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Molecular Phylogeny and Evolution of Mosquito Parasitic Microsporidia (Microsporidia: Amblyosporidae)[1]

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ABSTRACT. *Amblyospora* species and other aquatic Microsporidia were isolated from mosquitoes, black flies, and copepods and the small subunit ribosomal RNA gene was sequenced. Comparative phylogenetic analysis showed a correspondence between the mosquito host genera and their *Amblyospora* parasite species. There is a clade of *Amblyospora* species that infect the *Culex* host group and a clade of *Amblyospora* that infect the *Aedes*/*Ochlerotatus* group of mosquitoes. *Parathelohania* species, which infect *Anopheles* mosquitoes, may be the sister group to the *Amblyospora* in the same way that the *Anopheles* mosquitoes are thought to be the sister group to the *Culex* and *Aedes* mosquitoes. In addition, by sequence analysis of small subunit rDNA from spores, we identified the alternate copepod host for four species of *Amblyospora*. *Amblyospora* species are specific for their primary (mosquito) host and each of these mosquito species serves as host for only one *Amblyospora* species. On the other hand, a single species of copepod can serve as an intermediate host to several *Amblyospora* species and some *Amblyospora* species may be found in more than one copepod host. *Intrapredatorus barri*, a species within a monotypic genus with *Amblyospora*-like characteristics, falls well within the *Amblyospora* clade. The genera *Edhazardia* and *Culicospora*, which do not have functional meiospores and do not require an intermediate host, but which do have a lanceolate spore type which is ultrastructurally very similar to the *Amblyospora* spore type found in the copepod, cluster among the *Amblyospora* species. In the future, the genus *Amblyospora* may be redefined to include species without obligate intermediate hosts. *Hazardia*, *Berwaldia*, *Larssonia*, *Trichotuzetia*, and *Gurleya* are members of a sister group to the *Amblyospora* clades infecting mosquitoes, and may be representatives of a large group of aquatic parasites.

Key Words. *Aedes*, *Amblyospora*, copepod, *Culex*, insect pathology, *Ochlerotatus*, parasites.

MICROSPORIDIA belonging to the genus *Amblyospora* are a large and diverse group of obligate parasites of mosquitoes, and possess the most complex life cycles known among the phylum (Becnel and Andreadis 1999). This life cycle includes the production of three morphologically and functionally distinct spore types, vertical (transovarial) and horizontal transmission, and utilization of copepods as intermediate hosts. Two mosquito hosts and one copepod host are required to complete the entire *Amblyospora* life cycle. Over 90 species and/or isolates have been described worldwide from 79 different species of mosquitoes in nine genera (*Aedeomyia*, *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, *Ochlerotatus*, *Psorophora*; see Andreadis 1994 for partial host list). At least five additional *Amblyospora* species have been described from amphipods, blackflies, and caddisflies (Friedrich, Kepka, and Ingolic 1992; Hazard and Oldacre 1975;), but nothing is known of their life cycles.

Because of their early phylogenetic divergence, it has been suggested (Baker et al. 1997) that the complex life cycles exhibited by *Amblyospora* may be a primitive trait among the ''higher'' Microsporidia. This would imply that the simpler life cycles (i.e. one host with fewer sporulation sequences) observed in Microsporidia such as *Endoreticulatus*, *Nosema*, and *Vairimorpha* are the result of losses of various life cycle features and/or functions. Comparative small subunit ribosomal DNA (SSrDNA) data have further demonstrated (Baker et al. 1998) that *Amblyospora* and related mosquito-parasitic taxa (i.e. *Culicosporella*, *Edhazardia*, and *Parathelohania*) form a monophyletic group of mosquito parasites. An evolutionary correlation between parasite and host is supported by the high level of host specificity for their mosquito hosts exhibited among *Amblyospora* and closely related species, including *Culicospora magna*, *Culicosporella lunata*, *Edhazardia aedis*, and *Intrapredatorus barri* (Andreadis 1989; Becnel and Andreadis 1998; Sweeney, Doggett, and Piper 1990).

The identification of intermediate hosts for *Amblyospora* species has relied on reciprocal laboratory bioassays wherein various infection-free copepod and mosquito species are exposed to spores procured from potential alternate hosts (Andreadis 1989; Sweeney, Doggett, and Piper 1990). However, new molecular methods have recently been developed that can rapidly and reliably determine the identity and/or conspecificity of Microsporidia isolated from aquatic Crustacea and mosquitoes and thus reveal the identity of an intermediate host (Vossbrinck et al. 1998).

The objectives of this study were: (1) to examine the phylogenetic relationships among the *Amblyospora* species in relationship to their mosquito and copepod hosts, (2) to develop a better understanding of the phylogenetic relationships of the *Amblyospora* clade to closely related Microsporidia from other aquatic arthropod hosts and (3) to use SSrDNA sequence analysis to determine the intermediate copepod and definitive mosquito host relationships of various *Amblyospora* species.

MATERIALS AND METHODS

Field collections and host identification. All of the microsporidian isolates sequenced in this investigation were obtained from naturally infected hosts that were field-collected from a variety of aquatic habitats (Table 1). Mosquito larvae were identified according to Darsie and Ward (1981); copepods were identified according to Dussart and Defaye (1995) and Einsle (1996); black flies were identified according to Knoz (1965); and *Daphnia* were identified according to Floessner (2000). Specimens were initially screened for ''patent'' infection (white opaque coloration) in black photographic pans. This screening was followed by microscopic examination of the specimens for mature spores. Spores were isolated for sequencing from fourth

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[1] The small subunit rDNA sequences of the following Microsporidia have been deposited in the GenBank database: *Amblyospora canadensis* (AY090056), *Amblyospora cinerei* (AY090057, AY090058, AY090059, AY090060), *Amblyospora crenifera* (AY090061), *Amblyospora excrucii* (AY090043, AY090044), *Amblyospora ferocious* (AY090062), *Amblyospora indicola* (AY090051), *Amblyospora khaliulini* (AY090045, AY090046, AY090047), *Amblyospora opacita* (AY090052), *Amblyospora stictici* (AY090049), *Amblyospora weiseri* (AY090048), *Amblyospora* sp.1 (AY090053), *Amblyospora* sp.2 (AY090055), *Culicospora magna* (AY090054), *Hazardia* sp. (AF090066), *Parathelohania obesa* (AF090065).

