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PHYLOGENETIC POSITION OF *EIMERIA ANTROZOI,* A BAT COCCIDIUM (APICOMPLEXA: EIMERIIDAE) AND ITS RELATIONSHIP TO MORPHOLOGICALLY SIMILAR *EIMERIA* SPP. FROM BATS AND RODENTS BASED ON NUCLEAR 18S AND PLASTID 23S RDNA SEQUENCES

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ABSTRACT: Partial plastid 23S and nuclear 18S rDNA genes were amplified and sequenced from 2 morphologically similar *Eimeria* species, *E. antrozoi* from a bat (*Antrozous pallidus*) and *E. arizonensis* from deer mice (*Peromyscus* spp.), as well as some other *Eimeria* species from bats and rodents. The phylogenetic trees clearly separated *E. antrozoi* from *E. arizonensis.* The phylogenies based on plastid 23S rDNA data and combined data of both plastid and nuclear genes grouped 2 bat *Eimeria* and 3 morphologically similar *Eimeria* species from rodents into 2 separate clades with high bootstrap support (100%, 3 rodent *Eimeria* species; 72–97%, 2 bat *Eimeria* species), which supports *E. antrozoi* as a valid species. The rodent *Eimeria* species did not form a monophyletic group. The 2 bat *Eimeria* species formed a clade with the 3 morphologically similar rodent *Eimeria* species (*E. arizonensis, E. albigulae, E. onychomysis,* all from cricetid rodents) with 100% bootstrap support, whereas 2 other rodent *Eimeria* species (*E. nieschulzi, E. falciformis,* from murid rodents) formed a separate clade with 100% bootstrap support. This suggests that the 2 *Eimeria* species from bats might be derived from rodent *Eimeria* species and may have arisen as a result of lateral host transfer between rodent and bat hosts.

The genus *Eimeria* is, by far, the most speciose genus of all the coccidia (~1,700 species, see http://biology.unm.edu/biology/coccidia/list.html). Members of this large group of parasitic protists infect all vertebrates and sometimes can produce significant pathology or mortality (Levine, 1988). Traditionally, the identification of *Eimeria* species has relied primarily on oocyst morphology, but also on host specificity, host geographic distribution, and sometimes life cycle details (Levine, 1982; Duszynski and Wilber, 1997). However, the identification of some morphologically similar or less host-specific *Eimeria* species cannot be based solely on morphologic features if definitive conclusions are to be made. Two species, *E. arizonensis* and *E. antrozoi,* described in the parasitological literature provide such an example. *Eimeria antrozoi* was first reported in pallid bats (*Antrozous pallidus*) from collection localities in New Mexico and Mexico by Scott and Duszynski (1997). They initially called it ''*E. arizonensis*-like,'' rather than naming it because of the structural similarity of its sporulated oocysts to those of *E. arizonensis,* a rodent coccidium. The lack of strict host specificity of *E. arizonensis,* which is now known to infect at least *Peromyscus maniculatus, P. eremicus, P. truei, Reithrodontomys megalotus,* and *R. montanus* (Reduker et al., 1985; Duszynski et al., 1992; Hnida and Duszynski, 1999a), made the formal naming of *E. antrozoi* even more tenuous. Scott and Duszynski (1997) suggested that cross-infection or molecular studies between these species should be done to demonstrate distinctiveness. Duszynski et al. (1999) reexamined this coccidium and provided a specific epitaph because its regularity and high prevalence in some bat populations strongly suggested that these were not spurious infections with *E. arizonensis*; nonetheless, a comparative molecular study on these 2 morphologically similar species has never been done. Here, partial nuclear 18S and plastid 23S rDNA from these 2 species, as well as additional *Eimeria* species from bats and rodents, were amplified using polymerase chain reaction (PCR), and phylogenetic analyses among these species were conducted to try to determine their relationships.

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MATERIALS AND METHODS

Parasites and DNA extraction

The oocysts of *E. nieschulzi, E. falciformis,* and *E. arizonensis* maintained in the laboratory of D.W.D. were obtained by inoculating 100–1,000 sporulated oocysts of each species into laboratory-reared, coccidia-free hosts (*Rattus norvegicus* for *E. nieschulzi, Mus musculus* for *E. falciformis, Peromyscus maniculatus* for *E. arizonensis*) following methods described by Upton et al. (1992). The oocysts were collected, sporulated, and stored as described by Duszynski and Wilber (1997). The oocysts of *E. antrozoi* and *E. rioarribaensis* were obtained from naturally infected, wild-caught hosts collected by Scott and Duszynski (1997) and Duszynski et al. (1999). Oocysts were purified by floating in Sheather's solution (Dubey, 1996) and stored in 2% (wt/v) aqueous $K_2Cr_2O_7$ solution at ~4 C until used for DNA extraction.

