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Sequence diversity of internal transcribed spacer-1 (ITS-1) region of *Eimeria* infecting chicken and its relevance in species identification from Indian field samples

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Abstract Conventional method of species identification in *Eimeria* employs phenotypic characters of the oocysts and the site of infection in the chicken intestine, which are subjective analyses. PCR-based identification of *Eimeria* spp. is known to be specific and sensitive. We used internal transcribed spacer 1 (ITS-1)-based nested PCR to follow the distribution of *Eimeria* spp. in the field, which may be of significant value in the management of coccidiosis in chickens. In the present study, intestinal samples of chicks from commercial poultry farms, in India, suspected of having contracted *Eimeria* infections were analyzed using ITS-1 PCR. The PCR-amplified ITS-1 regions were also sequenced from these samples. Of 26 field samples analyzed, 19 showed the presence of multiple infections of *Eimeria* spp. Incidence of *Eimeria tenella* (80%) was found to be highest in these samples followed by *Eimeria mitis* (53%), *Eimeria acervulina* (42%), *Eimeria brunetti*, and *Eimeria maxima* (23%). Incidence of *Eimeria necatrix* was found to be the lowest (15%) in the samples analyzed, while none of the samples analyzed showed the presence of ITS-1 sequence from *Eimeria praecox*. The ITS-1 sequences amplified from *Eimeria* spp. in the present study showed few variations from the ITS sequences available in the GenBank database. Further studies will be required to determine whether these differences are unique to geographical locations.

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Introduction

Coccidiosis is one of the most economically important diseases of poultry (Ruff 1999). The disease is prevalent wherever intensive rearing of poultry birds is practiced. Coccidiosis in chicken is caused by seven species of *Eimeria*, viz. *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mittis*, *Eimeria necatrix*, *Eimeria tenella*, and *Eimeria praecox*.

Identification of species in the infected birds has important implications for the disease management as well as for studying their epidemiology and population biology (Woods et al. 2000). Conventional methods of identification, based on morphological features of the sporulated oocysts and infection site on the intestine, are tedious to perform and have serious limitations due to overlapping characteristics among different species (Long and Joyner 1984).

In eukaryotic cells, multiple copies of the highly conserved ribosomal RNA (rRNA) genes are located in tandem. A single cluster contains 18S, 5.8S, and 28S rRNA genes and the rRNA sequences are interspersed by internal transcribed spacers (ITS-1 and ITS-2). The ITS-1 sequences are highly variable between species and are relatively conserved within species. ITS sequences, therefore, have provided genetic markers for the identification of species in various organisms including *Eimeria* (Hnida and Duszynski 1999; Mugridge et al. 2000; Stucki et al. 1993; Barta et al. 1997; Schnitzler et al. 1998). In particular, primers specific

for ITS-1 have been developed for the detection of *Eimeria* spp. from fecal and intestinal samples using PCR assays (Lew et al. 2003; Schnitzler et al. 1998, 1999).

One of the major challenges faced by the poultry industry worldwide in controlling coccidiosis is the species-specific, at times strain-specific, nature of the immunity (Smith et al. 2002). Strain-specific protective immunity is particularly prevalent in *E. maxima* (Smith et al. 2002). Identification of immunoprotective antigens for organisms like *Eimeria* is far from complete, and the absence of a robust assay system complicates such a search (Blake et al. 2004). The lack of defined immunoprotective antigens has hindered the identification of polymorphism between the species and strains by genotypic analysis (Beck et al. 2009). Though the evolutionary relationships based on internal transcribed spacer sequence did not correlate well with the demonstrated immunological cross-reactivities among different strains of *E. maxima*, these strains could be differentiated based on the changes in the internal transcribed spacer nucleotide sequences (Barta et al. 1998). Thus, sequencing the ITS region of field isolates of *Eimeria* may be useful in assessing the strain diversity.

Indian poultry industry contributes nearly $2.2 billion annually to the national economy (Mohanty and Rajendran 2003). Despite the magnitude of the problem posed by coccidiosis to the poultry industry in India, the use of PCR-based technique for species identification is not reported thus far. Sequence information of ITS-1 regions of Indian isolates is therefore lacking. To our knowledge, this is the first ever report on the use of ITS-PCR for the identification of *Eimeria* in India. We report the ITS-1 sequence of Indian *Eimeria* isolates and have compared their phylogenetic relationships with the published sequences of other exotic isolates.

Materials and methods

Samples

Birds from farms that had either suffered mortality or showed symptoms of *Eimeria* infections were analyzed. The intestinal contents were squeezed out or intestinal scrapings were obtained from birds showing gut lesions during postmortem. The samples were stored and transported in 2% potassium dichromate solution. Presence of the coccidial oocysts was confirmed by microscopic examination. A total of 26 samples were obtained from commercial poultry farms in various parts of southern India.