Table **1**. Host, location and accession number of Microsporida sequenced for phylogenetic analyses.

Organism	Host	Geographic locale	Accession #
Amblyospora bracteata	*Odagamia ornata*	Czech Republic	AY090068
Amblyospora californica	*Culex tarsalis*	California, USA	U68473
Amblyospora canadensis	*Ochlerotatus canadensis*	Connecticut, USA	AY090056
Amblyospora cinerei	*Aedes cinereus*	Connecticut, USA	AY090057
Amblyospora cinerei	*Acanthocyclops vernalis*	Connecticut, USA	AY090058
Amblyospora cinerei	*Acanthocyclops vernalis*	Connecticut, USA	AY090059
Amblyospora cinerei	*Acanthocyclops venustoides*	Connecticut, USA	AY090060
Amblyospora connecticus	*Ochlerotatus cantator*	Connecticut, USA	AF025686
Amblyospora connecticus	*Acanthocyclops vernalis*	Connecticut, USA	AF025685
Amblyospora crenifera	*Ochlerotatus crinifer*	Argentina	AY090061
Amblyospora excrucii	*Acanthocyclops excrucians*	Connecticut, USA	AY090043
Amblyospora excrucii	*Acanthocyclops vernalis*	Connecticut, USA	AY090044
Amblyospora ferocious	*Psorophora ferox*	Argentina	AY090062
Amblyospora indicola	*Culex sitiens*	India	AY090051
Amblyospora khaliulini	*Ochlerotatus communis*	Connecticut, USA	AY090045
Amblyospora khaliulini	*Acanthocyclops vernalis*	Connecticut, USA	AY090046
Amblyospora khaliulini	*Acanthocyclops vernalis*	Connecticut, USA	AY090047
Amblyospora opacita	*Culex territans*	Connecticut, USA	AY090052
Amblyospora salinaria	*Culex salinarius*	Florida, USA	U68474
Amblyospora salinaria	*Culex salinarius*	Connecticut, USA	AY326270
Amblyospora stictici	*Ochlerotatus sticticus*	Connecticut, USA	AY090049
Amblyospora stimuli	*Ochlerotatus stimulans*	Connecticut, USA	AF027685
Amblyospora stimuli	*Diacyclops bicuspidatus*	Connecticut, USA	AY090050
Amblyospora weiseri	*Ochlerotatus cantans*	Czech Republic	AY090048
Amblyospora sp. 1	*Culex nigripalpus*	Florida, USA	AY090053
Amblyospora sp. 2	*Cyclops strenuus*	Czech Republic	AY090055
Amblyospora sp. 3	*Simulium* sp.	Palearctic	AJ252949
Berwaldia schaefernai	*Daphnia galeata*	Czech Republic	AY090042
Brachiola algerae	*Anopheles stephensi*	Illinois, USA	AF069063
Culicospora magna	*Culex restuans*	Connecticut	AY090054
Culicospora magna	*Culex restuans*	Connecticut	AY326269
Culicosporella lunata	*Culex pilosus*	Florida, USA	AF027683
Edhazardia aedis	*Aedes aegypti*	Thailand	AF027684
Endoreticulatus schubergi	*Lymantria dispar*	Portugal	L39109
Flabelliforma magnivora	*Daphnia magna*	Moscow, Russia	AJ302318
Gurleya daphniae	*Daphnia pulex*	Austria	AF439320
Marssoniella elegans	*Cyclops vicinus*	Czech Republic	AY090041
Gurleya vavrai	*Daphnia longispina*	Finland	AF394526
Hazardia milleri	*Culex quinquefasciatus*	Argentina	AF090067
Hazardia sp.	*Anopheles crucians*	Florida, USA	AF090066
Hyalinocysta chapmani	*Culiseta melanura*	Connecticut, USA	AF483837
Hyalinocysta chapmani	*Orthocyclops modestus*	Connecticut, USA	AF483838
Intrapredatorus barri	*Culex fuscanus*	Taiwan	AY013359
Janacekia debaisieuxi	*Odagoamia ornata*	Czech Republic	AY090070
Larssonia obtusa	*Daphnia pulex*	Sweden	AF394527
Nosema whitei	*Tribolium confusum*	Illinois, USA	AY305325
Parathelohania anophelis	*Anopheles quadrimaculatus*	Florida, USA	AF027682
Parathelohania obesa	*Anopheles crucians*	Florida, USA	AF090065
Polydispyrenia simulii	*Odagamia ornata*	Czech Republic	AY090069
Tritrichomonas foetus	*Homo sapiens*	cosmopolitan	M81842
Trichotuzetia guttata	*Cyclops vicinus*	Czech Republic	AY326268
Vairimorpha sp.	*Solenopsis richteri*	Florida, USA	AF031539
Vairimopha necatrix	*Pseudaletia unipunctata*	Illinois, USA	Y00266
Vavraia culicis	*Culex pipiens*	Czech Republic	AJ252961
Vavraia oncoperae	*Weiseana sp.*	New Zealand	X74112

instar mosquito larvae, last instar black fly larvae, adult female copepods, and adult female *Daphnia*.