DNA was extracted following methods described by Zhao et al. (2001). Briefly, purified oocysts stored in 2% $K_2Cr_2O_7$ solution were washed 4 times with sterile high-salinity phosphate-buffered saline (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4). The oocyst pellet was resuspended in 200 μl 5.25% sodium hypochlorite and incubated on ice for 30 min. After washing 4 times with the above saline, the oocyst pellet was resuspended in 60 μl lysis buffer (660 mM edetic acid [EDTA], 1.3% *N*-lauroylsarcosine, 2 mg/ml proteinase K, pH 9.5) and incubated at 65 C for 45 min. Then, 350 μl cetyltrimethylammoniumbromide (CTAB) was added and incubated at 60 C for another 1 hr. The DNA was extracted from the lysate with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and precipitated by 100% cold ethanol.

Gene amplification and sequencing

Nuclear 18S and plastid 23S rDNA were partially amplified by PCR. The specific primers were designed based on the published corresponding sequences of related species: for 18S rDNA, 18sF1 GCTTGTCTC AAAGATTAAGCC and 18sR2 AGCGACGGGCGGTGTGTACAA; for 23S rDNA, 23sIF CCTTTAAARAGTGCGTWAWAGCT and 23sIR CCCTAGAGTAACTTTTATCCGTT. PCR amplifications were carried out in 25-μl reactions under standard conditions on a T-gradient thermocycler (Scimetrics, Inc. Missouri City, Texas). The reaction mixture contained 1 unit of Taq polymerase, 10× PCR buffer, 0.04 mM of each deoxynucleotide, 2.5 mM M_gCl (PCR kit, Perkin Elmer, Foster City, California), 1 μm of each amplification primer, 1 μl DNA template, and MiliQ H_2O to volume. The cycling profile was as follows: 95 C for 4 min in precycle, followed by 35 cycles of 92 C denaturation for 45 sec, primer annealing at 60 C for 45 sec, and elongation at 72 C for 1.5 min. Final primer extension continued for an additional 7 min to allow the complete elongation of all amplifications.

The PCR products of 18S and 23S rDNA were directly cloned using

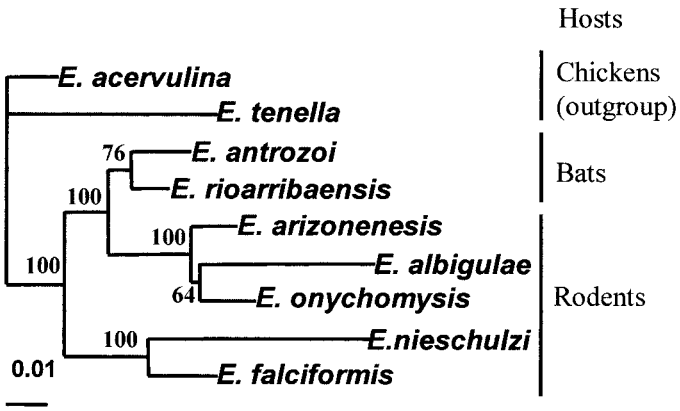

FIGURE 1. Phylogenetic tree inferred from plastid 23S rDNA sequences under parsimony, distance, and likelihood criteria. *Eimeria tenella* and *E. acervulina* (chicken parasites) are used as outgroup taxa. The numbers on the tree are bootstrap values under parsimony criterion with 1,000 replicates. Parsimony steps: 318, CI: 88%, CI excluding uninformative characters: 78%, RC: 67%, RI: 77%. Distance score: 0.3. Likelihood −ln: 3,290. This tree also was identical to the tree inferred from the combined data. For combined data, parsimony steps: 492, CI: 87%, CI excluding uninformative characters: 76%, RC: 65%, RI: 75%. Distance score: 0.3. Likelihood −ln: 6,325.

