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J.A. Hnida · D.W. Duszynski

Taxonomy and phylogeny of some *Eimeria* (Apicomplexa: Eimeriidae) species of rodents as determined by polymerase chain reaction/restriction-fragment-length polymorphism analysis of 18S rDNA

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Abstract The 18S rDNA genes of 10 *Eimeria* species from rodents (*E. albigulae*, *E. arizonensis*, *E. falciformis*, *E. langebarteli*, *E. nieschulzi*, *E. onychomysis*, *E. papillata*, *E. reedi*, *E. separata*, *E. sevilletensis*) were polymerase-chain-reaction (PCR)-amplified, digested with 12 restriction endonucleases, and electophoresed in agarose gels. The resulting fragment patterns (riboprints) distinguished all species except *E. sevilletensis* from *E. falciformis*, and *E. arizonensis* from *E. albigulae*; the sporulated oocysts of the latter two species and of *E. onychomysis* are often indistinguishable morphologically. When the restriction fragment data were analyzed using distance and parsimony phylogenetic methods a clade was found consistently, which contained *E. arizonensis*, *E. albigulae*, *E. onychomysis*, *E. reedi*, and *E. papillata*. This finding and other results of the phylogenetic analyses agreed and supplemented previous phylogenetic work on the *Eimeria* of rodents. Riboprinting appears to provide useful data for taxonomic and phylogenetic studies on the genus *Eimeria* and may be especially practical when samples do not contain enough oocysts for other molecular-based methods.

Introduction

The 1100+ described species of *Eimeria* (Apicomplexa: Eimeriidae) are obligate intracellular parasites of invertebrates and vertebrates (Levine 1988). Most species have been described from vertebrates (Duszynski and Upton 1999; Pellérdy 1974), and some are of considerable veterinary importance because they cause coccidiosis in domestic animals (Levine 1985). The taxonomy of this genus primarily has been based on the morphology of the sporulated oocyst and the identity of the host from which the oocysts have been recovered (Current et al. 1990; Duszynski and Upton 1999; Joyner 1982; Levine 1982). However, this approach can be compromised because qualitative and quantitative features of oocyst morphology often overlap among, and vary within, species of *Eimeria* (DeVos 1970; Duszynski 1971; Gardner and Duszynski 1990; Joyner 1982; Long and Joyner 1984; Parker and Duszynski 1986; Pellérdy 1965; Sommer 1998), and cross-transmission and field studies have indicated that some species of *Eimeria* are not as host-specific as previously thought (DeVos 1970; Hill and Duszynski 1986; Mayberry et al. 1982; Todd and Hammond 1968a,b; Thomas and Stanton 1994; Upton et al. 1992). Consequently, molecular techniques have become increasingly important for the identification and characterization of these parasites; e.g., isoenzymes (Chapman 1982; Johnston and Fernando 1997; Kučera 1991; Shirley 1975, 1978) and the random amplified polymorphic DNA (RAPD) assay (Cere et al. 1995; Johnston and Fernando 1995; MacPherson and Gajadhar 1993; Procunier et al. 1993; Shirley and Bumstead 1994) have been used to distinguish between species and strains of *Eimeria* infecting domestic fowl and a few mammalian hosts. Nevertheless, these techniques are constrained by the need for relatively large numbers of oocysts. For instance, although based on the polymerase chain reaction (PCR), the RAPD assay requires ~10,000 oocysts per reaction and, consequently, millions of sporulated oocysts are needed to screen for taxonomically informative RAPD primers and then use them to fingerprint *Eimeria* species (see Cere et al. 1995; Johnston and Fernando 1995; MacPherson and Gajadhar 1993; Procunier et al. 1993; Shirley and Bumstead 1994). Consequently, studies employing isoenzymes or RAPDs typically use coccidia species that infect domestic or laboratory animals, i.e., species that

J.A. Hnida (✉)
Division of Science and Technology, Peru State College,
P. O. Box 10, Peru, NE 68421, USA
e-mail: hnida@bobcat.peru.edu
Tel.: +1-402-8722231; Fax: +1-402-8722375

D.W. Duszynski
Department of Biology, The University of New Mexico,
Albuquerque, NM 87131-1091, USA

can be passaged to provide the large numbers of oocysts required by these methods.

Reduker et al. (1987) noted that although *Eimeria* is taxonomically diverse, "virtually nothing" was known about the systematic relationships of taxa within this genus. These authors used isoenzymes, sporulated oocyst morphology, and life history traits to examine the phylogeny of nine species parasitizing murid rodents. More recently, Barta et al. (1997) inferred the phylogenetic relationships of eight *Eimeria* species from domestic fowl by analyzing their 18S rDNA sequences. In addition, although it was not the focus of their study, Cere et al. (1995) included a parsimony analysis of RAPD data derived from a number of geographic isolates of two *Eimeria* species infecting domestic rabbits. These three studies are the beginnings of an evolutionary approach to understanding these parasites, but much more systematic work is needed to rectify Reduker et al.'s (1987) concern about the paucity of information on eimerian phylogenetics.

