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Alexandra Martynova-Van Kley
Stephen F Austin State University, avankley@sfasu.edu

A. Syvyk
Stephen F Austin State University

I. Teplova
Stephen F Austin State University

M. E. Hume
USDA, ARS, Southern Plains Agricultural Research Center

A. Nalian
Stephen F Austin State University

Rapid Detection of Avian *Eimeria* Species Using Denaturing Gradient Gel Electrophoresis

A. Martynova-VanKley,*[1] A. Syvyk,* I. Teplova,* M. Hume,† and A. Nalian*

**Department of Biotechnology, Stephen F. Austin State University, P.O. Box 6132, Nacogdoches, TX 75965; and †USDA, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, College Station, TX, 77845*

ABSTRACT A denaturing gradient gel electrophoresis (DGGE) assay was developed to rapidly discriminate species of avian *Eimeria*. Amplification by PCR of the small subunit ribosomal RNA gene (approximately 1,600 nucleotides) with *Eimeria* genus-specific primers followed by cloning and sequencing allowed us to carry out phylogenetic analyses and identify clone sequences to species level in most cases. Clones were subsequently used to amplify a smaller fragment (approximately 120 nucleotides) suitable for DGGE. The fragments were separated on denaturing gradient gel and bands with unique migration distances were mixed to obtain an identification ladder. The identification ladder and PCR products obtained from DNA extracted from fecal samples from several poultry farms were compared. Applying the DGGE method in this study allowed a rapid differentiation of *Eimeria* species present in fecal samples collected from poultry farms.

Key words: denaturing gradient gel electrophoresis, *Eimeria*, coccidiosis, species identification

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INTRODUCTION

Coccidiosis, caused by the protozoan *Eimeria*, is a common disease in poultry. There are 9 described species of *Eimeria* infecting chickens: *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, *Eimeria tenella*, *Eimeria mivati*, and *Eimeria hagani*. A severe infection may lead to weight loss and sometimes high mortality, depending on the species or strain present. The severity of the disease and clinical characteristics of the infection differ among *Eimeria* species, and precise identification of the species affecting the flock is essential for monitoring and control of coccidiosis and plays a key role in selection of appropriate treatment measures. Existing methods for detection and identification of *Eimeria* species include morphological and physiological evaluation of parasites and their sporulated oocysts (Long et al., 1976), isozyme-based tests (Shirley, 1975), and antibody-based tests. However, these tests are not always accurate, are inconsistent, and have low sensitivity (Long and Joyner, 1984). In recent years, several PCR-based *Eimeria* identification methods have been developed with varying degrees of success and only limited use of field samples. Methods such as amplified fragment length polymorphism (Blake et al., 2003) and random amplified polymorphic DNA (Shirley and Bumstead, 1994) lack reproducibility due to the low specificity of PCR. Multiplex PCR assays (Fernandez et al., 2003) that utilize species-specific primers for internal transcribed spacer (**ITS**)-1 (Su et al., 2003) or ITS-2 (Lien et al., 2007) genes are difficult to apply to multiple samples, especially when birds are simultaneously infected with several species of the parasite and with possibly unknown strains. Other PCR assays, including capillary electrophoresis (Gasser et al., 2005), use genus-specific primers and utilize length variation of the ITS-2 fragment, which is the most variable of the ribosomal RNA (**rRNA**) genes, to differentiate between species. To the best of our knowledge extensive studies investigating genetic diversity of avian *Eimeria* within and among species in field samples using rRNA genes have not been conducted. Therefore, in the current study, we explored *Eimeria* biodiversity based on a more conserved fragment of the 18S rRNA gene and attempted to identify multiple species in field samples using PCR followed by denaturing gradient gel electrophoresis (**DGGE**) with genus-specific primers. Denaturing gradient gel electrophoresis separates DNA fragments of the same length but different composition and allows screening of a large number of samples. The technique has been successfully used in numerous microbiological studies

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[1]Corresponding author: avankley@sfasu.edu

to estimate genetic biodiversity (Walter et al., 2000; Cocolin et al., 2002). Recently, DGGE was also applied to discriminate species of another apicomplexan, *Cryptosporidium* (Satoh and Nakai, 2007). The purpose of this study was to apply the DGGE method to rapidly discriminate among and identify *Eimeria* species. We designed one set of *Eimeria* genus-specific primers that amplify a small fragment (approximately 120 nucleotides) of the small subunit (**SSU**) rRNA gene. Subsequently, we used DGGE to separate the fragments based on their nucleotide composition and identified several bands to species level by phylogenetic analysis of the nearly complete SSU rRNA gene (approximately 1,600 nucleotides).

