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Uplatnění metod molekulární a buněčné biologie ve výzkumu prvoků *Eimeria*

Application of molecular and cellular biology methods in research of protozoa *Eimeria*

Disertační práce

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Abbreviations list

qPCR – quantitative polymerase chain reaction

rRNA – ribosomal RNA

rDNA – ribosomal DNA (encoding rRNA)

18S – eukaryotic small ribosomal subunit

ITS-1 – internal transcribed spacer 1, region of ribosomal DNA between 18S rRNA and 5.8S rRNA

COI – mitochondrial cytochrome *c* oxidase subunit I gene

RAPD - random amplified polymorphic DNA

SCAR - sequence-characterized amplified region

Keywords

Eimeria, coccidiosis, diagnostics, chicken, turkey, ribosomal RNA

Abstrakt

Eimerie jsou jednobuněčné prvoci z kmene *Apicomplexa* způsobující nemoc kokcidiózu, která je příčinou velkých ekonomických ztrát především v drůbežářském průmyslu. Cílem této práce bylo vyvinout nové molekulární metody a vyřešit některé problémy, což by bylo cenným příspěvkem v oboru, využitelným jak ve výzkumu tak i v praxi. Protože imunita proti eimeriím je druhově přísně specifická, je důležité znát jednotlivé druhy a dokázat je rozpoznávat. Tradiční diagnostické postupy spoléhají na klasické metody jako je určování morfologie oocyst pod mikroskopem, stanovování prepatentní periody nebo hodnocení lézí způsobených parazitem. Určování druhů těmito způsoby je však časově velice náročné a často nespolehlivé, hlavně v případech, kdy analyzujeme směs více druhů, jejichž parametry se překrývají. I když metody určování druhů využívající klasickou PCR již existují, tyto metody postrádají výhody nabízené real-time kvantitativní PCR (qPCR). Prvním cílem této práce bylo vyvinout qPCR metody pro detekci a kvantifikaci sedmi druhů kuřecích eimerií. Cílem byla vysoká specifita a maximální pokrytí všech různých kmenů každého druhu, proto byly jako cílové sekvence hledány jednokopiové nepolymorfní oblasti. Užitečnost metody byla demonstrována analýzou vzorků z terénu. Dalším cílem práce bylo vyřešení postavení druhu kuřecí kokcidie *Eimeria mivati*, jehož platnost je často zpochybňována. Pomocí izolace čistých kmenů a následné analýzy sekvencí malé ribozomální podjednotky (18S) jsme dokázali, že v rámci jednoho genomu tohoto parazita existují dva typy 18S sekvence a že tyto typy odpovídají sekvencím *E. mitis* a *E. mivati*. Existence dvou typů 18S tak byla u eimerií pozorována poprvé a znamená, že druh *E. mivati* je stejný druh jako *E. mitis*. Toto zjištění má důležité dopady pro diagnostiku kuřecích eimerií, veterinární praxi a výrobu živých vakcín. Další oblastí práce byly kokcidie krůt, kde jsme objasnili otázku dvou kmenů druhu *E. adenoeides*, které se lišily morfologií oocyst do takové míry, že jeden kmen byl původně považován za jiný druh. Pomocí analýzy genu 18S a testů křížové imunity jsme dokázali, že oba tyto kmeny představují jeden a tentýž druh.

Abstract

Eimeria is an apicomplexan parasite causing disease coccidiosis that is most prominent in poultry farming industry. This thesis is aimed to develop new molecular tools and resolve issues that would be a valuable contribution in the field from both research and industry perspective. Because immunity to *Eimeria* is strictly species-specific, it is important to know and recognize correctly all species that parasitize the host. Traditional diagnostic approaches rely on classical methods such as oocyst morphology determination under the microscope, measurement of prepatent period or in-vivo assessment of lesions caused by this parasite. However, diagnostics of individual species using these methods is very time-consuming and it is often unreliable, especially when mixture of multiple species whose parameters overlap is analyzed. Methods utilizing conventional PCR to distinguish species already exist, however, they lack advantages offered by quantitative real-time PCR (qPCR). The first aim of this thesis was to develop qPCR assays for detection and quantification of seven *Eimeria* species which infect chicken utilizing single-copy non-polymorphic targets in order to ensure maximal specifity and coverage of all strains of each species. Usefulness of this method was demonstrated by analysis of field samples. Another aim was to resolve status of *Eimeria mivati* that was considered doubtful species. We have analyzed small ribosomal subunit (18S) sequences of single-oocyst derived strains of *E. mitis* and we have found that two types of 18S co-exist within single genome that correspond to sequences of *E. mitis* and *E. mivati*. This implies that *E. mitis* and *E. mivati* represent the same species. The phenomenon of two types of 18S within single genome was not observed in *Eimeria* until now and it has important implications for diagnostics and vaccine production. The last aim was related to turkey coccidia *E. adenoeides* where we encountered two strains that differed in oocyst morphology to the extent never described before. We have resolved their status by molecular phylogenetics using 18S gene and cross-immunity tests.

1. Introduction

Eimeria is an apicomplexan parasite causing coccidiosis, the disease most prominent in poultry farming industry. It is single-celled protozoa with complex life cycle which ends with formation of tough oocysts excreted in faeces. This parasitic organism invades intestinal cells at defined locations depending on *Eimeria* species and it replicates massively within the cells causing substantial damage to the intestinal mucosa. Infections can result in high mortality, haemorrhagic disease and poor growth performance that cause significant economic losses. Eimerian oocysts are very resistant to common disinfectants and reside in environment for very long time. *Eimeria* is a close relative of important human pathogens *Toxoplasma* and *Plasmodium* and it possess similar cellular machinery and similar genes unique to apicomplexans. Numerous species of *Eimeria* which are adapted to the specific host exist. Often multiple species parasitize each host and the immunity of the host is always strictly species-specific (Shirley et al., 2007) or even strain-specific (Smith et al., 2002).

The current strategies to prevent this disease are based on using in-feed anticoccidial drugs or application of live vaccines. Anticoccidial drugs added to the feed are relatively easy to use and cheap, however, new problems arise from increased resistance of field strains of coccidia and there is also a pressure from public health authorities to reduce drug residuals. Many of the anticoccidial drugs are already ineffective against resistant strains and there is a low support for development of a new drugs from companies. On the other hand, live vaccines do not have a problem with emergence of resistance and do not bear a risk of any residuals. Live vaccines contain either virulent or attenuated line of each species being targeted. Although production of live vaccines is very expensive and they can pose a risk from residual pathogenicity, these vaccines are preferred for long-lived flocks where they elicit effective life-long protection. Therefore, the significant part of present research on coccidiosis focuses on improvement of current live vaccines and also novel types of vaccine are sought which might

be composed of recombinant protein or vector and would enable production of safer and cheaper vaccines.

Because the immunity of the host is always species-specific it is important to know and understand all species that parasitize the host in order to decide about their inclusion into any vaccine. For the live vaccines it is important to include all individual species which are known to be pathogenic and the similar situation would be with the recombinant vaccine where antigens from multiple species will be necessary.

The aims of the present work were to develop new molecular tools and to carry out specific molecular analyses of chicken and turkey *Eimeria* species. We present results based on three published papers from this field. More specifically, the first aim was development of reliable quantitative real-time PCR (qPCR) assays for identification and quantification of all seven chicken *Eimeria* species (Paper 1). This method serves as a support for currently used live vaccines and it is also utilized to screen farms for presence of coccidia in litter and it can be also used in research to quantify parasitemia during the course of infection. Second aim related to this field was to resolve issue with doubtful species *E. mivati* that was not until now analysed on molecular level (Paper 2). It was important also from the perspective to ensure that qPCR identification assays cover all chicken species occurring in farms. Another aim which emerged during study of turkey *Eimeria* species was resolution of conflict arising from classical diagnostics methods by molecular analysis (Paper 3).

2. Current state of the art

2.1 Methods for identification of coccidian species

Research on coccidia has been traditionally carried out using mainly in-vivo experiments that consisted of infection followed by isolation of oocysts and their analysis by light microscopy. Species of *Eimeria* were distinguished by oocyst morphology, prepatent period, minimum sporulation time or site of their infection (Long et al., 1976). However, these procedures take weeks to perform and the results are often ambiguous, especially when more species similar in these parameters are present. The most often used diagnostic parameter is the oocyst morphology because it is readily accessible and its assessment can be done quickly. Multiple species differ in oocyst size and shape, however, differences are often only slight and parameters often overlap as they are spread according to a Gaussian distribution. Hence, mistakes in identification often occur and quantitative analysis of mixtures containing multiple species is almost impossible. On the other hand, only simple laboratory methods that did not require expensive equipment are needed. With the advent of PCR the new methods for molecular characterization of *Eimeria* species at the DNA level emerged. The main aim of the first works was to develop PCR identification assays that could identify individual species which parasitize the host because the traditional methods were and are still unreliable and time-consuming. The first PCR methods were developed using internal transcribed spacer (ITS) of ribosomal DNA as an target (Schnitzler et al., 1999; Haug et al., 2007). However, there are known issues with ITS sequence which complicate its use in these methods. This sequence is known to be present in genome in multiple copies and these copies might differ significantly in sequence and it is known that ITS might differ significantly also within strains of the same species (Lew et al., 2003). Though their advantage of increased PCR sensitivity because of their multi-copy state, ITS sequences might be not reliable species-discriminating target in this fast evolving single-celled parasite which is known to having multiple different strains. Alternative methods of classical PCR were developed which use single-copy target sequences instead of ITS. These targets were

developed from RAPD-derived (Random Amplification of Polymorphic DNA) SCAR (Sequence Characterized Amplified Region) markers and were tested on multiple strains (Fernandez et al., 2003). Although this method is considered to have higher reliability, all these classical PCR methods lacks advantages offered by novel quantitative real-time PCR (qPCR) methods. The first and the only one (excluding ours) qPCR method for identification of chicken coccidia species was developed using ITS sequences as targets with all its disadvantages mentioned previously (Morgan et al., 2009). Impaired specifity of this method manifested as a false negativity in case of one strain of *E. praecox*. Moreover, as the copy number of ITS sequence varies and is not known in each *Eimeria* species, these assays cannot be used for exact quantification in complex mixtures. Therefore, we attempted to develop qPCR assays for all seven chicken *Eimeria* species that are based on single-copy RAPD-derived markers which were confirmed to be non-polymorphic in multiple strains of each species (Paper 1).

2.2 Chicken coccidia

In chicken, there are seven recognized species of *Eimeria* – *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. These species have been well studied and there are no doubts about their validity. However, there are also two species which were also described – *E. mivati* and *E. hagani* – but these species are not accepted by all experts in the field, although species designated as *E. mivati* is included in one commercial vaccine (Coccivac, Schering-Plough, USA). Chicken coccidia have the longest history of research and are the best studied *Eimeria* species. Their phylogenetic relationships were already studied to some extent using small subunit ribosomal RNA (18S) sequence (Barta et al., 1997) and there are no doubts about taxonomical status of the aforementioned seven species. The methods utilizing classical PCR (Fernandez et al., 2003) as well as qPCR (Vrba et al., 2010) to identify these species already exist. Although coccidian species which infect chicken host are the best studied *Eimeria* species, there are still doubts about validity of the two species mentioned previously – particularly *E. mivati*. While some researchers concluded that this is not a

valid species (Shirley et al., 1983; McDonald et al., 1983), another researchers advocated for this species (Fitz-Coy et al., 1989). The phylogeny studies that included some sequences from presumed *E. mivati* never concluded firm statements. Resolution of issue with *E. mivati* was important for decision whether should it be considered for inclusion into vaccine and it was also important to ensure that previously developed qPCR method for species identification covers this species. Our qPCR method identified this species as *E. mitis* and we have analysed status of this species by sequencing 18S rDNA and phylogeny reconstruction (Paper 2). It was the first report of molecular evidence for its taxonomical status – and it was confirmed that *E. mivati* is the same species as *E. mitis.* Previous attempts to resolve status of this species by 18S sequence failed because it was not known that there might be two polymorphic variants of 18S gene present within single genome of *Eimeria*. We reported this in *Eimeria* for the first time, although it was known from *Plasmodium* that such phenomenon exist, mainly among *Apicomplexa*. Finding of two variants of 18S gene within single genome have significant implications for population genetics and molecular diagnostic methods which utilize this gene.

2.3 Turkey coccidia

Eimeria species infecting turkeys are much less studied than its chicken counterparts. There are seven species described in the literature, namely: *E. adenoeides*, *E. dispersa*, *E. gallopavonis*, *E. innocua*, *E. meleagridis*, *E. meleagrimitis* and *E. subrotunda*. However, validity of most of these species has yet to be confirmed as there are multiple doubts which await to be resolved using molecular phylogenetics. For example, *E. subrotunda* and *E. innocua* were described many years ago but there are no reports of encountering these species nowadays (Chapman, 2008), species *E. gallopavonis* has never been encountered outside USA although all chicken *Eimeria* species are spread worldwide (Chapman, 2008), validity of *E. dispersa* has been questioned (Joyner, 1978), and finally notion that multiple species might exist under the name *E. meleagrimitis* were reported (Ruff et al., 1980; Chapman, 2008). The diagnostics according to the

morphology and other biological parameters that is well established in chicken coccidia is thus very limited in turkey coccidia because available literature is not consistent in description of individual species and there are no molecular tools for distinguishing these species. There are two commercial vaccines which include some species of turkey coccidia although there are no research papers concerning or supporting these vaccines. These vaccines utilize virulent strains and are thus marketed only in USA and Canada because virulent coccidiosis vaccines are not permitted in Europe. Only recently the paper was published that deals with characterization of ITS-1 sequences from four turkey coccidia species (Cook et al., 2010) and it also found discrepancies in current taxonomy and it suggests that two different species exist under name *E. meleagrimitis*. Although literature was quite consistent in description of species *E. adenoeides*, we have unexpectedly found that there are two different strains of this species that differ in oocyst morphology to the extent never observed in coccidiosis before. Such finding together with molecular analysis of 18S and cytochrome oxidase sequence is subject of our publication (Paper 3).

3. Aims of the work

The aims of the work were to develop a new tools and to resolve issues that would be a valuable contributions in the field of coccidiosis from both industry and research perspectives.

