Cloning and nucleotide sequencing of the second internal transcribed spacer of ribosomal DNA for three species of *Eimeria* from chickens in Taiwan


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Abstract

Coccidiosis of chickens caused by protozoan parasites of the genus *Eimeria* (Coccidia: Eimeriidae) is an enteric disease that results in great economic losses throughout the world, including Taiwan. Using polymerase chain reaction (PCR) with primers specific for the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA), three species of *Eimeria*, *E. tenella*, *E. maxima*, and *E. acervulina* have been successfully characterised from chickens in Taiwan. The sizes of PCR products from various isolates representing these three species were between 370 and 580 base pairs (bp). After cloning and sequencing of the PCR products, high nucleotide sequence identity (96.8–100%) was observed within a species. In addition, ITS-2 nucleotide sequences for *E. tenella* had higher homology (98.5–99.3%) than *E. maxima* (81.6–96.5%) when compared with appropriate sequences deposited in GenBank. To our knowledge, this is the first report of a 412-bp ITS-2 sequence for *E. acervulina* from chickens.

Keywords: Coccidiosis; Eimeria; Ribosomal DNA; Nucleotide sequencing; Internal transcribed spacer

1. Introduction

Coccidiosis in chickens is a worldwide disease caused by protozoan parasites of the genus *Eimeria*. It is responsible for significant economic losses to the livestock and poultry industries (Fernandez et al., 2003). Currently, seven species of *Eimeria*, *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis* are recognised as pathogenic to chickens (McDougal and Reid, 1997). Identification of the species has important implications for the diagnosis and control of the disease, as well as for studying their epidemiology and population biology (Woods et al., 2000b). However, conventional methods of identification, based on physiological and morphological features of the sporulated oocysts, require skilled personnel and have limitations due to overlap of characteristics among different species (Long and Joyner, 1984). For example, identification of *E. maxima* and *E. brunetti* based on oocyst shape and size may be unreliable as these two features can be the same or very similar between the two species (Woods et al., 2000b).

Molecular approaches for improving the accuracy of *Eimeria* species identification have been developed to overcome the limitations of traditional methods (Woods et al., 2000b). One such molecular approach involves analysing enzyme variation through electrophoresis...
and has been reported to be useful for the identification of species, strains and clones of *Eimeria* (Johnston and Fernando, 1997). However, this technique provides a limited number of variable enzymes and low level of polymorphisms and it is difficult to apply to a large number of samples (Fernandez et al., 2003). Another approach involves the random amplified polymorphic DNA (RAPD) technique based on the amplification of anonymous targets and the use of arbitrary primers (Welsh and McClelland, 1990; Williams et al., 1990). This generates fingerprints of multiple bands but does not yield reproducible results due to low specificity during polymerase chain reaction (PCR) (MacPherson et al., 1993).

In eukaryotic cells, multiple copies of the highly conserved ribosomal RNA (rRNA) genes are located in a tandem arranged series in which each gene is separated from the next by spacer DNA regions. A single cluster contains the rRNA genes for 18S, 5.8S, and 28S rRNA molecules that are separated by internal transcribed spacers (ITS-1 and ITS-2). Adjacent clusters are separated by external transcribed spacer (ETS) regions. Sequencing of rDNA regions including the small subunit 5S and ITS have provided genetic markers for the identification of *Eimeria* species (Stucki et al., 1993; Barta et al., 1997; Schnitzler et al., 1998). Some of these genetic markers have been applied to developing diagnostic assays and, in particular, primers specific for ITS-1 have been developed for PCR assays for the detection of *Eimeria* species in faecal and intestinal samples (Schnitzler et al., 1998, 1999). The rDNA ITS-2 has recently been sequenced for *E. tenella* and *E. maxima* (Barta et al., 1998, GenBank Accession Nos. AF026388 and AF027722-AF027726) and also appears in the rDNA region of *E. acervulina* (Schnitzler et al., 1999). The rDNA ITS-2 was identified by their ITS-2 rDNA and report here the first ITS-2 sequence of *E. acervulina* (GenBank Accession No. AJ742227).

2. Materials and methods

2.1. Parasites

Parasite populations were obtained from different geographical regions of Taiwan during a disease outbreak in 2001–2003. Faeces or intestinal contents from hosts were processed in 2.5% (w/v) K$_2$Cr$_2$O$_7$ at 28°C for 72 h to allow oocyst sporulation as described by Duszynski and Wilber (1997). Oocysts were isolated by centrifugation in a saturated NaCl solution (Shirley, 1995), washed three times in 30 mL of sterile deionised water, and purified using a sucrose-gradient centrifugation method (Gasser et al., 1987). Based on the morphology (i.e., size, shape and colour) of sporulated oocysts, pre-patent period, and locations of gross lesions in the intestines (Gasser et al., 2001), *E. tenella*, *E. maxima* and *E. acervulina* were identified (data not shown).

