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RESEARCH ARTICLE

Characterization of internal transcribed spacer-1 and apical membrane antigen-1 sequences provides insights into the genetic diversity of *Eimeria tenella* strains

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ABSTRACT

Coccidiosis is a major recurring problem in the poultry industry and is caused by infection of one or more of the seven *Eimeria* species known to infect chickens, with *Eimeria tenella* among the best studied and economically important. Studies on the genetic diversity of *E. tenella* strains is essential for the development of universally acceptable diagnostic markers and vaccines against the disease. *Eimeria tenella* internal transcribed spacer-1 (ITS-1) and apical membrane antigen-1 (AMA-1) sequences from different parts of the world are available in the public domain and therefore provide suitable comparative markers for genetic diversity study. In this study, the ITS-1 and AMA-1 sequence diversity of two local *E. tenella* strains, namely EtNSN6 and EtSGR6 were characterized. Both ITS-1 and AMA-1 sequences for EtNSN6 and EtSGR6 were retrieved by mapping to their respective genome sequences generated using next generation sequencing. Multiple sequence alignment of the ITS-1 and AMA-1 sequences with selected homologous sequences revealed the presence of a total of five and 13 single nucleotide polymorphisms (SNPs) respectively. All SNPs appeared to occur at random and did not show any unique pattern based on geographical regions while no insertions and deletions (indels) was found to occur in the aligned sequences. However, unique bases that defined certain strains were detected. Phylogenetics analyses performed with Maximum Parsimony (MP) and Maximum Likelihood (ML) methods revealed similar topology for the internal groups with all the *E. tenella* ITS-1 and AMA-1 sequences grouped in the same clade supported by high bootstrap confidence. This confirmed that both EtNSN6 and EtSGR6 samples are *E. tenella* strains. Sequence comparison and phylogenetics analyses further suggest the possibility of low genetic diversity among *E. tenella* strains.

Keywords: Broiler chicken; coccidiosis; inter-strain variation; ITS-1; AMA-1.

INTRODUCTION

The chicken production industry is one of the most important economic activity in the agricultural sector. However, it faces several disease threats, including coccidiosis, which is caused by the apicomplexan protozoa of the genus *Eimeria*. Coccidiosis has been reported to cause worldwide economic losses, which are associated with drop in egg production, poor weight gain or feed conversion and costs of prevention and treatment (Blake *et al*., 2020). Globally, the prevalence of coccidiosis is estimated to occur at 5% for clinical infections while subclinical infections are as high as 20% (Shirley *et al*., 2005). In Malaysia, a study of 35 fecal samples from local chicken farms located in four states of Peninsular Malaysia (Selangor, Negeri Sembilan, Pahang and Johor), reported the prevalence of coccidiosis infection to be 26% (Loo *et al*., 2022). Additionally, a study of 135 village chicken fecal samples from Kelantan reported a prevalence of coccidiosis infection of 7.4% (Norulhuda *et al*., 2017), while a study of 240 fecal samples from Penang and Perak reported a higher prevalence of coccidiosis infection of 27.1% (Haziqah & Irwan-Izzauddin, 2019). This intestinal disease is caused by infection of one or more of the seven *Eimeria* species known to infect chickens with each species having its own characteristics with respect to prevalence, site of infection, pathogenicity and immunogenicity (Chapman *et al*., 2013; Blake & Tomley, 2014). Of the *Eimeria* species that infect the chickens, *E. tenella* is considered to be one of the most economically important based on its prevalence and pathogenicity (Chapman, 2014).

Successful commercialization of chicken production relies on effective control of *Eimeria* parasites. The current approach to control *Eimeria* species is largely achieved through prophylactic chemotherapy. Nevertheless, long-term exposure and overdose of drugs have proven to drive the development of resistance in *Eimeria*, causing a few available drugs to be withdrawn or banned from the market (Shirley *et al*., 2007; Ahmad *et al*., 2016). Alternatively, coccidiosis control is also achieved to a lesser extend through vaccination as *Eimeria* species can generate a potent protective immune response in the host. However, anticoccidial vaccines available in the market are commonly produced using non-local strains that could be less compatible to provide complete protective immunity for the use in the local farms, if inter-strain variation