MATERIALS AND METHODS

Field Samples and DNA Extraction

Individual droppings from ten 30-d-old chickens from 10 poultry houses in east Texas were collected and stored at 4°C to prevent DNA degradation. The samples from each poultry house were pooled and 1 g of pooled sample was washed and vortexed with glass beads (710 to 1,180 μm, Sigma, Oakville, Ontario, Canada) for 15 min twice in 5 mL of water and then filtered through cheese cloth. After centrifugation, the samples were resuspended in stool lysis buffer. To extract the DNA we used the QIAamp DNA Stool Mini Kit (Qiagen Inc., Mississauga, Ontario, Canada) following the instructions of the manufacturer.

Primer Design

To design *Eimeria* genus-specific primers to amplify a fragment suitable for DGGE, 27 sequences of the nearly complete SSU rRNA gene were obtained from GenBank (Benson et al., 2006). The GenBank accession numbers and the species names of the sequences used in this study were as follows: *E. acervulina* (DQ136187.1 and DQ538351.1), *Eimeria bovis* (U77084.1), *E. brunetti* (U67116.1), *E. maxima* (DQ136186.1, DQ538350.1, EF210322.1, U67117.1, DQ538348.1, DQ640012.1, DQ538349.1, and EF122251.2), *E. mitis* (U40262.1), *E. mivati* (U76748.1), *E. necatrix* (DQ136185.1 and U67119.1), *E. praecox* (U67120.1), and *E. tenella* (EF210325.1, DQ136181.1, AF026388.1, DQ640011.1, U40264.1, DQ136183.1, DQ136184.1, DQ136180.1, DQ136179.1, and EF210326.1). Sequences were aligned with ClustalW (Thompson et al., 1994) and used to calculate the theoretical melting profiles with MELT94 software (Michikawa et al., 1997). The melting profiles and the multiple sequence alignment were overlaid to select a fragment suitable for DGGE analysis. The fragment was required to have a single melting domain and to be flanked by conserved regions to accommodate annealing of genus-specific DGGE primers. In addition, multiple sequence alignment was used to design another pair of *Eimeria* genus-specific SSU primers to amplify the nearly complete SSU rRNA gene for phylogenetic analysis.

PCR and Cloning of the Nearly Complete SSU rRNA Gene

To obtain PCR products suitable for cloning and sequencing, we carried out PCR with cloned Pfu DNA Polymerase AD (Stratagene Inc., La Jolla, CA) and *Eimeria* genus-specific SSU primers: forward primer, 5′-TTG TCT CAA AGA TTA AGC C-3′; reverse primer, 5′-AGC GAC GGG CGG TGT GTA CAA-3′. The extracted DNA (3 to 30 ng) with the SSU primer pair (0.5 μ*M*) was used to amplify a long fragment (~1,600 bp) in a final volume of 25 μL. The PCR program included an initial denaturation cycle of 95°C for 3 min followed by 25 cycles of denaturation at 94°C for 35 s, annealing at 56°C for 25 s, and an extension at 72°C for 2 min. The final extension step was increased to 4 min at 72°C. The PCR product was cloned with a pPCR Script-Amp cloning kit (Stratagene Inc.) into a pPCR Script-Amp SK(+) vector according to the manufacturer's protocol (Stratagene Inc.). The vector was transformed into *Escherichia coli* XL10 competent cells (Stratagene Inc.). Selection of transformants was done by blue-white screening. Colony PCR was performed for 350 clones to amplify the SSU rDNA insert. The screening yielded 58 positive transformants. Thirty randomly chosen clones were sent for sequencing. Two clones that failed to sequence and 5 clones that were only partially sequenced were not included in further analysis.