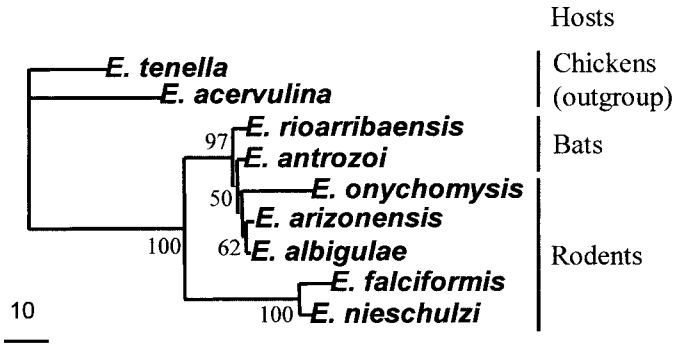

FIGURE 2. One of the 2 best MP trees inferred from nuclear 18S rDNA sequences, which was identical to a single distance and likelihood tree. Parsimony steps: 145, CI: 94%, CI excluding uninformative characters: 90%, RC: 86%, RI: 91%. Distance score: 0.08. Likelihood −ln: 2,821. Numbers on the tree are bootstrap support values under parsimony criterion with 1,000 replicates (<50% not labeled).

Original TA Cloning Kit (Invitrogen, Carlsbad, California) following the manufacturer's instructions. The plasmids with expected DNA inserts were isolated using QIAprep Miniprep Kit (QIAGEN Inc., Valencia, California). Sequencing of clones was performed on both strands using BigDye terminator cycle sequencing ready reaction kit (ABI PRISM, Perkin Elmer). PCR primers were used as sequencing primers. To cover the full length of the inserted DNA fragments, some inner primers were designed to overlap the boundaries of primers. The sequences have been deposited in the GenBank database. The accession numbers of 18S rDNA sequences of *E. antrozoi, E. rioarribaensis, E. arizonensis, E. onychomysis,* and *E. albigulae* are AF307876–AF307880 and 23S rDNA sequences of *E. antrozoi, E. rioarribaensis, E. arizonensis, E. onychomysis, E. albigulae, E. nieschulzi, E. falciformis, E. tenella,* and *E. acervulina* are AF307881–AF307889. The 18S rDNA sequences of *E. tenella* (accession no. U67121), *E. acervulina* (accession no. U67115), and *E. falciformis* (accession no. AF080614) were retrieved from the GenBank database.

Sequence alignment and phylogenetic analysis

The sequences were aligned using ClustalW in default alignment parameters, and the alignments were refined by eye to maximize the similarities (Barta, 1997). The phylogenetic analysis was carried out using Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0b3 (Swofford, 1999). Maximum parsimony (MP), distance with minimum evolution (ME) model, and maximum likelihood (ML) criteria were employed to do the analyses. Transition/transversion ratios (ti/tv) were estimated from each data set via likelihood for MP and ML analyses; ti/tv = 0.99 for 23S rDNA data set, 2.1 for 18S rDNA data set, and 1.2 for the combined data set. The F84 model was chosen for ME and ML analyses to ameliorate AT-rich biases of both data sets. Gamma model was chosen to correct site-to-site heterogeneity of both data sets (Yang, 1996). The gamma parameter α (0.3 for 23S rDNA data set, 0.09 for 18S data set, 0.05 for combined data set) was estimated from each data set. To determine if combining the data was necessary, a partition–homogeneity test (also called incongruence length difference test [ILD], Farris et al., 1984; Cunningham 1997) was done. The *P*-value (0.01) of the partition–homogeneity test was calculated by PAUP*. The best trees were searched by evaluating all possible trees using an exhaustive search for all analyses. A bootstrap with 1,000 replicates was performed for all the analyses. The software TreeView version 1.5 (Page, 1996) was used for printing phylogenetic trees.

RESULTS

Phylogenetic analysis for plastid 23S rDNA sequences

Phylogenetic analysis for the plastid 23S rDNA sequences from *E. albigulae, E. arizonensis, E. falciformis, E. nieschulzi,* and *E. onychomysis* (from rodents) and *E. antrozoi* and *E. rioarribaensis* (from bats) was conducted using PAUP* under different criteria. Two chicken *Eimeria* spp., *E. tenella* and *E. acervulina,* were used as outgroup taxa. For this data set, the alignment contained 1,146 total positions including gaps. A single best parsimonious tree was inferred (Fig. 1). The figures on the tree are bootstrap values with 1,000 replicates under the parsimony criterion. In this best tree with 318 steps, an 88% consistency index (CI), a 78% CI excluding uninformative characters, a 77% retention index (RI), and a 67% rescaled consistency index (RC), the ingroup taxa were divided into 2 major groups. The 2 bat *Eimeria* species (*E. antrozoi, E. rioarribaensis*) and 3 rodent *Eimeria* species (*E. albigulae, E. arizonensis, E. onychomysis*) formed a clade with 100% bootstrap support. In this subtree, the 2 bat *Eimeria* and 3 rodent *Eimeria* species formed sister groups with 76 and 100% bootstrap support, respectively. *Eimeria antrozoi* and *E. arizonensis* are separated clearly and placed in different groups with high bootstrap support. For the same data set, both ME and ML generated a single tree (distance score: 0.3; log likelihood: −ln 3,290) that was identical to the MP tree (Fig. 1). The bootstrap support for the *E. antrozoi* and *E. rioarribaensis* branch was 97% under the distance criterion, with 1,000 replicates.