of the pathogenic species affects the immune-protective role of the vaccine. This is because antigenic diversity and incompatible population structure would undermine vaccine development and raise doubts on the vaccines' efficacy (Blake *et al*., 2015; Clark *et al*., 2016). This is evident in *E. maxima* strains, which showed specific antigenic and immune diversity observed in different field samples (Allen *et al*., 2005). Furthermore, cross protection against heterologous strain infection between 54.3% and 100% was reported in the study of four strains of *E. maxima* in South Korea (Lee *et al*., 2010). Therefore, studies on the genetic diversity of *Eimeria* strains from field samples population are crucial and could contribute to a better understanding of the antigenic diversity of chicken coccidiosis (Morris & Gasser, 2006).

Genetic diversity studies on *E. tenella* populations require suitable gene locus candidates i.e. those with publicly available sequences derived from different geographical regions. As such, internal transcribed spacer-1 (ITS-1) and apical membrane antigen-1 (AMA-1) gene fragment sequences have been used in several previous studies (Lew *et al*., 2003; Blake *et al*., 2015; Kumar *et al*., 2015). In this study, ITS-1 and AMA-1 sequences were generated from two samples, namely EtNSN6 and EtSGR6 isolated from local broiler chicken farms that practiced different production system and raised different chicken breeds. Analysis of both sequences were subsequently carried out to determine the variation and phylogenetic relationship among related sequences in *E. tenella* strains from different parts of the world. The determination of inter-strain variation between the local *E. tenella* strains and other strains that infect chickens in different geographical regions would have important implications for the management of coccidiosis as well as supporting the progress in the control of the disease in Malaysia.

MATERIALS AND METHODS

E. tenella ITS-1 and AMA-1 sequence generation

E. tenella populations from locally collected samples, namely EtNSN6 and EtSGR6 were established as previously reported (Loo *et al*., 2022). Genomic DNA was extracted from purified oocysts based on the protocol previously described by Fernandez *et al*. (2003a, 2003b) with minor modifications (Loo *et al*., 2019). The concentration of the extracted genomic DNA was quantified using a Qubit fluorometer (Life Technologies) while purity control was performed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The genomic DNA was then used in the construction of next generation sequencing library following the manufacturer's protocol (NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®). The whole genome sequencing was outsourced to GeneWiz and performed using a 2 x 150 paired-end configuration on the HiSeq instrument. Further image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina). Sequences generated were subjected to pre-processing and quality screening using SolexaQA DynamicTrim dan SolexaQA LenghtSort (Cox *et al*., 2010). Quality reads obtained after the pre-processing and trimming steps were then screened and trimmed for PhiX library sequence contamination using Bowtie 2 (Langmead & Salzberg, 2012). The cleaned quality reads were assembled using MEGAHIT (Li *et al*., 2015) to produce contigs which were then formed in scaffolds using SSPACE (Boetzer & Pirovano, 2014). *E. tenella* ITS-1 and AMA-1 sequences were mapped against the assembled genome sequences using BLASTn (Altschul *et al*., 1990; Boratyn *et al*., 2013). *E. tenella* ITS-1 sequences were mapped with the sequence reported by Lew *et al*. (2003) (GenBank accession number AF446074.1) while *E. tenella* AMA-1 sequences were mapped using the coding sequence of the *AMA-1* gene (Reid *et al*., 2014) (GenBank accession number XM_013374032.1).

Sequence homology and phylogenetics analysis

E. tenella ITS-1 and AMA-1 sequences from various geographical regions were mined from the GenBank database using MegaBLAST (Chen *et al*., 2015). Parameters were set at ≥95% percentage identity with the minimum expected value of $E \leq 10^{-100}$ to screen for high similiarity sequences. Multiple sequence alignment of *E. tenella* ITS-1 and AMA-1 sequences was then performed using ClustalW (Thompson *et al*., 1994). The aligned sequences were then analyzed to identify single nucleotide polymorphisms (SNPs), as well as insertions and deletions (indels).

