gamma distribution with four variable rate categories plus invariable sites. The shape parameter alpha and proportion of invariable sites were estimated by maximum likelihood from a distance tree topology using PAUP 4.0 b10. Support of nodes was assessed with 1,000 bootstrap replicates for the NJ tree, and 100 replicates for the ML analysis.

Transmission electron microscopy. A microwave procedure was employed for spore fixation and infiltration. Purified spores of *N. locustae* were fixed in 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) using a Pelco 3450 microwave (Ted Pella, Redding, CA). The cells were fixed by placing at the cold spot of the microwave and irradiated at 115 W for a cycle of 2 min on, 2 min off, 2 min on. This cycle was repeated twice more. After three rinses in 0.1 M cacodylate buffer at 115 W for 40 s, cells were stained with osmium tetroxide using the microwave by irradiating for 2 min on, 2 min off, 2 min on, again at 115 W. Cells were rinsed three times in double distilled water. Fixed cells were dehydrated using the microwave at 212 W for 40 s in increasing concentrations of ethanol, using 50%, 70%, 95%, and three changes of 100% ethanol. Cells were then infiltrated with Epon-Spurr's resin in the microwave, by irradiating for 3 min in 1:1 resin-100% ethanol at 212 W twice, and 3 min in pure Epon-Spurr's resin at 212 W twice. The resin was polymerized by heating for 12 h at 60 °C. Sections ranging 50–90 nm were cut using a Ultracut T ultramicrotome (Leica) and then stained using lead citrate and 2% uranyl acetate. Stained grids were observed using a Hitachi 7600 transmission electron microscope.

RESULTS AND DISCUSSION:

Small subunit rRNA Phylogeny. The previously uncharacterized SSU rRNA was completely sequenced from a fragment of the *N. locustae* genome containing the rRNA operon. The fragment included about 2,500 bp with no significant similarity upstream of the SSU gene, suggesting that the rRNA units may be located next to telomeric DNA, as was found in *Encephalitozoon cuniculi* (Katinka et al. 2001). The GC content of the SSU gene is 64%, which is significantly higher that the GC content typical of the SSU rRNAs of other *Nosema* species, which range between 33.9% and 39%, but very similar to that of *Antonospora scoticae*, which has 62.5% of G and C (Fries et al. 1999). The *N. locustae* sequence shares 92.5% identity with that of *A. scoticae*, but only 59% with *N. bombycis*.

To determine the phylogenetic affinity of *N. locustae*, we analysed a comprehensive set of microsporidian SSU rRNA sequences, maximizing the diversity of microsporidian lineages represented to avoid misplacements due to incomplete sampling. Phylogenetic analyses recovered trees similar to other published microsporidian SSU rRNA phylogenies (Bell et al. 2001; Canning et al. 2002; Fries et al. 1999; Lom and Nilsen 2003; Morris and Adams 2002; Muller et al. 2000). Most importantly, this included a consistent and strongly supported clade comprising all ''true'' *Nosema* species (as well as *Vairimorpha* species and *Oligosporidium occidentalis*). This in turn is the sister-group of the *Encephalitozoon* group and more distantly related to the *Enterocytozoon*/*Endoreticulatus* group, as has been observed previously [e.g. (Baker et al. 1995)]. This large group is in turn associated with that composed mostly of fish-infecting species *Glugea*, *Loma*, *Spraguea*, and allies. *Nosema locustae* never branched with the ''true'' *Nosema* clade (Fig. 1 and 2), and instead was consistently and strongly associated with *A. scoticae* (with 100% bootstrap support in all analyses). It is doubtful that the position of *N. locustae* is unduly influenced by its GC-content, since the distance tree was constructed using Log-Det and closely resembled the ML tree in the important features, namely the relationship between *N. locustae* and *A. scoticae* and lack of affinity between *N. locustae* and the ''true'' *Nosema* clade (some of the poorly supported parts of the tree differed, but these are not directly relevant to the position of *N. locustae*).