The first aim was to develop a qPCR assays for all seven chicken *Eimeria* species. These assays can be used by researchers to advance their research efforts in coccidiosis as well as companies focusing on diagnostics of field samples.

The second aim was to resolve issue with *E. mivati* using molecular tools. That would be a valuable and persuading contribution to a longstanding debate about this species in the field of chicken coccidiosis as it is important to know what species really exist in order to do correct decisions in vaccine production and field diagnostics.

The third aim was to resolve unknown status of the two different strains of turkey coccidia *E. adenoeides*, where we encountered variations that were never described before. Consequently, this has an important implications for diagnostics of turkey coccidia and it is a contribution to the whole image of turkey coccidia taxonomy that we trying to complete in our further research in this field.

4. Comments on published papers

4.1 Development of qPCR for detection and quantification of all seven Eimeria species infecting chicken (Paper 1)

This paper presents development and validation of seven qPCR assays specific to seven chicken *Eimeria* species – *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. The aim was to choose the target sequence that would be single-copy and non-polymorphic within species in order to ensure compatibility with a broad spectrum of various strains that exist in coccidia. For this purpose we have utilized SCARdb (Fernandez et al., 2004) database of known SCAR (sequence-characterized amplified region) markers and we have utilized and validated four previously developed markers for *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* (Blake et al., 2008). SCARdb contains multiple RAPD-derived sequences tested on several strains in Brazil. These sequences were found to be species-specific but sequence identity among different strains was confirmed only in some of these sequences. We have selected multiple candidate SCAR markers for each species and designed qPCR markers within their sequence. SCAR sequences were first checked for presence of repetitions by Tandem Repeat Finder (Benson, 1999) in order to avoid repetitious regions. Then, qPCR markers 100 – 150 bp in length were designed using Primer3 software (Rozen and Skaletsky, 2000) and secondary structure of resulting candidate qPCR markers was checked using mfold DNA folding software (Zuker, 2003). We aimed to avoid qPCR markers that would be easily folded into strong secondary structure that would hinder correct annealing of primers and probe. At the beginning we have ordered all primers (without expensive probes) for such designed candidate qPCR markers. We obtained all available strains of each of seven *Eimeria* species and isolated the DNA for further tests of polymorphism of these candidate markers. At first we validated species-specifity of each marker and its convenience for qPCR using Sybr GREEN qPCR assays that do not require fluorescent probes. Using these Sybr GREEN reactions we also screened for marks of cross-reactivity with foreign DNA that is present in huge amount in

DNA samples from faces (bacteria, yeast, plant, host). Then we PCR-amplified each candidate qPCR marker from each strain of respective species and then cloned these markers into pBluescript vector and sequenced them. Polymorphisms of each candidate marker among various strains was assessed by alignment of resulting sequences by BLAST bl2seq algorithm (Altschul et al., 1997). The ultimate goal was to find qPCR marker for each species that would be identical in sequence with SCAR from which it was derived and also identical among all strains tested. After testing one or more candidate markers for each species we have found such markers. Markers previously developed for *E. maxima*, *E. necatrix* and *E. tenella* were found to be non-polymorphic in all tested strains so there was no need to test another candidate markers for these species. However, marker previously developed for *E. acervulina* was found to be polymorphic so we have tested another candidate markers for this species. Polymorphism of markers was avoided to lower probability of false negativity or positivity in yet unknown strains and also in order to ensure correct annealing of fluorescent probe in the middle of marker sequence. Imperfect probe annealing could lead to underestimation of species quantity or it could prevent detection entirely. For the final set of markers, fluorescent probes were ordered using 6-carboxyfluorescein (6-FAM) at the 5'-end and Black Hole Quencher (BHQ-1, replacement of TAMRA) at the 3'-end. The standard curves were then generated using both plasmid-encoded markers as well as genomic DNA. These curves demonstrated repeatability across wide linear range of measurements – between 10^1 - 10^8 for plasmid DNA and between 10^1 - 10^6 for genomic DNA. It indicates sensitivity at the level of 10 genomes which is approximately one sporulated oocyst (8 genome copies). Coefficients of regression exceeded 0.99 in all assays. Precision of these assays was measured as an average standard deviation of mean triplicate Ct values and was less than 0.19 which means that these assays can distinguish between two-fold dilutions in 99.2% cases. Efficiency of PCR was found to be similar among assays and was measured to be over 1.93. Specifity, sensitivity and standard curves were validated also in independent laboratory at Royal Veterinary College, University of London by Dr. Damer Blake. In the paper we

show how to calibrate these seven assays using genomic DNA from known number of oocysts and such calibrated assays can be then used for reliable relative quantification of individual species in mixture. This approach is demonstrated and assays are also validated by analysis of field samples from multiple farms. Relative abundance of each species was measured using DNA isolated from litter and faces collected at farms. The species which were present in sufficient amount to be detected by microscopy were confirmed by this traditional approach. Moreover, in one selected sample from Angola we confirmed each species also using 18S amplification, cloning and sequencing.

The developed assays provide quick and reliable tool for specific detection and quantification of seven *Eimeria* species infecting chicken. Single-celled *Eimeria* parasites possess huge replicative potential and thus have ability to mutate genotype relatively quickly, especially under the pressure of host immunity and anticoccidial drugs. This is favourable for development of many genetically different strains worldwide. For this reason we aimed for global validity and we did thorough analysis of each qPCR marker using as many strains as possible. Although we cannot guarantee that some different strain of some species exists that might escape from detection using our primers and probes, our approach aimed to minimize such risk. Along with seven species assays we also routinely include additional *Eimeria* genus-specific assay targeting universal multi-copy 5S region. This assay is able to detect any *Eimeria* species with high sensitivity and is useful in cases where sample contains non-chicken species of *Eimeria*. In such cases all seven species assays can be negative and *Eimeria*-5S assay will be positive indicating presence of non-chicken *Eimeria* (for example turkey coccidia). This assay might prove helpful also in some rare case where some strain of chicken *Eimeria* escapes detection. Sensitivity of our assays is sufficient to detect genomic DNA from single sporulated oocyst, however, in practice the sensitivity is limited by efficiency of DNA isolation. Using the dedicated kit we can isolate DNA directly from litter and faeces, but the sensitivity is lower compared to the method where the oocysts are first isolated from faeces using flotation and then the DNA is isolated from purified oocysts. The isolation of DNA from eimerian oocysts is critical step because the methods

to efficiently disintegrate tough oocysts are not easy. We have found highest and consistent efficiency by utilizing mechanical disruption by vigorous shaking with glass beads in Mini-BeadBeater (BioSpec, USA) machine. Samples are then processed by standard spin column DNA isolation. In cases when oocysts contained in litter and faeces need to be disintegrated we utilize silicon-carbide sharp particles instead of glass beads. Apart from oocysts, the DNA can be isolated from any developmental stage of *Eimeria* such as sporozoites or merozoites. These developmental stages are present in infected tissues or cell culture and do not require mechanical disruption step. DNA can be thus more easily isolated using standard protocols developed for tissues.

We have demonstrated usefulness of this qPCR technique for screening of farms for presence of coccidia and their quantification. Mixed-species infections and mixed-species vaccines are most common, therefore quantification of individual species is needed, for example in order to do distinguish problem-causing species from ubiquitous species present in small amount. Relative quantification is particularly important in live vaccine manufacture where the inocula need to be tested for species-purity in order to prevent immunization with other species, both in vaccine production as well in quality control process. Final multi-species formulation of live vaccines can be checked as well. Also in veterinary practice, samples of infected intestinal tissue can be taken from multiple locations and analyzed for species abundance and this can help in cases where there are doubts about what species caused the observed lesions or in cases of suspected subclinical coccidiosis that facilitates development of necrotic enteritis. In research it is also advantageous to check species-purity of samples and measure their level of contamination with other species. We can also quantify parasitemia (per gram tissue) in various parts of intestine during the course of infection, for example when studying life cycle of attenuated species. Given the high economical significance of chicken coccidiosis it is very probable that qPCR techniques will become routine diagnostic method in many laboratories even though these methods are not currently cheap.

4.2 Discovery of two types of 18S sequence within E. mitis that contests the existence of E. mivati (Paper 2)

In this paper we analyze species *E. mitis* using sequencing of 18S and cytochrome oxidase (COI) gene in order to resolve issue of related species *E. mivati* whose validity was considered doubtful. While some researchers believed that this species is valid, other researchers considered this species to be the same species as *E. mitis*. During the course of thorough 18S sequencing of pure strains of *E. mitis* we have found that it contains two types of 18S gene and that the sequences of these two types correspond to sequences of *E. mitis* and *E. mivati*. Such result was not expected because it was not known that organisms from *Eimeria* genus might contain more than one type of 18S gene, although it was observed in some other apicomplexan organisms, eukaryotes normally have these genes homogenized and maintain only single type of 18S sequence.

Species *E. mitis* that was used for our analyses originated from mixed field isolate where *E. mitis* was detected and quantified by our qPCR method. It was a mixture of five species. We have first selectively enriched this mixture by passaging on birds which were immunized with other four species (*E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*). This way the content of *E. mitis* was increased from 30% in original sample to 98% in enriched sample. Such mixture was then used for single-oocyst isolations. In order to ensure analysis of pure strains we have carried out five independent single-oocyst isolations that consisted of picking-up single oocyst by micromanipulator and infecting chicken with that single oocyst. This technique is analogous to colony picking in microbiology that is done for the similar reasons – to ensure that we are working with single-cell derived line and not mixture of species. The oocysts of these strains were then characterized by measurement under the microscope and we have found that all five strains have similar distribution of length and width and that average

dimensions (15.0 μm x 13.8 μm) correspond to known dimensions of both *E. mitis* and *E. mivati*.

Oocysts of each of five strains were then used for DNA isolation and amplification of 18S and COI genes. PCR fragments were cloned into pBluescript vector and resulting purified plasmids were sequenced. Six bacterial colonies with 18S and two colonies with COI for each *E. mitis* strain were subjected to sequencing. We have found two types of 18S sequence in each strain in roughly equal ratio. The first type (mitis-type) have high homology to already known sequences of *E. mitis* and the second one (mivati-type) is homologous to known sequences attributed to *E. mivati*. Phylogenetic analysis using all 18S sequences of *E. mitis* and *E. mivati* showed that these two types segregate into two clades with high statistical support. This suggest that these two sequences do not evolve according to traditional model of concerted evolution. Grouping observed in phylogenetic analysis also confirmed that the differences between these two sequence types are not just random polymorphisms but are rather fundamental. Mitochondrial cytochrome *c* oxidase subunit I gene sequences were identical among all five strains and are highly homologous to the only one known sequence of *E. mivati*. COI sequence of *E. mitis* was not published until now. Phylogenetic analysis utilizing COI sequences of *E. mitis*, *E. mivati* and other chicken *Eimeria* species showed grouping into clades that correspond to species. In this analysis *E. mitis* and *E. mivati* COI sequences are grouped into single lineage which is in agreement with our hypothesis that this two sequences come from the same species. We employed Bayesian inference and maximum likelihood method and both provided the same topology of phylogenetic tree with high statistical support.

We also investigated predicted secondary structure of these two types of small ribosomal subunit in order to check whether differences at the DNA level might have some significant impact on structure. Using superposition with *Toxoplasma gondii* 18S structure, we have indentified structure (helix 43 in V7 region) that differs in these two types of 18S. This helix is elongated in mitis-type 18S rRNA by four nucleotide pairs compared to mivati-type 18S

rRNA. Although we cannot predict what is the functional impact of this difference in secondary structure, we have shown that the differences in these sequences cannot be regarded as random single-nucleotide polymorphisms (SNPs) that are otherwise common in 18S sequences from various strains. We can only hypothesize that different 18S types might play different roles in parasite life cycle as it was observed in *Plasmodium*.

Our findings oppose existence of *E. mivati* as an independent species, because if the strains carrying one or the other type of 18S gene exist, they will likely cross-breed and still represent single species. However, more probable explanation is that all strains of *E. mitis* contain two types of 18S gene and that the sequences detected previously and attributed to either *E. mitis* or *E. mivati* come from the same species. It is probable that detection of only one type of sequence in the past was caused by insufficient sampling where only one or two 18S clones were sequenced. When field samples containing multiple species were analysed, researchers found multiple 18S sequences including mitis-type and mivati-type from *E. mitis* which were thought to be from two species. Our work presents persuading molecular evidence that contests existence of *E. mivati* and reports two types of 18S sequence within single eimerian genome. These findings are important for both *Eimeria* taxonomy as well as for molecular diagnostics utilizing 18S gene.

4.3 Description of two strains of E. adenoeides with remarkable morphological variability (Paper 3)

The paper deals with turkey coccidia *E. adenoeides* which is considered as one of the most pathogenic and most often encountered coccidia of the turkey. Although literature is relatively consistent in description of this species compared to other turkey *Eimeria* species, we have found unexpected morphological variability of oocysts that has never been observed before in coccidiosis. At the beginning of our work with turkey coccidia we thought that the smaller strain (KCH) is a species *E. meleagrimitis* because of its oocyst morphology and dubious pathogenicity, however, after molecular

analysis of 18S gene we hypothesized that it might be the same species as *E. adenoeides* because their sequences shared high similarity. Utilizing additional molecular and biological tests we concluded that these two strains represent single species.

After preliminary analysis of 18S sequences from mixed field samples from small turkeys farms in Czech Republic we performed single-oocyst isolations of desired species. We have isolated strains now designated *E. adenoeides* KR and *E. adenoeides* KCH. At the beginning the KCH strain was though to be *E. meleagrimitis*. We measured oocyst dimensions for each strain. While the KR strain had large and ellipsoidal oocysts, the KCH strain had small and ovoid oocysts. Oocyst morphology of KR strain corresponded to *E. adenoeides* as described in literature. Oocysts of KCH strain corresponded to *E. meleagrimitis* or some other turkey coccidia species. DNA from both purified strains was subjected to PCR amplification of 18S, ITS-1 and COI genes. PCR fragments were cloned into vector and purified plasmids from multiple clones were sequenced. Here, we have found only one type of 18S sequence within each genome. The 18S sequences of KR and KCH strain shared very high similarity and differed only in two tranversions. Despite this similarity we expected these two strains to be still two different species because some examples of 18S from rabbit coccidia show that such small difference between species is possible. ITS-1 sequences were quite different between these two strains, however, it is known that, because of its polymorphism, ITS-1 is not appropriate for inference of phylogenetic relationships in *Eimeria*. Mitochondrial cytochrome oxidase sequences were relatively different at the DNA level though they are translated into identical protein.