DNA extraction from oocyst samples

Intestinal contents of the suspected field samples were analyzed for the presence of *Eimeria* oocysts. Samples containing oocyst were processed for DNA extraction as described by Zhao et al. (2001). Briefly, the intestinal contents were passed through a sieve to remove coarse materials. Then, the filtrate was centrifuged at 2,000×*g* and the pellet was washed thrice using distilled water. The washed pellet was reconstituted into phosphate-buffered saline and the oocyst wall broken by vortexing in the presence of glass beads (1-mm diameter; Sigma Chemical Company, USA). The vortexed material was recovered from the glass beads and pelleted by centrifugation at 1,000×*g*. The pellet was resuspended in 0.5-ml lysis buffer containing 660 mM EDTA, 1.3% *N*-lauryl sarcosine (Sigma Chemical Company), and 2 mg/ml proteinase K (Sigma Chemical Company), and the suspension was incubated at 65°C for 45 min. Then, genomic DNA was extracted by adding an equal volume of mixture containing phenol/chloroform/isoamyl alcohol in the ratio of 25:24:1 (Invitrogen, USA). Genomic DNA was precipitated from the aqueous phase by adding an equal volume of isopropyl alcohol (Merck, India). The precipitated DNA was washed twice using 70% ethanol. Some of the intestinal samples did not contain oocysts. The epithelial lining from such intestines were scraped and genomic DNA extracted using DNazol reagent (Invitrogen) as per the manufacturer's instruction.

Identification of *Eimeria* spp. using PCR

The DNA extracted from intestinal sample was used to amplify the ITS-1 region. Genus-specific primers were used for the amplification of ITS-1 region from all pathogenic *Eimeria* spp. of chicken. PCR was performed using conditions as described by Lew et al. (2003). Briefly, the PCR reaction mix was prepared using 50–100 ng of oocyst DNA, 50 pmol each of genus-specific primers, 1 U of *Taq* polymerase (Bangalore Genei, India), 200 μM each of dNTPs (Eppendorf, Germany), and PCR buffer containing 1.5 mM $MgCl_2$ (Bangalore Genei). The thermal cycling was done with an initial denaturing step at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 30 s and a final extension step at 72°C for 7 min. The amplified product from this PCR step (1 μl) was used as template to identify the species of *Eimeria* in a nested PCR. A similar PCR reaction mix as described above was used for the nested PCR also. The primer sequences and the annealing temperature used in the nested PCR are provided in Table 1.

Sequencing of ITS-1 region

The amplified products of the first PCR were cloned into pCR 2.1 TOPO TA (Invitrogen) cloning vector. At least ten colonies were picked randomly for each sample. The insert sizes of ITS-1 were checked by digesting the plasmids with

Table 1 Primers used for the identification and differentiation of prevalent *Eimeria* spp. of chicken (Lew et al. 2003)

S. no.	Species	Primer sequence 5′-3′ (forward and reverse primers)		Annealing temperature	Expected product size (bp)
1	*Eimeria* spp. (universal primer)	F	AAGTTGCGTAAATAGAGCCCT	56	400–750
		R	AGACATCCATTGCTGAAAG		
2	*E. acervulina*	F	GGCTTGGATGATGTTTGCTG	72	321
		R	CGAACGCAATAACACACGCT		
3	*E. brunetti*	F	GATCAGTTTGAGCAAACCTTCG	72	311
		R	TGGTCTTCCGTACGTCGGAT		
4	*E. maxima*	F	GCGGTTTCATCATCCATCATCG	70	145
		R	CGTTGTGAGAAG/AACTGA/GAAGGG		
5	*E. maxima* US	F	GTGAT/ATCGTTC/TGG/AG/AAGTTTGC	70	145
		R	CT/ACACCACTCACAATGAGGCAC		
6	*E. mitis*	F	GGGTTTATTTCCTGTCC/GTCGTCTC	58	328
		R	GCAAGAGAGAATCGGAATGCC		
8	*E. necatrix*	F	TACATCCCAATCTTTGAATCG	61	383
		R	GGCATACTAGCTTCGAGCAAC		
9	*E. tenella*	F	AATTTAGTCCATCGCAACCCT	65	278
		R	CGAGCGCTCTGCATACGACA		

*Eco*RI which flanks the insert. We anticipated mixed infections in the field samples. Though originating from different species, some of the ITS-1 amplicons may have very similar size. An agarose gel electrophoresis cannot resolve such small differences with sufficient clarity. Therefore, minimum of two plasmids were randomly selected for each identical insert size for sequencing with M13 forward and M13 reverse primers.