Riboprinting, the analysis of restriction-fragment-length polymorphisms (RFLP) of PCR-amplified 18S rDNA, has been used to differentiate species or strains of free-living (Brown and De Jonckheere 1994; De Jonckheere 1994) and parasitic protozoa (Clark 1992, 1997; Clark and Diamond 1991; Clark and Pung 1994; De Jonckheere 1994). In addition, data from riboprints have been used in phylogenetic analyses of some parasitic protozoa (Clark 1997; Clark and Diamond 1997; Clark et al. 1995; De Jonckheere 1994; Noyes et al. 1997; Pomport-Castillon 1997). This technique is considered useful because only small amounts of DNA are required to PCR-amplify the 18S rDNA (Clark 1997; White et al. 1990), and digestion with 12 restriction enzymes often provides sufficient data for taxonomic or phylogenetic analyses (Clark 1992, 1997).

In this work we used the riboprinting technique in an attempt to characterize and differentiate ten species of *Eimeria* from rodents. The taxonomic status of three of these species (*E. arizonensis*, *E. albigulae*, *E. onychomysis*) has been questioned because they infect closely related murid rodents, and their sporulated oocysts are often morphologically indistinguishable (Upton et al. 1992). In addition, we compared the results of phylogenetic analyses of the riboprint data with Reduker et al.'s (1987) phylogenetic hypotheses for some of these *Eimeria* species. To enable methodological comparisons with other riboprinting studies we used 12 restriction enzymes each with 4-bp recognition function as recommended by Clark (1992, 1997) and Clark et al. (1995).

Materials and methods

Parasites

Eimeria species were obtained from wild-caught hosts or naturally infected laboratory animals or were laboratory-maintained isolates originating from various regions of the United States or Europe (Table 1). Host feces or intestinal contents were processed in 2.5% (w/v) $K_2Cr_2O_7$ to allow oocysts to sporulate (Duszynski and Wilber 1997). Most sporulated oocysts were identified to species using coverslip flotation with modified Sheather's sucrose solution (Barnard and Upton 1994); identification of *E. arizonensis*, *E. albigulae*, and *E. onychomysis* was further verified using cross-transmission experiments (Hnida and Duszynski 1999). Because some samples contained few oocysts, more were obtained by inoculation of ~20–100 sporulated oocysts into laboratory-reared, coccidia-free hosts (*Mus musculus*, *Neotoma albigula*, *Onychomys leucogaster*, *Peromyscus maniculatus*, *P. truei*) using methods described by Upton et al. (1992). Isolates were concentrated, purified of large fecal debris by centrifugation in Sheather's solution (Dubey 1996), and stored in 2.5% $K_2Cr_2O_7$ at ~4 °C until used for DNA extraction.

DNA extraction

For each isolate, ~10,000–500,000 oocyts were washed 2–3 times in sterile dH_2O, incubated on ice in 20% NaOCl (10–13% active chlorine) for 1 h (Cere et al. 1995), rinsed 3 times in sterile dH_2O, and suspended in 500 µl of TE buffer [10 m*M* TRIS, 1 m*M* ethylenediaminetetraacetic acid (EDTA, pH 8.0)]. The oocysts and sporocysts were ruptured by vortexing with sterile 4-mm glass beads for 10 min (MacPherson and Gajadhar 1993); for most samples, 2–3 beads were used in a 1.5-ml microcentrifuge tube. The vortexed suspension was added to 1.0 ml of CTAB buffer (2% CTAB, 1.4 *M* NaCl, 0.2% β-mercaptoethanol, 20 m*M* EDTA, 100 m*M* TRIS/HCl) containing proteinase K (100 µg/ml) and incubated at 65 °C for 1 h. Afterward the DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), precipitated by ethanol, air-dried, and redissolved in 40–50 µl of TE buffer.