Although molecular data indicated that these two strains might represent single species, we could not do any firm conclusions from these results without further biological tests. We have compared prepatent period, macroscopic lesions and pathogenic effect of both strains and we have carried out cross-immunization tests. Prepatent period was measured to be same in both strains (103-108 hours). Observed intestinal macroscopic

lesions were typical for *E. adenoeides* and were without noticeable differences between the two strains. Pathogenicity was measured as a body weigh gain depression after challenge infection. There were no significant differences in pathogenic effect measured this way. Both strains were similarly pathogenic thus their virulence can be considered equal. Cross-immunization tests consisted of immunization of turkeys with one strain and then challenge infection with other strain. Oocyst output was then measured after challenge infection and compared with control (non-immunized) group. Both cross-immunization tests showed significant decrease of oocyst output after heterologous challenge and indicate that these two strains should be considered as single species. Combined data from molecular analysis and biological tests provide strong support for hypothesis that these two strains represent single species.

We have also analyzed two commercial turkey coccidiosis vaccines that exist on market using 18S sequencing. We have sequenced multiple 18S clones from each vaccine and we were looking for sequences homologous to sequences from our strains of *E. adenoeides*. Using this approach we have found that each vaccine contains different strain of *E. adenoeides* and that these strains correspond to our strains KR and KCH. Vaccine Coccivac-T produced by Schering-Plough (USA) contains strain homologous to our *E. adenoeides* KR and vaccine Immucox-T produced by Vetech (Canada) contains strain homologous to our *E. adenoeides* KCH. These two vaccines were never analysed thoroughly and it was not known that such difference exist, however, our cross-immunization tests show that both strains can protect turkeys against *E. adenoeides* challenge and that it is not necessary to include both strains in vaccine.

In conclusion, our results showed that species diagnostics according to oocyst morphology is unreliable in turkey coccidia and should be carried out only with combination with other parameters or should be abandoned entirely. New qPCR diagnostic methods are being developed by our research group in order to bring new tools for this purpose.

5. Results summary

We have achieved the following results:

1. We have developed quantitative real-time PCR assays for all seven chicken *Eimeria* species using single-copy non-polymorphic targets. These assays can be used both in research as well as in veterinary practice for specific pathogen detection and precise quantification.

2. We have resolved the doubtful taxonomical status of *E. mivati* using molecular analysis of 18S rRNA. This species has been questioned for more than 30 years and we brought the molecular evidence that clarifies its status. We confirmed that it is the same species as *E. mitis* and we have discovered for the first time in *Eimeria* that two types of 18S co-exist within genome. Moreover, we showed that these two rRNA types differ significantly in secondary structure and might play different roles in parasite life cycle.

3. We have resolved the status of the two strains of *E. adenoeides* that differ in oocyst morphology to the extent never observed before in coccidiosis. The smaller strain resembled species *E. meleagrimitis* but molecular evidence and further cross-immunity tests showed that these are the two strains of the same species.

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I.

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II.

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III.

Poplstein, M. and Vrba, V. (2011). Description of the two strains of turkey coccidia *Eimeria adenoeides* with remarkable morphological variability. *Parasitology*, **138**, 1211-6. (IF=2.522)

I.

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Quantitative real-time PCR assays for detection and quantification of all seven *Eimeria* species that infect the chicken

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ABSTRACT

The development and validation of real-time quantitative PCR (qPCR) assays specific to all seven *Eimeria* species that cause coccidiosis in the chicken is described. The presented work utilizes previously published assays for *Eimeria maxima*, *E. necatrix* and *E. tenella* and adds assays for *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox*. These assays target unique single copy sequences derived from sequence characterized amplified region (SCAR) markers. All seven qPCR markers were sequenced from multiple strains and confirmed to be non-polymorphic and identical to the original SCAR sequence. Sequences conserved within each species were chosen with the aim of developing genuinely universal markers, providing global coverage. An exact match for the primers and TaqMan® probe during PCR cycling enables precise relative quantification of multiple species in a mixture regardless of the strains present. All markers utilized in these qPCR assays are absolutely species-specific and support reproducible quantification across a wide linear range, unaffected by the presence of non-target species or other contaminating DNA. The sensitivity of these assays indicates that DNA equivalent to a single sporulated oocyst can be consistently detected. These assays will be a valuable tool from both industry and research perspectives. Comparison of our panel of qPCR assays with results derived by microscopy, the traditional Gold Standard, using poultry farm field samples support their efficacy.

1. Introduction

The *Eimeria* species are apicomplexan parasites that cause the disease coccidiosis, most notably in chickens. Coccidiosis has a massive economic impact on the poultry production industry and is one of the most common diseases faced by poultry (Shirley et al., 2005). Eimerian infection can cause severe damage to the host intestine, resulting in impaired feed intake and increased mortality. The life cycle of these parasites is complex and ends with the formation of oocysts excreted in feces. Oocysts are readily accessible for both classical morphological diagnostics and serve as a source of DNA for molecular analysis. Traditionally, seven *Eimeria* species are recognized to infect the chicken, exhibiting variable levels of pathogenicity. Species can be distinguished by oocyst morphology, pre-patent period, site of infection or minimum sporulation time, but all of these methods are labour intensive, time consuming and can be very difficult and unreliable with a mixed sample, prompting the development of DNA-based molecular methods.

Classical non-quantitative PCR methods for molecular diagnosis of the seven *Eimeria* species that infect chickens have been developed using targets including internal transcribed spacer or ITS-1 sequences (Schnitzler et al., 1999; Haug et al., 2007) or sequence characterized amplified regions – SCARs (Fernandez et al., 2003a). However, these methods lack the ability to quantify the level of

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infection and methods utilizing internal transcribed spacer sequences suffer in terms of breadth of strain coverage per species since they represent a polymorphic region (Lew et al., 2003). Polymorphism within the target region can affect sensitivity or at worst increase the risk of false negativity (Morgan et al., 2009).

This work builds upon four previously published real-time quantitative PCR (qPCR) assays developed for *Eimeria* that infect the chicken (Blake et al., 2008), utilizing SCAR targets which have been confirmed to be non-polymorphic by sequencing multiple strains of each species and are present as a single-copy per genome. An alternative real-time qPCR method for the detection and quantification of the seven chicken *Eimeria* species exists, using the ribosomal ITS-2 DNA sequence as the target (Morgan et al., 2009). Importantly, although ITS regions are present in multiple copies per genome and many share the same sequence, there is some degree of variability both within a genome as well as between strains and species (Cantacessi et al., 2008). Also, the number of copies per genome is unknown, probably varies between species and maybe strain, which in combination with sequence heterogeneity results in an unpredictable number of matching targets for each specific strain and species. This has been highlighted by the inability of such an assay to detect the *Eimeria praecox* strain present in the Paracox® vaccine. Despite the advantage of higher sensitivity when using a multi-copy target, the non-polymorphic single-copy target can be better suited for specific detection and precise quantification of multiple species in a mixture.

We present qPCR assays for detection and quantification of the seven *Eimeria* species that infect the chicken, representing an improved coverage of globally diverse strains together with high specificity and sensitivity. The successful application of these assays to field samples collected from poultry farms provides preliminary practical validation.

2. Materials and methods

2.1. Parasites

Several strains and field isolates of each *Eimeria* species were used in order to assess polymorphism for each candidate marker (Table 1). The majority of the strains and field isolates listed were passaged in our laboratory. Livacox® (BIOPHARM, Czech Republic) and Paracox® (Intervet, United Kingdom) vaccines were used as sources of four or seven species, respectively. The vaccinal strains from Livacox® were also available separately. Oocysts were harvested as described previously (Long et al., 1976) and stored in 2.5% potassium dichromate or 1% chloramine at 4 °C.

2.2. Genomic DNA preparation

Purified oocysts were washed in PBS, disrupted in a Mini-BeadBeater-16 (BioSpec, USA) using 0.5 mm glass beads and DNA was extracted from the lysate using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Chicken genomic DNA was prepared from muscle cells using the same kit.

2.3. Design of candidate markers

Quantitative real-time PCR targets were designed using the SCARdb database of species-specific SCARs (Fernandez et al., 2004). The markers were designed in non-repetitive regions of each SCAR identified using Tandem repeats finder version 4.00 as a screen (Benson, 1999). Putative marker secondary structure was investigated using the mfold DNA folding predictor (Zuker, 2003). The length of each PCR target was chosen to be between 100 and 150 bp. Primers and probes were designed using Primer3 software (Rozen and Skaletsky, 2000). TaqMan® hybridization probes were labeled with 6–carboxyfluorescein (6-FAM™) at the 5′ end and with Black Hole Quencher (BHQ-1™) at the 3′ end. The primers and probes were synthesized by Sigma–Aldrich (Germany). Primers were initially designed for all of the proposed new markers (Table 2). Fluorescent probes were synthesized only for the final best-performing non-polymorphic markers. Primers and probes targeting four *Eimeria* species described previously (Blake et al., 2008) were ordered as published with the exception that the TAMRA™ quencher was replaced with BHQ-1™.

2.4. PCR amplification of candidate markers

Each marker was first tested for species-specificity against the panel of *Eimeria* species and host (chicken) genomic DNA using SYBR® Green real-time PCR and agarose gel electrophoresis. The final non-polymorphic markers were also tested for species-specificity against the turkey coccidia *E. adenoeides*, *E. meleagrimitis*, *E. dispersa* and *E. gallopavonis*. Reactions were performed using qPCR 2× SYBR® Master Mix (Top-Bio, Czech Republic), 500 nM forward and reverse primers and 3 μl template DNA in a 20 μl volume and run using a Stratagene Mx3005P® real-time PCR cycler. The thermal cycling program consisted of initial denaturation at 95 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 15 s and combined annealing and extension at 60 °C for 30 s. Dissociation curves were generated from measurement of fluorescence between 55 °C and 95 °C in the smallest possible increments. Threshold cycle and dissociation curve data were analyzed to assess species-specificity in addition to agarose gel electrophoresis. Each candidate marker, including the four previously published markers, were then amplified from every available strain or isolate of each respective species. All of the PCR products were blunted, phoshorylated and cloned into EcoRV digested dephoshorylated pBluescript vector (Stratagene, USA) using home-made (Inoue et al., 1990) chemically competent DH5α™ cells (Invitrogen, USA). Minipreps were done with the QIAprep Spin Miniprep Kit (Qiagen, Germany) and the purified plasmids were sequenced (Macrogen, Korea).

2.5. Real-time quantitative PCR

Quantitative real-time PCR reactions were performed in a Stratagene Mx3005P® real-time qPCR cycler in a

Table 1
Eimeria strains and isolates used for the assessment of candidate marker polymorphism and species-specificity. Strains marked with * were not used directly in our work but were used during the derivation and confirmation of the appropriate SCAR marker (Fernandez et al., 2003b, 2004).

Host	Species	Strain or field isolate	Geographical origin
Chicken	*E. acervulina*	*E. acervulina* CH-p-74/89 (Livacox®)	Czech Republic
		E. acervulina R-92	Czech Republic
		E. acervulina Weybridge	United Kingdom
		E. acervulina HP (Paracox®)	United Kingdom
		E. acervulina field isolate 1	Czech Republic
		E. acervulina field isolate 2	Czech Republic
		E. acervulina field isolate 3	Czech Republic
		E. acervulina field isolate 4	Czech Republic
		E. acervulina field isolate 5	Czech Republic
		E. acervulina field isolate 6	Angola
		E. acervulina BO*	Brazil
		E. acervulina PEFA*	Brazil
		E. acervulina I*	Brazil
		E. acervulina SC*	Brazil
		E. acervulina SP*	Brazil
		E. acervulina MG*	Brazil
Chicken	*E. brunetti*	*E. brunetti* Weybridge	United Kingdom
		E. brunetti C	Brazil
		E. brunetti HP (Paracox®)	United Kingdom
		E. brunetti field isolate 1	Croatia
Chicken	*E. maxima*	*E. maxima* JMN (Livacox®)	Czech Republic
		E. maxima G-94	Czech Republic
		E. maxima Weybridge	United Kingdom
		E. maxima CP or MFP (Paracox®)	United Kingdom
		E. maxima field isolate 1	Angola
		E. maxima Houghton	United Kingdom
Chicken	*E. mitis*	*E. mitis* HP (Paracox®)	United Kingdom
		E. mitis field isolate 1	Czech Republic
		E. mitis field isolate 2	Czech Republic
		E. mitis field isolate 3	Angola
Chicken	*E. necatrix*	*E. necatrix* NHUK (Livacox®)	Czech Republic
		E. necatrix CV	Czech Republic
		E. necatrix field isolate 1	Czech Republic
		E. necatrix HP (Paracox®)	United Kingdom
		E. necatrix Swe K-236*	Sweden
		E. necatrix C*	Brazil
Chicken	*E. praecox*	*E. praecox* Weybridge	United Kingdom
		E. praecox HP (Paracox®)	United Kingdom
		E. praecox field isolate 1	Czech Republic
		E. praecox field isolate 2	Angola
Chicken	*E. tenella*	*E. tenella* CH-E-A (Livacox®)	Czech Republic
		E. tenella M-98	Czech Republic
		E. tenella Houghton	United Kingdom
		E. tenella HP (Paracox®)	United Kingdom
		E. tenella field isolate 1	Czech Republic
		E. tenella MC*	Brazil
		E. tenella TA*	United Kingdom
		E. tenella Beltsville*	USA
		E. tenella LH2*	United Kingdom
		E. tenella Weybridge	United Kingdom
		E. tenella Wisconsin	USA
Turkey	*E. adenoeides*	*E. adenoeides* KR	Czech Republic
Turkey	*E. dispersa*	*E. dispersa* KR	Czech Republic
Turkey	*E. gallopavonis*	*E. gallopavonis* (Coccivac®-T)	Canada
Turkey	*E. meleagrimitis*	*E. meleagrimitis* KCH	Czech Republic

20 μl volume using qPCR 2× Blue Master Mix (Top-Bio, Czech Republic), 500 nM forward and reverse primers, 250 nM probe, 50 nM ROX™ passive reference dye (Invitrogen, USA) and 3 μl template DNA. Cycling conditions were 95 °C for 1 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Fluorescence data were collected at the end of each cycle. Each sample was run in triplicate. No-template negative controls were included. The resulting data were processed using MxPro™ software (Stratagene, USA). Threshold cycles (Ct) were calculated from baseline-corrected normalized fluorescence (dRn) data. The threshold fluorescence level was set to an arbitrary value (0.02) for all seven assays so that amplification curves were in log-linear phase at that level.