Sequence Analysis

Both strands of each of the clones were sequenced by Amplicon Express (Pullman, WA). The chromatograms obtained were assembled with the STADEN package (Staden et al., 2000). Sequences were checked for contamination using the VecScreen (http://www.ncbi.nlm.nih.gov/ VecScreen/) and segments of vector origin were removed manually. The sequences were deposited in GenBank under accession numbers EU044765–85. A search by BLAST (Altschul et al., 1990) was conducted against the GenBank database and the top 5 matching sequences for every clone were retrieved. The sequences were combined and after excluding redundant entries, aligned by ClustalW 1.81 (Thompson et al., 1994). Multiple alignment parameters such as gap opening and extension penalties available in ClustalW were modified to improve the quality of the alignment. The final alignment was manually examined for obvious errors. The alignment and additional information is available at http://biotech.sfasu.edu/bt/EimeriaID/. Phylogenetic trees were constructed by using maximum likelihood (**ML**) and Bayesian inference (**BI**) methods. The ML method was carried out in PHYML (Guindon and Gascuel, 2003) and Bayesian inference in MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). For both analyses

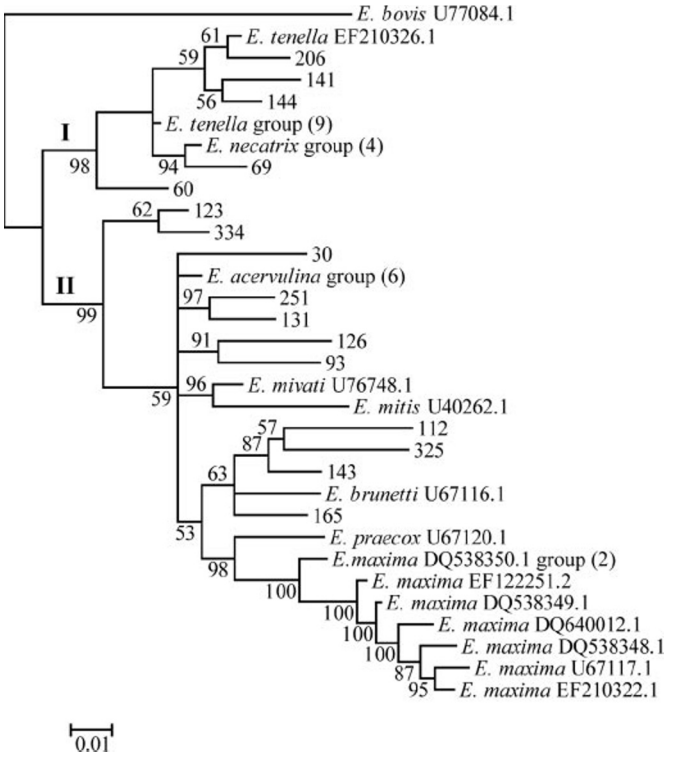

Figure 1. Bayesian inference 50% consensus trees from nearly complete *Eimeria* small subunit (SSU) ribosomal RNA genes. The numbers at the nodes are posterior probabilities expressed as percentages. Scale bar = 0.01 substitution per base. The numeral in parentheses represents the number of sequences present in the group. *Eimeria tenella* group: EF210325.1, DQ136181.1, AF026388.1, DQ640011.1, U40264.1, DQ136183.1, DQ136184.1, DQ136180.1, and DQ136179.1; *Eimeria necatrix* group: DQ136185.1, U67119.1, clones 200 and 176; *Eimeria acervulina* group: DQ136187.1, DQ538351.1, clones 151, 1, 21, and 95; *Eimeria maxima* group: DQ538350.1, DQ136186.1.