PCR amplification, restriction enzyme digestion, and gel electrophoresis

PCR amplifications were performed in 100-µl reaction mixtures using ~5–10 ng of template DNA (White et al. 1990), AmpliTaq DNA polymerase, and reagents in the PCR Core Kit (Perkin Elmer, USA) according to the manufacturer's instructions. The PCR primers for the 18S region were the "universal" eukaryotic primers A (5′-AACCTGGTTGATCCTGCCAGT-3′) and B (5′-TGATCCTTCTGCAGGTTC-ACCTAC-3′; see Hanelt et al. 1996). After an initial step of denaturation for 4 min at 94 °C, the PCR cycle was 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, with the primer extension time being increased by 3 s for each subsequent reaction cycle. After 30 cycles an additional 7-min period of extension at 72 °C was performed. Before digestion, PCR products were purified with Centricon-100 columns (Millipore) and further concentrated by vacuum centrifugation. The PCR products from each isolate were digested in 20-µl reaction volumes using the following 12 restriction enzymes: *Alu*I, *Bst*UI, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3AI, *Sau*96I, *Scr*FI, and *Taq*I (see Clark 1992). The digested PCR products were electrophoresed through 2.5% agarose gels, stained with ethidium bromide, and photographed (Polaroid 667, 3000 ISO film). The molecular sizes of the fragments were estimated using a 100-bp ladder (Gibco) run on the same agarose gels, and for verification of fragment comigration, amplified DNAs from different isolates were digested with the same restriction enzyme and separated in adjacent lanes on the same gel (Clark and Diamond 1995). The PCR/RFLP procedure was repeated a minimum of two times for each isolate/restriction enzyme combination.

Data analysis

Fragments were scored as present or absent (1/0 scoring; see Table 2); however, fragments measuring less than 100 bp in length were not included as they were not always clearly visible on all riboprints (see Clark and Diamond 1997; Clark et al. 1995). Ge-

Table 1 Parasite species list indicating the host, location, and date of origin of isolates (*LTER* Long Term Ecological Research site, *NM* New Mexico, *AZ* Arizona, *OR* Oregon, *AL* Alabama)

Eimeria spp.	Collection		
	Host	Locality	Date
E. albigulae	*Neotoma albigula*	Rio Salado, Sevilleta LTER, NM	1992
E. albigulae	*N. albigula*	Rio Salado, Sevilleta LTER, NM	1995
E. albigulae	*N. albigula*	Two-22, Sevilleta LTER, NM	1995
E. albigulae	*N. albigula*	Sandia Mountains, NM	1995
E. arizonensis	*Peromyscus eremicus*	Portal, AZ	1996
E. arizonensis	*P. leucopus*	Rio Grande bosque, Albuquerque, NM	1995
E. arizonensis	*P. maniculatus*	Corvallis, OR	1995
E. arizonensis	*P. truei*	Goat Draw, Sevilleta LTER, NM	1996
E. arizonensis	*P. truei*	Rio Salado, Sevilleta LTER, NM	1996
E. falciformis	*Mus musculus*	Tijeras, NM	1997
E. falciformis	*M. musculus*	Wuppertal, Germany	1961
E. langebarteli	*P. leucopus*	Two-22, Sevilleta LTER, NM	1996
E. nieschulzi	*Rattus norvegicus*	Tempe, AZ	1961
E. onychomysis	*Onychomys leucogaster*	Rio Salado, Sevilleta LTER, NM	1992
E. onychomysis	*O. leucogaster*	Rio Salado, Sevilleta LTER, NM	1993
E. oncyhomysis	*O. torridus*	Portal, AZ	1996
E. papillata	*Mus musculus*	Michigan	1976
E. reedi	*Perognathus flavus*	Five Points Larrea, Sevilleta LTER, NM	1997
E. separata	*R. norvegicus*	Auburn, AL	1968
E. sevilletensis	*O. leucogaster*	Rio Salado, Sevilleta LTER, NM	1993

netic distances were estimated from fragment comigration values by the method of Nei and Li (1979) and the resulting distance matrix (Table 3) was used for tree construction by the FITCH program in PHYLIP (Felsenstein 1989), which implements the Fitch-Margoliash method for phylogenetic inference from distance data (Fitch and Margoliash 1967). All FITCH searches were done with randomized input order of species ($n = 10$ replicates) and global rearrangements of taxa. The presence or absence of comigrating fragments were also treated as characters for maximum-parsimony (MP) analyses using prerelease test versions d63 and d64 of PAUP* 4.0 (Swofford 1996). Fitch parsimony and unrooted Dollo parsimony models were used (see Swofford et al. 1996 for a discussion of the use of Dollo parsimony with restriction site data); all MP analyses were done using the exhaustive search option. Bootstrap values (Felsenstein 1985) were obtained using 10,000 replicates with the branch-and-bound search option. Maximum-likelihood (ML) analysis (molecular-clock-enforced) of the internal transcribed spacer 1 (ITS1) sequences of the 20 *Eimeria* taxa (Table 1) revealed 2 major clades, 1 of which contained *E. arizonensis* and the *E. arizonensis*-like taxa (Hnida and Duszynski, unpublished data). Thus, in the present study, taxa from the second major clade (*E. falciformis*, *E. nieschulzi*, *E. separata*, and *E. sevilletensis*) were used for outgroup comparisons in the distance and MP analyses. Because *E. sevilletensis* was not distinguished from *E. falciformis* by RFLP analysis (see below), it was not used as an outgroup taxon. The amount of phylogenetic signal in the RFLP data set was assessed for 10,000 trees using the Random Trees analysis in PAUP* (Hillis and Huelsenbeck 1992).