Isolation of DNA. Methods of DNA isolation were similar to those previously published by Vossbrinck et al. (1998). Field-collected specimens were brought to the laboratory and examined for microsporidial spore infection. Single host specimens were homogenized briefly in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and filtered through 50-μm nylon mesh. The supernatant was then removed and the pellet was resuspended in 150 μl of TAE buffer and placed in a 0.5-ml micro-centrifuge tube. A 10-μl aliquot of spore suspension was removed and examined under phase-contrast microscopy (100–400×) to confirm the presence of viable spores, which appear highly refractive. One-hundred-fifty milligrams of glass beads were then added to the spore suspension and the tube was shaken in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 50 s and then immediately put at 95 °C for 3 min. A 10-μl aliquot of the solution was removed and inspected under phase-contrast microscopy for ruptured spores, which do not appear refractive.

DNA amplification, sequencing, and phylogenetic analysis. One to five microliters of the TAE/ruptured spore solution was removed and used in a standard PCR reaction (94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 90 s) using primers 18f and 1492r (see below). The PCR product, usually 1,250–1,400 nucleotides in length, was then purified on a Qiaquick PCR purification kit (Qiagen Company,Valencia, CA) and prepared for sequencing. Sequencing was done at the Keck Biotechnology Resource Laboratory at Yale University with the following microsporidian primers: 18f, 5′-CACCAGGTTGATTCTGCC-3′; SS350f, 5′-CCAAGGA(T/C)GGCAGCAGGCGCGAAA-3′; 350r, 5′-TTTCGCGCCTGCTGCC(G/A)TCCTTG-3′; SS530f, 5′-GTGCCAGC(C/A)GCCGCGG-3′; SS530r, 5′-CCGCGG(T/G)GCTGGCAC-3′; 1047r, 5′-AACGGCCATGCACCAC-3′; 1061f, 5′-GGTGGTGCATGGCCG-3′; and 1492r, 5′-GGTTACCTTGTTACGACTT-3′.

Sequences were aligned using the Clustal X program (Thompson et al. 1994) and the 3′-end of the molecule was flush trimmed to a final length of 1,510 characters including gaps (alignment available from corresponding author upon request). No other portions of the alignment were changed or eliminated. We selected *Tritrichomonas foetus* as the eukaryotic outgroup. It has been well established, based on both genotypic and phenotypic characters, that *T. foetus* is not a member of the microsporidian clade. Aligned sequences were analyzed by Maximum Parsimony and Neighbor Joining analyses using PAUP version 3.1b (Swofford 1993). Neighbor Joining analysis was done using 100 bootstrap replicates. Maximum Parsimony analysis was done using the heuristic search method. All characters were unordered and had equal weight, no topological constraints were enforced and 838 characters were parsimony informative.

RESULTS AND DISCUSSION

Genbank accession numbers for the SSrDNA sequences obtained in this study and for previously published sequences used in the analyses are shown in Table 1. Identical parasite sequences from mosquito and copepod hosts have been given separate Genbank listings. While there is not total agreement between the two phylogenetic analyses (Fig. 1A, 1B), they yield new insight into a number of relationships among these genera and species.

A remarkable degree of correlation was observed between host and parasite at the generic level for the *Amblyospora* species infecting mosquitoes, as well as for *Culicospora*, *Edhazardia*, and *Intrapredatorus*. Species that parasitize *Aedes* and *Ochlerotatus* (formerly a subgenus of *Aedes*) mosquitoes (Reinert 2000) may form a distinct group, as do those species that parasitize *Culex* mosquitoes. In the Neighbor Joining analysis (Fig. 1A) the *Aedes*/*Ochlerotatus* parasites form a monophyletic group while in the Maximum Parsimony analysis (Fig. 1B) parasites of the *Aedes*/*Ochlerotatus* are a paraphyletic grouping. It is unlikely, although possible, that the *Aedes*/*Ochlerotatus* hosts also represent a paraphyletic grouping. Additional sequence data from other molecules will have to be obtained to resolve the differences seen between the two analyses used in this study.

A discrepancy exists concerning the relative positions of *Amblyospora ferocious* and the *Hyalinocysta*/*Culicosporella* clade. Neighbor joining analysis (Fig. 1A) indicates *Amblyospora ferocious*, a parasite of the mosquito *Psorophora ferox*, to be the sister group of the *Culex* and *Aedes*/*Ochlerotatus* parasites with the *Hyalinocysta chapmani*/*Culicosporella lunata* group as the next most closely related taxon. Maximum Parsimony analysis (Fig. 1B) reverses the relative position of these two taxa, indicating the *Hyalinocysta*/*Culicosporella* taxon to be the sister group to the *Culex* and *Aedes*/*Ochlerotatus* parasites, and *Amblyospora ferocious* to be the next most closely related taxon. Again more data will be needed to resolve this discrepancy. The final and potentially most significant unresolved discrepancy, indicated by the trichotomy in Fig. 1A, is whether the *Parathelohania* clade from *Anopheles* mosquitoes is the sister group to the *Amblyospora* parasites of mosquitoes or whether the ''Aquatic Outgroup'' is the sister group to the *Amblyospora* parasites of mosquitoes. We hypothesize that microsporidian parasites of *Anopheles* and the Culicinae evolved from parasites of crustaceans and that parasitism of mosquitoes by *Parathelohania*, *Amblyospora*, and *Hyalinocysta* arose from a single event. While the ''Aquatic Outgroup'' includes Microsporidia of a variety of shapes and sizes, the morphology and life cycles of *Parathelohania* and *Amblyospora* are nearly identical except for the shape of the meiospore found in patently infected mosquito larvae (Avery and Undeen 1990; Hazard and Weiser 1968). Both *Parathelohania* and *Amblyospora* have copepod intermediate hosts in which uninucleate spores are produced which infect mosquito larvae orally. In both genera, gametogenesis and plasmogamy occur in the larval mosquito host and binucleate spores responsible for transovarial transmission are produced in adult females. *Hyalinocysta chapmani* has a life cycle that similarly includes meiospore production in mosquito larvae and obligatory development in a copepod host. However, *H. chapmani* lacks a developmental sequence leading to transovarial transmission in adult female mosquitoes. Transovarial transmission and an intermediate host are thought to represent the ancestral state (Andreadis and Vossbrinck 2002). Alternatively, the parasitism of mosquitoes by *Parathelohania* could represent a separate evolutionary event. The parasitism of mosquitoes by the two *Hazardia* species probably represents such a separate event.