Phylogenetic analyses were performed using MEGA X (Kumar *et al*., 2018) with Maximum Parsimony (MP) dan Maximum Likelihood (ML) methods. In the MP method, a heuristic search with tree bissection reconnetion (TBR) branch swapping was used to infer the shortest trees. As for the ML method, nucleotide substitution model for best fit to the data set was evaluated in MEGA X. The Kimura 2-parameter (K2P) model was found to be the model of choice for ITS-1 sequences while the Kimura 2-parameter model with invariant distribution (K2P + I) was best fit for AMA-1 sequences. Both phylogenetic analyses were performed with 1 000 bootstrap replications. Bootstrap values ≥70 were considered significant and indicated the confidence with which the same topotype would be formed and maintaining the topology (Hillis & Bull, 1993; Park *et al*., 2010).

RESULTS

The genomic DNA isolated from two local *E. tenella* strains, namely EtNSN6 and EtSGR6 that were previously established by Loo *et al*. (2022), were prepared with the concentration of ≥30 ng/µL and the total mass of ≥1 µg. The DNA purity was ensured to be in the range of 1.8-2.0 for $A_{260/280}$ and 2.0-2.2 for $A_{260/230}$. The genomic DNA were then subjected to next generation sequencing using the Illumina HiSeq platform. Quality reads obtained after the pre-processing, trimming and screening steps were assembled to produce contigs and scaffolds (Table 1). A total of 83 674 scaffolds was produced for

Table 1. Statistics of EtNSN6 and EtSGR6 genome sequence assembly

	EtNSN6		EtSGR6	
	Contig	Scaffold	Contig	Scaffold
Total number of sequences	85 318	83 674	61 194	59 870
Total sequence length (bp)	128 237 335	128 363 032	93 699 248	93 838 308
GC content (%)	49.84	49.80	50.76	50.71
Maximum sequence size (bp)	363 671	372 522	449 337	449 374
Minimum sequence size (bp)	200	200	200	200
Average sequence size (bp)	1 503	1 534	1 531	1 567
Total number of sequences >1kb	17 237	16 991	9 593	9 424
Total sequence length >1kb (bp)	95 453 112	95 954 232	69 199 918	69 627 189

EtNSN6, with the maximum size of 372 552 bp and average size of 1 534 bp. As for EtSGR6, the total scaffolds produced was 59 870 with the maximum size of 449 374 bp and average size of 1 567 bp. Analyses of the *E. tenella* ITS-1 produced a 279 bp sequence that mapped to scaffold number 1 916 (8 263 bp) of the EtNSN6 genome and to scaffold number 704 (18 782 bp) of the EtSGR6 genome. Subsequently, analyses of the *E. tenella* AMA-1 produced a 3 580 bp sequence which consisted of eight exon and seven intron regions. All exons were found to obey the adopted intron-AG-/exon/-GT splicing rule, as were also observed in previously described *E. tenella* phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Ling *et al*., 2007) and glucose-6-phosphate isomerase (G6-PI) (Loo *et al*., 2010). The sequence mapped to scaffold number 389 (37 477 bp) of the EtNSN6 genome and to scaffold number 126 (90 315 bp) of the EtSGR6 genome. The average length of the exons was 201 bp which is shorter than the average length of introns 305 bp. The combination of all the exon regions in the sequential order resulted in a full length coding sequence of 1 611 bp.

E. tenella ITS-1 sequences from various geographical regions were mined from the GenBank database and a total of 84 sequences with query coverage of ≥90% and identity of 96.4-99.6% were retrieved. The mining of *E. tenella* AMA-1 sequences resulted in 59 sequences with query coverage of ≥78% and identity of 99.2-100.0%. Analyses of the *E. tenella* ITS-1 and AMA-1 sequences revealed several sequences from the same country with 100% similarity. Therefore, only unique sequences were screened to represent a particular country. As a result, a total of 22 and 18 sequences were selected for ITS-1 and AMA-1 respectively for subsequent multiple sequence alignment analyses. Alignment of ITS-1 EtNSN6 and EtSGR6 sequences with *E. tenella* ITS-1 sequences from China (two strains), Korea, Japan, India (four strains), Pakistan (two strains), Australia, Nigeria, Libya (two strains), Egypt, Uganda, South Africa (two strains), Germany (two strains), France, United Kingdom and United States revealed five SNPs at base positions 61 (T-G), 64 (A-T), 93 (A-C), 115 (G-A) and 154 (C-T) as shown in Table 2. Among these SNPs, one was found to involve purine substitution (A and G base) at base position 115 while one was found to involve pyrimidine substitution (C and T base) at base position 154. Base substitutions between the purine and pyrimidine groups occurred at three sites (base positions 61, 64 and 93). All SNPs appeared to occur at random and do not show any unique pattern based on geographical regions. However, a unique base A occurred at base position 64 for samples NSN6 and Australia compared to other sequences that were represented with base T. In addition, a unique base T occurred for samples Pakistan and USA compared to other sequences that were represented with base C at base position 154. No indels was found to occur in the aligned *E. tenella* ITS-1 sequences.