Results

Altogether, 10 of the 12 restriction enzymes produced 18S fragment patterns that were polymorphic among most of the 10 *Eimeria* species (Fig. 1A,B; Table 2); the enzymes *Hin*f I and *Sau*3A I produced identical banding patterns in all taxa (Table 2). However, the riboprints of *E. arizonensis* and *E. albigulae* were identical, as were those of *E. falciformis* as compared with *E. sevilletensis*. No intraspecific variation was observed between the two isolates of *E. falciformis* or among the multiple isolates of *E. onychomysis*, *E. albigulae*, and *E. arizonensis* (Fig. 1B). Of the 64 restriction fragments observed, 32 were found in all species and 8 were unique fragments (autapomorphies) found in the following 5 species: *E. langebarteli*, *E. onychomysis*, *E. papillata*, *E. reedi* and *E. separata* (Table 2).

All pairwise distance estimates (Nei and Li 1979) were less than 0.1 (Table 3), indicating that the fragment comigration data were reliable indicators of branch order for the distance-based trees (Clark and Diamond 1997). Because the FITCH program in the PHYLIP package allows only one outgroup taxon per analysis, three distance-based trees were inferred using either *E. nieschulzi*, *E. separata*, or *E. falciformis* as an outgroup. The resulting trees (Fig. 2) had similar topologies but differed in the relationships among the outgroup taxa and in their locations relative to the ingroup taxa. In all trees, *E. langebarteli*, an ingroup taxon, was placed closest to the lineage of *E. falciformis* and *E. sevilletensis* (outgroup taxa), and the remaining ingroup taxa retained the same relationships to each other.

The random trees procedure detected significant phylogenetic signal (Hillis and Huelsenbeck 1992) in the RFLP data set with the presence/absence of a fragment treated as a binary character ($g1 = -0.527$, $P < 0.05$). The Fitch parsimony analysis, with *E. falciformis*, *E. separata*, and *E. nieschulzi* representing an outgroup, produced 2 equally parsimonious trees; each was 38 steps long with a consistency index (CI) of 0.84. The consensus tree is presented in Fig. 3 with values from the bootstrap analysis, which obtained a consensus tree with identical topology. The Dollo parsimony analysis (same

Enzyme	*Eimeria* spp.								
	MW	Eaz/Eal	Eon	Ere	Ela	Epa	Ese	Eni	Efa/Esv
*Bst*UI	900	0	1	0	1	0	1	1	1
	790	1	0	1	0	1	0	0	0
	330	1	1	1	1	1	1	1	1
	280	1	1	1	1	1	1	1	1
	150	1	1	1	1	1	1	1	1
*Sau*96I	570	1	1	1	1	1	1	1	1
	470	1	1	1	1	1	1	1	1
	400	0	0	0	1	0	1	1	1
	380	1	1	1	0	1	0	0	0
	280	1	1	1	1	1	1	1	1
	120	1	1	1	1	1	1	1	1
*Hae*III	960	1	1	1	1	0	1	1	1
	600	0	0	0	0	1[a]	0	0	0
	360	0	0	0	0	1[a]	0	0	0
	290	1	1	1	0	1	0	1	1
	280	1	1	1	1	1	1	1	1
	220	1	1	1	1	1	1	1	1
	210	0	0	0	1[a]	0	0	0	0
	180	0	0	0	0	0	1[a]	0	0
*Taq*I	880	1	1	1	1	1	1	1	1
	490	1	1	1	1	1	0	1	0
	390	0	0	0	0	0	1	0	1
	320	1	1	1	1	1	1	1	1
*Rsa*I	1600	0	0	0	1	1	1	1	1
	1000	1	1	1	0	0	0	0	0
	680	1	1	1	0	0	0	0	0
	170	1	1	1	1	1	1	1	1
*Alu*I	630	1	1	1	1	1	1	1	1
	370	1	1	1	1	1	1	1	1
	280	1	1	1	0	0	0	0	0
	260	0	0	0	1	1	1	1	1
	200	1	1	0	1	1	1	1	1
	130	0	0	0	1	0	1	1	1
	100	0	0	1[a]	0	0	0	0	0
*Msp*I	860	0	0	0	1	0	1	1	1
	780	1	1	1	0	1	0	0	0
	380	1	1	1	1	1	1	1	1
	290	1	1	1	1	1	1	1	1
*Hha*I	1050	0	1[a]	0	0	0	0	0	0
	770	1	0	1	1	1	1	1	1
	300	1	0	1	0	0	1	0	0
	260	1	1	1	1	1	1	1	1
	190	1	1	1	1	1	1	1	1
	170	1	1	1	1	1	1	1	1
	130	1	1	1	1	1	1	1	1
*Scr*FI	780	0	0	0	1	0	0	0	1
	590	1	1	1	1	1	1	1	1
	490	1	1	1	1	1	1	1	1
	440	1	1	1	0	1	1	1	0
	230	1	1	1	0	1	1	1	0
*Dde*I	520	0	0	0	0	1[a]	0	0	0
	460	0	0	0	1	0	1	1	1
	370	1	1	1	1	1	1	1	1
	350	1	1	1	1	1	1	1	1
	270	0	0	1[a]	0	0	0	0	0
	130	1	1	1	0	0	0	0	0
*Hin*fI	820	1	1	1	1	1	1	1	1
	550	1	1	1	1	1	1	1	1
	240	1	1	1	1	1	1	1	1
	120	1	1	1	1	1	1	1	1
*Sau*3AI	1040	1	1	1	1	1	1	1	1
	320	1	1	1	1	1	1	1	1
	270	1	1	1	1	1	1	1	1
	140	1	1	1	1	1	1	1	1