Phylogenetic analysis of ITS-1 sequence

The ITS-1 sequences obtained from Indian isolates were compared with that of European, Australian, American, and Chinese isolates available in the GenBank database (Table 2). The sequence alignment was performed using ClustalW program, and phylogenetic and molecular evolutionary analyses were conducted using *MEGA*, version 4 (Tamura et al. 2007). A maximum parsimony tree was created using the ITS-1 sequences from Indian isolates and other published sequences. The pairwise percentage identity was calculated using GeneDoc multiple sequence alignment editor, version 2.6.002 (Nicholas and Nicholas 1997).

Results

Identification of *Eimeria* spp. using PCR

Species-specific nested PCR was used to identify *Eimeria* spp. All 26 intestinal samples, which were sent for

Table 2 List of the ITS-1 sequences used in the study

No.	Isolate ID	GenBank accession no.	Origin
1	*E. tenella* isolate D	AF446074	Australia
2	*E. tenella* Shanghai	FJ449692	China
3	*E. tenella* ET 25	AY779514	USA
4	*E. tenella* Houghton	AF446075	Laboratory strain
5	*E. maxima* Shanghai	FJ449682	China
6	*E. maxima* USDA 68	AF027722	USA
7	*E. maxima* Europe	AF065094	Europe
8	*E. maxima* Isolate B	AF446059	Australia
9	*E. acervulina* EA-JJ-30	AY779492	USA
10	*E. acervulina* Isolate M1	AF026384	Europe
11	*E. acervulina* Isolate C	AF446056	Australia
12	*E. acervulina* Shanghai	FJ449688	China
13	*E. brunetti* Ebr-Swe1	AF026383	Europe
14	*E. brunetti* Isolate C	AF446058	Australia

Table 3 Summary of species-specific nested PCR results from the clinical coccidiosis samples from various farms

Region	Total no. of farms tested	No. of farms positive for a species in nested PCR						Maximum no. of species found in single sample
		E. acervulina	*E. brunetti*	*E. maxima*	*E. mitis*	*E. necatrix*	*E. tenella*	
Tamil Nadu	16	7	6	1	7	3	12	5
Andhra Pradesh	10	4	–	5	7	1	9	4
Cumulative	26	11 (42%)	6 (23%)	6 (23%)	14 (53%)	4 (15%)	21 (80%)	5

postmortem analysis, were analyzed using the nested PCR. The samples originated from two major commercial poultry-rearing regions in India. Sixteen samples from Namakkal in Tamil Nadu and ten samples from various parts of Andhra Pradesh were tested for the presence of *Eimeria* spp. Six species out of seven tested in the nested PCR were detected from the field samples. Nineteen samples contained oocysts from multiple species of *Eimeria*. As many as five species could be detected from a single sample collected from a farm in Tamil Nadu. The incidence of *E. tenella* (80%) was the highest in those samples tested. *E. necatrix* was detected in relatively lesser number of farms (15%). *E. praecox* was not detected from any of the samples tested. The summary of the result is provided in Table 3. A representative gel picture showing the PCR amplification products from one of the field isolates is provided in Fig. 1. ITS-1 region from few of these samples was sequenced to ascertain the sequence homology between the isolates.

Sequencing of ITS-1 region

The samples showing amplification product with universal primers were further processed for sequencing. The PCR products amplified with the universal primers were cloned into pCR 2.1 TOPO vector. Minimum of ten clones per plate were selected, and the insert size was checked using *Eco*RI restriction enzyme. The insert sizes varied from ~400 to ~700 bp. A total of 26 ITS-1 sequences could be generated from the samples analyzed. Twelve of these sequences were from *E. tenella*, seven from *E. brunetti*, four from *E. acervulina*, and three from *E. maxima*. The length of the ITS-1 region (in bp) for each sequence is provided in Table 4.

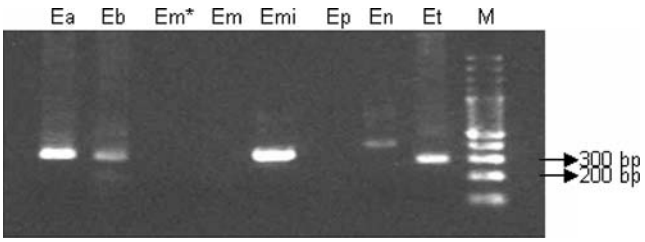

Fig. 1 Nested ITS-PCR products from a representative field sample resolved on 2% agarose gel. PCR amplification was found using primers specific for *E. acervulina* (*Ea*, ~321 bp), *E. brunetti* (*Eb*, ~311 bp), *E. mitis* (*Emi*, ~328 bp), *E. necatrix* (*En*, ~383 bp), and *E. tenella* (*Et*, ~278 bp). No amplification was observed in *E. maxima* (*Em*), *E. maxima* US (*Em**), and *E. praecox*-specific primers in this particular field sample. *M*, 100-bp DNA ladder