2.6. DNA standards

Standard curves were constructed using both plasmid and genomic DNA dilutions. Plasmids containing

Table 2
Genomic DNA sequences tested for intra-specific polymorphism.

Species	Marker ID	Size (bp)	SCARdb or gene ID	Reference marker sequence (Accession no.)	Polymorphic variants found (Accession no.)
E. acervulina	ACE1	124	Ac-R01-1731	AY571542	FN985063, FN985064
	ACE2[a]	150	Ac-A04-462	AY571521	nd[a]
	ACE3	103	Ac-AD18-953	AY571534	none
E. brunetti	BRU1[b]	152	Br-A03-1060	AY571547	nd[b]
	BRU2	112	Br-E04-961	AY571553	FN985077, FN985078
	BRU3	101	Br-K04-601	AY571557	FN985071, FN985072
	BRU4	150	Br-A09-1184	AY571550	FN985074, FN985075
	BRU5[c]	127	Br-AD16-637	AY571551	nd[c]
	BRU6[a]	112	Br-J18-626	AY571556	nd[a]
	BRU7	118	Br-J18-626	AY571556	none
E. maxima	MAX1	138	*Em*MIC1	M99058	none
E. mitis	MIT1	107	Mt-E03-538	AY571511	FN985080, FN985081
	MIT2	117	Mt-A03-460	AY571503	FN985083
	MIT3	115	Mt-A09-716	AY571506	none
E. necatrix	NEC1	134	Nc-AD10-702	AY571565	none
E. praecox	PRA1	117	Pr-A09-1108	AY571603	none
	PRA2	155	Pr-AD16-901	AY571607	FN985087
E. tenella	TEN1	100	Tn-E03-1161	AY571629	none

nd = not done.
[a] Not sequenced following non-specific amplification generating multiple bands.
[b] Not sequenced because the PCR product was longer than expected.
[c] Not sequenced since the PCR yielded a weak product, possibly indicating a suboptimal primer pair.

cloned single copies of one qPCR target were linearized by digestion with BamHI (Fermentas, Lithuania) and diluted into 10-fold series containing 10^8–10^1 copies per 3 μl. The concentration of genomic DNA extracted from each species was measured using a generic *Eimeria* species real-time PCR assay targeting the 5S ribosomal DNA (Blake et al., 2006) and diluted into 10-fold series representing 10^6–10^1 genomes per 3 μl template. A mixed genomic template serial dilution series was also created containing 10^5–10^1 genomes of each species per 3 μl. PCR efficiency was expressed as $10^{(-1/\text{slope})}$.

2.7. Sequence characterization

Each candidate real-time quantitative PCR marker sequence generated from every strain and isolate tested was compared by alignment to the relevant reference sequence using the BLAST (bl2seq) algorithm (Altschul et al., 1990). Reference marker sequences were obtained using the original SCAR marker sequence. All polymorphic variants identified here have been submitted to GenBank (Table 2). Candidate sequence analyses were supplemented by bioinformatic comparison using tBLASTx with the NCBI non-redundant nucleotide collection (Altschul et al., 1997).

2.8. Application to field samples

Samples of feces plus bedding were collected from 13 poultry farms in Angola, Poland and the Czech Republic. Each sample was made by pooling samples collected at multiple randomly selected sites within the test poultry house. The average weight of each pooled sample was 350 g. The total oocyst content of each sample was determined microscopically as described previously (Long et al., 1976). The number and identity of *Eimeria* species present was assessed based upon oocyst morphology. Oocysts were isolated by sucrose flotation as described previously (Long et al., 1976) and sporulated in 2.5% potassium dichromate. DNA was extracted post-sporulation as described above using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany). Quantitative real-time PCR assays for all seven *Eimeria* species, plus the universal genus-specific (5S rDNA) assay, were run in triplicate for each sample. Average Ct's measured at the same arbitrary fluorescence threshold level for each species were extracted using MxPro™ software. Three aliquots of the mixed template dilution series were included in each qPCR plate to provide positive control, enable comparison between plates and support quantification by comparison with previous calibration based upon standard curves derived from triplicate dilution series (as described above). Thus, regression equations established using mixed genomic templates were used for conversion of Ct cycles into number of target copies. The data were entered into Microsoft Excel and the number of copies in each qPCR reaction for each species was calculated using the formula $N = \text{Efficiency}^{(C_0 - C_t)}$ where N is the number of copies, C_0 is zero-copies intercept cycle and C_t is the measured mean threshold cycle. Efficiency in this formula is expressed as a number between 1 and 2 (usually around 1.94) and zero-copies intercept cycle is the point at which the standard curve crossed the vertical axis at the zero-copies point (usually 35–40). These two parameters were established from the relevant standard curve for each species and were used in all subsequent qPCR runs. Assays which did not reach threshold cycle and indicated absence of target species were assigned zero copies. The relative abundance was then calculated as the number of copies of the target species divided by the sum number of copies of all seven species and represented as a percentage.

3. Results

3.1. Species-specificity of the qPCR markers

All of the proposed quantitative PCR markers tested here were derived from species-specific SCAR markers. Absolute species-specificity was confirmed since each primer pair tested was able to amplify the expected product from all available strains of the relevant *Eimeria* species but no other species or the host. This was further confirmed by an early threshold cycle (Ct) and single-peak dissociation curve using the SYBR® Green qPCR assay. Agarose gel electrophoresis confirmed the correct amplicon size of each product and the absence of products from non-target species.

3.2. Polymorphism of the qPCR markers

Every candidate marker, including the four previously published markers (Blake et al., 2008), was amplified in duplicate from all available strains and isolates, cloned and sequenced to assess intra-specific polymorphism (Table 2). We found the published marker targeting *E. acervulina* to be polymorphic, including both deletions and substitutions in the probe area. Despite this polymorphism we were still able to detect *E. acervulina* genomic DNA using these published primers and probe in all tested strains. But, in order to prevent possible underestimation during quantification and avoid the risk of assay failure with other, potentially more polymorphic as yet unknown strains, we decided to design additional candidate markers for *E. acervulina*. The remaining three published markers which target *E. maxima*, *E. necatrix* and *E. tenella* were found to be non-polymorphic (Table 2). Candidate markers were tested for *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox* in a stepwise manner until we found a non-polymorphic marker for each species. The highest level of polymorphism (and thus the highest number of candidate markers) was found working with *E. brunetti*. The final non-polymorphic markers identified for these four species were developed as qPCR markers and combined with the three previously published non-polymorphic markers to form a new panel of chicken *Eimeria* species qPCR assays (Table 3).

3.3. Assay sensitivity

All seven species-specific qPCR markers were tested in dilution series experiments using both genomic and plasmid DNA. Standard curves revealed that each assay was highly reproducible using single-species or mixed-species genomic DNA (Table 4). Coefficients of regression exceeded 0.99 in all assays over at least six orders of magnitude for genomic DNA or over at least eight orders of magnitude for plasmid DNA. Assay precision measured as the average standard deviation of the mean triplicate Ct values was $\leq$ 0.19 (i.e. able to distinguish between 2-fold dilutions in 99.2% cases). PCR efficiency was found to exceed 1.93 and was directly comparable between all seven assays. Standard curves also confirmed that all seven assays were sensitive enough to detect 10 copies of the target genome. All seven markers appear to be single-copy per haploid genome based upon the comparison of standard curves constructed from normalized genomic DNA template and plasmid DNA.

3.4. Field sample validation

Field samples collected from poultry farms in Angola, Poland and the Czech Republic were tested for the presence of *Eimeria* species parasites using qPCR diagnostic protocols (Table 5). All samples were collected and tested as part of a routine screening program or to diagnose a suspected subclinical coccidiosis problem. Parallel micro-

Table 3
The final panel of qPCR primer and probe sequences specific for the seven *Eimeria* species that infect the chicken. The markers for *E. maxima*, *E. necatrix* and *E. tenella* remain unchanged from those published previously following additional validation here (Blake et al., 2008).

Species	Sequence source (SCARdb ID)	Primer and probe sequences	Amplicon size [bp]
E. acervulina	Ac-AD18-953	ACE-F: GCAGTCCGATGAAAGGTATTTG ACE-R: GAAGCGAAATGTTAGGCCATCT ACE-P: [6-FAM]ACAGTCCCCGCTGATGGTGTAACG[BHQ1]	103
E. brunetti	Br-J18-626	BRU-F: AGCGTGTAATCTGCTTTTGGAA BRU-R: TGGTCGCAGACGTATATTAGGG BRU-P: [6-FAM]CAACCGCAGCAAGCGAAGTTGA[BHQ1]	118
E. maxima	*Em*MIC1	MAX-F: TCGTTGCATTCGACAGATTC MAX-R: TAGCGACTGCTCAAGGGTTT MAX-P: [6-FAM]ATTGTCCAGCCAAGGTTCCCTTCG[BHQ1]	138
E. mitis	Mt-A09-716	MIT-F: CAAGGGGATGCATGGAATATAA MIT-R: CAAGACGAATGGAATCAATCTG MIT-P: [6-FAM]CCCGCGAGGGTTTCAGTTGATG[BHQ1]	115
E. necatrix	Nc-AD10-702	NEC-F: AACGCCGGTATGCCTCGTCG NEC-R: GTACTGGTGCCAACGGAGA NEC-P: [6-FAM]CCGTAGCATAGCTCAGGCAGCCAC[BHQ1]	134
E. praecox	Pr-A09-1108	PRA-F: CACATCCAATGCGATATAGGG PRA-R: ACAGAAAAACGCAAAGAGCAA PRA-P: [6-FAM]AGCAGCAGCTGCCTCTCATTGACC[BHQ1]	117
E. tenella	Tn-E03-1161	TEN-F: TCGTCTTTGGCTGGCTATTC TEN-R: CAGAGAGTCGCCGTCACAGT TEN-P: [6-FAM]CTGGAAAGCGTCTCCTTCAATGCG[BHQ1]	100

Table 4
Parameters of *Eimeria* species-specific quantitative real-time PCR standard curves.

Species	Template	Linear range	Coefficient of regression	PCR efficiency	Average standard deviation of the mean
E. acervulina	Genomic DNA	10^6–10^1	0.994	1.95	0.14
	Plasmid DNA	10^8–10^1	1.000	1.99	0.07
	Mixed genomic DNA	10^5–10^1	0.996	1.94	0.17
E. brunetti	Genomic DNA	10^6–10^1	0.994	1.94	0.16
	Plasmid DNA	10^8–10^1	0.997	2.00	0.12
	Mixed genomic DNA	10^5–10^1	0.995	1.93	0.15
E. maxima	Genomic DNA	10^6–10^1	0.992	1.94	0.18
	Plasmid DNA	10^8–10^1	1.000	1.98	0.13
	Mixed genomic DNA	10^5–10^1	0.997	1.95	0.16
E. mitis	Genomic DNA	10^6–10^1	0.999	1.96	0.12
	Plasmid DNA	10^8–10^1	0.998	2.00	0.08
	Mixed genomic DNA	10^5–10^1	0.998	1.93	0.11
E. necatrix	Genomic DNA	10^6–10^1	0.997	1.93	0.19
	Plasmid DNA	10^8–10^1	1.000	1.97	0.15
	Mixed genomic DNA	10^5–10^1	0.996	1.94	0.18
E. praecox	Genomic DNA	10^6–10^1	0.993	1.96	0.14
	Plasmid DNA	10^8–10^1	0.997	1.99	0.11
	Mixed genomic DNA	10^5–10^1	0.995	1.95	0.15
E. tenella	Genomic DNA	10^6–10^1	0.993	1.95	0.17
	Plasmid DNA	10^8–10^1	0.999	1.99	0.10
	Mixed genomic DNA	10^5–10^1	0.992	1.95	0.12

Table 5
Real-time quantitative PCR validation using field samples collected from poultry farms in Angola (AO), Poland (PL) and the Czech Republic (CZ). OPG = oocysts per gram, cs = caecal swab, nd = not detected. *E.ac* = *E. acervulina*, *E.br* = *E. brunetti*, *E.ma* = *E. maxima*, *E.mi* = *E. mitis*, *E.ne* = *E. necatrix*, *E.pr* = *E. praecox* and *E.te* = *E. tenella*.

Farm	OPG	Oocysts used for qPCR	Relative abundance						
			E.ac	*E.br*	*E.ma*	*E.mi*	*E.ne*	*E.pr*	*E.te*
Kuito, AO	730	180000	10%	nd	21%	4%	nd	65%	nd
Jinosov, CZ	80000	102000	35%	0,05%	14%	24%	1%	5%	21%
Habry 1, CZ	300	7592	19%	nd	2%	21%	37%	12%	8%
Habry 2, CZ	90	2463	18%	nd	nd	11%	69%	2%	nd
Holotin, CZ	1000	1664	5%	nd	nd	6%	84%	4%	nd
Biskupice, CZ	600	23310	48%	nd	12%	6%	26%	3%	5%
Cuemba, AO	380	330	67%	nd	nd	33%	nd	nd	nd
Farm 1, PL	5100	34700	61%	nd	8%	5%	nd	23%	3%
Zidlochovice, CZ	cs	587000	0,2%	nd	nd	2%	0,2%	nd	97%
Farm 2, PL	513	2052	15%	12%	6%	12%	7%	11%	36%
Cernovice, CZ	cs	100000	0,01%	nd	nd	1%	0,1%	nd	99%
Libis 2, CZ	4893	44600	88%	2%	1%	7%	nd	1%	nd
Hornatky, CZ	657	5300	86%	nd	7%	nd	nd	nd	7%

scopic validation successfully identified all species that were detected by qPCR to represent at least 10% of each sample. The relatively large size of the *E. maxima* oocyst supported microscopic validation (identification and enumeration) of all samples identified as positive by qPCR. Additionally, eight samples underwent comparative microscopic morphological identification where oocysts from each morphological group were counted in a McMaster chamber. Morphological groups corresponded to individual species with the exception of the *E. brunetti*/*E. maxima*-like and *E. necatrix*/*E. tenella*-like groups. The results corresponded well with the qPCR results (compare Tables 5 and 6; statistical significance using Pearson's correlation varied from $p < 0.05$ to $p < 0.001$ by farm, $p < 0.01$ to $p < 0.001$ by morphological group). Additional validation was undertaken for the sample from Kuito (Angola), where PCR amplification of the 18S rDNA, followed by cloning and sequencing of multiple random clones, identified all species found using qPCR and no other species (data not shown).