While the *A. scoticae* and *N. locustae* clade shows no affinity to the ''true'' *Nosema* clade, it shows little affinity to any other microsporidian group either. However, a relationship with a group composed of *Brachiola algerae*, *Thelohania solenopsae*, *Nosema acridophagus*, *Janacekia debaisieuxi*, *Bacillidium* sp., and the bryozoan parasites *Trichonosema pectinatellae*, *Pseudonosema cristatellae*, *Schroedera plumatellae* and *Bryonosema plumatellae* was observed in distance and ML trees, but this was not strongly supported (Fig. 1 and 2). The two last species, whose descriptions were published almost simultaneously, are probably members of the same genus since they were assigned to microsporidia taken from the same host, their associated SSU sequences are highly similar to one another and to *Bryonosema tuftyi*, and they share significant morphological features (Canning et al. 2002; Morris and Adams 2002). An association between *A. scoticae* and the same group was previously shown by Canning et al. (2002). Interestingly, two species of this group were until very recently, classified within *Nosema*. *Brachiola algerae* was transferred to *Brachiola* on the basis of its wide host range and ability to develop at 37 °C in mammalian cell cultures and humans (Lowman et al. 2000). *Pseudonosema cristatellae* was originally described as *N. cristatellae* (Canning 1997) following a morphological diagnosis, but was later transferred to *Pseudonosema* because of its placement in the SSU rRNA tree (Canning et al. 2002). An SSU sequence (AF024658) deposited in Genbank under *Visvesvaria acridophagus* also belongs to this group. To our knowledge, it corresponds to *Nosema acridophagus*, a species that infects acridid grasshoppers (Streett and Henry 1993) and is related to *B. algerae* and *T. solenopsae* (Canning et al. 2002).

The possibility that this large and diverse assemblage represents a newly recognised division within the microsporidia suggests it would be of interest to examine all *Nosema* species that infect orthopterans. Sokolova and Lange (2002) pointed out several similarities among *N. locustae* and some of these species, especially *N. grylli* which shares with *N. locustae* the same number of polar filament coils, the deposition of electron-dense material on the membrane and a meront-sporont transition characterized by the presence of electron-dense bodies inside the nuclei. Sequencing of SSU rRNA may be a simple way to determine the taxonomic status of these species suspected to be members of the genus, at least until a reliable set of morphological characters is found.

Ultrastructure of the *N. locustae* exospore. Huger (1960) reported that *N. locustae* spores have a multi-layered exospore. However, Sokolova and Lange (2002) reported that it is electron dense with no differentiated structure. Since typical *Nosema* exospores are thick and simple, and Fries et al. (1999) showed that *A. scoticae* has a distinctive multi-layered exospore composed of four thin layers, it is important to determine the exact nature of the *N. locustae* exospore. We therefore examined the structure of the exospore of *N. locustae* using TEM (Fig. 3) and found the exospore construction of *N. locustae* to