[a] Autapomorphic fragment

◀

Table 2 Sizes of restriction fragments found after digestion of the 18S rDNA gene (*1* Present, *0* = absent, *MW* molecular weight, *Eaz E. arizonensis*, *Eal E. albigulae*, *Eon E. onychomysis*, *Ere E. reedi*, *Ela E. langebarteli*, *Epa E. papillata*, *Ese E. separata*, *Eni E. nieschulzi*, *Efa E. falciformis*, *Esv E. sevilletensis*)

Table 3 Estimated genetic distances between species. Values represent the average number of changes per nucleotide position estimated from the data in Table 2 (*Eaz E. arizonensis*, *Eal E. albigulae*, *Eon E. onychomysis*, *Ere E. reedi*, *Ela E. langebarteli*, *Epa E. papillata*, *Ese E. separata*, *Eni E. nieschulzi*, *Efa E. falciformis*, *Esv E. sevilletensis*)

	Eaz/Eal	Eon	Ere	Ela	Epa	Ese	Eni
Eaz/Eal	–						
Eon	0.0168	–					
Ere	0.0097	0.0271	–				
Ela	0.0768	0.0734	0.0894	–			
Epa	0.0387	0.0511	0.0497	0.0646	–		
Ese	0.0660	0.0713	0.0778	0.0279	0.0627	–	
Eni	0.0544	0.0511	0.0660	0.0173	0.0431	0.0168	–
Efa/Esv	0.0768	0.0735	0.0894	0.0139	0.0646	0.0206	0.0173

outgroup taxa) produced 2 equally parsimonious trees; each was 40 steps long with a CI of 0.80. The consensus of these trees was identical to the Fitch parsimony consensus tree, and the Dollo parsimony bootstrap values are given in Fig. 3.

Both MP phylogenies (Fig. 3) were congruent with those produced using pairwise distances (Fig. 2) for the clade containing *E. papillata*, *E. onychomysis*, *E. arizonensis*, *E. albigulae*, and *E. reedi*. However, the bootstrap support for the internode separating the latter three taxa from *E. onychomysis* was 61% in the Dollo and 79% in the Fitch parsimony analyses; thus, the relationships among these four taxa are best considered unresolved. Distance and MP analyses concurred in finding *E. langebarteli* to be more closely related to the outgroup taxa, with weak bootstrap support for the branch connecting *E. langebarteli* to *E. falciformis* and *E. sevilletensis* (Fig. 3).

Discussion

Most of the 10 *Eimeria* species from rodents were distinguishable when riboprinted with 12 restriction enzymes. In addition, the riboprints of 5 of the species included autapomorphic fragments that could be used as characters for species identification. However, *E. albigulae* was not differentiated from *E. arizonensis*, although the other *E. arizonensis*-like taxon, *E. onychomysis*, was discriminated (Table 2). Nevertheless, it would be premature to consider the RFLP data as evidence that *E. arizonensis* and *E. albigulae* are conspecifics, because cross-transmission experiments (Upton et al. 1992; Hnida and Duszynski 1999) and ITS1 sequencing (Hnida and Duszynski, unpublished data)