2.2. Parasite propagation

One-day-old Arbor Acres chicks were supplied by a commercial hatchery in Southern Taiwan and raised in a coccidia-free environment with an ad libitum supply of filtered water and food that was free of anti-coccidial agents and antibiotics. At two weeks of age, the chicks were transferred to wire-mesh cages that were cleaned with water at 80°C and sterilised at 180°C for 10 min. The animals were checked for the absence of coccidia by faecal examinations between one day and three weeks of age and immediately before experimental infection. At three weeks of age, the chicks were orally infected with a single oocyst based on the procedures suggested by Fernandez et al. (2003).

Experimental procedures employing animals followed the National Pingtung University of Science and Technology Guidelines for the care and use of animals for research purposes.

2.3. Genomic DNA extraction

The *Eimeria* isolates obtained from infected chickens were identified, purified and propagated as described above. Genomic DNA was isolated from oocysts using the methods reported by Fernandez et al. (2003) with some modifications. In brief, approximately 5–6×10$^7$ purified oocysts were cleaned with 5.75% sodium hypochlorite solution at 4°C for 20 min, washed twice with sterile deionised water and resuspended in lysis buffer (600 mM EDTA, 1.3% N-lauroylsarcosine, 2 mg/mL proteinase K, pH 9.5) at 65°C for 45 min. The lysate was then mixed with cetlytrimethyl ammonium bromide (CTAB) buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% 2-mercapto-ethanol, 20 mM EDTA, 100 mM Tris–Cl, pH 8.0], incubated at 60°C for 1 h, and added to an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove proteins. These CTAB-treated mixtures were centrifuged at 13,000 g for 15 min and an equal volume of chloroform was added to the supernatant which was then re-centrifuged. The supernatant was mixed with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol to precipitate DNA by centrifugation at 13,000 g for 10 min. The DNA pellet was washed with 70% ethanol, resuspended in TE (10 mM Tris–Cl, pH 8.0, 0.1 mM EDTA), and quantified and visualised as described elsewhere (Fernandez et al., 2003).
2.4. PCR amplification and gel electrophoresis

Genomic DNA isolated from oocysts of infected chickens was used as a template to amplify the ITS-2 by PCR performed in 50 µL of solution containing 50 ng of template DNA, 2.5 mM dNTP, 1 U Taq DNA polymerase (Promega), 1× reaction buffer [10× stock solution: 500 mM KCl, 100 mM Tris–Cl pH 8.3, 15 mM MgCl₂ and 0.01% (w/v) gelatin], and 25 µM each of WW2 (forward: 5’-ACGTCTGTTCAGTGTCT-3’) and WW4r primers (reverse: 5’-AAATTCAGCGGTAACCTCG-3’) under the following conditions: 94 °C – 30 s (denaturation), 55 °C – 30 s (annealing) and 94 °C – 30 s (extension) for 30 cycles.

The WW2 and WW4r primers were previously used to amplify the ITS-2 of E. tenella and E. maxima as described by Woods et al. (2000b). For E. tenella, these primers were used to anneal to the 5.8S and 28S rRNA (GenBank Accession Nos. AF026388 and AF026388, respectively) and the expected size of ITS-2 is 550 base pairs (bp). For E. maxima, the WW2 primer was used to anneal to the 5.8S rRNA (GenBank Accession Nos. AF027722, AF027723, AF027724, AF027725, and AF027726); whilst the WW4r primer was used to anneal to the 28S rRNA (GenBank Accession Nos. AF027722, AF027723, AF027724, AF027725, and AF027726). The expected sizes of ITS-2 for E. maxima are 338, 345, 369, 345 and 342 bp. Using WW2 and WW4r to anneal to 5.8S and 28S rRNA, respectively, the expected size of ITS-2 for E. maxima is 342 bp.

PCR products were detected in 2.5% agarose-TBE (100 mM Tris, pH 8.0, 90 mM borate and 1 mM EDTA) gel, stained with ethidium bromide and photographed. The size markers used to estimate PCR products were the Bio-100 DNA ladders (Protech Inc.).