Table 6
Microscopic morphological diagnosis of eight selected field samples. Oocyst counts were distributed into five morphological groups representing *E. acervulina* (*E.ac*), *E. brunetti*/*E.maxima-like* (*E.br*/*E.ma*), *E. mitis* (*E.mi*), *E. necatrix*/*E. tenella-like* (*E.ne*/*E.te*) and *E. praecox* (*E.pr*). nd = none detected.

Farm	Relative abundance				
	E.ac	*E.br*/*E.ma*	*E.mi*	*E.ne*/*E.te*	*E.pr*
Kuito, AO	11%	16%	5%	nd	68%
Jinosov, CZ	44%	9%	15%	23%	9%
Habry 1, CZ	27%	5%	23%	38%	7%
Habry 2, CZ	14%	nd	22%	64%	nd
Farm 2, PL	11%	14%	17%	42%	16%
Cernovice, CZ	nd	nd	nd	100%	nd
Libis 2, CZ	96%	4%	nd	nd	nd
Hornatky, CZ	79%	7%	nd	14%	nd

4. Discussion

We have developed a panel of quantitative real-time PCR assays which targets all seven *Eimeria* species that

infect the chicken, combining absolute specificity with precise sensitivity. Our aim was to identify markers defined by the complete absence of polymorphism, based upon sampling as many strains as possible, in an attempt to develop an assay with global validity. For this purpose multiple strains were sequenced across at least one locus until suitable non-polymorphic markers were identified for all seven species. Moreover, these markers were identical in sequence to the reference SCAR regions from which they were derived. These SCARs were originally amplified and sequenced using multiple geographically distant strains (Fernandez et al., 2004). The absence of polymorphism guarantees an exact match of primers and probe during PCR and thus enables precise relative quantification of multiple species in a mixture, regardless of the strains present. A partial primer or probe mismatch could lead to underestimation of the target species while a larger mismatch could disable detection and quantification entirely. Although it is recognized that any assay targeting a single region of a genome may be vulnerable to unexpected genetic diversity, the benefits associated with a target with a defined genome copy number and no pre-existing polymorphism outweigh such a risk. Single nucleotide polymorphisms are unlikely to have a dramatic effect on these assays. Supplementation with a genus specific assay, for example targeting the ribosomal 5S repeat (Blake et al., 2006), can insure against a parasite population with the unlikely loss or rearrangement of the entire target region escaping detection. The ribosomal 5S genus-specific assay has about 100-fold higher sensitivity and can help in identifying additional non-target eimerian parasites in a mixture.

Species-specificity was confirmed using all seven species of *Eimeria* that infect the chicken as well as four turkey *Eimeria* species and genomic DNA extracted from the host (the chicken). The use of a simple oocyst purification process without a sodium hypochlorite step prior to DNA purification suggests the absence of cross priming with likely environmental contaminants more distantly related to *Eimeria* (i.e. within the hosts feed and its natural gut microflora). Comparative BLAST analyses identified no notable homology with any other publicly available sequence. Although we confirmed the limit of sensitivity to be below ten copies of the target sequence, the practical limit of detection will also depend on other factors including the efficiency of oocyst recovery from feces, DNA extraction and the presence of excess contaminants and PCR inhibitors in a sample. Since one sporulated oocyst contains eight genome copies these assays are shown to be able to detect and quantify DNA from the equivalent of a single oocyst. All seven assays are based on markers which showed high and comparable PCR efficiency and precision measured as the average standard deviation of replicate Ct values from the mean. Although PCR efficiency can vary depending on sample contaminants or different reaction conditions, it was important that all PCR efficiencies were similar, supporting the precise measurement of relative abundance of individual species in a mixture. Importantly, utilization of these assays requires proper calibration of the specific quantitative real-time PCR instrument and master-mix as well as a positive standard for comparison between plates. Upon calibration, the slope of the regression curve (the efficiency of PCR) should remain almost unchanged while the zero cross points will be different for each marker. Calibration using a serially diluted mixed genomic DNA template presenting equal amounts of each species is recommended. Such a template can be prepared by mixing equal amounts of fully sporulated oocysts of each species counted using the McMaster method (Long and Rowell, 1958) or purified genomic DNA. Aliquots of the template series should then be included in all subsequent qPCR tests as a positive standard. It should be noted that while the fully sporulated oocyst has eight genome copies, a non-sporulated oocyst contains only two genomes and intermediate stages caused by bad sporulation in suboptimal conditions may contain between two and eight genomes. When absolute quantification is required sporulation factor should be considered in all calculations as described elsewhere (Morgan et al., 2009).

The adoption of highly conserved single copy sequences as qPCR targets provided high specificity and precision compared to polymorphic multi-copy targets such as ITS-2, used in other recent work (Morgan et al., 2009), albeit with some loss of sensitivity. Since the repeated ITS-2 regions are known to be highly variable between many *Eimeria* strains (Cantacessi et al., 2008), and since the precise number of copies in each genome is unknown, the specificity and precision of any associated qPCR assay is likely to be impaired. For example, the *E. praecox* ITS-2 targeted qPCR assay failed to quantify the *E. praecox* strain included in the Paracox® vaccine (Morgan et al., 2009). Nevertheless, the large number of ITS-2 copies per parasite can provide a very high level of sensitivity where sequence homology allows, reducing the impact of possible sample loss during collection and processing and facilitating quantification from very small samples. Our decision to select highly conserved single copy targets improved specificity and species coverage at the cost of some sensitivity. Nonetheless, the sensitivity of the assays described here was still more than adequate for diagnostic use.

The uptake of qPCR assays to enumerate eimerian parasites in the field will depend to a large extent on cost, the requirement for technical input and robustness in different laboratories. The comparable PCR efficiencies of the assays described here suggest that they could be multiplexed and by using four different fluorescent dyes they would fit into two tubes. However, the relatively higher cost of probes with dyes other than FAM™ compared to the rather lower costs of extra master-mix and associated consumables indicate no requirement for multiplexing at this time. Optimizing and validating assays based upon a series of multicolor probes is also likely to require a higher level of technical input from the user. The availability of relatively cheap separate assays provides a diagnostic 'toolkit' from which a client can select only those assays that they require. For example, only two of the 13 field samples tested here required the full panel of seven *Eimeria* species-specific qPCR assays (Table 5). The results of these first field trials correlated well with traditional microscopic diagnosis in all examples except where minority sub-populations were detected by qPCR. The detection of these sub-populations by qPCR could possibly indicate false-positive results, although the sensitivity of the qPCR

assays and the difficulty of oocyst speciation by microscopy make it much more likely that these parasites were missed by routine microscopy, a feature common to many qPCR assays targeting apicomplexan parasites (Khairnar et al., 2009). Output from these samples has informed the choice of anticoccidial control strategy on several farms, identifying a requirement to adjust their anticoccidial drug program or vaccinate in some examples.

The use of three previously published *Eimeria* species-specific assays in this study provides further validation for each, identifying no target sequence polymorphism from an expanded sample set and demonstrating their utility in a different laboratory with different equipment. Similarly, duplication of the four new assays using serially diluted genomic DNA in a separate laboratory with a 7500 FAST Real-Time PCR System and the associated TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, data not shown) support the broad stability and robustness of these assays.

A key advantage of the qPCR method is that it can be done quickly and resulting decisions can be made immediately, while traditional diagnostic methods could last for weeks and can be labor-intensive. The choice of template material used for qPCR can have a significant impact not only on the duration of the process but also on the data generated. In this study we employed a pre-DNA extraction purification step based upon oocyst flotation in sugar solution in parallel with traditional microscopy. Oocyst purification can improve assay sensitivity, enhancing detection of minority parasite sub-populations, although there is a risk of uneven oocyst purification. As an alternative the use of a procedure supporting the extraction of total DNA from unpurified fecal material can remove the requirement for oocyst purification (i.e. QIAamp DNA stool kit), reducing the potential for bias and further streamlining the protocol at a slight cost to sensitivity.

The real-time quantitative PCR assays described here for all seven *Eimeria* species that infect the chicken provide a valuable modern tool as a successor to traditional diagnostic methods. They are both fast and reliable and can be applied in coccidiosis research, live vaccine manufacture or veterinary practice. They can be incorporated into quality control processes in the laboratory and during manufacture of live vaccines as a method to check both inocula purity and the final formulation of mixed species. The efficiency of any anticoccidial strategy can now be tested by measuring the relative abundance of each studied species. Importantly, there is no obligate requirement to purify a defined stage of the eimerian life cycle. Any eimerian developmental stage can be used for DNA extraction and quantification, for example in post-mortem diagnostics in the field. As the demand for more efficient global food production increases and qPCR becomes cheaper and more available in the field it is likely that assays such as the ones described here will become increasingly valuable.

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The discovery of the two types of small subunit ribosomal RNA gene in *Eimeria mitis* contests the existence of *E. mivati* as an independent species

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ABSTRACT

Although the validity of the coccidian species, *Eimeria mivati*, has been questioned by many researchers for a long time there has not been any molecular analysis that would help resolve this issue. Here we report on the discovery of the two types of small ribosomal subunit (18S) gene within the *Eimeria mitis* genome that correspond to the known 18S sequences of *E. mitis* and *E. mivati*, and this is in conflict with the existence of *E. mivati* as an independent species. We have carried out five single oocyst isolations to obtain five single-oocyst-derived strains of *E. mitis* and these were analyzed by the sequencing of 18S and mitochondrial cytochrome *c* oxidase subunit I genes. The two types of 18S gene were found to be present in each strain in roughly equal ratios. This indicates that if the strains carrying only one or the other 18S type exist, they will likely cross-breed and still represent a single species. However, the more probable explanation is that all strains of *E. mitis* contain two types of 18S gene and that the occasional detection of only one or the other type by sequencing might be caused by insufficient sampling. This is also the first report of the two types of 18S gene in *Eimeria*, which has already been described in some other apicomplexan species, most notably *Plasmodium*. We also found that these two types of ribosomal RNA differ significantly in their secondary structure. The biological significance of the two 18S gene variants in *E. mitis* is not known, however, we hypothesize that these variants might be used in different stages of the parasite's life-cycle as it is in other apicomplexan species investigated so far.

1. Introduction

Eimeria is a genus of apicomplexan parasites that includes various species responsible for coccidiosis, one of the most challenging diseases in the chicken farming industry. Significant effort and money is spent every year on the prevention and treatment of chicken coccidiosis. The disease is prevented either by administering in-feed anticoccidials or by applying vaccines. Owing to the increase in resistance of field strains of *Eimeria* and due to the pressure from the public health authorities and consumers, to reduce the amount of drug residuals in the food, vaccination is becoming the preferred option. Apart from one sub-unit vaccine that utilises maternal immunity (Sharman et al., 2010), all the other coccidiosis vaccines currently on the market contain live parasites which are either virulent or attenuated. The important feature shared among coccidia is that the host immunity acquired after infection is strictly species-specific. This affects the formulation of live vaccines as they must contain each *Eimeria* species that is being targeted. Also, the vaccine production cost significantly increases with every species that is added to the

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vaccine. It is evident that it is necessary to identify all the eimerian species that infect the chicken host, in order to fight coccidiosis effectively.

There are seven species of *Eimeria* infecting the chicken host that are well-known and have been studied thoroughly, namely: *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox* and *Eimeria tenella*. These species are universally accepted and there are no doubts about their validity. However, there are two species that are also described in the literature but their existence is questioned by many researchers (Shirley et al., 1983; McDonald and Ballingall, 1983), namely: *Eimeria mivati* and *Eimeria hagani*. These two species are also overlooked in papers which deal with species discrimination techniques (Fernandez et al., 2003; Morgan et al., 2009; Vrba et al., 2010).

E. mivati was first described by Edgar and Seibold (1964) as a pathogenic coccidium having oocyst morphology similar to *E. mitis*. Later it was suggested to refer to this species as *E. acervulina* var. *mivati* because the inoculum being studied was contaminated with *E. acervulina* (Long, 1973; Shirley, 1979). Studies on resistance transfer confirmed that *E. mivati* is not an *E. acervulina* variant (Ryley and Hardman, 1978). The subsequent cross-immunization study revealed that *E. mivati* is the same species as *E. mitis* (Shirley et al., 1983) while another study concluded that according to the ultrastructure of schizonts these are two distinct species (Fitz-Coy et al., 1989).

Molecular characterization could help to resolve this issue but the original isolate of *E. mivati* is no longer available. However, the small subunit ribosomal RNA (18S rRNA) gene of *E. mivati* was sequenced (Barta et al., 1997) and is available for comparison. Here, we report that during molecular characterization of our strains of *E. mitis* by sequencing the 18S rRNA gene we have identified two types of this gene to be present within the genome, and that these types correspond to the known sequences of *E. mitis* and *E. mivati*. This suggests that there is only one species characterized by the presence of these two types of 18S gene. This is also the first report of the discovery of two types of the18S rRNA gene in *Eimeria*.

2. Materials and methods

2.1. Parasites

The parasite in question originates from a litter and faeces sample collected during routine screening at a chicken farm in the Czech Republic. The farm was not using any anticoccidials but instead applied the live attenuated vaccine LIVACOX® Q (Biopharm, Czech Republic). Oocysts were isolated by using the modified salt flotation method (Long et al., 1976) and were stored in 2.5% potassium dichromate at 4 °C. The contents of *Eimeria* species present in the oocysts sample were analyzed by quantitative real-time PCR (Vrba et al., 2010).