ITS-1 sequences of Indian isolates were compared with the published nested primer sequences used for species identification (Lew et al. 2003). This was done to determine the relevance of using the primers, which were essentially designed for the sequences of ITS-1 from American and Australian isolates, in the identification of *Eimeria* species in India. The ITS-1 sequences of all *E. acervulina* and *E. brunetii* isolates from India matched the published nested primer sequences of *E. acervulina* and *E. brunetti*, respectively. Two of the *E. maxima* isolates (AP_6 and TN_2) matched with the nested primer sequences of *E. maxima* from USA, while other *E. maxima* isolates (TN_9) matched with the nested primer sequences of *E. maxima* from Australia (Lew et al. 2003). With respect to *E. tenella*, six out of 12 ITS-1 sequences had a mismatch near the 5′ end of the forward primer (A→C) and one (AP_10), out of the 12 sequences had one base pair mismatch (A→G) at the 3′ end of the reverse primer. The published sequences of *E. tenella* nested primers and the corresponding mismatches found in the Indian isolates are underlined below.

E. tenella forward: 5′ AA(C)T TTA GTC CAT CGC AAC CCT 3′
E. tenella reverse: 5′ CGA GCG CTC TGC ATA CGA CA(G) 3′

The mismatch near the 5′ end of the forward primer sequence has obviously not affected the nested PCR results. However, considering the fact that many isolates had the mismatch, it is desirable to use degenerate primers incorporating both the nucleotides. In contrast, one of the 12 isolates had a mismatch with the 3′ end of the reverse primer, which is expected to affect the PCR amplification. Given the limited number of samples examined, it would be premature to conclude that this mismatch at the 3′ end reflects a general trend in Indian *E. tenella* isolates. Analysis of a larger sample size would help determine

Table 4 Length of ITS-1 sequences from *Eimeria* spp. isolated in India

S. no.	Sequence ID	Species	Genbank accession no.	Length of ITS-1 (bp)
1	AP1_acervulina	*E. acervulina*	GQ856311	403
2	TN1_ acervulina	*E. acervulina*	GQ856312	407
3	TN3_ acervulina	*E. acervulina*	GQ856303	403
4	TN6_ acervulina	*E. acervulina*	GQ856306	404
5	AP5_brunetti	*E. brunetti*	GQ856292	449
6	AP7_brunetti	*E. brunetti*	GQ856294	449
7	AP8_brunetti	*E. brunetti*	GQ856295	449
8	AP9_brunetti	*E. brunetti*	GQ856296	449
9	TN4_ brunetti	*E. brunetti*	GQ856304	449
10	TN5_ brunetti	*E. brunetti*	GQ856305	449
11	TN11_ brunetti	*E. brunetti*	GQ856314	449
12	AP6_maxima	*E. maxima*	GQ856293	322
13	TN2_ maxima	*E. maxima*	GQ856313	322
14	TN9_ maxima	*E. maxima*	GQ856309	446
15	AP2_tenella	*E. tenella*	GQ856289	564
16	AP3_tenella	*E. tenella*	GQ856290	564
17	AP4_tenella	*E. tenella*	GQ856291	564
18	AP10_tenella	*E. tenella*	GQ856297	564
19	AP11_tenella	*E. tenella*	GQ856298	564
20	AP12_tenella	*E. tenella*	GQ856299	564
21	AP13_tenella	*E. tenella*	GQ856300	561
22	AP14_tenella	*E. tenella*	GQ856301	564
23	AP15_tenella	*E. tenella*	GQ856302	564
24	TN7_tenella	*E. tenella*	GQ856307	564
25	TN8_tenella	*E. tenella*	GQ856308	564
26	TN10_tenella	*E. tenella*	GQ856310	564

whether the sequence difference is a common occurrence or an isolated case.

Phylogenetic analysis of *Eimeria* spp. using ITS-1 sequences

A maximum parsimony tree was created using the ITS-1 sequences from Indian *Eimeria* spp. by comparing the available ITS-1 sequences of exotic isolates from Europe, America, Australia, and China (Fig. 2). There was a clear species-wise clustering, irrespective of the geographical location, for all the ITS-1 sequences of *E. tenella*, *E. acervulina*, and *E. brunetti*. The *E. maxima* sequences formed two separate groups. Two (AP_6 and TN_2) of the three *E. maxima* sequences in the present study grouped distantly compared to the third (TN_9) sequence. The sequence diversity and strain variation in *E. maxima* was reported earlier by other authors as well (Schnitzler et al. 1999; Lew et al. 2003; Cantacessi et al. 2008).