2.5. Cloning and nucleotide sequencing of the ITS-2 rDNA

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc.) following the manufacturer’s recommendations. Purified PCR products were cloned into 3015 bp of the pGEM-T Easy Vector (Promega). The ligated product was transformed into Escherichia coli INVαF competent cells (Invitrogen), and the transformed cells were allowed to recover in SOC medium (Invitrogen). These recovered cells were plated onto LB agar (MBBio) plates containing ampicillin, IPTG (MBBio) and X-Gal (MBBio) to facilitate blue/white selection of transformants. Fifteen white colonies were randomly selected from each agar plate and inoculated in a LB medium (MBBio) containing ampicillin, and the plasmid DNA was purified as previously described (Su et al., 2004) and then digested with EcoRI (Promega) (Sambrook et al., 1989) to verify whether the plasmid DNA contains the ITS-2 insert.

Plasmids from positive colonies were purified using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced by MDBio. The complete nucleotide sequence data of the recombinant plasmids were determined by aligning both strands of sequence data using the DNASTAR Program (Version 2.5, Lynnon BioSoft) and tested for homology to known sequences in the GenBank databases using a basic local alignment search tool (BLAST) (Altschul et al., 1990). The phylogenetic relationship between Eimeria species isolated from Taiwan and other geographic areas was analysed according to the percentages of similarity and difference for ITS-2 sequences revealed by the BLAST search. Sequence data were compared and the phylogenetic trees were generated with the MegAlign program (DNASTAR Inc.) using the J. Hein method with weight residue weight table in the program. Analysis of the phylogenetic trees was based on the length of each pair of branches that represents the distance between sequence pairs. The second pair may present and switch the view between balanced branches or unbalanced branches (DNASTAR Inc.). With the balanced display, the distances between ancestors in the tree can be averaged. Dotted lines seen in the balanced view indicate a negative branch length: that is, one introduced by averaging the tree. In the unbalanced view, branch distances correspond to sequence divergence (DNASTAR Inc.).

3. Results

3.1. PCR amplification of Eimeria ITS-2

A total of nine isolates representing three different Eimeria species, E. tenella, E. acervulina, and E. maxima were purified from field samples in Taiwan and propagated from single-oocyst derived infections. Fig. 1 shows the PCR amplification of ITS-2 from seven isolates representing these three species, E. tenella (lanes 5–7), E. acervulina (lanes 3 and 4), and E. maxima (lanes 1 and 2). Based on the observation after ethidium bromide staining under the UV light, the other two isolates representing each of E. acervulina and E. maxima show similar sizes to those of their corresponding species on lanes 3–4 and 1–2 (Fig. 1), respectively. However, the DNA bands produced from these two isolates were faint, could not be detected on the photographs, and were thus excluded from Fig. 1. No variation in size or number of bands was detected among isolates representing each species (Fig. 1). In addition, there was only one band for the ITS-2 of E. maxima, E. acervulina, and E. tenella (Fig. 1, lanes 1–7).
3.2. Cloning and nucleotide sequence analysis of PCR products

To determine the nucleotide sequences of the ITS-2 amplicon, the PCR products of the ITS-2 were purified and cloned. After cloning into the pGEM vector (Promega), the released ITS-2 inserts representing *E. tenella* (Fig. 2, lanes 7–9), *E. acervulina* (lanes 1–3), and *E. maxima* (lanes 4–6) were similar in sizes to their corresponding ITS-2 amplicons (Fig. 1). Both strands of the ITS-2 amplicons were sequenced and their sequence alignment showed 100% complementarity between the two strands (data not shown). Different colonies isolated from the same agar plate representing each ITS-2 amplicon showed 100% identity in their nucleotide sequences (Table 2), suggesting no difference in sequences within an ITS-2 amplicon.

Nucleotide sequence analysis of ITS-2 regions revealed high homology of 96.8%, 98.0%, and 98.3% between three isolates of *E. maxima*. The ITS-2 sequences determined for isolates of *E. maxima* showed homology of 81.6–96.5% with appropriate sequences deposited in GenBank (GenBank Accession Nos. AF027722-AF027726) (Gasser et al., 2001; Woods et al., 2000a) (Table 1). Phylogenetic analysis showed that the *E. maxima* Guelph strain from Canada (GenBank Accession No. AF027726) had the greatest homology to the Taiwan isolates (Fig. 3). The results of sequencing showed homology of 98.9%, 98.9%, and 99.6% between the three isolates of *E. tenella* from Taiwan. The ITS-2 sequences from these three isolates (isolates 1–3) and *E. tenella* Houghton strain (GenBank Accession No. AF026388) showed 98.5%, 99.3%, and 99.3% homology, respectively (Table 1), suggesting they have close phylogenetic relationships. Construction of a phylogenetic tree places *E. tenella* isolates 2 and 3 from Taiwan and Houghton strain on the same branch (Fig. 4).