2.2. Single oocyst isolations

The isolate containing five different species, including *E. mitis*, was passaged on chickens immunized with four other species in order to obtain an isolate enriched in *E. mitis*. Five chickens were immunized at the one-day of age with LIVACOX® Q that contains *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*. The chickens were infected with the mixed isolate two months later and the faeces were collected at days 5–7 after infection. Inoculum enriched in *E. mitis* was subjected to five single oocyst isolations to obtain five pure strains. Single oocyst isolations were completed with a micromanipulator (Narishige, Japan) that was mounted to an inverted microscope (Nikon, Japan) using glass microcapillaries filled with silicone oil. Single oocysts were selected, aspirated into a micropipette, moved between droplets of PBS and then embedded into agar cubes that were subsequently used for inoculating the chickens. Chickens were kept in a coccidia-free environment and monitored for the presence of oocysts in their faeces before inoculating with single oocysts. Another three passages of each single-oocyst-derived strain were completed to obtain a sufficient amount of oocysts. Final oocyst suspensions were subjected to quantitative real-time PCR analysis to confirm their species-purity (Vrba et al., 2010).

2.3. Biological characterization

Each single-oocyst-derived strain of *E. mitis* was measured under the microscope to determine the average oocyst dimensions. One hundred oocysts from each strain were measured, the longest and the shortest perpendicular dimensions were recorded, a histogram was generated in Microsoft Excel and the average dimensions were determined.

2.4. PCR amplification, cloning and sequencing of 18S and COI genes

Oocysts for isolation of DNA were washed in PBS, disrupted in a Mini-BeadBeater-16 (BioSpec, USA) using 0.5 mm glass beads, and the DNA was extracted from the lysate using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Small subunit ribosomal DNA was amplified using primers ERIB1 and ERIB10 (Barta et al., 1997), and the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified using primers KM204 and KM205 (Schwarz et al., 2009). PCR reactions were carried out using high-fidelity Phusion Hot Start DNA polymerase (Finnzymes, Finland). These reactions contained 200 μM dNTP each, 500 nM forward and reverse primers, 1 μl of template DNA and 0.02 U/μl of Phusion enzyme in Phusion HF buffer. The thermal cycling program consisted of an initial denaturation at 98 °C for 1 min, followed by 25 cycles of denaturation at 98 °C for 15 s, annealing at 69 °C for 20 s and extension at 72 °C for 1 min. PCR products were blunted and cloned into EcoRV digested dephoshorylated pBluescript vector (Stratagene, USA) using home-made (Inoue et al., 1990) chemically competent DH5α™ cells (Invitrogen, USA). Minipreps were prepared with the QIAprep Spin Miniprep Kit (Qiagen, Germany) and the purified plasmids were sequenced (Macrogen, Korea). Six bacterial colonies carrying the cloned 18S gene and two colonies with the COI gene were picked for each *E. mitis* strain and subjected to

sequencing. In total, 30 random clones of 18S and 10 clones of COI gene were analyzed from five single-oocyst derived strains of *E. mitis*.

2.5. Sequence analysis

The sequences of 18S and COI genes were trimmed to the regions between primers using BioEdit software (Hall, 2005). All available 18S sequences of *E. mitis* and *E. mivati* were retrieved from GenBank and used for alignment with our sequences. Similarly, multiple chicken *Eimeria* species COI sequences were selected from GenBank, including all sequences of *E. mivati*, for alignment with our COI sequence. Two COI sequences of *Eimeria cf. mivati* retrieved from GenBank were excluded from analysis due to suspected errors originating from PCR – the sequence FJ236441 is probably a chimeric molecule containing a part from *E. maxima* and the sequence FJ236434 contains frameshift mutation. Sequences were aligned using Clustal W (Larkin et al., 2007) and phylogenetic relationships were analyzed in TOPALi software (Milne et al., 2004). Maximum likelihood (ML) inference was carried out using PhyML (Guindon and Gascuel, 2003) and Bayesian inference (BI) was performed using MrBayes (Ronquist and Huelsenbeck, 2003). The optimal nucleotide substitution models for phylogenetic analyses were chosen using ModelGenerator (Keane et al., 2006) according to Bayesian Information Criterion (BIC). The reliability of ML tree topology was evaluated by bootstrap resampling with 100 replicates. Bayesian inference was performed using two independent runs for 500,000 generations with the first 30% discarded as burn-in and sampling every 10 generations. For the COI coding sequence, the substitution models were selected independently for each codon position and used in partitioned BI implemented in MrBayes and partitioned ML implemented in RAxML (Stamatakis, 2006). Phylogenetic trees were midpoint rooted and visualized in FigTree software (Rambaut, 2009). The secondary structures of the two types of small ribosomal subunit RNA from *E. mitis* were predicted by superposition with *Toxoplasma gondii* (X75453) structure retrieved from The Comparative RNA Web Site (Cannone et al., 2002). Sequences were structurally aligned with the aid of Sequence to Structure (S2S) software (Jossinet and Westhof, 2005) and the extra nucleotides present in *E. mitis* and missing in the *Toxoplasma* model were refolded using the RNAfold on the Vienna RNA Web Server (Hofacker, 2003). Structures were visualized in PseudoViewer3 (Byun and Han, 2009). The numbering of helices is based on the general eukaryotic model of small ribosomal subunit (Neefs et al., 1993).

3. Results

3.1. Isolation of E. mitis strains

From the original field isolate containing five species, namely: *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. tenella*, we have successfully isolated five pure strains of *E. mitis* using selective enrichment on immunized birds and single-oocyst isolations. While the original contents of *E. mitis* in the mixed sample was 30%, after the enrichment using LIVACOX® Q immunized birds, the contents of *E. mitis* according to quantitative PCR rose to 98%. After the single-oocyst isolations and their amplification by three passages in chickens we have obtained five strains of *E. mitis* that were all confirmed 100% species-pure by quantitative PCR.

3.2. Biological properties of E. mitis isolates

All five strains of *E. mitis* shared the same range of oocyst dimensions, that is, 12.0–19.2 μm × 10.8–16.8 μm, and an average oocyst dimension of 15.0 μm × 13.8 μm. These dimensions correspond to both the published *E. mitis* dimensions (15.6 μm × 14.2 μm) as well as to the *E. mivati* dimensions (15.6 μm × 13.4 μm).

3.3. Sequencing of 18S and COI genes

We have found two types of 18S gene to be present in each of the five single-oocyst-derived strains. While the first type is homologous to the sequence of *E. mivati* in GenBank, the second type is homologous to the sequences of *E. mitis*. On average, half of the randomly picked colonies carried the *E. mivati* type and the other half carried *E. mitis* type. The sequencing resulted in a total of six unique 18S sequences. Three sequences belong to the *E. mivati* group and share 99.6–99.7% sequence identity to each other and 99.4–99.5% sequence identity to the published *E. mivati* 18S sequence (accession no. U76748). Another three sequences belong to the *E. mitis* group and share 99.5–99.7% sequence identity to each other and 98.9–99.7% sequence identity to the three published *E. mitis* 18S sequences (accession nos. U40262, U67118 and FJ236379). Sequences from the *E. mivati* group share 98.3–98.7% sequence identity with the sequences from *E. mitis* group. GenBank contains also six sequences named *Eimeria cf. mivati* gathered during broiler farm screenings (accession nos. FJ236373–FJ236378, Schwarz et al., 2009) and these sequences share 99.5–99.9% identity with our sequences from the *E. mivati* group. All our 18S sequences were deposited into GenBank under accession numbers FR775302–FR775307. Mitochondrial cytochrome *c* oxidase subunit I gene sequences from all five strains were found to be identical and share 99.8% identity with the GenBank COI sequence assigned to *E. mivati* (accession no. EF174185). There was not any COI sequence of *E. mitis* in GenBank at the time of this manuscript preparation. Our sequence of *E. mitis* COI gene was submitted to GenBank under the accession number FR796699.

3.4. Phylogenetic analysis

The phylogenetic trees constructed from all known as well as newly sequenced 18S sequences of *E. mitis* and *E. mivati* showed grouping of these two sequence types into separate lineages (Fig. 1). Both maximum likelihood method and Bayesian inference method resulted in a similar tree topology that clustered these two sequence types into two separate clades with high statistical support (ML bootstrap support = 100%, BI posterior probability = 1.00).

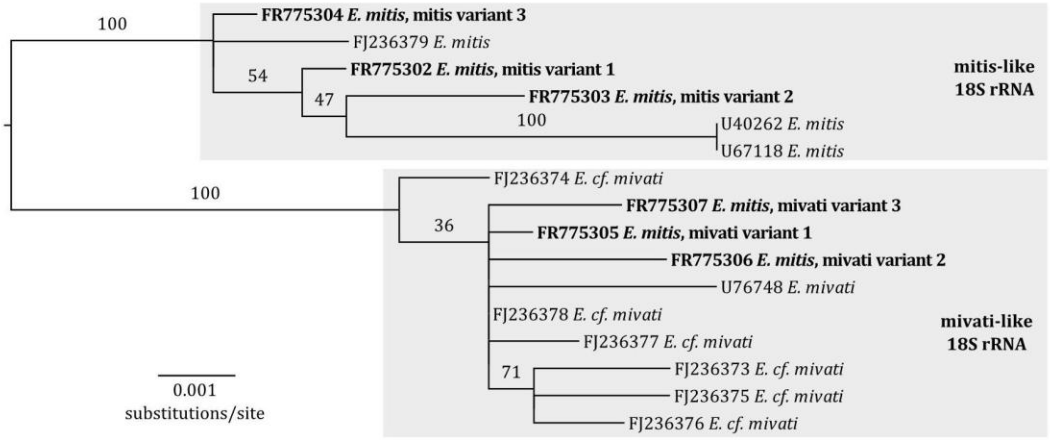

Fig. 1. Maximum likelihood phylogenetic tree constructed from all available 18S sequences of *E. mitis* and *E. mivati* showing two clades corresponding to the two sequence types. For each sequence, accession number and species name is shown. Values above branches show bootstrap support based on 100 replications. Sequences from this study are highlighted in bold.

Phylogenetic analysis based on COI sequences resulted in a tree where the sequences from each *Eimeria* species, except for *E. mitis* and *E. mivati*, were clustered in separate lineages (Fig. 2). The sequences of *E. mitis* and *E. mivati* form a single lineage in the tree. Both phylogenetic estimation methods provided the same tree topology. There was a high statistical support for monophyly of the group of COI sequences from *E. mitis* and *E. mivati* (ML bootstrap support = 90%, BI posterior probability = 1.00).

3.5. Secondary structure of 18S rRNA

Structural alignment of the two types of 18S rRNA found in *E. mitis* showed that the major difference between the mivati-type and the mitis-type is the insertion/deletion of nine nucleotides in the V7 region corresponding to helix 43 of the eukaryotic small subunit ribosomal RNA model. This insertion elongates helix 43 in mitis-type RNA by four nucleotide pairs (Fig. 3).

4. Discussion

The small ribosomal subunit (18S) is a well-known gene used to infer phylogenetic relations and to distinguish between species. It is organized into transcriptional units with other ribosomal genes and eukaryotes generally have more copies of this gene arranged in tandem repeats (about 140 in *E. tenella*; Shirley and Tomley, Sanger Institute *E. tenella* genome project website). Traditionally, it is assumed that these genes undergo concerted evolution where the copies are continuously homogenized by gene conversion and an unequal crossover, and thus remain virtually identical. However, exceptions to this rule exist mainly among the apicomplexan species like *Plasmodium* (McCutchan et al., 1988), *Cryptosporidium* (Le Blancq et al., 1997) and *Babesia* (Reddy et al., 1991), where the two types of 18S gene coexist in a single genome. It is hypothesized that these genes evolve according to a birth-and-death model of evolution where the new variants are created by gene duplication and some duplicates are maintained in

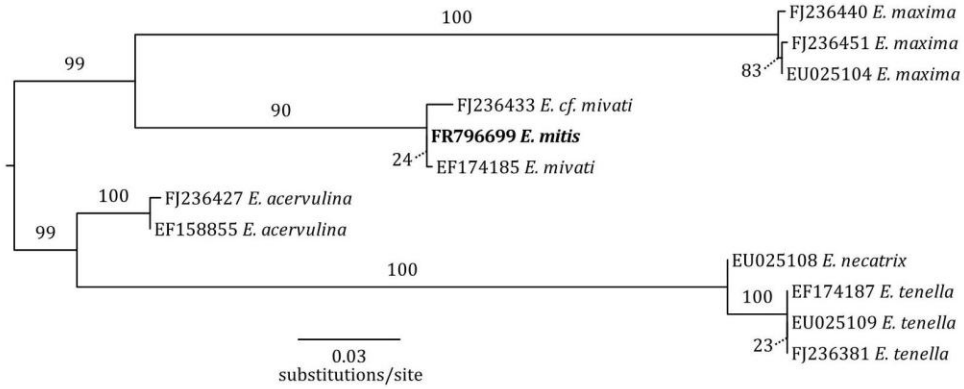

Fig. 2. Maximum likelihood phylogenetic tree constructed from multiple COI sequences of chicken *Eimeria* species including all available sequences of *E. mivati* and our sequence of *E. mitis*. Values above branches and values connected with dotted lines show bootstrap support based on 100 replications. Sequence from this study is highlighted in bold.