Phylogenetic analysis for nuclear 18S rDNA sequences

Phylogenetic analyses were carried out using partial nuclear 18S rDNA sequences for the same taxa as in the plastid 23S rDNA phylogenies. Almost the full length of the 18S rDNA was used. The aligned sequence length is 1,540, including gaps. The 2 equal best MP trees—a single ME and a single ML tree, respectively—were generated by different search criteria. The single ME tree (ME score: 0.08) and ML tree (−ln 2,821) were identical to each other and also identical to 1 of the 2 MP trees (Fig. 2). The 2 MP trees had 164 steps and were similar in that the ingroup formed 2 major lineages as in 23S rDNA tree. The 2 trees were different with respect to the positions of *E. antrozoi*

and *E. onychomysis*. The bootstrap analysis cannot resolve *E. antrozoi* from *E. onychomysis* and the branch of *E. albigulae* and *E. arizonensis* (Fig. 2).

Combining data analysis

The *P*-value (0.01) of the partition–homogeneity test calculated by PAUP* suggested that combining these 2 data sets may improve the phylogenetic accuracy (Huelsenbech et al., 1996; Cunningham, 1997). The phylogenetic analyses of the combined data were carried out under parsimony, distance, and likelihood criteria. All 3 methods resulted in a single identical tree, which has the same topology as the plastid 23S rDNA tree (see Fig. 1). The MP steps were 492, CI was 87%, CI excluding uninformative characters was 76%, RI was 75%, and RC was 65%; ME score was 0.3, and likelihood was −ln 6,325. Bootstrap support under the likelihood criterion with 1,000 replicates was 72% for the bat *Eimeria* spp. branch and 100% for the *E. albigulae* and *E. onychomysis* clade. Bootstrap support for other branches was the same as in the 23S rDNA tree.

DISCUSSION

Within *Eimeria,* most species names have been based solely on the morphology of their sporulated oocysts, the host identity, and the geographic distribution of the host species (Joyner, 1982; Duszynski and Wilber, 1997). However, oocyst morphological features, both qualitative and quantitative, can overlap among, and vary within, species of *Eimeria* (Duszynski, 1971; Joyner, 1982; Long and Joyner, 1984; Parker and Duszynski, 1986; Gardner and Duszynski, 1990; Hnida and Duszynski, 1999b). Cross-transmission and field studies have indicated that some *Eimeria* species, including *E. arizonensis,* can infect several host genera, at least within the same host family (Mayberry et al., 1982; Upton et al., 1992; Hnida and Duszynski,1999a). Thus, the morphology of sporulated oocysts and host specificity are not always completely reliable for differentiating some *Eimeria* species that may be more euryxenous in their host requirements (also see Wilber et al., 1998). Phylogenetic analyses based on molecular data have provided a powerful tool for the more certain identification and characterization of the more cryptic species. Hnida and Duszynski (1999c) successfully differentiated 3 morphologically similar *Eimeria* species from rodents in the family Cricetidae, *E. arizonensis* (from *Peromyscus*), *E. albigulae* (from *Neotoma*), and *E. onychomysis* (from *Onychomys*), using a phylogenetic analysis based on ITS1 sequences. In the present study, the plastid 23S rDNA sequences are shown to be a good genetic yardstick for phylogenetic analysis for these *Eimeria* species. On the other hand, the nuclear 18S rDNA is not as good a choice to determine the phylogenetic relationships among very closely related *Eimeria* species because of its high conservation. The sequence comparisons showed that the differences of the nucleotides in 18S rDNA sequences are only 8/1,539 between *E. arizonensis* and *E. albigulae,* 12/1,523 between *E. albigulae* and *E. onychomysis,* 13/1,528 between *E. arizonensis* and *E. onychomysis,* and 9/1,548 between *E. antrozoi* and *E. rioarribaensis.* These changes are so small that they may not contain enough phylogenetic information, especially for Parsimony analysis, because only part of the variable characters are parsimony-informative and used to infer trees.