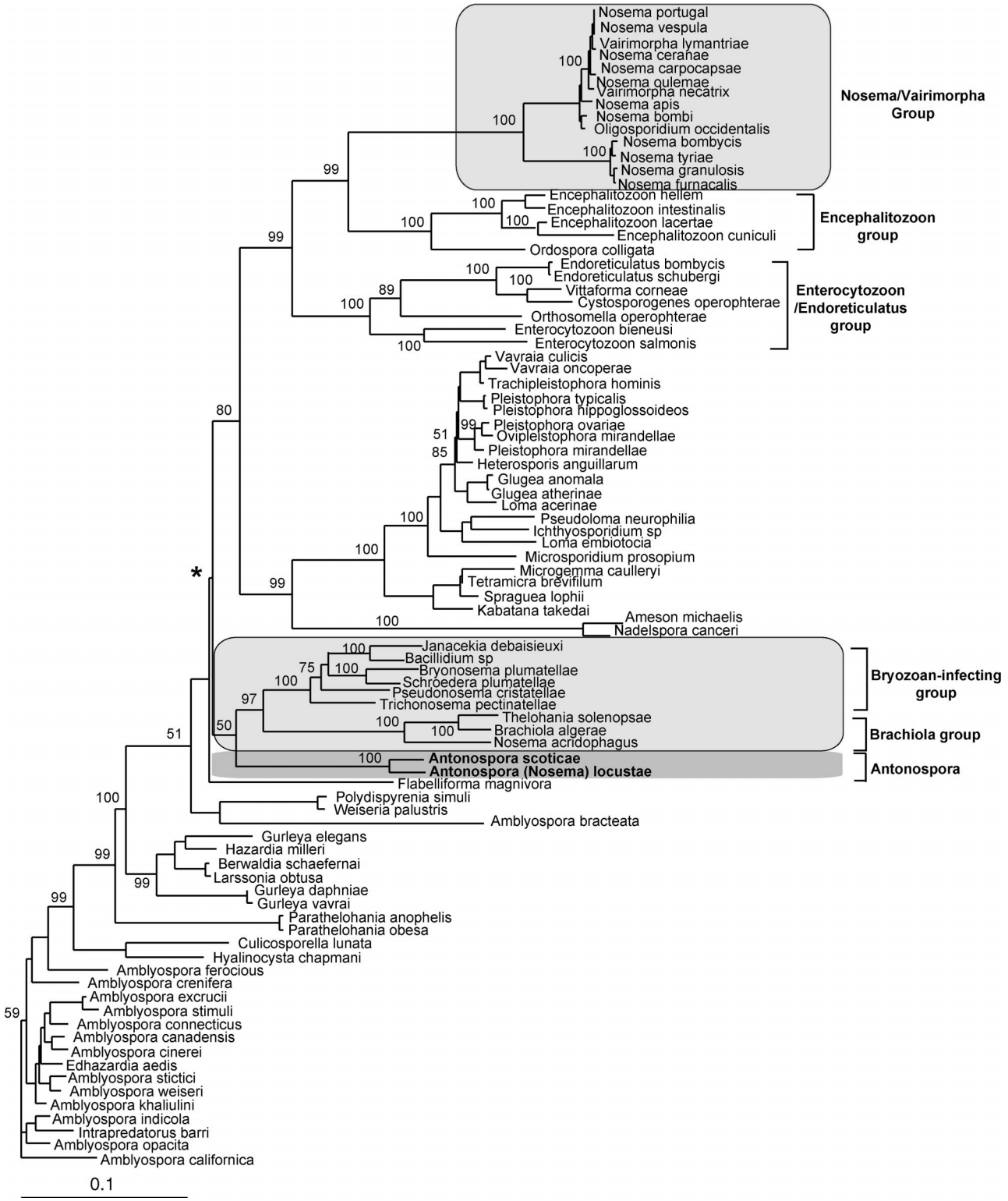

Fig **1.** Phylogeny of Microsporidia encompassing all currently recognized lineages. The tree is based on the complete SSU rRNA sequences using the Neighbor-Joining algorithm with LogDet distances. Numbers represent bootstrap support (1,000 replicates). Asterisks indicate nodes with less than 50% of bootstrap support.