![Fig. 1. Agarose gel showing ITS-2 PCR products representing *Eimeria maxima* (lanes 1–2), *E. acervulina* (lanes 3–4), and *E. tenella* (lanes 5–7). Lane M represents a 100-bp DNA size marker (Protech Inc.).](image1)

![Fig. 2. Cloning of ITS-2 PCR products into the pGEM-T easy vector (Qiagen Inc., CA). Ligated products were transformed into *Escherichia coli* and the plasmid DNA was subsequently purified as described in Materials and Methods. Lanes 1–3, 4–6, and 7–9 represent cloned ITS-2 for *E. acervulina*, *E. maxima* and *E. tenella*, respectively, after EcoRI digestion to release the inserted PCR products. Lane M represents a 100-bp DNA size marker (Protech Inc.).](image2)
Table 1
Comparison of nucleotide sequence identity of the ITS-2 rDNA of *E. maxima* and *E. tenella* isolates from Taiwan to those deposited in GenBank

<table>
<thead>
<tr>
<th>E. maxima</th>
<th>E. tenella</th>
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</thead>
<tbody>
<tr>
<td>USDA strain (AF027722)</td>
<td>93.8–95.2%</td>
</tr>
<tr>
<td>Maryland strain (AF027723)</td>
<td>94.7–96.2%</td>
</tr>
<tr>
<td>North Carolina strain (AF027724)</td>
<td>81.6–82.8%</td>
</tr>
<tr>
<td>Florida strain (AF027725)</td>
<td>92.4–93.5%</td>
</tr>
<tr>
<td>Guelph strain (AF027726)</td>
<td>95.3–96.5%</td>
</tr>
<tr>
<td>Houghton strain (AF026388)</td>
<td>98.5–99.3%</td>
</tr>
</tbody>
</table>

* Three isolates each for *E. maxima* and *E. tenella* from Taiwan.
* GenBank Accession number.
* Percentages of nucleotide sequence identity.

Table 2
Comparison of nucleotide sequence identity of the ITS-2 rDNA between *E. acervulina*, *E. maxima*, and *E. tenella* from Taiwan

<table>
<thead>
<tr>
<th>E. acervulina</th>
<th>E. maxima</th>
<th>E. tenella</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>68.4</td>
<td>52.9</td>
</tr>
<tr>
<td>68.4</td>
<td>100%</td>
<td>58.2</td>
</tr>
<tr>
<td>52.9</td>
<td>58.2</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Sequence identity measured was based on the comparison between three isolates within one of the three species and among different colonies isolated from the same agar plate representing each ITS-2 amplicon.

4. Discussion

Fig. 1 shows that the sizes of the amplicons were within the range 370–580 bp and similar to the findings of Woods et al. (2000a) suggesting comparable sizes of the ITS-2 sequences from isolates of the three species from Taiwan and Australia. Fig. 1 also shows only one band for the ITS-2 of *E. maxima* and *E. acervulina* from Taiwan (Fig. 1, lanes 1–4), whereas isolates from Australia produced additional bands with sizes of 420 and 490 bp for *E. maxima* and *E. acervulina*, respectively (Woods et al., 2000a). Multiple ITS bands on an agarose gel for some *Eimeria* species were reported due to the presence of different sequence types within an amplicon (Woods et al., 2000a).

Our results also support the previous findings of Woods et al. (2000a) who detected only one band for the ITS-2 of each *Eimeria* species on the agarose gel. For example, a size similarity was found between inserted ITS-2 of three *Eimeria* species after cloning (Fig. 2) and their corresponding ITS-2 amplicons (Fig. 1). In addition, no difference in nucleotide sequences within an ITS-2 amplicon was found from inserts sequenced from different colonies isolated from the same agar plate representing each ITS-2 amplicon (Table 2). Further, 100% sequence complementarity was found between the two strands of the ITS-2 amplicons (data not shown).

The higher sequence identity of nucleotide sequences for *E. tenella* isolates (98.9–99.6%) from Taiwan compared to *E. maxima* (96.8–98.3%) may be related to the differences in epidemiology or evolution between various pathogens. In addition, based on the ITS-2 sequence analysis, a closer phylogenetic relationship...
for isolates of *E. tenella* compared to *E. maxima* was observed between Taiwan and other geographic areas (Table 1). Taken together, high homology of the ITS-2 nucleotide sequences was found between isolates within an *Eimeria* species (Table 1), whilst low sequence identity (Table 2) was noticed between species, suggesting a lower phylogenetic variation for isolates within a species compared to those between species.

In summary, our data demonstrate that the ITS-2 regions of rDNA are useful for differentiating between species for at least some *Eimeria* species and, based on their sequence analysis, phylogenetic relationships between different species and between isolates within a species can be determined.

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