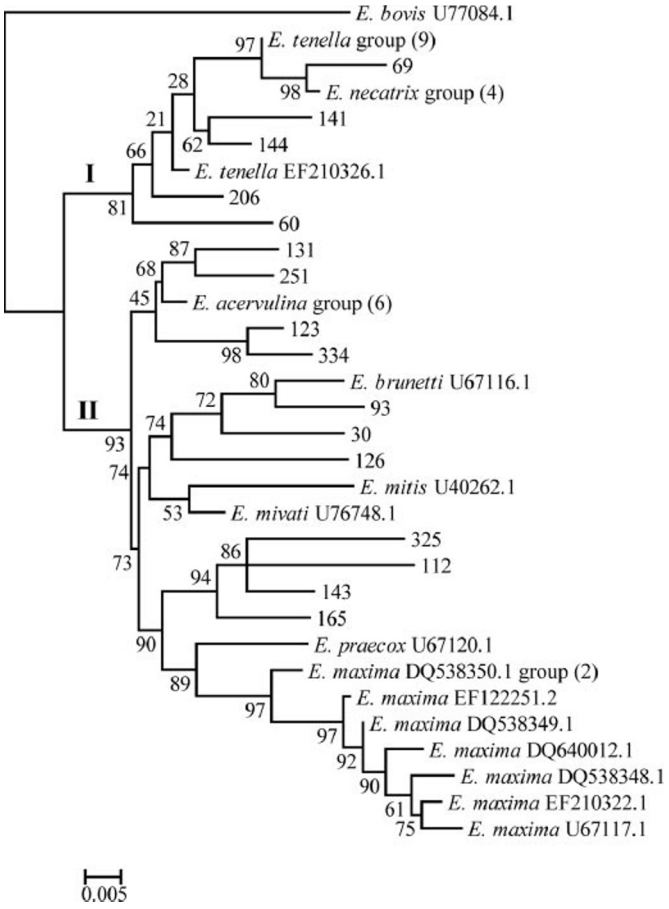

Figure 2. Maximum likelihood tree of *Eimeria* small subunit (SSU) ribosomal RNA genes constructed using Phyml; *Eimeria bovis* was used as the outgroup. The robustness of species groups was assessed using the approximate likelihood ratio test method; the numerals above or below branches represent the nonparametric branch support based on the Shimodaira-Hasegawa-like procedure. Scale bar = 0.005 substitution per base. The number of sequences in that group is given in parentheses. *Eimeria tenella* group: EF210325.1, DQ136181.1, AF026388.1, DQ640011.1, U40264.1, DQ136183.1, DQ136184.1, DQ136180.1, and DQ136179.1; *Eimeria necatrix* group: DQ136185.1, U67119.1, clones 200 and 176; *Eimeria acervulina* group: DQ136187.1, DQ538351.1, clones 151, 1, 21, and 95; *Eimeria maxima* group: DQ538350.1, DQ136186.1.

we used a general-time-reversible model for nucleotide substitution allowing both programs to estimate the proportion of invariable sites and the gamma distribution parameter. The BIONJ option was selected to generate an initial tree for the ML method and the BI was started with a random tree. The reliability of the ML tree was estimated by the approximate likelihood ratio test method (Anisimova and Gascuel, 2006). For the BI analysis, 4 Markov chains were run for 5,000,000 generations and sampled every 1,000 generations. After elimination of the first 25% of the trees as burn-in, 3,750 trees were used to generate a 50% majority-rule consensus-tree.

DGGE Identification Ladder Preparation

The 23 successfully sequenced plasmids were used to carry out PCR (final volume of 50 µL). One microliter of each purified recombinant plasmid was used as template to amplify an ~120-bp fragment with the genus-specific DGGE primers (0.5 µ*M*) that were previously designed: forward primer containing GC-clamp 5′-GCC CGC CGC GCC CGC GCC CGT CCC GCC GCC CCC GCC CGG ATT AGA TAC AAA ACC AAC CC-3′, and reverse primer 5′-GCT GAT AGG TCA GAA ACT TG-3′. The amplification process was performed using 25 µL of JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma Chemical Co., St. Louis, MO) in a Mastercycler (Eppendorf Scientific Inc., Westbury, NY) according to the following program: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 60°C for 35 s, and an extension at 72°C for 2 min. A final extension was performed at 72°C for 7 min. Three microliters of each PCR product was tested on a 2% agarose gel. The remaining 42 µL was mixed to obtain the DGGE identification ladder.