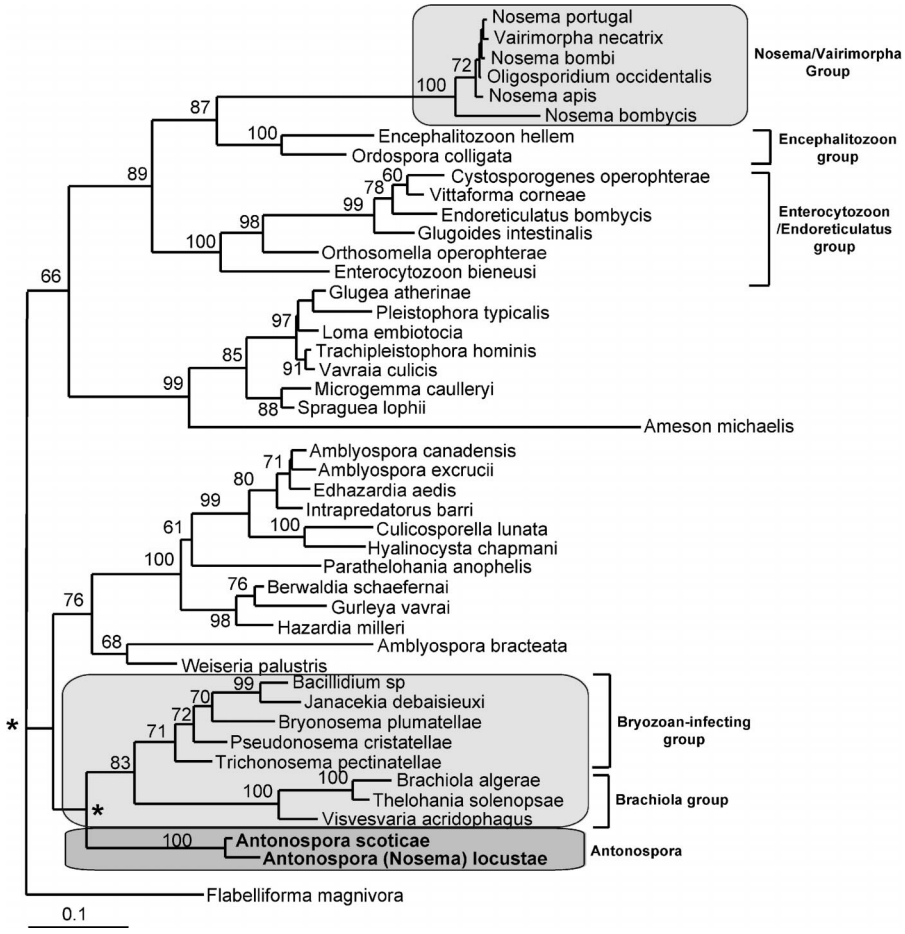

Fig **2.** Maximum Likelihood tree of microsporidian species based on the complete SSU rRNA sequences. Numbers represent bootstrap support (100 replicates). Asterisks indicate nodes with less than 50% of bootstrap support.

resemble that reported for *A. scoticae*, unlike the typical monolayered exospore characteristic of ''true'' *Nosema* species (Figure 3A–C). This observation is consistent with Huger's (1960) but contrasts with the Sokolova and Lange (2002) report, perhaps due to the lower magnification in the latter photomicrographs. In addition, the polar filament of *N. locustae* was found to be made up of many coils organised in several rows (Fig. 3A,C–E), as is found in *A. scoticae*, rather than a small number of coils in a single row, as is characteristic of most ''true'' *Nosema* species.

Antonospora scoticae, *N. locustae*, and the group containing *B. algerae* also share some common features that reinforce this broader, hypothetical relationship. Canning et al. (2002) observed a double-layered exospore in *B. tuftyi*, a parasite of bryozoans that grouped close to *N. locustae* in some trees (not shown). Moreover, all these species possess polar filaments with a high number of coils, with the exception of *B. algerae*, which has 8–12 coils (Lowman et al. 2000). Detailed ultrastructural studies in *Brachiola* have revealed a precocious deposition of electron-dense material on the plasmalemma during merogony (Koudela et al. 2001; Lowman et al. 2000). Conspicuous deposition of electron-dense material on the plasmalemma also occurs in *N. locustae*, in other *Nosema* species from orthopterans, and in some of the bryozoan-infecting species (Canning et al. 2002; Sokolova and Lange 2002). However, it is difficult to determine whether the depositions are precocious in these species from published studies because that event is often viewed as the indication of the switch between merogony and sporogony. Precocious deposition of electron-dense material on the plasma membrane surface of the meronts, if demonstrated, could be a characteristic shared by *A. scoticae*, *N. locustae* and the group of species related to *B. algerae*, and thus represents an indication of a relationship among them.