The pairwise percentage identity among the sequences was checked using GeneDoc multiple sequence alignment editor, version 2.6.002 (Table 5). The *E. acervulina, E. brunetti*, and *E. tenella* ITS-1 sequences were >96% identical when compared within the species. However, the TN_9 *maxima* sequence had only 30% identity with the other two *E. maxima* (TN_2 and AP_6) isolates. The ITS-1 sequence identity between different species of *Eimeria* ranged from 22% to 57%.

Discussion

Diversity in *Eimeria* spp. is traditionally studied by its phenotypic characters of the oocysts and life cycle stages. The published data on molecular diversity among *Eimeria* spp. are limited and population genetic studies have been scarce. Molecular studies of population diversity in *Eimeria* species remain elementary in the poultry industry. Importantly, most studies with *Eimeria* have been based on a few well-characterized laboratory strains, many of which were isolated more than 30 years ago and have a strong European/North American bias (Beck et al. 2009). ITS sequence information on chicken *Eimeria* are now beginning to emerge from the field isolates of Australia and Taiwan (Lew et al. 2003; Lien et al. 2007; Su et al. 2003). Similar sequence diversity studies for Indian isolates have

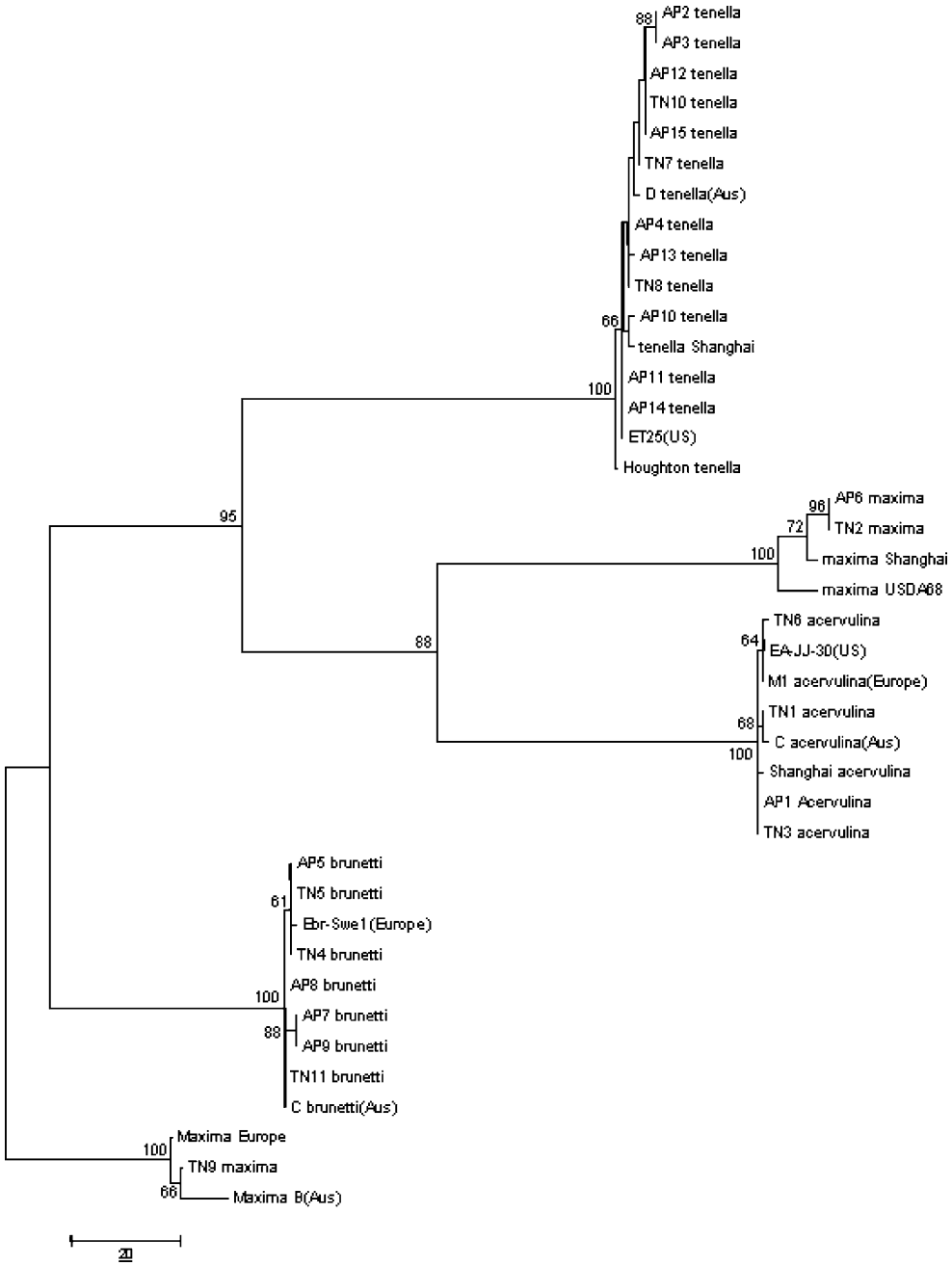

Fig. 2 Maximum parsimony tree comparing the ITS-1 sequences of Indian isolates with the sequences of Australian, American, European, and Chinese isolates. Percentage of bootstrap (1,000 replicates) values is shown at each node. The tree indicates the species-wise grouping of isolates except for *E. maxima*

not been reported. In the present study, we have determined the ITS-1 sequence of *Eimeria* field isolates in India. Using these sequence information, we also evaluated the utility of the available species-specific nested primers in identifying *Eimeria* spp. from Indian field samples.

In our study, six out of seven species tested in a nested ITS-PCR could be detected from the field samples (Table 3). Among the pathogenic species, the incidence of *E. tenella* was the highest followed by *E. acervulina*. *Eimeria mitis*, which is not known to cause severe infection in chicks, was also detected in 53% of the samples. *E. praecox* could not be detected in any of the samples tested. Since the samples were taken from birds showing intestinal lesion, it is possible that birds harboring single infection of *E. praecox* (which does not produce apparent lesion) were not sampled at all. Many of the samples contained multiple infections from *Eimeria* spp. Our results seem to agree to the observations made previously by other investigators that suggest the occurrence of mixed infection with more than one species of *Eimeria* being very common and that infection of *E. tenella* and *E. acervulina* are the most prevalent species worldwide (Shirley et al. 2005; McDougald et al. 1986).

The ITS-1 regions from a few samples were PCR-amplified and sequenced. Sequence information were obtained for four *Eimeria* spp. The ITS-1 length of *E.*

Table 5 Pairwise percentage identity between the ITS-1 sequences of different *Eimeria* species

isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1 . AP1_Acervulina	97	100	98	50	50	50	50	50	50	50	50	33	33	47	30	30	30	30	30	30	30	30	30	30	30	30	98	99	97	99	50	49	46	33	47	33	30	30	26	30
2 . TN1_acervulina		97	97	50	50	50	50	50	50	50	50	33	33	47	30	30	29	30	30	30	29	29	30	30	30	30	98	98	96	97	50	49	46	33	47	33	30	29	25	30
3 . TN3_acervulina			98	50	50	50	50	50	50	50	50	33	33	47	30	30	30	30	30	30	30	30	30	30	30	30	98	99	97	99	50	49	46	33	47	33	30	30	26	30
4 . TN6_acervulina				50	49	49	49	50	50	50	50	33	33	47	30	30	29	30	30	30	29	29	30	30	30	30	97	98	97	98	50	49	46	33	47	33	30	29	25	30
5 . AP5_brunetti					98	99	98	100	99	99	30	30	59	35	35	34	35	35	35	34	34	35	35	35	35	50	50	50	50	99	97	59	30	59	30	35	34	31	35
6 . AP7_brunetti						99	100	98	98	99	30	30	59	35	35	34	35	35	35	34	34	35	35	35	35	50	50	49	50	99	96	58	30	58	30	35	34	31	35
7 . AP8_brunetti							99	99	99	99	30	30	59	34	34	34	34	34	35	34	34	34	35	34	34	50	50	49	50	99	97	58	30	58	30	35	34	31	34
8 . AP9_brunetti								98	98	98	99	30	30	59	35	35	35	34	34	35	35	34	34	35	35	35	50	50	49	50	99	96	58	30	58	30	35	34	31	35
9 . TN4_brunetti									99	99	30	30	59	35	35	34	35	35	35	34	34	35	35	35	35	50	50	50	50	99	97	59	30	59	30	35	34	31	35
10. TN5_brunetti										99	30	30	59	35	35	35	34	34	34	35	34	34	35	35	35	35	50	50	50	50	99	96	58	30	58	30	35	34	31	34
11. TN11_brunetti											30	30	60	35	35	34	35	35	35	34	34	35	35	35	35	50	50	49	50	100	96	59	30	59	30	35	34	31	35
12. AP6_maxima												100	30	22	22	21	22	22	22	21	22	22	22	22	22	33	33	32	33	30	30	30	97	30	93	22	22	20	22
13. TN2_maxima													30	22	22	21	22	22	22	21	22	22	22	22	22	33	33	32	33	30	30	30	97	30	93	22	22	20	22
14. TN9_maxima														31	31	31	31	31	31	31	31	31	31	31	31	47	47	47	47	60	58	92	31	94	30	31	31	27	31
15. AP2_tenella															100	98	98	99	99	97	98	99	99	99	99	30	29	29	30	35	34	31	22	30	21	98	98	67	98
16. AP3_tenella																98	98	99	99	97	98	99	99	99	99	30	29	29	30	35	34	31	22	30	21	98	98	67	98
17. AP4_tenella																	97	99	98	98	98	98	99	99	98	29	29	29	30	34	34	31	22	30	21	98	98	67	98
18. AP10_tenella																		98	98	97	98	98	98	98	98	30	30	30	30	35	34	31	22	30	21	97	97	66	98
19. AP11_tenella																			99	98	99	99	99	99	99	30	29	29	30	35	34	31	22	30	21	99	99	67	99
20. AP12_tenella																				97	98	99	99	98	99	30	30	30	30	35	35	31	22	31	21	98	98	67	98
21. AP13_tenella																					98	97	98	98	98	29	29	29	30	34	34	31	22	30	21	97	97	66	97
22. AP14_tenella																						98	99	99	99	29	29	29	30	34	34	31	22	30	21	98	99	67	99
23. AP15_tenella																							99	99	99	30	30	30	30	35	34	31	22	31	21	98	98	67	98
24. TN7_tenella																								99	99	30	30	30	30	35	34	31	22	31	21	99	98	67	99
25. TN8_tenella																									99	30	29	29	30	35	34	31	22	31	21	99	98	67	99
26. TN10_tenella																										30	30	30	30	35	34	31	22	31	21	99	98	67	99