DGGE Analysis of Field Samples

Five microliters of DNA extracted from field samples was used to perform PCR with the pair of DGGE primers using the same PCR conditions described above.

The electrophoresis was performed both with the PCR products (20 µL) and the DGGE identification ladder (20 µL) for 4.5 h at 250 V in 1 × Tris-acetate-EDTA (TAE) buffer at 60°C using a DGGE-2001 apparatus (CBS Scientific Co., Del Mar, CA). Polyacrylamide gels (8%, 0.75-mm thick) were prepared with a denaturing gradient of urea-formamide mix (Myers et al., 1987) ranging from 35 to 50%. After electrophoresis, the gels were stained with SYBR Green I (Invitrogen Inc., Carlsbad, CA) for 30 min and subsequently photographed using a BioRad Imager System equipped with a Gel Doc XR camera and Quantity-One software (Bio-Rad Inc., Hercules, CA).

RESULTS

Species Identification and Phylogenetic Analysis

To identify the species of *Eimeria* present in field samples we cloned and sequenced 22 nearly complete SSU rRNA genes. The BLAST queries against GenBank identified several related sequences of *Eimeria*. However, we could not identify all sequences to species level. For example, a query of clone 93 returned an *E. brunetti* sequence with 98% identity (1,565/1,587) to our sequence as the first match and *E. acervulina* with 98% (1,559/1,591) as the second match. We attempted to clarify relationships among the sequences by using a character-based phylogenetic approach in which phylogenetic trees were inferred by ML and BI methods. All trees were rooted with the outgroup species of *E. bovis* (Figures 1 and 2). As expected, the avian *Eimeria* species formed a monophyletic clade in both trees and addition of our sequences did not substantively change the previously published tree topology (Barta et al., 1997). In the first clade (Figures 1 and 2), marked by Roman numeral I, there was a high likelihood for a close relationship between *E. necatrix* and *E. tenella*. Both analyses grouped *E. tenella* sequences with the sequence of *E. necatrix*; however, the branching patterns within the clade were less resolved. In the ML tree *E. necatrix* appeared to be more closely related to *E. tenella* (EF210325.1) by excluding the *E. tenella* (EF210326.1) sequence. The BI analysis did not resolve the branching pattern in this clade and collapsed to polytomy. Nonetheless, both phylogenetic trees showed affiliation of clone 69 with *E. necatrix* and clones 141, 144, and 206 with *E. tenella* (EF210326.1). In the second clade (II), *E. maxima* was strongly affiliated with *E. praecox* and *E. mitis* with *E. mivati*. However, the position of *E. brunetti* (U67116.1) according to ML was different from that resulting from BI analyses. The BI analysis supported identification of clones 112, 143, 165, and 325 by grouping them with the *E. brunetti* sequence, whereas other clones collapsed in an unresolved polytomy. The ML tree provided a different affiliation for *E. brunetti*; it was grouped with clones 30, 93, and 126, and clones 131 and 251 were associated with *E. acervulina*.