At present we have not determined how closely the mosquito and parasite phylogenies parallel each other; however, the parasite phylogeny does not conflict with the conventional classification of the mosquito hosts (Harbach and Kitching 1998; Knight and Stone 1977). The *Anopheles* mosquitoes (Subfamily Anophelinae) are thought to be the sister group to the culicine mosquitoes (Subfamily Culicinae) as the *Paratheohania* appear to be the sister group to the *Amblyospora* Microsporidia.

The position of *Culicosporella lunata*, a parasite of *Culex pilosus*, does not support a close correlation between mosquito and parasite phylogenies. However, *Culex pilosus* is a member of the subgenus *Melanoconion*. With the exception of *Culex fuscanus* (host of *I. barri*), all the other *Culex* hosts of *Amblyospora* spp. in this study belong to the subgenus *Culex*. Also, *Amblyospora crenifera*, a parasite of *Ochlerotatus crinifer*, does not group within the *Aedes*/*Ochlerotatus* group of parasites. Further analysis of additional sequence data will be needed to resolve these discrepancies.

Our phylogenetic analyses demonstrate clearly that the monotypic genera *Culicospora*, *Edhazardia*, and *Intrapredatorus* fall within the *Amblyospora* clade, making *Amblyospora* a paraphyletic taxon. Both *Culicospora magna* and *E. aedis* have morphologies and life cycles similar to those of the *Amblyospora*, but lack functional meiospores and do not require an intermediate copepod host. The absence of an intermediate host in the life cycles of these two Microsporidia most likely reflects an ecological adaptation to the habitat of the larval host (Becnel et al. 1989) and is not a reflection of evolutionary relatedness. The hosts for both of these Microsporidia, *Culex restuans* (*Culicospora magna*) and *Aedes aegypti* (*E. aedis*), develop rapidly under ephemeral conditions and typically exhibit overlapping generations. In the absence of a readily available intermediate

host and with a continuous supply of larval mosquito hosts, these parasites have probably adapted by eliminating the intermediate host from the life cycle. Our findings suggest that these Microsporidia species are adjusting their life cycle to accommodate host ecological conditions. Andreadis (2002) noted a similar situation with *H. chapmani*, where ecological conditions appear to have favored the production of meiospores in female mosquitoes while eliminating transovarial transmission for greater success in transmisson.

The genus *Intrapredatorus* was recently erected by Chen, Kuo, and Wu (1998) to describe a microsporidium from *Culex fuscanus* that is very similar to *Amblyospora trinus* from *Culex halifaxi* (Becnel and Sweeney 1990). Both species have two concurrent sporulation sequences involving meiosis and nuclear dissociation to produce two uninucleate spore types in a predaceous larval host. Nilson and Chen (2001) compared SSrDNA sequences among *I. barri* and other species belonging to the Amblyosporidae and justified the establishment of *Intrapredatorus* as a genus based on the ''relatively large'' number (129 to 262) of nucleotide differences between *I. barri* and other species of *Amblyospora*. They identified four groups within the Amblyosporidae: (1) *P. anophelis*, (2) *Culicosporella lunata*, (3) *A. californica* and *A. salinaria* and (4) *A. connecticus*, *A. stimuli*, *E. aedis*, and *I. barri*. Nilson and Chen's (2001) argument regarding the clustering of the clade is ambiguous. Their phylogeny showed *I. barri* to cluster well within the *Amblyospora*. However, based on their recommendation of four groups, the only true *Amblyospora* species would be *A. californica* (the type species for *Amblyospora*) and *A. salinaria*. The remaining species of *Amblyospora* would have to be transferred to new genera. Our analysis of more species from additional hosts supports defining *Amblyospora* as a much broader group of mosquito parasites, which includes *I. barri* as well as *Culicospora magna* and *E. aedis*. If further sequence analyses of other genes support these findings based on SSrDNA, strong consideration should be given to reassigning these three monotypic genera to the genus *Amblyospora*.

The consensus tree (Fig. 1A) shows the *Culex* and *Aedes*/*Ochlerotatus* parasites to be separate groups, while Maximum Parsimony analysis shows the *Culex* parasite group to be a specialized subgroup of the *Aedes*/*Ochlerotatus* Microsporidia, making the *Aedes*/*Ochlerotatus* Microsporidia group paraphyletic. However, bootstrap analysis using the Maximum Parsimony heuristic search (100 replicates) does not support a paraphyletic relationship and we conclude that the *Amblyospora*, which infect *Culex* and *Aedes*/*Ochlerotatus*, are separate groups.

Identical SSrDNA sequences were obtained from *Amblyospora salinaria* from *Culex salinarius* and an undescribed *Amblyospora* species from *Culex nigripalpus*. *Culex nigripalpus* and *Culex salinarius* are closely related species that occur in the same aquatic habitat in Florida, USA, but are separated temporally: *Culex nigripalpus* is present in summer and fall, *Culex salinarius* is present in winter and spring. Whether these two microsporidian parasites are separate species, different populations of the same species or a single population present throughout the year remains to be determined and will require analysis of a more rapidly changing region of the microsporidial genome.

In two cases we were able to collect isolates of Microsporidia from the same mosquito species at widely separated locations (from Florida and Connecticut, USA) and in both instances the SSrDNA sequences were identical (see Table 1 for *Amblyospora salinaria* and *Culicospora magna*). This provides further evidence of the specificity of the Amblyosporidae for their mosquito hosts.