Taxonomic status of *N. locustae*. *Nosema locustae* shows no relationship to the type-species of *Nosema* (*N. bombycis*) in SSU phylogenies and also differs in lacking the single-layer exospore and small number and single row of polar filament coils typical of *Nosema* species. These observations, along with previous suggestions (e.g. Baker et al. 1994) undoubtedly support the exclusion of *N. locustae* from the genus *Nosema*. Instead, *N. locustae* is closely related to *A. scoticae* in phylogenetic analysis based on SSU rRNA and the genetic distance between their SSU rRNA genes falls within the range of interspecific divergence observed in several genera, including *Amblyospora*, *Encephalitozoon*, *Enterocytozoon*, and *Nosema* itself. This is not to say that *N. locustae* should be considered a member of *Antonospora* simply because they are closely related: a large number of species placed in distinct genera also share this level of similarity, and genetic distance is generally a poor criterion for microsporidian systematics because the relationship between that character and morphological differentiation shows extensive variation. Nevertheless, *N. locustae* also meets all the morphological characters included in the diagnosis of *Antonospora*: presence of a four-layered exospore (Huger 1960; this work), diplokaryotic merogony, disporoblastic development, direct contact with the host cytoplasm, and ovocylindrical, binucleate spores (Sokolova and Lange 2002). Unfor-