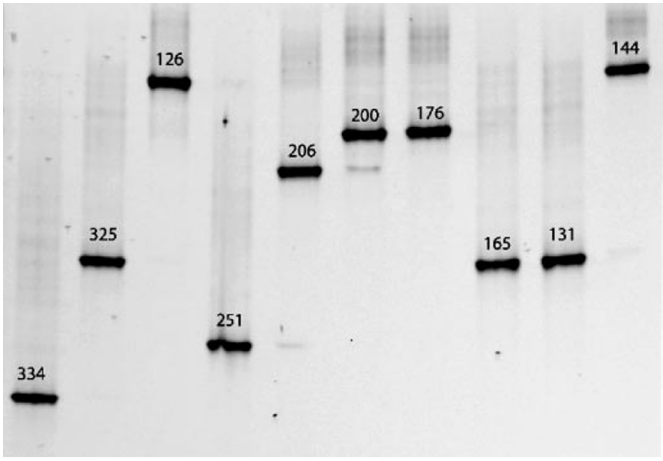

Figure 3. Example of denaturing gradient gel electrophoresis analysis (35 to 50% denaturant) of products obtained from amplification of *Eimeria* clones. Clones were identified by phylogenetic analysis as follows: 200, 176 = *Eimeria necatrix*; 141, 206 = *Eimeria tenella*. Clones 165 and 325 were identified differently by Bayesian inference (BI) and maximum likelihood methods as *Eimeria brunetti* and *Eimeria maxima*, respectively. Clones 131 and 251 were identified as *Eimeria acervulina* and 126 as *E. brunetti* by BI method only. Clone 334 was determined to be a chimeric artifact.

Clones 123 and 334 were excluded from the first clade by both phylogenetic trees but did not specifically affiliate with any other clades. These clones were identified as possible chimeric products when the sequences obtained from both the 5′ and 3′ regions of the clones were examined. Sequence analysis of 900 nucleotides from the 5′ end of these clones revealed that this portion of the sequences was closely related to *E. acervulina* sequences (data not shown). Sequences of the remaining 697 nucleotides from the 3′ end of the clones suggested strong affiliation to *E. necatrix* (DQ136185.1). The clone 123 sequence was nearly identical to that of clone 334 with only 3 mismatches (data not shown).

In addition, pairwise comparison of the portion of the sequences that corresponds to the DGGE fragment revealed that following clones and GenBank sequences are identical: clone 206 and *E. tenella* (EF210326.1); clones 144 and 126 and *E. tenella* (EF210325.1, DQ136181.1, AF026388.1, DQ640011.1, U40264.1, DQ136183.1, DQ136184.1, DQ136180.1, and DQ136179.1); clones 112, 176, and 200, and *E. necatrix* (DQ136185.1 and U67119.1); clones 1, 151, 21, 95, and 123 and *E. acervulina* (DQ136187.1 and DQ538351.1). With the exception of clones 123, 126, and 112, species identification by fragment sequence alone was supported by both phylogenetic analyses. The DGGE fragment sequence of clones 131, 325, and 165 was identical to that of *E. maxima* (DQ538350.1 and DQ136186.1) and the sequence of clone 93 to *E. maxima* (EF122251.2).

In conclusion, from sequences of 22 clones, 2 sequences were identified as chimeric, 11 were unambiguously