Intermediate copepod hosts were identified for four species of *Amblyospora* from mosquitoes: *Amblyospora excrucii* from *Acanthocyclops vernalis*; *Amblyospora khaliulini* from *A. vernalis*; *Amblyospora cinerei* from *A. vernalis* and *Acanthocyclops venustoides*; and *Amblyospora stimuli* from *Diacyclops bicuspidatus*. The sequence data confirmed previous laboratory transmission studies implicating the two copepods *A. vernalis* and *D. bicuspidatus* as intermediate hosts for *A. cinerei* and *A. stimuli*, respectively (Andreadis 1994). With the addition of the above four *Amblyospora* species, a copepod intermediate host has now been identified for twelve *Amblyospora* species. This study shows that the copepod *A. vernalis* serves as an intermediate host for several different *Amblyospora* species in nature, and that some *Amblyospora* species can use more than one species of copepod as the intermediate host. These findings are consistent with experimental laboratory bioassays (Andreadis 1989; Becnel and Andreadis 1998; Sweeney, Doggett and Piper 1990) and provide further evidence that *Amblyospora* species do not exhibit the same high level of specificity for the intermediate host as they do for the definitive mosquito host. Also presented is an undescribed *Amblyospora* species (*Amblyospora* sp. 2) isolated from the copepod *Cyclops strenuus*. This was collected from a pool in the Czech Republic where *Amblyospora weiseri* was previously isolated from the mosquito *O. cantans*. *Amblyospora weiseri* and *Amblyospora* sp. 2 were initially thought to be isolates of the same species, but clearly represent separate species whose alternate/definitive host, respectively, remains to be discovered. The finding of multiple *Amblyospora* species in the same habitat is common. In Connecticut, USA, for example, *A. excrucii* and *A. stimuli* have been isolated from mosquitoes (*O. excrucians* and *O. stimulans*, respectively) and copepods (*A. vernalis* and *D. bicuspidatus*, respectively) inhabiting the same pool at the same time (Andreadis 1994). Concurrent epizootics of *A. canadensis* and *A. cinerei* have also been reported (Andreadis 1993) to occur in their respective host mosquitoes in the same pools. These findings further reaffirm the high levels of host specificity exhibited by the *Amblyospora* for their definitive mosquito hosts (Andreadis 1989; Becnel and Andreadis 1998; Sweeney, Doggett, and Piper 1990).

Hyalinocysta chapmani and *Culicosporella lunata* are sister taxa to the *Amblyospora*. The genus *Hyalinocysta* is distinguished from the *Amblyospora* by the diplokaryotic meronts, which are formed by karyokinesis rather than by plasmogamy, and by the absence of a developmental sequence leading to the production of binucleate spores and transovarial transmission, a universal trait in *Amblyospora* (Andreadis and Vossbrinck 2002). *Culicosporella* is distinguished from *Amblyospora* by its production of binucleate-lanceolate spores rather than uninucleate-lanceolate spores for the oral infection of the mosquito host (Becnel and Fukuda 1991). While these differences may not be indicative of taxonomic divisions, the phylogenetic placement of *Hyalinocysta* and *Culicosporella* outside of all ''true'' *Amblyospora* (''true'' defined here as *Amblyospora* species from *Aedes*/*Ochlerotatus* and *Culex* hosts) justifies their taxonomic designations (Andreadis and Vossbrinck 2002).

Members of the Culicidae can be infected by Microsporidia unrelated to the *Amblyospora*. For example, *Hazardia* sp. and *Hazardia milleri*, members of the ''Aquatic outgroup'', infect *Anopheles crucians* and *Culex quinquefasciatus*, respectively (Table 1). *Hazardia milleri* can be transmitted from mosquito to mosquito directly without the need for an intermediate host. Other parasites of mosquitoes analyzed in this study are *Vavraia culicis* (a close relative of *Vavraia oncoperae* from the Porina moth, *Weiseana* sp.) and *Brachiola algerae* from *Anopheles stephensi*. We conclude that Microsporidia have invaded members of the Culicidae several times independently.