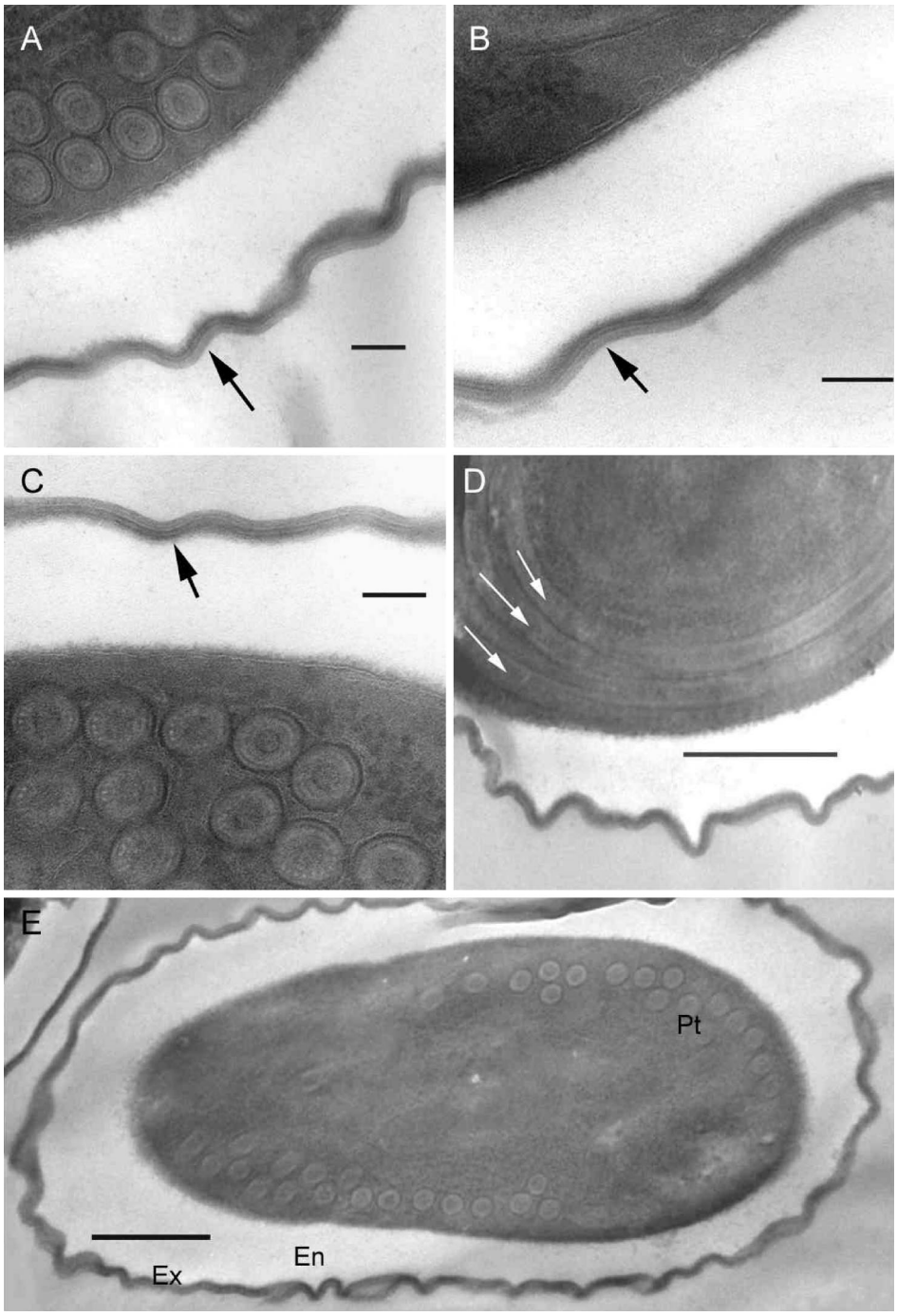

Fig **3.** Transmission electron micrographs of the mature spore of *Nosema locustae*. Images **A, B** and **C** all show the detail of the spore wall displaying the distinctive multi-layers in the exospore (indicated by arrows) which are common to both *N. locustae* and *Antonospora scoticae* (scale bar = 100 nm). *Antonospora* is described as having a four-layer exospore (Fries et al. 1999). The actual number of layers is somewhat subjective, but the cross-sectional stratification is identical in appearance between *N. locustae* and *A. scoticae*. Image **D** shows a latitudinal section of a *N. locustae* spore at a section where the spore has three laterally apposed polar tube coils (indicated by arrows) (scale bar = 500 nm). Image E shows a whole spore with labeling of structures discussed above. Ex, exospore; En, endospore; Pt, polar tubes (scale bar = 500 nm).

tunately, characters from pre-spore stages in *A. scoticae* are not available for comparison (Fries et al. 1999).

In summary, the morphological similarities and the close relatedness represent sufficient evidence to consider *A. scoticae* and *N. locustae* co-generic. An independent study based on SSU rRNA data has also concluded *N. locustae* and some closely related species are not related to ''true'' *Nosema*, and proposes a new genus for these taxa (Sokolova, pers. commun.). Since this is a very poorly studied but potentially highly diverse group, we favour using the existing genus at this time. Molecular data are only known from a handful of species in this group, and the differences that may be evoked to create a new genus could become irrelevant when other species are analysed. Accordingly, we propose to transfer *N. locustae* into the genus *Antonospora*, with the new designation *Antonospora locustae* (Canning 1953) Slamovits, Williams, and Keeling, 2003.

Note added in proof: During the proof stage of this manuscript, Sokolova et al. (2003, *J. Invert. Pathol.*, **84**:159–172) independently showed that *N. locustae* and other species are related to *Antonospora.* They have proposed a new genus for these species, *Paranosema.* It is presently not clear whether the traits that define this new genus (spore shape, host range, ge-

netic distance) distinguish it from *Antonospora*. Additional molecular and ultrastructural studies on related species are needed to settle this question.

ACKNOWLEDGMENTS

We thank M&R Durango Inc., Bayfield, CO for purified spores, J. Law and N. Fast for genome sequence survey data, and two anonymous colleagues for useful suggestions. This work was supported by a New Investigator Award in Molecular Pathogenic Mycology from the Burroughs-Wellcome Fund to PJK. PJK is a Scholar of the Canadian Institute for Advanced Research and a New Investigator of the Canadian Institutes for Health Research and Michael Smith Foundation for Health Research.

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Received 08/31/03, 11/05/03, 11/08/03; accepted 11/10/03

APPENDIX

Table **1**. Supplementary Data.