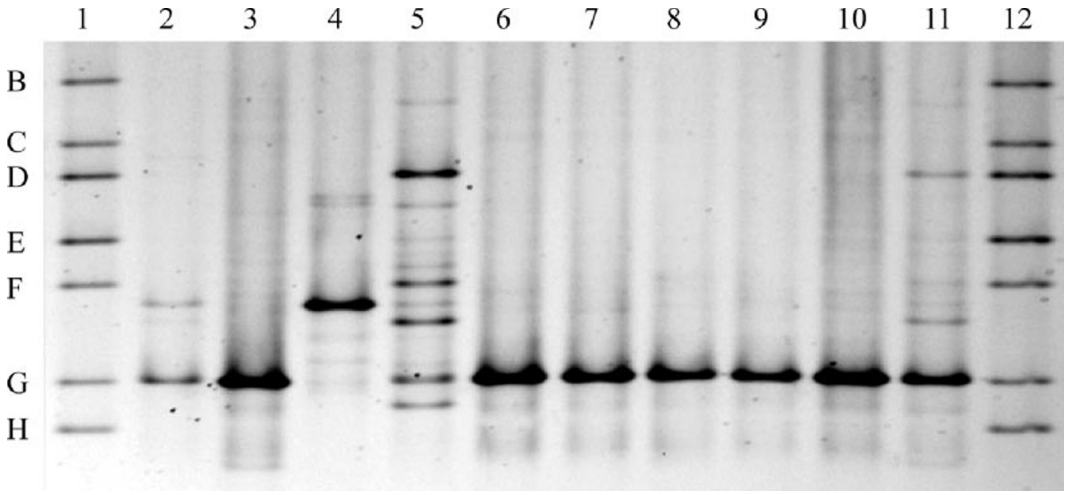

Figure 4. Example of *Eimeria* small subunit (SSU) ribosomal RNA fragment profiles collected from 10 poultry farms in east Texas. Lanes 1 and 2 contain the identification ladder: B = *Eimeria tenella*, C = *Eimeria necatrix*, D = *E. tenella*, E = *Eimeria brunetti*, F = *E. brunetti*, G = *E. tenella*/*Eimeria acervulina*, and H = unidentified.

identified to species, and the remaining 9 were identified differently by ML and BI methods. The summary of the results obtained from phylogenetic analysis is shown in Table 1.

PCR-DGGE Analysis

Based on multiple sequence alignment of 8 avian *Eimeria* SSU rDNA sequences from GenBank, a genus-specific primer set was designed to amplify a 114- to 118-bp fragment. The forward primer had a single nucleotide mismatch in its priming site with the sequences from *E. mitis* and *E. maxima*. In contrast, the reverse primer was completely homologous to all reference sequences. The fragment contained significant differences in the sequences of the species of avian *Eimeria* indicating the feasibility of using this fragment for species identification. The PCR amplification carried out with the set of primers was successful in all tested samples, which included cloned SSU rDNA as well as DNA extracted from fecal field samples. The expected size of the PCR-amplified fragments was verified on agarose gel (data not shown). To test the specificity of the primers we carried out a PCR with template DNA extracted from fecal samples obtained from coccidia-free 2-d-old chicks: no PCR product was obtained (data not shown). The PCR products obtained from amplification of clones were subjected to DGGE analysis. An example of a DGGE profile is shown in Figure 3. For 22 sequenced clones we obtained 9 sequence-specific migrations on the 35 to 50% gradient gel. Clone 334 was disregarded, because both BI and ML analyses failed to identify its phylogenetic position. Clone 30 was also disregarded, because its position in the BI tree was unresolved and it was the only sequence that exhibited band position A. Sequences that could not be identified or were identified differently by BI and ML methods were disregarded for identification of ladder bands. The PCR products from other clones were mixed to obtain an identification ladder with 7 bands (Figure 4; lanes 1 and 12) and were identified as follows: band B = *E. tenella*; C = *E. necatrix*; D = *E. tenella*; E = *E. maxima*/*E. praecox*/*E. brunetti*; F = *E. maxima*/*E. praecox*/*E. brunetti*; G = *E. tenella*/*E. acervulina*; H = *E. acervulina*.

The DNA samples extracted from fecal field samples obtained from 10 east Texas farms with different histories of coccidiostat and vaccine use were subjected to PCR-DGGE analysis. The gel in Figure 4 includes the identification ladder on both sides of the gel to aid interpretation of the results. Comparison of the DGGE band profiles shows that this method can indeed detect differences in populations of *Eimeria* present at different farms. Comparison of migration distances of fragments enabled identification of several bands to species level. Samples 3, 6, 7, 8, 9, and 10 contained a dense band that corresponded to band G in the identification ladder. Six clones that exhibited the same migration distance as band G (*E. tenella*/*E. acervulina*) were sequenced (Table 1). Four sequences were unambiguously identified as *E. acervulina* and 2 sequences as *E. tenella*. The high frequency of *E. acervulina* can be explained by the administration of *E. acervulina*-specific vaccine in those farms. Sample 5 had the most diverse variety of fragments, 2 of which corresponded to bands D and F of the identification ladder. The same bands were present in sample 11. The bands in the ladder corresponded to *E. tenella* and *E. maxima*/*E. brunetti* respectively. Sample 4 contained another very dense band that did not correspond to any band in the identification ladder. The same fragment (more faint) was present in samples 2 and 5; however, excision and sequencing of the ~120-bp fragment did not provide a reliable phylogenetic identification. Other faint frag-