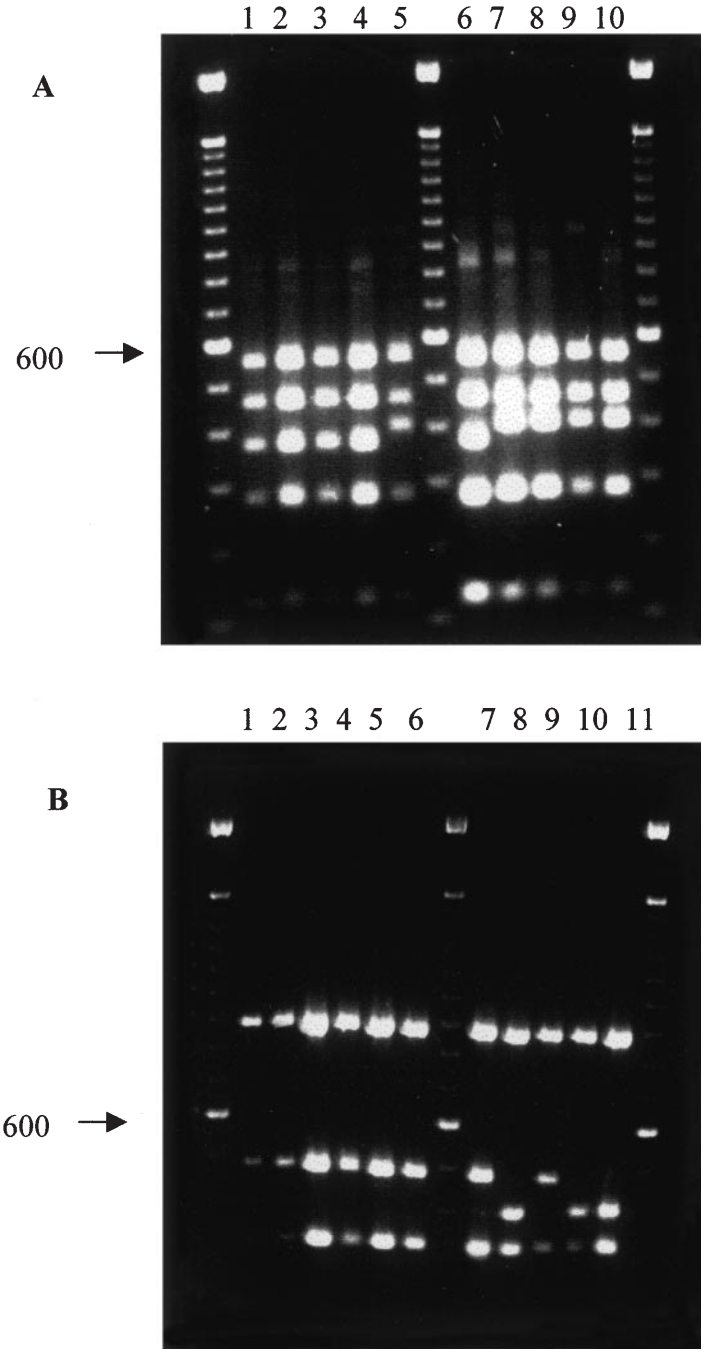

Fig. 1A,B Riboprints of *Eimeria* spp. analyzed on 2.5% agarose gels. **A** Digestions with *Sau*96 I: *E. arizonensis* (*lane 1*), *E. albigulae* (*lane 2*), *E. onychomysis* (*lane 3*), *E. reedi* (*lane 4*), *E. langebarteli* (*lane 5*), *E. papillata* (*lane 6*), *E. separata* (*lane 7*), *E. nieschulzi* (*lane 8*), *E. falciformis* (*lane 9*), *E. sevilletensis* (*lane 10*). **B** Digestions with *Taq*I: *E. arizonensis* Rio Salado, NM isolate (*lane 1*); *E. arizonensis* Corvallis, OR isolate (*lane 2*); *E. albigulae* (*lane 3*); *E. onychomysis* (*lane 4*); *E. reedi* (*lane 5*); *E. langebarteli* (*lane 6*); *E. papillata* (*lane 7*); *E. separata* (*lane 8*); *E. nieschulzi* (*lane 9*); *E. falciformis* (*lane 10*); *E. sevilletensis* (*lane 11*); 100-bp molecular-size markers (*outside and center lanes*)