	Taxon	Accession
1	*Amblyospora bracteata*	U68473
2	*Amblyospora californica*	U68473
3	*Amblyospora canadensis*	AY090056
4	*Amblyospora cinerei*	AY090060
5	*Amblyospora connecticus*	AF025685
6	*Amblyospora crenifera*	AY090061
7	*Amblyospora excrucii*	AY090044
8	*Amblyospora ferocious*	AY090062
9	*Amblyospora indicola*	AY090051
10	*Amblyospora khaliulini*	AY090046
11	*Amblyospora opacita*	AY090052
12	*Amblyospora stictici*	AY090049
13	*Amblyospora stimuli*	AF027685
14	*Amblyospora weiseri*	AY090048
15	*Ameson michaelis*	L15741
16	*Antonospora scoticae*	AF024655
17	*Bacillidium* sp	AF104087
18	*Berwaldia schaefernai*	AY090042
19	*Brachiola algerae*	AF069063
20	*Bryonosema plumatellae*	AF484691
21	*Culicosporella lunata*	AF027683
22	*Cystosporogenes operophterae*	AJ302320
23	*Edhazardia aedis*	AF027684
24	*Encephalitozoon hellem*	AF177920
25	*Encephalitozoon intestinalis*	U09929
26	*Encephalitozoon lacertae*	AF067144
27	*Encephalitozoon cuniculi*	Z19563
28	*Endoreticulatus bombycis*	AY009115
29	*Endoreticulatus schubergi*	L39109
30	*Enterocytozoon bieneusi*	AF023245
31	*Enterocytozoon salmonis*	AF186003
32	*Flabelliforma magnivora*	AJ302318
33	*Glugea anomala*	AF044391
34	*Glugea atherinae*	U15987
35	*Gurleya daphniae*	AF439320
36	*Gurleya elegans*	AY090041
37	*Gurleya vavrai*	AF394526
38	*Hazardia milleri*	AY090067
39	*Heterosporis anguillarum*	AF387331
40	*Hyalinocysta chapmani*	AF483838
41	*Ichthyosporidium* sp	L39110
42	*Intrapredatorus barri*	AY013359
43	*Janacekia debaisieuxi*	AJ252950
44	*Kabatana takedai*	AF356222
45	*Larssonia obtusa*	AF394527
46	*Loma acerinae*	AF356224
47	*Loma embotocia*	AF320310
48	*Microgemma caulleryi*	AY033054
49	*Microsporidium prosopium*	AF151529
50	*Nadelspora cancerii*	AF305706
51	*Nosema apis*	U26534
52	*Nosema bombi*	AY008373
53	*Nosema bombycis*	AF240347
54	*Nosema carpocapsae*	AF426104
55	*Nosema ceranae*	U26533
56	*Nosema furnacalis*	U26532
57	*Nosema granulosis*	AJ011833
58	*Nosema oulemae*	U27359
59	*Nosema portugal*	AF033316
60	*Nosema tyriae*	AJ012606
61	*Nosema vespula*	U11047
62	*Oligosporidium occidentalis*	AF495379
63	*Ordospora colligata*	AF394529
64	*Orthosomella operophterae*	AJ302317
65	*Ovipleistophora mirandellae*	AF356223
66	*Parathelohania anophelis*	AF027682
67	*Parathelohania obesa*	AY090065
68	*Pleistophora hippoglossoideos*	AJ252953
69	Pleistophora mirandellae	AJ252954
70	*Pleistophora ovariae*	AJ252955
71	*Pleistophora typicalis*	AJ252956
72	*Polydispyrenia simuli*	AJ252960
73	*Pseudoloma neurophilia*	AF322654
74	*Pseudonosema cristatellae*	AF484694
75	*Schroedera plumatellae*	AY135024
76	*Spraguea lophii*	AF104086
77	*Tetramicra brevifilum*	AF364303
78	*Thelohania solenopsae*	AF134205
79	*Trachipleistophora hominis*	AJ002605
80	*Trichonosema pectinatellae*	AF484695
81	*Vairimorpha lymantriae*	AF033315
82	*Vairimopha necatrix*	Y00266
83	*Vavraia culicis*	AJ252961
84	*Vavraia oncoperae*	X74112
85	*Nosema ''Visvesvaria'' acridophagus*	AF024658
86	*Vittaforma corneae*	U11046
87	*Weiseria palustris*	AF132544