Table 1. Comparison of identification results obtained from Bayesian inference (BI) and maximum likelihood (ML) analysis for 22 sequenced *Eimeria* clones[1]

Band ID	Clone no.	BI identification	ML identification
A	30	—	*Eimeria brunetti*
B	144	*Eimeria tenella*	*E. tenella*
	126	—	*E. brunetti*
C	200†	*Eimeria necatrix*	*E. necatrix*
	176†	*E. necatrix*	*E. necatrix*
	69	*E. necatrix*	*E. necatrix*
	112	*E. brunetti*	*Eimeria maxima/Eimeria praecox*
D	206	*E. tenella*	*E. tenella*
E	143	*E. brunetti*	*E. maxima /E. praecox*
	165	*E. brunetti*	*E. maxima /E. praecox*
	325	*E. brunetti*	*E. maxima /E. praecox*
F	131	—	*Eimeria acervulina*
G	141*	*E. tenella*	*E. tenella*
	60*	*E. tenella*	*E. tenella*
	93	—	*E. brunetti*
	123	—	—
	151‡	*E. acervulina*	*E. acervulina*
	1‡	*E. acervulina*	*E. acervulina*
	21‡	*E. acervulina*	*E. acervulina*
	95‡	*E. acervulina*	*E. acervulina*
H	251	—	*E. acervulina*
I	334	—	—

[1]Clones marked with †, *, and ‡ had identical sequences as determined by pairwise comparison.

ments, which can be observed on the gel, are probably PCR artifacts (Mathieu-Daude et al., 1996).

DISCUSSION

To our knowledge this is the first molecular study aimed at identification of avian *Eimeria* from field samples using the PCR-DGGE approach. Our study provides clear evidence that the PCR-DGGE approach has potential to be used for identification of *Eimeria* taxa. We have identified a single melting domain region suitable for DGGE analysis that showed *Eimeria* population differences between 10 sampled farms. It is important to note that comparison of selected DGGE fragment sequences of SSU rRNA genes from GenBank showed differences in sequence composition for each species. These differences suggested the possibility of using the selected fragment for DGGE-based *Eimeria* species identification. However, during this study we found several clones that exhibited identical migration distances on 35 to 50% DGGE, but were identified as different species by phylogenetic analyses. For example, clones 200 and 69 had identical migration distances on DGGE but were identified as *E. necatrix* and *E. brunetti*, respectively. Comparison of their respective fragment sequences showed only a single nucleotide mismatch. Similarly, co-migrating clones 141 and 151 were identified as *E. tenella* and *E. acervulina*, respectively, and had 4 nucleotide mismatches. These bands could possibly be resolved by performing DGGE on a different gradient of denaturants. Although topologies of both phylogenetic trees were largely the same, some of the clones were grouped differently. Notably, clones 112, 143, 165, and 325 were grouped to the single *E. brunetti* sequence available in GenBank by BI analysis (Figure 1), whereas the ML method showed strong affiliation among the clone sequences and excluded the sequence of *E. brunetti* (Figure 2). Additional sequences of SSU rRNA from *E. brunetti* could clarify phylogenetic relationships among these field isolates. Comparison of profiles from field samples (Figure 4) showed that the identification ladder has an acceptable range on the gel and allowed rapid evaluation of diversity of *Eimeria* in the samples. Further cloning and sequencing is required to identify major bands that displayed different migration distances compared with bands in the ladder. Minor bands could represent either unidentified strains or be an artifact of PCR-DGGE such as the heteroduplexes commonly found in mixed-template samples. In conclusion, the results of this study demonstrate the utility of this approach for rapid discrimination of *Eimeria* species in field samples and its possible use as a "coccidiosis load" monitoring tool despite the problems of conclusive species identification. This study also suggests that a multi-gene sequencing approach for the identification of avian *Eimeria* species may lead to better discrimination of samples at the species or strain level. Consensus analysis with combined sequencing data could also lead to identification of drug-resistant strains and possibly to a re-evaluation of traditional species circumscriptions.

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