have shown that they are taxonomically and phylogenetically distinct. Identical riboprints also were found for *E. falciformis*, a parasite of *Mus* species, and *E. sevilletensis*, a parasite of *Onychomys* species. The ITS1 sequences of these species indicate that they are closely related (Hnida and Duszynski, unpublished data). Although the sporulated oocysts of these coccidia are morphologically similar, they can readily be distinguished by several other characters (Hnida et al. 1998). Thus, the restriction enzymes used in this study failed to differentiate between the 18S sequences of two pairs of *Eimeria* species, and they detected no intraspecific variation in the multiple isolates of four species, even when these originated from hosts separated by thousands of kilometers (e.g., *E. arizonensis*, *E. falciformis*; Table 1). Other workers have reported the lack of interspecific (e.g., *Nosema* spp., *Glugea* spp.; Pomport-Castillon et al. 1997) and intraspecific (e.g., *Trypanosoma fallisi*, *T. ranarum*; Clark et al. 1995) variation in the riboprints of parasitic protozoa (the former study used 20 restriction enzymes and the latter used 12). In contrast, extensive intraspecific variation has been found in some parasitic protozoa, e.g., *Blastocystis hominis*, *Entamoeba* spp., and *T. cruzi*, which has enabled "ribodemes" to be distinguished within these species (Clark 1997; Clark and Diamond 1997; Clark and Pung 1994). Although

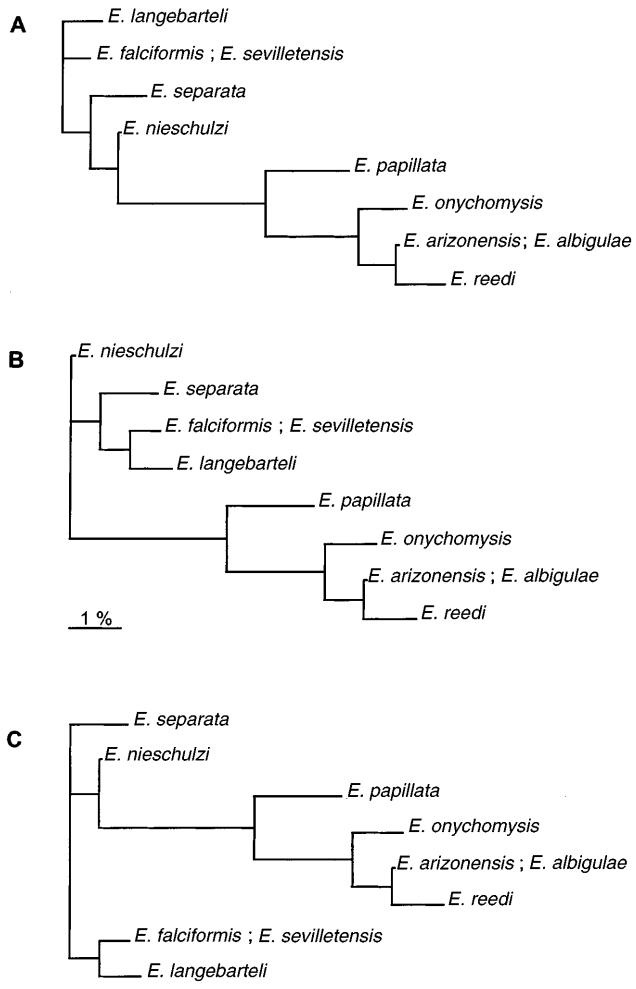

Fig. 2A–C Fitch-Margoliash analyses. Branch lengths are proportional to percentages of divergence. *Bar* 1% divergence. **A** Outgroup (OG) = *E. falciformis*. **B** OG = *E. nieschulzi*. **C** OG = *E. separata*

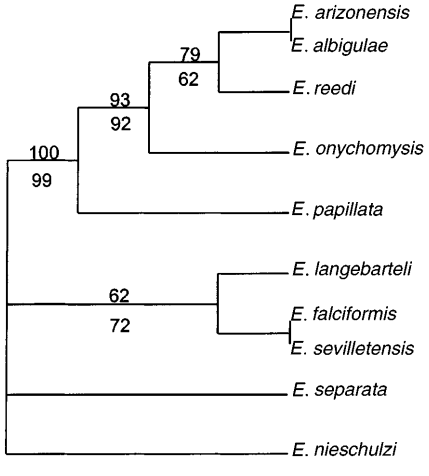

Fig. 3 Consensus tree for Fitch and Dollo parsimony analyses. *Numbers above a line* represent the percentage of 10,000 bootstrap replicates supporting that portion of the tree for the Fitch parsimony analysis; *numbers below a line* represent bootstrap values (10,000 replicates) for the Dollo parsimony analysis

limited to 10 species, our results indicate that riboprinting with a battery of 12 restriction enzymes (Clark 1992, 1997) may provide an "oocyst-efficient" alternative to other molecular-based methods for taxonomic differentiation of some, but not all, *Eimeria* infecting a common host taxon. If riboprinting were done with more restriction enzymes, or in conjunction with denaturing gradient-gel electrophoresis (Dowling et al. 1996; Gasser 1998; Gasser et al. 1996; Stothard et al. 1997), then it should allow more *Eimeria* species and, perhaps, isolates within species to be differentiated.