Multiple sequence alignment of AMA-1 EtNSN6 and EtSGR6 sequences with *E. tenella* AMA-1 sequences from China, Japan, India (two strains), Nigeria (four strains), Libya (two strains), Egypt, Germany (two strains), United Kingdom (two strains), United States and Venezuela (two strains) revealed 13 SNPs at base positions 77 (T-C), 177 (G-T), 779 (T-G), 796 (T-G), 805 (C-G), 806 (A-G), 808 (C-G), 926 (C-A), 930 (T-G), 978 (G-T), 1 064 (A-G), 1 088 (T-A) and 1 251 (C-T) as shown in Table 3. Among these SNPs, two were found to involve purine substitution (A and G bases) at base positions 806 and 1064 while another two were found to involve pyrimidine substitution (C and T bases) at base positions 77 and 1 251. Base substitutions between the purine and pyrimidine groups occurred at nine sites (base positions 177, 779, 796, 805, 808, 926, 930, 978 and 1 088). Similar to the *E. tenella* ITS-1 sequences, all SNPs in the AMA-1 sequences appeared to occur at random and do not show any unique pattern based on geographical regions. Similar to ITS-1, no indels was found to occur in the aligned AMA-1 sequences. Nonetheless, unique bases that defined certain samples were detected. These occurred at base position 77, where Nigeria 4, UK Weybridge and USA showed base C compared to base T in other samples. A unique base, which specifically defined the sample from China was detected at base positions 1 064 and 1 088 while samples from Japan, Nigeria 3 and Nigeria 4 shared unique bases at base positions 779, 796, 805, 806, 808, 926 and 930. A total of 10 SNPs incurred as non-synonymous substitutions at base positions 77, 779, 796, 805, 806, 808, 926, 930, 1 064 and 1 088 (Table 4). However, the frequency of non-synonymous substitutions appeared to be low among the samples investigated with 3/20 (15%) at base positions 77, 779, 796, 805, 806, 808, 926 and 930. At base positions 1 064 and 1 088, the non-synonymous substitutions were detected only with 1/20 (5%).

In the phylogenetic analysis, the MP and ML trees were constructed using *E. tenella* ITS-1 and AMA-1 sequences from the local strains and selected homologous sequences of others *Eimeria* and apicomplexan species retrieved from GenBank. The apicomplexans species (*Neospora caninum* and *Toxoplasma gondii*) were used as outgroup species to root the trees. The topology of MP and ML trees illustrated all the *E. tenella* ITS-1 sequences were grouped in the same clade supported by ≥97% bootstrap confidence (Figure 1). This confirmed that both EtNSN6 and EtSGR6 samples are *E. tenella* strains. All the ITS-1 sequences of *E. tenella*, *E. acervulina*, *E. brunetti*, *E. mitis* and *E. necatrix* were clustered in separate clades, irrespective of their geographical location while the sequences of *E. maxima* formed two separate clades. Both *E. tenella* and *E. necatrix* were closely related and placed in the same sister clade with high bootstrap support (≥93%). Similar to the *E. tenella* ITS-1 sequences, the topology of MP and ML trees illustrated all the *E. tenella* AMA-1 sequences were grouped in the same clade supported by 100% bootstrap confidence (Figure 2) and confirmed that both EtNSN6 and EtSGR6 samples are *E. tenella* strains. All the AMA-1 sequences of *E. tenella* and *E. maxima* were also clustered in separate clades, irrespective of their geographical location.