Phylogenetic analyses based on both plastid 23S and nuclear 18S rDNA sequences in the present study clearly separated *E. antrozoi* and *E. arizonensis.* In general, plastid 23S and nuclear 18S rDNA phylogenies of the relationships among the 7 ingroup taxa were consistent: the 5 morphologically similar *Eimeria* species (3 from rodents, 2 from bats) formed a clade with high bootstrap support (100% from 23S rDNA data, 97% from 18S rDNA data). In this subtree, the plastid phylogeny placed the 2 bat *Eimeria* and 3 rodent *Eimeria* species in parallel groups (Fig. 1), whereas the nuclear phylogeny grouped these 5 *Eimeria* in a single clade (Fig. 2). The phylogenies based on the combined data of both plastid 23S and nuclear 18S rDNA grouped the 2 bat *Eimeria* and 3 rodent *Eimeria* species into 2 parallel clades with high bootstrap support (100%, 3 rodent *Eimeria* species; 72%, 2 bat *Eimeria* species), which provides solid support for Duszynski et al.'s (1999) conclusion, based principally on host differences, that *E. antrozoi* is a valid species, even though its oocysts are morphologically similar to those of *E. arizonensis.*

Reduker et al. (1987), Hnida and Duszynski (1999b), and Zhao and Duszynski (2001a, 2001b) noted that there were 2 lineages of *Eimeria* spp. in rodents. The 5 rodent *Eimeria* species used in this study represent both lineages. The results showed that the 5 rodent *Eimeria* species did not form a monophyletic group in either the plastid 23S or nuclear 18S rDNA phylogenies. The 2 bat *Eimeria* species formed a clade with 3 morphologically similar rodent *Eimeria* species (*E. arizonensis, E. albigulae, E. onychomysis*), whereas the other 2 rodent species, *E. nieschulzi* and *E. falciformis,* formed a separate group. The level of confidence in the branching topology was highly significant. Bootstrapping indicates that the 2 bat *Eimeria* species and 3 rodent *Eimeria* species are monophyletic in 100% of 1,000 sampled trees in the plastid 23S rDNA tree and 97% in the nuclear 18S rDNA tree. Recognizing that bats and rodents are separate orders of mammals, these results may suggest that, within *Eimeria,* morphological similarity of sporulated oocysts may be more significant in reflecting parasite–host phylogenetic/evolutionary relationships than is host specificity.

It is clear from the results that the 2 bat *Eimeria* (*E. antrozoi, E. rioarribaensis*) cluster more closely to the 3 morphologically similar rodent *Eimeria* (*E. arizonensis, E. albigulae, E. onychomysis*) than they do to the other 2 rodent *Eimeria* species (*E. nieschulzi, E. falciformis*). This suggests that the 2 bat *Eimeria* species might be derived from rodent *Eimeria* species. They may have arisen as a result of lateral host transfer between rodent and bat hosts. Scott and Duszynski (1997) pointed out the possibility that *E. antrozoi* might be derived from *E. arizonensis* because of the feeding habit and behavior of *A. pallidus,* its host. Regularly landing on the ground to feed on large insects such as millipedes, scorpions, ground crickets, and beetles may bring pallid bats in regular contact with rodent feces carrying infective *E. arizonensis* oocysts, which might result in *E. arizonensis* being able to adapt to the gut cells of *A. pallidus* and eventually evolve into a new species (Scott and Duszynski, 1997). However, this explanation may not apply to *E. rioarribaensis.* Its hosts, myotid bats, often are found in trees, shrubs, and rock crevices, but rarely on the ground (Duszynski et al., 1999). Such roosting behavior would not seem to facilitate transfer of this *Eimeria* between bats and rodents.

Another possibility would be that the morphologically similar

Eimeria species may share a common ancestor that existed before their hosts diverged. Actually, the *Eimeria* species are thought by some to be much more ancient than their hosts. According to Escalante and Ayala (1995), the coccidia diverged about 800 million years ago (mya), whereas the mammals diverged about 300 mya, and rodents diverged less than 100 mya (Kumar and Hedges, 1998; Huchon et al., 2000). It is possible that the ancestors of different *Eimeria* lineages existed in the common ancestor of mammals, or even invertebrates, and they also experienced independent divergence when their hosts radiated and diverged, which resulted in independent *Eimeria* lineages that expanded the host range. Because rodents and bats are not sister groups in mammal phylogeny and because of the limitation of host diversity of *Eimeria* taxa used in this study, analysis on more *Eimeria* species from different hosts would help to clarify this problem.

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