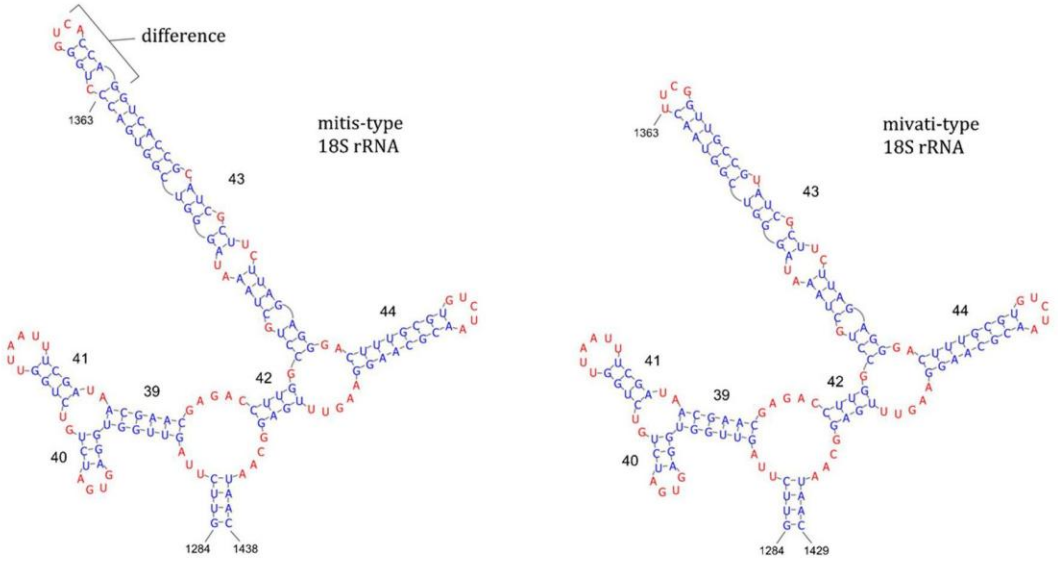

Fig. 3. Predicted secondary structures of the V7 region of small ribosomal subunits corresponding to the two types of sequences found within the *E. mitis* genome. The region from helix 39 to helix 44 is shown. Sequences FR775302 (mitis-type) and FR775305 (mivati-type) are displayed from base 1284 to 1438 and 1429, respectively.

the genome for a long time while the others are deleted (Rooney, 2004; Nei and Rooney, 2005).

There have been doubts about the validity of *E. mivati* species for more than 30 years. This species was often questioned in works utilising classical coccidiosis methods like oocyst morphology and cross-immunity but there was no molecular evidence to support this. Our discovery of the two types of 18S gene within the *E. mitis* genome is the first of such molecular evidence that supports conclusions of some previous studies, which stated that *E. mivati* and *E. mitis* are the same species (Shirley et al., 1983). The two types of 18S gene which correspond to the published sequences of *E. mivati* and *E. mitis* indicate that if the strains carrying only one or the other 18S type exist, they will likely cross-breed and still represent a single species. However, the more probable explanation is that all strains of *E. mitis* contain two types of 18S gene and that the occasional detection of only one or the other form by sequencing is caused by insufficient sampling, where only a single or a few random clones are sequenced. It is also highly probable that the original author who sequenced the 18S gene of presumed *E. mivati* (Barta et al., 1997) only obtained the *E. mivati* sequence variant due to the low number of clones sequenced (probably two) while the species investigated contained both variants. Also, in the course of sequencing 18S gene from mixed species isolates (like in Schwarz et al., 2009) the sequence originally from *E. mitis* can be mistakenly assigned to *E. mivati*. Our mitochondrial cytochrome *c* oxidase subunit I gene sequence of *E. mitis* is almost identical to the sequence in GenBank assigned to *E. mivati* (although this COI gene sequence was not derived from the original *E. mivati* strain), which suggests that this gene comes from the same species. The phylogenetic tree constructed from multiple COI sequences showed that the sequences of *E. mitis* and *E. mivati* form a single lineage distinct from other chicken *Eimeria* species and this is in agreement with the hypothesis that these sequences are derived from the same species. This hypothesis is also supported by the recently published work concerning *Eimeria* DNA barcoding (Ogedengbe et al., 2011), which concludes that the COI is a reliable marker for species delimitation. Our finding that both 18S and COI sequences of *E. mitis* and *E. mivati* come from the same species implies that the phylogenetic analysis based on COI by Ogedengbe et al. (2011) clusters each actual eimerian species into a separate monophyletic clade. The real-time quantitative PCR assay (that is based on the genomic sequence-characterized amplified region – SCAR-markers) used in this study for species identification and quantification, recognized these coccidian species correctly as *E. mitis*. This indicates that this quantitative PCR assay also covers species thought to be *E. mivati*.

Although intragenomic polymorphism of the 18S gene was already described in several apicomplexan species (most notably *Plasmodium*) this is the first time it was found in *Eimeria*. The sequence divergence between the two gene variants found in *E. mitis* is not as high as in *Plasmodium* but is higher than in *Cryptosporidium* and *Babesia* (Table 1). The phylogenetic tree reconstruction (Fig. 1) shows that these two sequence types form separate clades, which suggests that these genes might not evolve in concert and do not undergo homogenization. The predicted secondary structure of the two types of 18S rRNA found in *E. mitis* showed that these two types differ in the length of helix 43 in the V7 region, which means that the differences observed in primary structure have a significant impact on their secondary structure. The biological significance of the two 18S gene types in *E. mitis* is not known, however, we hypothesize that these types might be used in different stages of the parasite's life-cycle as it is in other apicomplexan species investigated so far.

Table 1
Sequence identity shared between the two 18S gene types in various apicomplexan species.

Species	18S types	Sequence identity	Reference
Plasmodium falciparum	A/C	89%	McCutchan et al. (1988)
Plasmodium vivax	A/C	92%	Qari et al. (1994)
Plasmodium cynomolgi	A/B	90%	Corredor and Enea (1994)
Cryptosporidium parvum	A/B	99%	Le Blancq et al. (1997)
Babesia bigemina	A,B/C	99%	Reddy et al. (1991)
Eimeria mitis	mit/miv	98%	

This work presents molecular evidence that contests the existence of *E. mivati* as an independent species and it is in agreement with previous studies that reached the same conclusion by using traditional methods (Shirley et al., 1983). On the basis of these findings, the authors would recommend that the *Eimeria* taxonomy is revised accordingly.

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III.

Description of the two strains of turkey coccidia *Eimeria adenoeides* with remarkable morphological variability

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SUMMARY

Although oocyst morphology was always considered as a reliable parameter for coccidian species discrimination we describe strain variation of turkey coccidia, *Eimeria adenoeides*, which remarkably exceeds the variation observed in any other *Eimeria* species. Two strains have been isolated – the first strain maintains the typical oocyst morphology attributed to this species – large and ellipsoidal – while the second strain has small and ovoid oocysts, never described before for this species. Other biological parameters including pathogenicity were found to be similar. Cross-protection between these 2 strains in 2 immunization and challenge experiments was confirmed. Sequencing and analysis of 18S and ITS1 ribosomal DNA revealed a close relationship according to 18S and a relatively distant relationship according to ITS1. Analysis of 18S and ITS1 sequences from commercial turkey coccidiosis vaccines Immucox®-T and Coccivac®-T revealed that each vaccine contains a different strain of *E. adenoeides* and that these strains have 18S and ITS1 sequences homologous to the sequences of the strains we have isolated and described. These findings show that diagnostics of turkey coccidia according to oocyst morphology have to be carried out with caution or abolished entirely. Novel PCR-based molecular tools will be necessary for fast and reliable species discrimination.

Key words: *Eimeria adenoeides*, coccidiosis, morphology.

INTRODUCTION

Coccidiosis is a widespread disease caused by several *Eimeria* species. This apicomplexan parasite is most notable in the chicken and turkey farming industry. Coccidiosis often runs a subclinical course with a negative effect on the performance of farmed poultry and clinical outbreaks of the most pathogenic species can cause significant economic losses. According to the literature, 7 species that infect the turkey host (*Meleagris gallopavo*) are recognized – *E. adenoeides*, *E. dispersa*, *E. gallopavonis*, *E. innocua*, *E. meleagridis*, *E. meleagrimitis* and *E. subrotunda* (for review see Chapman, 2008). However, there are doubts about the taxonomic integrity of *E. meleagrimitis* (Cook *et al*. 2010) and due to the inconsistency of data in the literature and the lack of molecular characterization, correct species identification can be difficult.

Eimeria adenoeides was first described by Moore and Brown (1951). It is considered the most pathogenic turkey coccidia and heavy infections can cause 100% mortality in young turkey poults (Clarkson, 1960). Low doses of oocysts lead to depressed body weight gains and the increasing doses progressively influence severity of infection (Hein, 1969). The pathogenic effect of *E. adenoeides* is greater than that of *E. meleagrimitis* in birds fed with an equivalent number of oocysts (Clarkson, 1958, 1959).

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Traditionally, *Eimeria* species are distinguished by oocyst morphology, pre-patent period, site of infection, pathogenic effects or minimum sporulation time based on the assumption that these parameters are fixed for each species. Oocyst morphology was always considered a reliable discriminating parameter to distinguish between species that differ significantly in oocyst size or shape.

Here we report strain variation in the oocyst morphology of *E. adenoeides* that is outside the traditional limits of strain variation observed in any *Eimeria* species. While the oocysts of the *E. adenoeides* reference strain, KR, are large and ellipsoidal we have found a strain (KCH) whose oocysts are small and ovoid. Other biological parameters including pathogenicity measured as a body weight gain depression after infection were similar. We also confirmed the cross-protection between these 2 strains and compared the sequences of the small ribosomal subunit (18S), the internal transcribed spacer 1 (ITS1) and the mitochondrial cytochrome *c* oxidase subunit I gene (COI).

MATERIALS AND METHODS

Parasites

Fecal samples were collected during coccidiosis outbreaks at small turkey farms in the Czech Republic where *E. adenoeides* was suspected

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according to macroscopic lesion diagnosis. These farms were not using in-feed anticoccidials. Oocysts were isolated using the modified salt flotation method (Long *et al*. 1976) and were stored in 2·5% potassium dichromate at 4 °C. Single oocyst isolations were completed with a micromanipulator (Narishige, Japan) that was mounted to an inverted microscope (Nikon, Japan) using glass microcapillaries filled with silicone oil. Single oocysts were selected, aspirated into a micropipette, moved between droplets of PBS and then embedded into agar cubes that were subsequently used for inoculating the turkeys. The isolated strains were passaged in British United Turkeys (BUT) Big 6 turkeys. Commercial vaccines Immucox®-T (Vetech, Canada) and Coccivac®-T (Intervet, USA), both of which contain multiple species of turkey coccidia, served as a source of DNA for comparison with our strains of *E. adenoeides*.

Biological characterization

The oocyst size and shape of each isolated strain were determined by measuring 100 oocysts under the microscope. The longest and the shortest perpendicular dimensions were recorded, a histogram was generated in Microsoft Excel and the average dimensions were determined. To measure the pre-patent period, turkeys in 2 groups of 5 birds per group were infected at 23 days of age with 10000 sporulated oocysts per bird with either KR or KCH strain, and feces were collected at 6-h intervals between 0 and 120 h post-infection. Oocysts isolated from feces were counted using the standard McMaster method (Long *et al*. 1976). To examine macroscopic lesions, turkeys at 30 days of age in 2 groups of 5 birds per group were infected with 200000 sporulated oocysts of either KR or KCH strain and intestinal lesions were inspected 6 days after infection.

Pathogenicity comparison

The pathogenic effect on body weight gain was studied using 17-day-old turkeys arranged into 8 groups of 5 birds per group, which were infected with various doses of sporulated oocysts (5, 10 and 20 thousand) of either KR or KCH strain. The control groups were not infected. Turkeys were individually weighed at days 0, 3, 6 and 9 after infection. Relative body weight gains among groups were compared using the ANOVA and Dunnett's multiple-comparison test with NCSS 2001 software. $P < 0{\cdot}05$ was considered significant.

Cross-immunization experiment

The first cross-immunization experiment included immunization with the KCH strain and a challenge with the KR strain. In this experiment 45-day-old turkeys arranged into 2 groups of 5 birds per group were either immunized with 500 sporulated oocysts of the KCH strain or not immunized and both groups were challenged 14 days later with 10000 sporulated oocysts of the KR strain. Single droppings were collected from each bird after massage of the cloaca at days 0, 5 and 6 after the challenge, and the oocysts were counted using the standard McMaster method. Oocyst counts were expressed as the number of oocysts per gramme of feces (OPG). The second cross-immunization experiment included immunization with the KR strain and challenge with the KCH strain. Here the 14-day-old turkeys were again arranged into 2 groups of 5 birds per group, and the birds were either immunized with 1000 sporulated oocysts of the KR strain or not immunized and both groups were challenged 14 days later with 10000 sporulated oocysts of the KCH strain. The OPG was counted using all feces collected between days 4 and 8 after the challenge. The homologous challenge was tested in a separate experiment where the 10-day-old turkeys were arranged into 4 groups of 5 birds per group. Two groups were immunized with 500 sporulated oocysts of each strain (KR and KCH strains, respectively) and the remaining 2 groups were not immunized. Each group was challenged 20 days later with 50000 sporulated oocysts of the corresponding homologous strain. Single droppings used for OPG calculation were collected from each bird after massaging of the cloaca 6 days after the challenge.