Table 2. SNP locations in *E. tenella* ITS-1 sequences

Sample[a]	Base position[b]				
	61	64	93	115	154
NSN6	T	**A**	A	G	C
SGR6	T	T	A	G	C
CHN 1 GQ153633.1	G	T	A	A	C
CHN 2 GQ153634.1	T	T	A	G	C
KOR FJ447468.1	T	T	A	G	C
JPN LN609809.1	T	T	A	G	C
IND 1 GQ856298.1	G	T	A	A	C
IND 2 GQ856307.1	T	T	C	A	C
IND 3 GQ856308.1	T	T	A	A	C
IND 4 GQ856310.1	G	T	C	A	C
PAK MN830382.1	G	T	C	A	**T**
AUS AF446074.1	T	**A**	C	A	C
NGA LN609828.1	G	T	C	A	C
LBY 1 LN609946.1	T	T	A	G	C
LBY 2 LN609947.1	G	T	A	A	C
EGY MF034720.1	G	T	A	A	C
UGA LN609880.1	T	T	A	G	C
ZAF 1 MN727040.1	G	T	A	G	C
ZAF 2 MN727041.1	G	T	A	A	C
DEU 1 LN609776.1	G	T	C	A	C
DEU 2 LN609777.1	T	T	A	G	C
FRA LN609774.1	G	T	C	A	C
UK AF026388.1	T	T	A	G	C
USA AY779504.1	G	T	A	A	**T**

[a]CHN (China); KOR (Korea); JPN (Japan); IND (India); PAK (Pakistan); AUS (Australia); UGA (Uganda); NGA (Nigeria); LBY (Libya); EGY (Egypt); ZAF (South Africa); DEU (Germany); FRA (France); UK (United Kingdom); USA (United States of America).

[b]Base positions refer to the reference sequence (EtNSN6). Underlined bases show unique SNPs.

Table 3. SNP locations in *E. tenella* AMA-1 sequences

Sample[a]	Base position[b]												
	77	177	779	796	805	806	808	926	930	978	1 064	1 088	1 251
NSN6	T	G	T	T	C	A	C	C	T	G	A	T	C
SGR6	T	G	T	T	C	A	C	C	T	G	A	T	C
CHN LN609982.1	T	G	T	T	C	A	C	C	T	G	**G**	**A**	C
JPN LN609980.1	T	T	**G**	**G**	**G**	**G**	**G**	**A**	**G**	T	A	T	T
IND 1 LN609983.1	T	G	T	T	C	A	C	C	T	G	A	T	C
IND 2 LN609985.1	T	T	T	T	C	A	C	C	T	T	A	T	T
NGA 1 LN609981.1	T	T	T	T	C	A	C	C	T	T	A	T	T
NGA 2 LN610002.1	T	T	T	T	C	A	C	C	T	G	A	T	C
NGA 3 LN610012.1	T	G	**G**	**G**	**G**	**G**	**G**	**A**	**G**	T	A	T	T
NGA 4 LN610013.1	**C**	T	**G**	**G**	**G**	**G**	**G**	**A**	**G**	G	A	T	C
LBY 1 LN609995.1	T	G	T	T	C	A	C	C	T	G	A	T	C
LBY 2 LN609996.1	T	T	T	T	C	A	C	C	T	T	A	T	T
EGY LN610000.1	T	T	T	T	C	A	C	C	A	T	A	T	T
DEU 1 LN609977.1	T	T	T	T	C	A	C	C	T	G	A	T	C
DEU 2 LN609994.1	T	T	T	T	C	A	C	C	T	T	A	T	T
UK Houghton LN609976.1	T	T	T	T	C	A	C	C	T	T	A	T	T
UK Weybridge LN609979.1	**C**	G	T	T	C	A	C	C	T	G	A	T	C
USA LN609978.1	**C**	G	T	T	C	A	C	C	T	G	A	T	C
VEN 1 LN609988.1	T	T	T	T	C	A	C	C	T	T	A	T	T
VEN 2 LN609990.1	T	T	T	T	C	A	C	C	T	G	A	T	C

[a]CHN (China); JPN (Japan); IND (India); NGA (Nigeria); LBY (Libya); EGY (Egypt); DEU (Germany); UK (United Kingdom); USA (United States of America); VEN (Venezuela).
[b]Base positions refer to the reference sequence (EtNSN6). Underlined bases show unique SNPs.