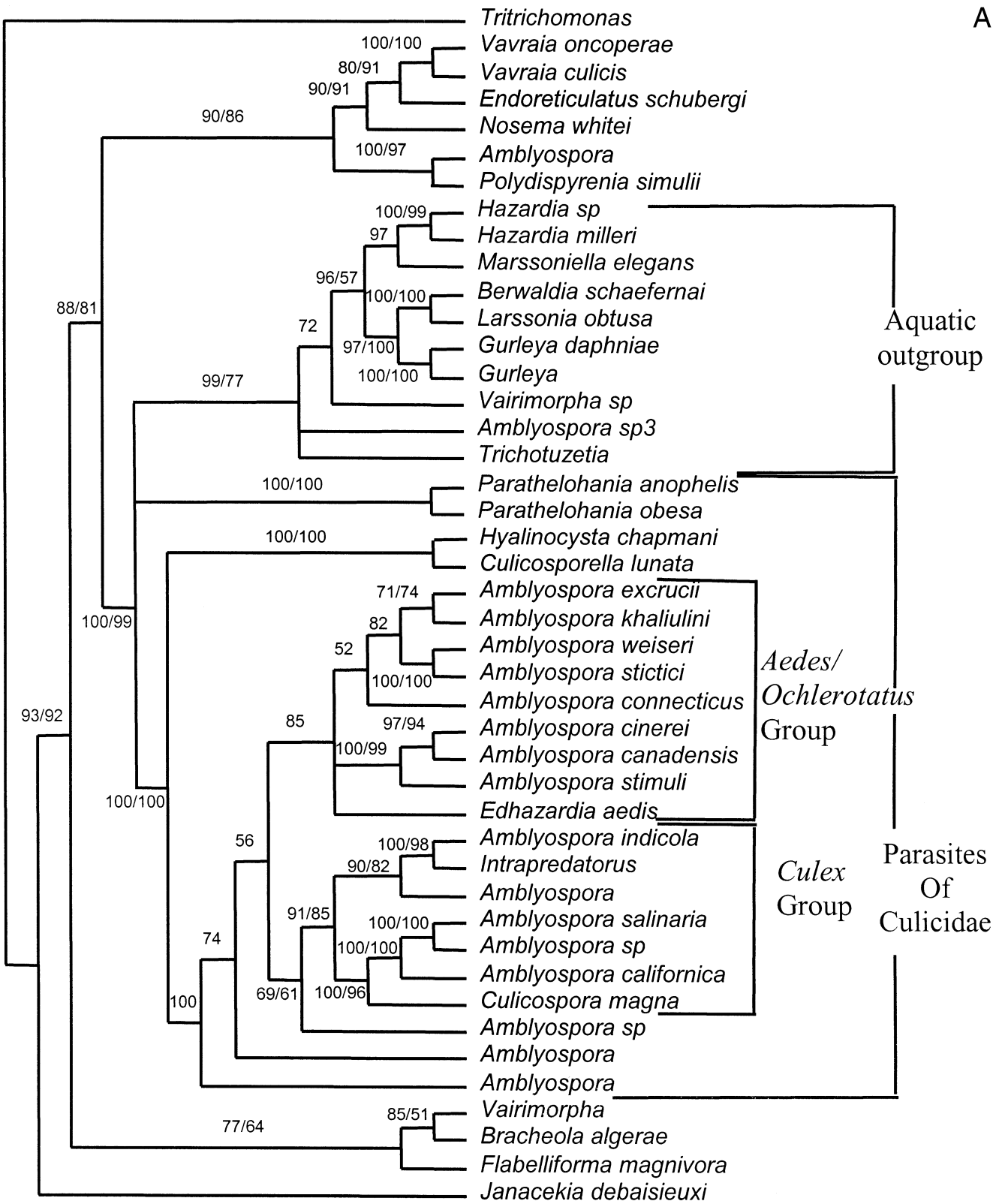

Fig. **1.** Phylogenetic analysis of 43 microsporidian taxa. *Tritrichomonas foetus* is included as an outgroup. **A)** Neighbor Joining consensus tree using 100 bootstrap replicates. The numbers represent Neighbor Joining bootstrap values; a second number, where applicable, indicates the maximum parsimony heuristic bootstrap value (100 replicates). **B)** Maximum Parsimony Analysis showing the single shortest tree of 5,762 steps. Bar indicates 100 nucleotide changes.

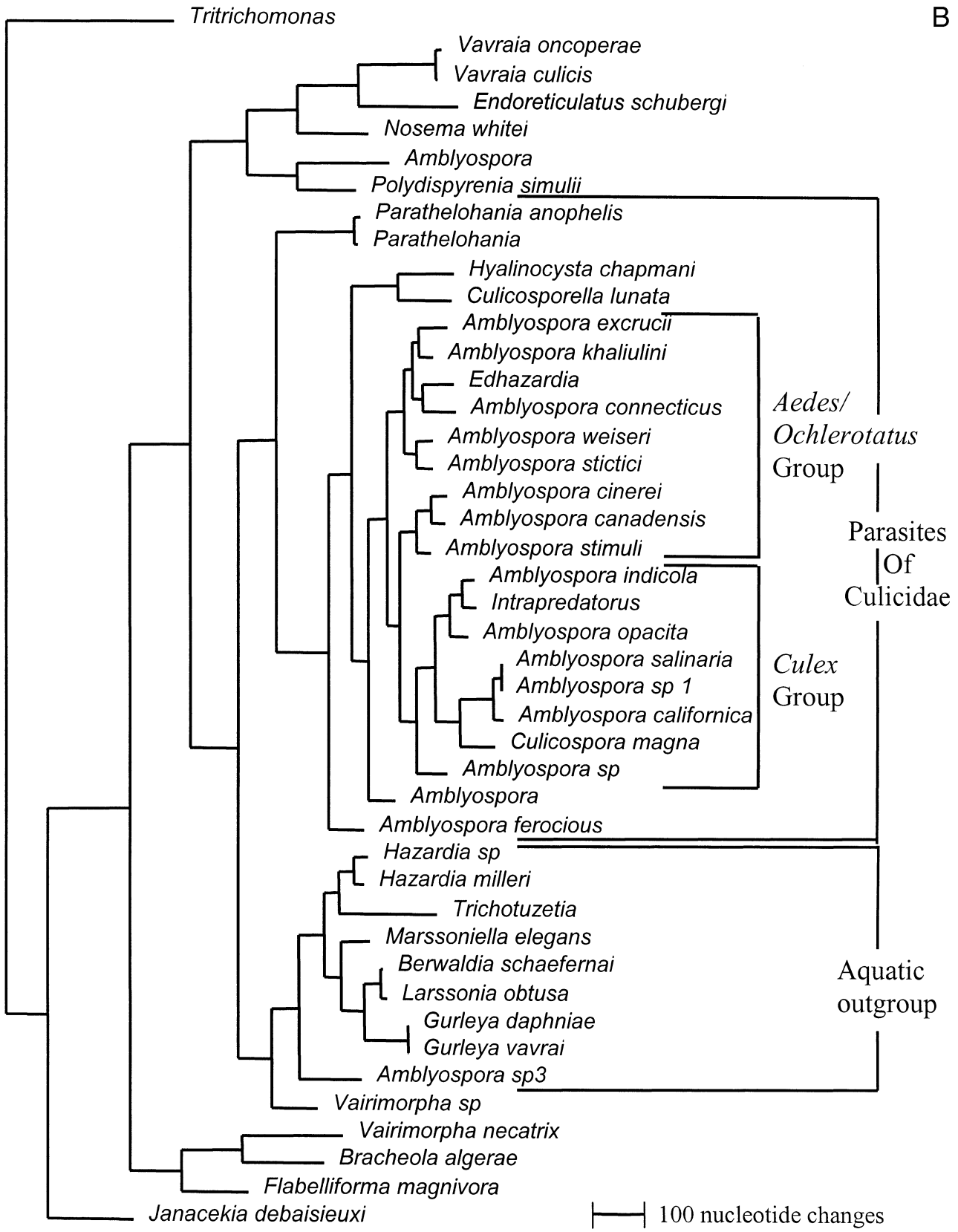

Fig. **1.** Continued.