Sequencing of 18S, ITS1 and COI

Oocysts for isolation of DNA were washed in PBS, disrupted in a Mini-BeadBeater-16 (BioSpec, USA) using 0·5 mm glass beads, and the DNA was extracted from the lysate using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Full-length small subunit ribosomal DNA was amplified using ERIB1 and ERIB10 primers (Barta *et al*. 1997) and the ITS1 region was amplified using custom degenerate primers designed according to the comparison of sequences from multiple *Eimeria* species using Clustal W software (Larkin *et al*. 2007, Table 1). Mitochondrial cytochrome *c* oxidase subunit I gene was amplified using primers KM204 and KM205 (Schwarz *et al*. 2009). PCR reactions to amplify the 18S rDNA and COI gene were carried out using high-fidelity Phusion Hot Start DNA polymerase (Finnzymes, Finland). These reactions contained 200 μM dNTP each, 500 nM forward and reverse primers, 1 μl of template DNA and 0·02 U/μl of Phusion enzyme in Phusion HF buffer. The thermal cycling program for 18S consisted of an initial denaturation at 98 °C for 1 min followed by 25 cycles of denaturation at 98 °C for 15 s, annealing at 69 °C

Table 1. Sequences of primers used for amplification of 18S, ITS1 and COI

Primer name	Sequence (5′–3′)	Length (bp)
ERIB1 (forward)	ACCTGGTTGATCCTGCCAG	19
ERIB10 (reverse)	CTTCCGCAGGTTCACCTACGG	21
ITS1-F (forward)	GTAAATAGAGCCCYCTAARGGAT	23
ITS1-R (reverse)	GCGTGAGCCAAGACATYCATTGC	23
KM204 (forward)	GTTTGGTTCAGGTGTTGGTTG	21
KM205 (reverse)	ATCCAATAACCGCACCAAGAG	21

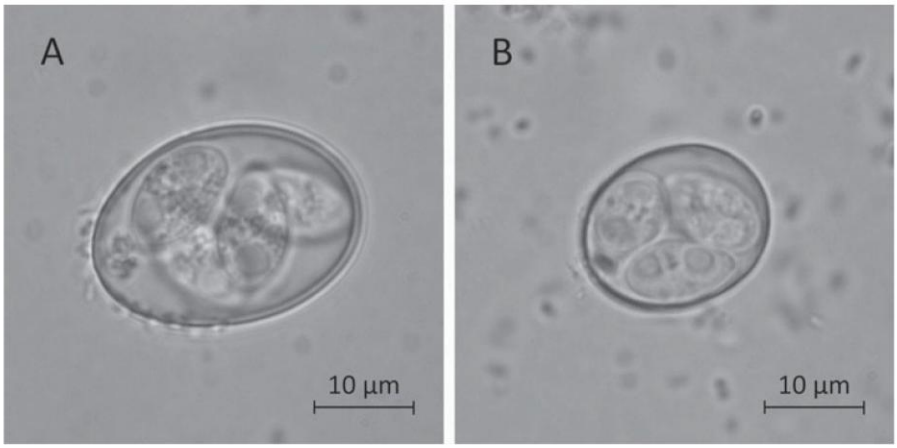

Fig. 1. Sporulated oocysts of *Eimeria adenoeides* KR (A) and *E. adenoeides* KCH (B).

for 20 s and extension at 72 °C for 1 min. The program for COI consisted of an initial denaturation at 98 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 15 s, annealing at 67 °C for 20 s and extension at 72 °C for 30 s. PCR reactions for ITS1 were carried out using Taq polymerase premixed in 2 × Blue Master Mix (Top-Bio, Czech Republic) with 500 nM forward and reverse primers and 1 μl of template DNA. The thermal cycling program for ITS1 consisted of an initial denaturation at 95 °C for 1 min followed by 25 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany). Taq-generated PCR products were blunted and all ribosomal DNA fragments were phoshorylated and cloned into EcoRV digested dephoshorylated pBluescript vector (Stratagene, USA) using home-made (Inoue *et al*. 1990) chemically competent DH5α™ cells (Invitrogen, USA). Minipreps were prepared with the QIAprep Spin Miniprep Kit (Qiagen, Germany). Amplified COI genes were sequenced directly from the PCR products using the same PCR primers and the ribosomal targets (18S and ITS1) were sequenced from the purified plasmids (Macrogen, Korea). Eight independent PCR, cloning and sequencing reactions were done for the 18S and ITS1 of both *E. adenoeides* strains. Two COI PCR reactions from each strain were bidirectionally sequenced. Consensual sequences were manually created in BioEdit software (Hall, 2005). Vaccine Immucox®-T was analysed by the sequencing of 48 clones of each 18S and ITS1, and Coccivac®-T was analysed using 96 clones of each 18S and ITS1.

RESULTS

Isolation and biological characterization of E. adenoeides *strains*

We have isolated 2 strains of *E. adenoeides* that differ significantly in oocyst morphology. The strain which has the standard oocyst morphology attributed to this species (large and ellipsoidal) was named *E. adenoeides* KR and the strain with small and oval oocysts was named *E. adenoeides* KCH. Size difference was easily seen under the microscope because the average oocyst length of the smaller strain is approximately the width of the larger strain (Fig. 1, Table 2). The pre-patent period was measured to be the same for both strains and it corresponds to the published data about *E. adenoeides* (Table 2). After experimental infection, both strains of *E. adenoeides* displayed typical macroscopic lesions for this species, most notably caecal caseous plugs. Pathological signs included caecal deformation and loss of flexibility, ulceration of the tubular part of the caeca, liquid or creamy caecal content with the presence of white caseous and fibrinous necrotic material. There were no notable differences in macroscopic lesions between these two strains.

Table 2. Biological characteristics of isolated strains of *E. adenoeides*

(HPI = hours post-infection.)

Strain	Oocyst dimensions width × length (μm) Average	Intervals	Index length/ width	Oocyst shape	Pre-patent period (HPI)
E. adenoeides KR	19·4 × 27·8	14·1–23·5 18·8–34·4	1·43	Ellipsoidal	103–108
E. adenoeides KCH	16·1 × 19·4	13·2–19·2 15·6–22·8	1·20	Broadly oval	103–108

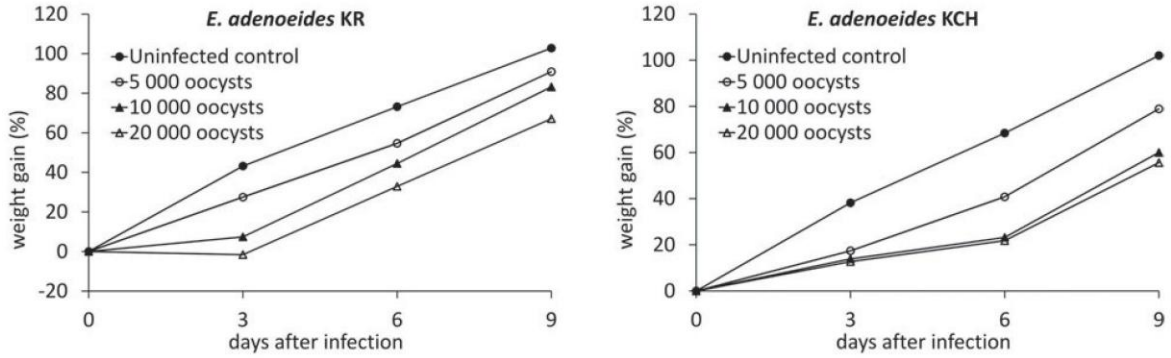

Fig. 2. Pathogenicity measured as a weight gain reduction after infection with various doses of *Eimeria adenoeides* sporulated oocysts.

Pathogenicity comparison

Comparison of the pathogenic effect on body weight gain after infection did not reveal any significant difference in pathogenicity between the KR and KCH strains (Fig. 2). The pathogenic effect on body weight gain was clearly dose-dependent at least in the range of 5 to 20 thousand sporulated oocysts used for infection.

Cross-immunization experiment

Data from the cross-immunization experiment indicate that the KR and KCH strains of *E. adenoeides* do induce cross-protection, i.e. immunization of turkey with the one strain protects against a challenge with the other strain. When immunized with the KCH strain, the oocyst output after the challenge with the KR strain was reduced by 95% and 79% on days 5 and 6 post-challenge, respectively. When immunized with the KR strain the oocyst output after the challenge with the KCH strain was reduced by 77% (Fig. 3). In the homologous immunization and challenge experiment the oocyst output was reduced by 98% in both strains (Fig. 4).

Sequencing of 18S and ITS1 rDNA and COI gene

The sequences of small ribosomal subunits of the KR and KCH strains were found to be almost identical differing in only 2 transversions but the ITS1 sequences share only 77% similarity. The 18S sequences of our *E. adenoeides* strains share 99·8% similarity to the sequence in GenBank named *Eimeria adeneodei* (Accession no. AF324212, Zhao and Duszynski 2001, *unpublished*). We also found the 18S and ITS1 sequences of our KR strain to be present in the Coccivac®-T vaccine and the sequences of KCH strain in the Immucox®-T vaccine. The sequences of 18S from our strains were identical to the sequences in these vaccines and the ITS1 sequences shared more than 99% similarity. The sequences of 18S and ITS1 were deposited into GenBank (Accession nos FR745913 – FR745916). Comparison of our ITS1 sequences with ITS1 sequences from the published work concerning diagnostic PCR (Cook *et al*. 2010) revealed that while the sequence of the KR strain corresponds to the published *E. adenoeides* sequence, the sequence of the KCH strain is absent and there is no sequence in that paper with a high homology to this sequence. The mitochondrial COI sequences of our *E. adenoeides* strains share 97·7% identity at the DNA level and 100% identity at the amino acid level, meaning that all nucleotide substitutions are synonymous. COI sequences of these two strains were also deposited into GenBank (Accession nos FR846201 and FR846202).

DISCUSSION

We have isolated and characterized 2 strains of *E. adenoeides* that differ remarkably in oocyst size

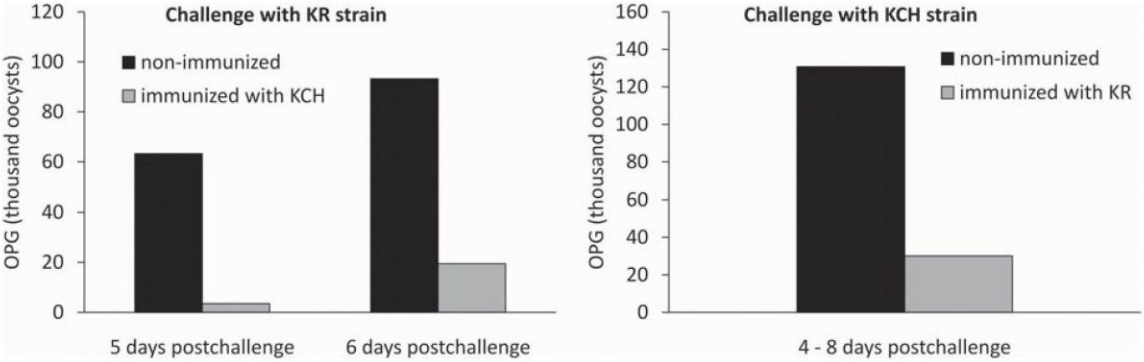

Fig. 3. Oocyst output results in experimental groups employing different immunization and challenge strains of *Eimeria adenoeides*.

and shape. This is the first description of such a substantial strain variation of oocyst morphology in any *Eimeria* species. The size of the oocysts was always considered as a fixed and relatively reliable parameter for distinguishing among *Eimeria* species but our findings indicate that this might not be true for all coccidian species. Although diagnostics according to oocyst morphology is well established in chicken coccidia it seems that such diagnostics will be problematic or even impossible in turkey coccidia.

Although strain variation of oocyst morphology was already described in *E. meleagrimitis* (Long *et al.* 1977) it was not to such an extent as we have observed in *E. adenoeides*. While the described variability of the length of *E. meleagrimitis* oocysts was 19% we have encountered a 43% difference in oocyst length between *E. adenoeides* strains. Such a difference is also readily seen under the microscope and it is easy to mistake the smaller strain of *E. adenoeides* for *E. meleagrimitis* which has oocysts of the same size and shape.

We have shown that the other biological parameters of the KR and KCH strains are similar and that their pathogenic effect also does not differ significantly, therefore their virulence (strength) can be considered equal. The cross-protection between the two strains was shown to be very high (77–95%) although not as high as in the homologous challenge experiment (98%), still suggesting, however, that there is no need to include both strains in a live turkey coccidiosis vaccine because immunization with one strain protects against a challenge with another strain.

Sequencing of the 18S ribosomal DNA revealed that both strains share almost the same small subunit sequence, which is also homologous to the sequence in GenBank incorrectly named *E. adeneodei*. However, sequences of ITS1 differed substantially, which might indicate a distant relation of the two strains. The ITS1 sequence divergence between these two strains is approximately as high as the ITS1 sequence divergence between chicken *Eimeria* species. Therefore, when looking only at ITS1 these two strains

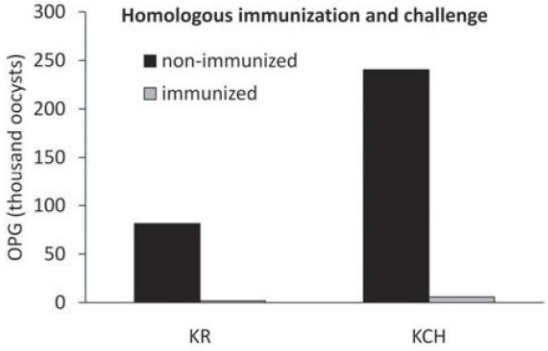

Fig. 4. Oocyst output results from the homologous immunization and challenge experiment.

could be considered as two different species but it is known that ITS1 varies strongly both between strains as well as between species, and it is not a reliable marker for inferring phylogenetic relationships of chicken *Eimeria* (Lew *et al.* 2003). Moreover, there can be more variants of an internal transcribed spacer present within a single genome and these copies can also differ significantly (Lew *et al.* 2003; Cantacessi *et al.* 2008; Cook *et al.* 2010). The recently published diagnostic PCR for turkey coccidia (Cook *et al.* 2010) that is based on ITS1 sequences includes only sequence homologous to the KR strain, and the alignment of the sequences of primers developed for *E. adenoeides* to ITS1 of the KCH strain indicates that this PCR will probably fail to detect the KCH strain. Comparison of COI sequences revealed that these genes are translated to the identical protein although the DNA sequences differ by multiple synonymous substitutions. These differences in the DNA sequence might also indicate a relatively distant relation of the two strains. Nevertheless, the identity at amino acid level (100%) is still higher than the identity shared between the closest chicken coccidia species (99·3%). The closest COI amino acid sequences found in chicken coccidia are those of *E. tenella* and *E. necatrix* and these sequences differ at 2 amino acid positions.

By analysis of the 18S and ITS1 sequences, which are present in the commercial live virulent turkey coccidia vaccines Immucox®-T and Coccivac®-T, we have revealed that each vaccine contains a different strain of *E. adenoeides* and that these strains correspond to our KR and KCH strains. Coccivac®-T contains a strain of *E. adenoeides* that is similar to the KR strain, and Immucox®-T contains a strain similar to the KCH. Furthermore, we have confirmed by microscopy that Immucox®-T does not contain large and ellipsoidal oocysts typical for the KR strain. Such microscopical evaluation was possible since Immucox®-T contains only 2 species (*E. adenoeides*, *E. meleagrimitis*), and *E. meleagrimitis* has small oocysts as well. Owing to the fact that Coccivac®-T contains 4 different species that are similar in oocyst morphology, we could not evaluate the oocyst morphology of an included *E. adenoeides* strain.

Our findings show that diagnostics of turkey coccidia according to oocyst morphology have to be carried out with caution and the same should be valid for other coccidian species that are much less studied. Currently, the best approach to fast and reliable diagnostics is to develop a PCR-based molecular method, which will cover all strains and species, as was completed for chicken coccidia. However, care should be taken when using polymorphic targets such as ITS1 or ITS2.

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