Table 4. *E. tenella* AMA-1 non-synonymous substitutions

Base position[a]	Amino acid substitution	
	Majoriti	Minoriti
77	V	A
779	V	G
796	C	G
805 dan 806	Q	G
808	R	G
926	T	K
930	S	R
1 064	Q	R
1 088	V	E

[a]Base positions refer to the reference sequence (EtNSN6).

DISCUSSION

Most studies involving *E. tenella* have been based on a few well-characterized laboratory strains with strong European/North American bias (Bhaskaran *et al*., 2010). The study on the gene sequence diversity of *E. tenella* strains would provide fundamental information on the inter-strain variation that infect local chicken populations. In this study, we report the characterization of gene sequences of two local *E. tenella* strains (EtNSN6 and EtSGR6) from local broiler chicken farms that practice different production system and raised different chicken breeds. EtNSN6 was sampled from a raised floor system farm that breeds the Cobb 500 chicken breed while EtSGR6 was sampled from a deep litter system farm that breeds the village hybrid chicken breed. Both EtNSN6 and EtSGR6 were sampled from broiler production systems and chicken

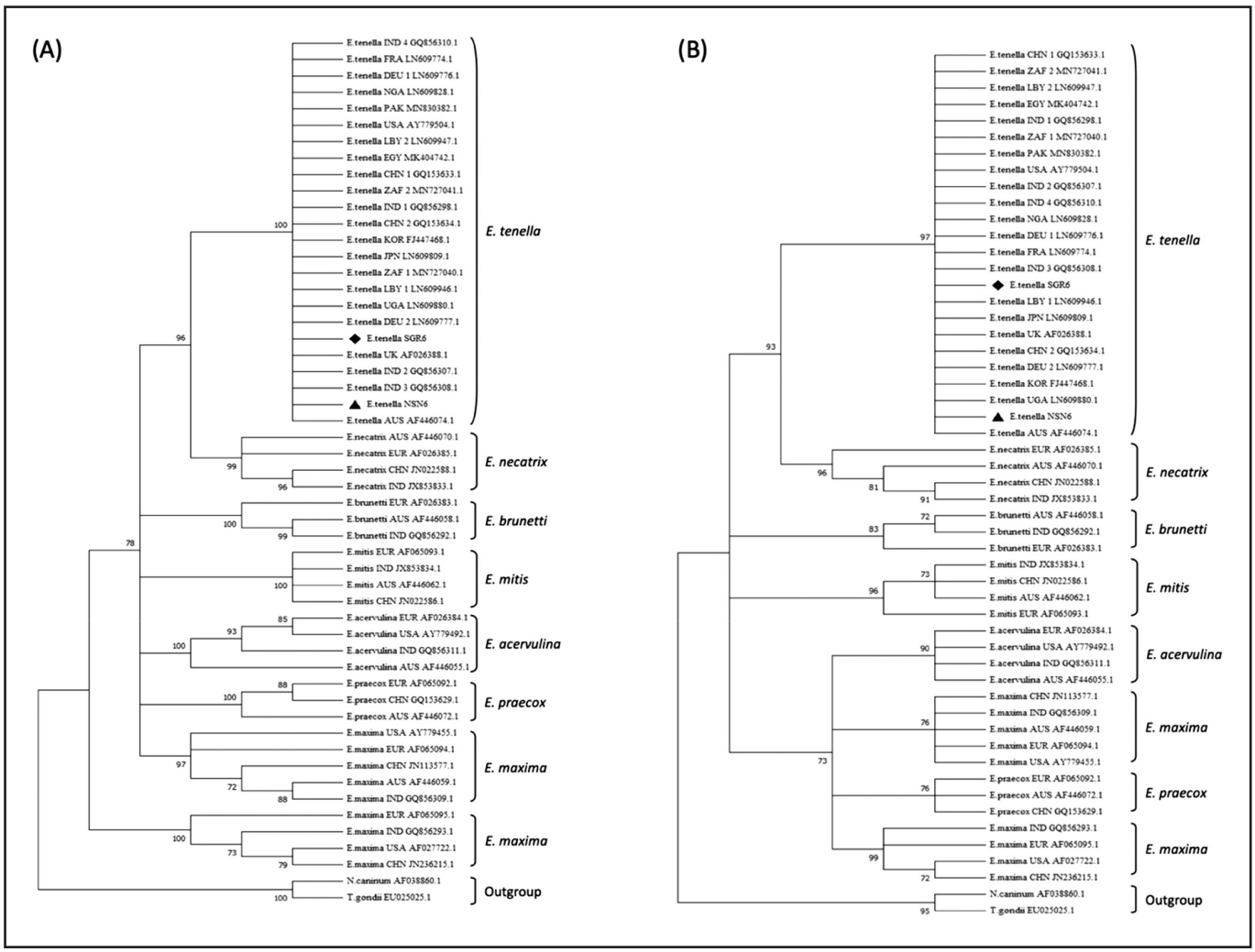

Figure 1. Consensus trees of *Eimeria* species ITS-1 sequences were generated by MP (A) and ML (B) analyses. Trees are rooted using *N. caninum* dan *T. gondii* as outgroup, and robustness was tested by 1 000 replicates of boostrapping with values shown next to the branches. The ▲ symbol indicates EtNSN6 and ◆ symbol indicates EtSGR6.

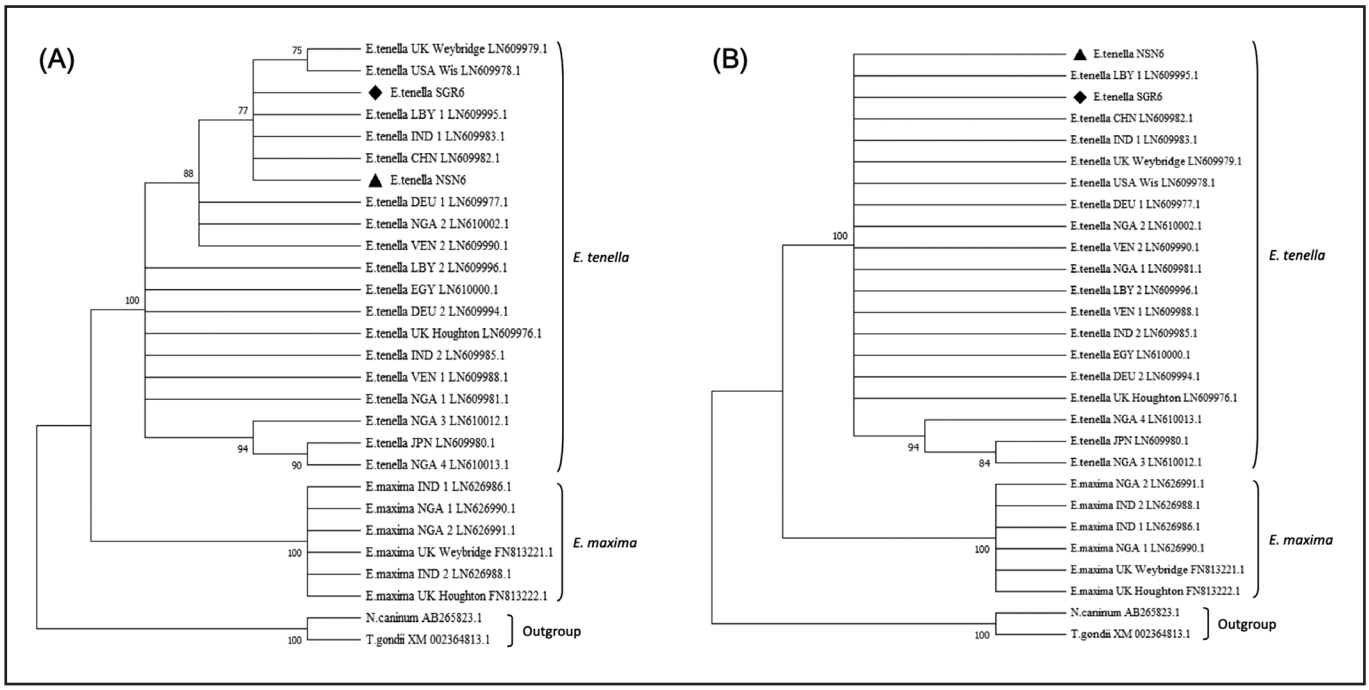

Figure 2. Consensus trees of *Eimeria* species AMA-1 sequences were generated by MP (A) and ML (B) analyses. Trees are rooted using *N. caninum* dan *T. gondii* as outgroup, and robustness was tested by 1 000 replicates of boostrapping with values shown next to the branches. The ▲ symbol indicates EtNSN6 and ◆ symbol indicates EtSGR6.