The RFLP data set was analyzed with phylogenetic methods that have been used in other riboprinting studies (e.g., Clark and Diamond 1997; Clark et al. 1995; De Jonckheere 1994; Pomport-Castillon et al. 1997), and the resulting distance and parsimony trees had similar topologies (Figs. 2, 3). In addition, portions of these phylogenies concur, for the most part, with those of Reduker et al. (1987, Figs. 2, 4), who used *E. nieschulzi* as an outgroup in cladistic analyses of the evolutionary relationships among *E. albigulae*, *E. arizonensis*, *E. langebarteli*, *E. papillata*, and a number of taxa not included in the present study. Reduker et al.'s (1987) analyses differentiated *E. arizonensis* from *E. albigulae*, whereas riboprinting did not; however, the riboprinting data support those authors' conclusion that the two species are closely related. In addition, Reduker et al.'s (1987) phylogenetic trees indicate that *E. arizonensis*, *E. albigulae*, and *E. papillata* belong to a lineage that includes the former two parasites as more highly derived taxa. We obtained the same result in the distance (Fig. 2) and MP analyses, the latter being supported by very high bootstrap values (Fig. 3). Finally, phylogenetic analyses of the ITS1 sequences of these species differentiated *E. albigulae* and *E. arizonensis* and found them to be closely related, highly derived taxa within a clade that includes *E. papillata* (Hnida and Duszynski, unpublished data). Because the results of these studies are congruent and derive from different types of data, there is strong support for the hypothesized evolutionary relationships of *E. arizonensis*, *E. albigulae*, and *E. papillata* (Quicke 1993).

The distance and MP analyses implied that *E. langebarteli* was not a member of the clade comprising *E. papillata*, *E. onychomysis*, *E. arizonensis*, *E. albigulae*, and *E. reedi* (Figs. 2, 3). This result agrees with Reduker et al.'s (1987; Fig. 2) phylogeny of *Eimeria* from murids. In contrast, MP and ML analyses of the ITS1 sequences of these and other rodent eimerian taxa have placed *E. langebarteli* within this clade (Hnida and Duszynski, unpublished data). Additional sequence or RFLP data, derived from genes not linked with the ribosomal gene complex (e.g., mitochondrial genes), could be used to resolve these conflicting hypotheses (Avise 1994; Baverstock and Moritz 1996).

Phylogenetic analyses placed *E. reedi*, which parasitizes heteromyid rodents (Ernst et al. 1970; Ford et al. 1990), within the clade containing *E. arizonensis*, *E. albigulae*, and *E. onychomysis* – a relationship well

supported by bootstrap analyses (Fig. 3; however, the relationships among these taxa should be considered unresolved). This was not expected, because the latter three taxa are parasites of murid rodents (Levine and Ivens 1990). Because the Muridae and Heteromyidae are within the Sciurognathi (Wilson and Reeder 1993) and the *Eimeria* of rodents are considered to be, with some exceptions, genus-specific (Levine and Ivens 1988), we anticipated that *E. reedi* would serve as an outgroup to the *Eimeria* from murids. Although unexpected, this result is probably not spurious because phylogenetic analyses of ITS1 rDNA sequences from the same *Eimeria* species have found that *E. reedi* is closely related to *E. arizonensis*, *E. albigulae*, and *E. onychomysis* (Hnida and Duszynski, unpublished data). Given that the sporulated oocysts of these species are morphologically similar (Upton et al. 1992) and that *E. falciformis* and *E. sevilletensis* appear to be closely related (see above) and have structurally similar sporulated oocysts (Hnida et al. 1998), we suggest that if two or more *Eimeria* species share a common host taxon (species, genus, family, suborder, and, perhaps, order) and their sporulated oocysts are morphologically similar, then the species may be closely related (see Barta et al. (1997) for an example from the *Eimeria* of domestic fowl).

Because riboprinting samples only a portion of the 18S rDNA sequence (Clark 1997), its utility for taxonomic and phylogenetic work with parasitic protozoa is determined by the amount of sequence variation in the taxa of interest. We have found it to be useful for differentiating some taxonomically vexing eimerian species and for studying the evolutionary relationships among a group of *Eimeria* infecting closely related hosts. Overall, the results agreed with prior taxonomic and phylogenetic analyses of these species. Although not without limitations (Clark 1997; Dowling et al. 1996; Hillis et al. 1994), riboprinting is an efficient way to obtain molecular data from samples containing as few as 10,000 oocysts, thus making it a useful method for work with *Eimeria* species that are not routinely passaged through domestic or laboratory animals.

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