27. C_Acervulina(Aus.)																											98	97	98	50	49	45	33	47	33	30	29	25	30
28. EA-JJ-30(USA)																												98	98	50	49	45	33	47	33	30	29	25	29
29. M1_ Acervulina(Eur.)																													97	49	48	45	33	46	32	30	29	25	29
30. Shanghai_ Acervulina																														50	49	45	33	47	33	30	30	26	30
31. C_brunetti(Aus.)																															96	59	30	59	30	35	34	31	35
32. Ebr-Swe12(Eur.)																																57	31	57	30	34	34	31	34
33. Maxima_B(Aus.)																																	31	88	30	31	31	27	31
34. maxima_Shanghai																																		30	93	22	22	20	22
35. Maxima_Eur.																																			30	30	30	27	30
36. maxima_USDA																																				21	21	20	21
37. D_tenella(AUS)																																					98	67	98
38. ET25(USA)																																						67	99
39. Houghton_tenella																																							67
40. tenella_Shanghai																																							

The sequence diversity within *E. maxima* was highlighted using a dotted box

acervulina varied from 404 to 407 bp. ITS-1 length of 398 to 404 bp is reported earlier for *E. acervulina* (Lew et al. 2003; Schnitzler et al. 1998, 1999). In the present study, all the *E. brunetti* ITS-1 sequences were 449 bp in length, whereas earlier reports suggest the length of 441 to 444 bp for *E. brunetti* ITS-1 sequence (Lew et al. 2003; Schnitzler et al. 1998, 1999). Similarly, the length of *E. tenella* ITS-1 sequences was 563 bp except in one sequence where it was 560 bp. The ITS-1 length of 562 and 563 was reported earlier for *E. tenella* (Lew et al. 2003; Schnitzler et al. 1998). Lew et al. (2003) reported a shorter sequence (456 bp) for ITS-1 of *E. tenella*. We did not encounter this short variant in the ITS sequences amplified in the present study.

For *E. maxima*, two different kinds of ITS-1 sequences were identified. One of the sequences (TN_9) was 446 bp in length, and this sequence was similar to the ITS-1 sequence reported by Lew et al. (2003) for the Australian isolates and a Swedish isolate reported by Schnitzler et al. (1999). The other two *E. maxima* ITS-1 sequences (TN_2 and AP_6) were 322 bp in length and were similar to the *E. maxima* sequences of American isolates (317–319 bp; Barta et al. 1998). Lew et al. (2003) and Barta et al. (1998) have published *E. maxima* ITS-1 sequences from Australian and American field isolates, respectively, suggesting the presence of either one of the two variant *E. maxima* ITS-1 sequences. On the contrary, ITS-1 sequences of *E. maxima* from our study showed the presence of both the sequence variants. A similar observation was made by Schnitzler et al. (1999) for the Swedish isolates. Therefore, for identification of *Eimeria* species in India, we suggest the use of nested primers specific for both the *E. maxima* variants.