breeds that are commonly found in Malaysian farms. Compared to the closed-house production management system where the farm hygiene and biosecurity are effectively controlled, both of these conventional production systems provide sub-optimal control. Previous studies have identified variable farm management practices as the predisposing factor towards *Eimeria* species occurrence and enhance the likelihood of the coccidiosis disease (Al-Natour *et al*., 2002; Gharekhani *et al*., 2014). Therefore, analysis of EtNSN6 and EtSGR6 would provide an insight on the influence of different farm management systems and chicken breeds towards the genetic diversity of the parasite.

Eimeria tenella ITS-1 and AMA-1 sequences were selected in this study because of the availability of these sequences from strains found in various parts of the world. This would enable the comparison of the genetic diversity of the two local strains with other geographically distant strains. Both ITS-1 and AMA-1 sequences were retrieved by mapping the EtNSN6 and EtSGR6 genome sequences generated using next generation sequencing. This method offers an alternative approach to the laborious method of obtaining gene sequences by PCR, cloning, purification and sequencing. The EtNSN6 and EtSGR6 genome sequences would also provide essential resource for comparative analysis when other genetic markers derived from various parts of the world are available. Additionally, as more *E. tenella* strain genomes are being sequenced, the EtNSN6 and EtSGR6 genome sequences would be useful for whole genome comparative analysis.

Analysis results showed that ITS-1 and AMA-1 sequences from EtNSN6 and EtSGR6 are highly similar, indicating that differences in farm management systems and chicken breeds do not appear to influence the genetic diversity of the parasites. Multiple sequence alignment of *E. tenella* ITS-1 and AMA-1 revealed all SNPs appeared to occur at random and did not show any unique pattern based on geographical regions while no indels was found to occur in the aligned sequences. This suggests that there was no relationship between the sequence variation and the location where the *E. tenella* strains were isolated. However, bases that defined specific strains were detected, as observed in previously described *E. tenella* G6-PI for Houghton, Weybridge and Wisconsin strains (Loo *et al*., 2010). These characteristics are useful in the development of suitable markers for DNA fingerprinting of the different strains, which could be applied to determine the purity of *E. tenella* strains used to prepare live anticoccidial vaccines and therefore avoiding potential cross-contamination (Fernandez *et al*., 2003b). Additionally, identification of strains could differentiate the vaccine strains from the field strains and this would be helpful in epidemiological studies of *E. tenella* infections in the chicken farms. The approach of using SNPs as DNA fingerprints has been performed to differentiate *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine strains from the virulent *M. bovis* strains (Garcia *et al*., 2009) and can be a valuable tool for *E. tenella*.

The phylogenetic analysis illustrated all the *E. tenella* ITS-1 and AMA-1 sequences in this study were grouped in the same clade irrespective of their geographical location supported with high bootstrap confidence and this confirmed that both EtNSN6 and EtSGR6 samples are *E. tenella* strains. ITS-1 and AMA-1 sequences from different *Eimeria* species were also clustered in separate clades, indicating low intra-species and high inter-species diversity, which is consistent with previous reports on ITS-1 (Schwarz *et al*., 2009; Bhaskaran *et al*., 2010; Kumar *et al*., 2015). This also implies that the impact due to the genetic diversity of the parasites on the development of vaccines may be limited.

CONCLUSION

The findings of this study suggest that ITS-1 and AMA-1 sequences from the local isolates (EtNSN6 and EtSGR6) and selected related sequences from other geographical regions are conserved and exhibit low genetic diversity. However, further investigation should be conducted with a larger local sample size, which would provide a better representation, to test the correlation of the results obtained in this study. This effort will provide fundamental information on the impact of inter-strain variation of *E. tenella* that infect local chicken populations on the occurrence and extent of resistance to anticoccidial drugs for the management of coccidiosis. Additionally, understanding the variations would be useful in the development of vaccines for more effective disease control.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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