There are two species of *Amblyospora* (*A. bracteata* and *Amblyospora* sp 3) from blackflies that are unrelated to the ''true'' *Amblyospora* from mosquitoes (Fig. 1). *Amblyospora* sp 3. was included in a recent report by Refardt et al. (2002) who demonstrated a polyphyletic origin for the Microsporidia that infect *Daphnia*. Refardt et al. (2002) use Maximum Likelihood analysis to place *Parathelohania* as the sister group to the *Amblyospora* and our ''Aquatic Outgroup'' whereas we show a trichotomy and believe that *Parathelohania* may be the sister group to the *Amblyospora*/*Hyalinocysta* clade.

In conclusion, these phylogenetic analyses clearly demonstrate that the host is an important indicator of relatedness among members of the genus *Amblyospora*. We define the ''true'' *Amblyospora* species as those parasites of mosquitoes that fall phylogenetically within the *Amblyospora* clade to the exclusion of designated *Amblyospora* species found in other arthropod hosts, such as the Simuliidae. *Edhazardia aedis*, *Culicospora magna*, and *I. barri* species, like many of the aquatic Microsporidia, have been described based on characteristics that are likely to be evolutionarily adaptive rather than indicative of a common origin. The loss of intermediate hosts within this clade lends more credibility to the idea that the ancestral state for Microsporidia may be that of the complex life cycle, and that portions of the life cycle can be lost relatively rapidly over evolutionary time (Baker et al. 1997). The *Amblyospora* are very specific for their definitive mosquito host but can infect multiple copepod intermediate hosts. We have shown that *Amblyospora* infections of mosquito species from disparate locations are the same parasite species. This study also defines more clearly a group of parasites of crustaceans and insects (*Hazardia*, *Berwaldia*, *Larssonia*, *Gurleya*, and *Trichotuzetia*) that we identify as our ''Aquatic Outgroup'', a likely sister group to the ''true'' *Amblyospora*.

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ERRATA

The title of the article, ''Observations on the Life Stages of *Sphaerothecum destruens* n. g., n. sp., a Mesomycetozoean Fish Pathogen Formally Referred to as the Rosette Agent''. 2003. *J. Eukaryot. Microbiol.*, **50**(6):430–438 by Kristen D. Arkush, Leonel Mendoza, Mark A. Adkison, and Ronald P. Hedrick, should be changed to read as follows:

''Observations on the Life Stages of *Sphaerothecum destruens* n. g., n. sp., a Mesomycetozoean Fish Pathogen Formerly Referred to as the Rosette Agent''.

The article by Charles R. Vossbrinck, Theodore G. Andreadis, Jiri Vavra, and James J. Becnel, 2004, Molecular Phylogeny and Evolution of Mosquito Parasitic Microsporidia (Microsporidia: Amblyosporidae), *J. Eukaryotic Microbiol.*, **51**(1):88–95, was printed with the omission of six species in Fig 1A and 1B. The correct versions of Fig. 1A and 1B are printed on the next two pages.