Given that our work marks the first attempt involving the use of ITS-PCR for differentiating *Eimeria* spp. in India, it was important to ascertain the appropriateness of the species-specific primers used in the present study. Therefore, the primers used in the species-specific nested PCR were aligned with the ITS-1 sequences obtained in the present study. The primer sequences of *E. acervulina* and *E. brunetti* matched 100% with the ITS-1 sequences of the present study. The *E. maxima* sequences matched either with the primers designed based on US isolates or on Australian isolate. With respect to *E. tenella* primers, we noticed a single base pair mismatch both in the forward as well as in the reverse primers. The mismatch in the forward primer was near the 5′ end of the primer (A→C) and was present in many ITS-1 sequences of the present study. Though the mismatch near the 5′ end may not be of much significance, it may be prudent either to use a shorter primer (that terminates before the polymorphic region at the 5′ end) or a degenerate primer consisting of both the bases (A and C) because this substitution was noticed in 50% of the *E. tenella* ITS-1 sequences. Another single base pair mismatch was seen at the 3′ end of the reverse primer. This

mismatch in the reverse primer was present in only one out of the 12 *E. tenella* ITS-1 sequences of the present study. It is not clear whether the mismatch is real or an error generated by Taq polymerase. Mismatches at the 3′ end of the primers are known to seriously affect the PCR amplification; therefore, it is important to sequence a larger number of *E. tenella* ITS-1 sequences to determine the presence or absence of this mismatch. Since the PCR product obtained using universal primer was sequenced to determine various ITS-1 sequences, we were in a position to detect the change. If this sequence mismatch at the 3′ end of the primer is real, the incidence of *E. tenella* may have been underreported.

The selection of clones from the universal PCR product for sequencing was completely random. Therefore, it is possible that the plasmid clones containing *E. mitis* and *E. necatrix* ITS-1 sequences were not selected for sequencing. Though the ITS-1 sequences of *E. mitis* and *E. necatrix* were not obtained in the present study, these two species were detected in the species-specific nested PCR (Fig. 1). Thus, the available primers may be used for species identification of *E. mitis* and *E. necatrix*. Since the minor variations in the primer binding sites may not be reflected in PCR amplification, our claim needs to be substantiated by sequencing the ITS-1 regions from *E. mitis* and *E. necatrix* of Indian isolates. Nevertheless, we conclude that the available nested primers for *E. mitis* and *E. necatrix* are relevant for the species identification of Indian isolates of *Eimeria.*

E. praecox was not detected in any of the field samples. It is premature to conclude whether the *E. praecox* incidence is undetectably low in India or the available nested primers are not suitable to identify the species. It is also possible that the birds with single infection of *E. praecox* were not sampled at all because *E. praecox* produce asymptomatic coccidiosis.

In the maximum parsimony tree (Fig. 2), we found a species-wise clustering for ITS-1 sequences from *E. tenella*, *E. acervulina*, and *E. brunetti*. The *E. maxima* sequences had two different lineages. Two of *E. maxima* sequences from India grouped along with the US sequences, and another sequence grouped with Australian sequences. These two different *E. maxima* sequences shared only 30% sequence similarity. Lew et al. (2003), while studying the ITS-1 sequence of Australian *Eimeria* isolates, found that Australian and American *E. maxima* grouped separately. PCR primers designed to amplify the American *E. maxima* did not amplify the Australian *E. maxima* tested, indicating that the two ITS-1 types do not coexist in the Australian *E. maxima.* However, in India, both the types of *E. maxima* seem to be prevalent. Our claim would require further validation using in vivo cross-protection studies to find out whether the two *E. maxima* lineages represent high strain variation within the Indian isolates of *E. maxima*. It is not known whether the two *E. maxima* lineages represent two species lineages (Lew et al. 2003). Lew et al. (2003) also proposed that these divergent ITS-1 sequences may represent rDNA pseudogenes.

In conclusion, six out of seven species of *Eimeria* were found in chicken intestinal samples collected in India. The available nested primer sequence can be used to identify *Eimeria* species in India with some minor modifications. *E. maxima* demonstrates apparent diversity in ITS-1 sequence. However, the immunological diversity of *E. maxima* isolates of India needs to be further verified because the strain variations in *E. maxima* may require addition of more than one strain of *E. maxima* in the live vaccine (Shirley et al. 2007).

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