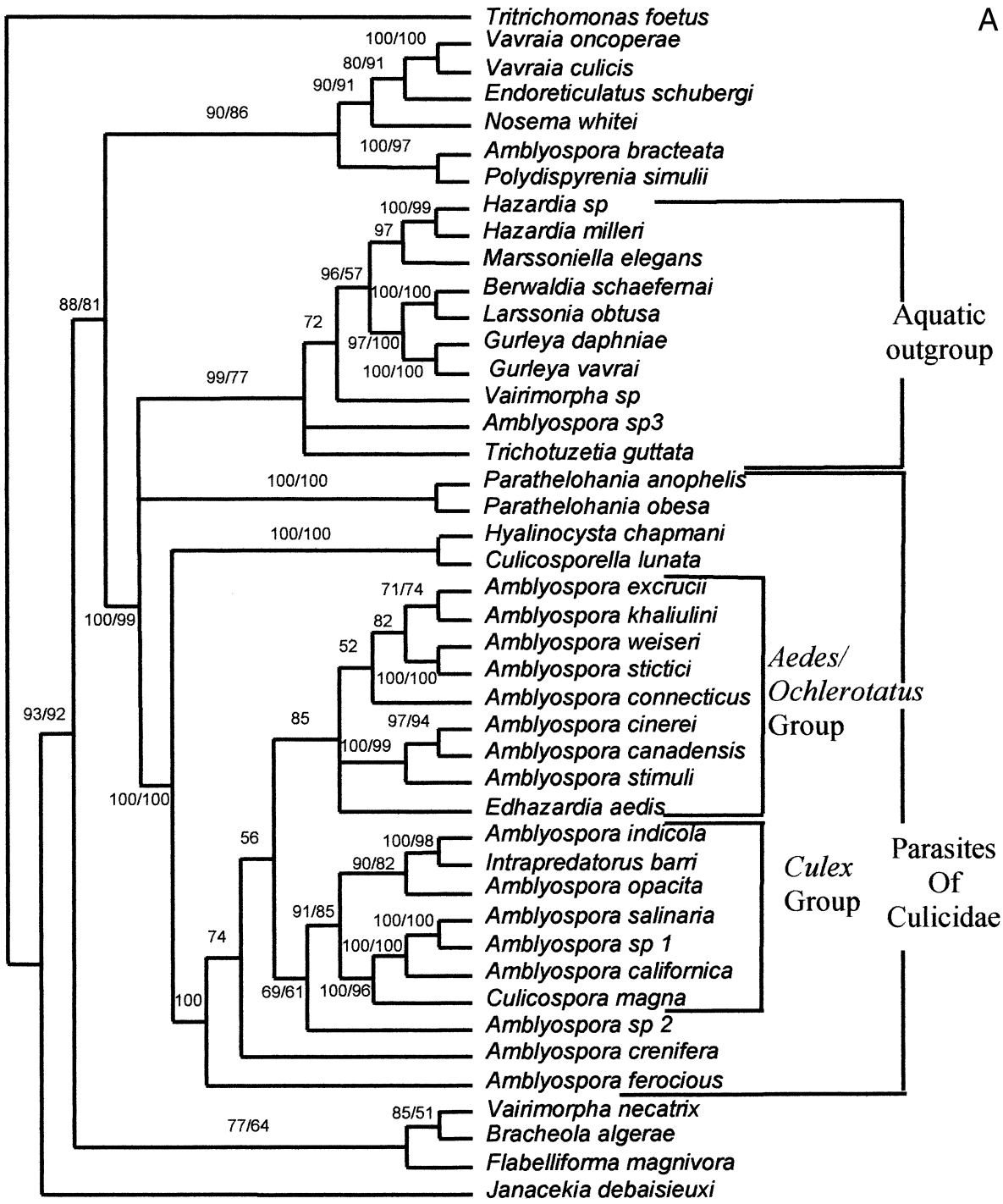

Fig. **1.** Phylogenetic analysis of 43 microsporidian taxa. *Tritrichomonas foetus* is included as an outgroup. **A)** Neighbor Joining consensus tree using 100 bootstrap replicates. The numbers represent Neighbor Joining bootstrap values; a second number, where applicable, indicates the maximum parsimony heuristic bootstrap value (100 replicates). **B)** Maximum Parsimony Analysis showing the single shortest tree of 5,762 steps. Bar indicates 100 nucleotide changes.

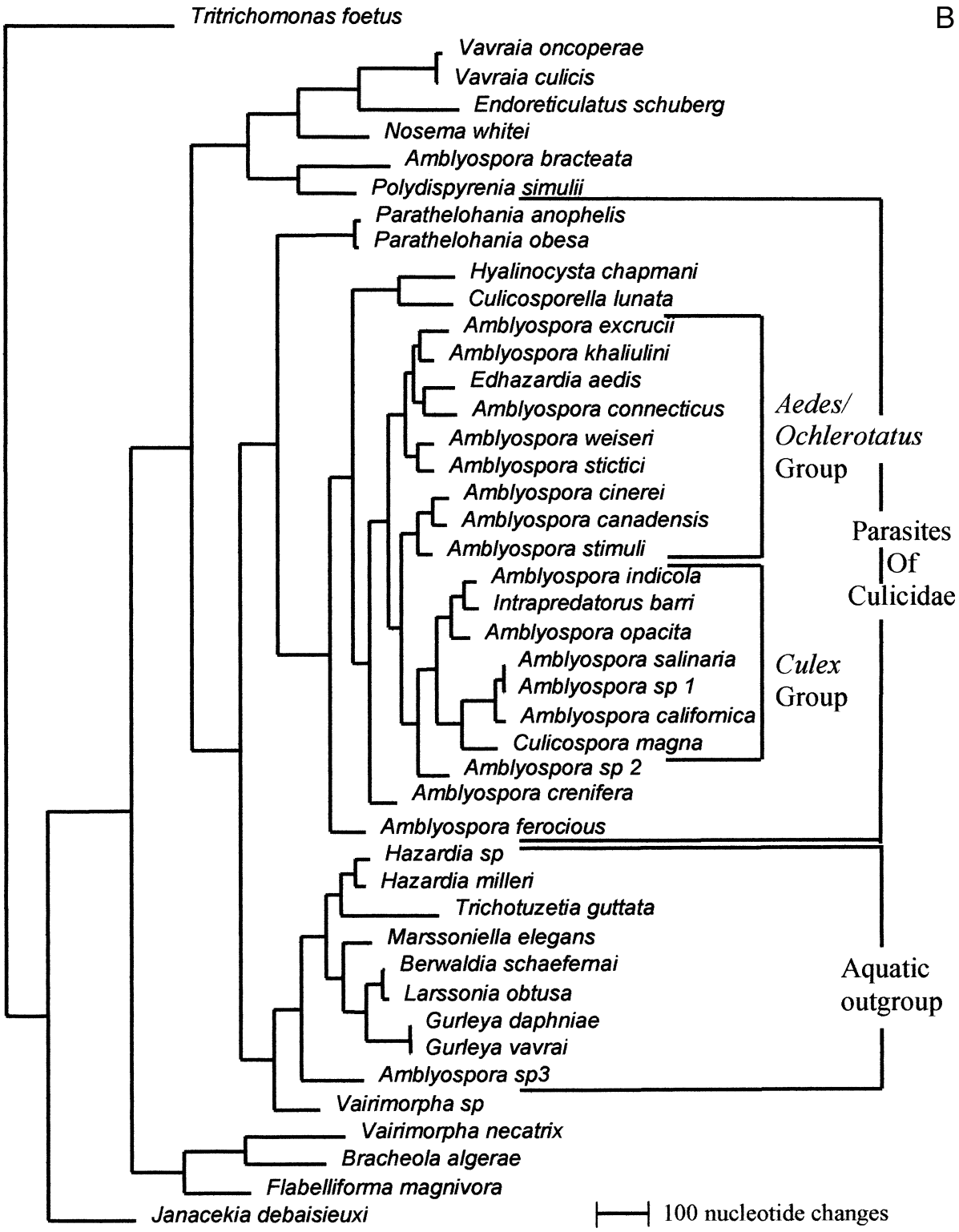

Fig. **1.** Continued.