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Exploring the genetic diversity of *Eimeria acervulina*: A polymerase chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) approach

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ABSTRACT

Eimeria, protozoan parasites that can cause the disease coccidiosis, pose a persistent challenge to poultry production and welfare. Control is commonly achieved using good husbandry supplemented with routine chemoprophylaxis and/or live parasite vaccination, although widespread drug resistance and challenges to vaccine supply or cost can prove limiting. Extensive effort has been applied to develop subunit anticoccidial vaccines as scalable, cost-effective alternatives, but translation to the field will require a robust understanding of parasite diversity. Using a new Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) panel we begin to describe the genetic diversity of *Eimeria acervulina* populations in Africa and Europe. PCR-RFLP genotyping *E. acervulina* populations sampled from commercial broiler and layer chickens reared in Nigeria or the United Kingdom (UK) and Republic of Ireland (RoI) revealed comparable levels of haplotype diversity, in direct contrast to previous descriptions from the close relative *E. tenella*. Here, 25 distinct PCR-RFLP haplotypes were detected from a panel of 42 *E. acervulina* samples, including 0.7 and 0.5 haplotypes per sample in Nigeria (n = 20) and the UK/RoI (n = 14), respectively. All but six haplotypes were found to be country-specific. The PCR-RFLP markers immune mapped protein 1 (IMP1) and heat shock protein 90 (HSP90) were most informative for Nigerian *E. acervulina*, while microneme protein 3 (MIC3) and HSP90 were most informative in UK/RoI populations. High haplotype diversity within *E. acervulina* populations may indicate frequent genetic exchange and potential for rapid dissemination of genetic material associated with escape from selective barriers such as anticoccidial drugs and future subunit vaccines.

1. Introduction

Eimeria are coccidian parasites of the family Eimeriidae, phylum Apicomplexa. Most *Eimeria* species are obligate intracellular parasites of the intestinal tissues of vertebrates (Jirku et al. 2013), where intracellular development within the mucosal and/or sub-mucosal layers of the intestine can result in tissue damage, enteritis, and malabsorption (Shirley et al. 2005). Under intensive farming conditions these pathogens can cause the enteric disease coccidiosis, undermining the growth, productivity, and welfare of chickens, and contributing to serious economic losses estimated at UK£ 10.4 billion per annum worldwide in 2016 (Shirley et al. 2005, Blake et al. 2020).

Seven *Eimeria* species are widely recognised to infect domestic chickens, namely *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*. Recently, three new cryptic species previously known as operational taxonomic units (OTUs) X, Y, and Z were discovered (Morris et al., 2007; Cantacessi et al., 2008) and confirmed to be distinct and widespread (Clark et al., 2016a, 2016b; Jatau et al., 2016; Hauck et al., 2019). The names *E. lata*, *E. nagambie*, and *E. zaria* have been suggested, respectively (Blake et al. 2021). These species differ in biology, pathogenicity, and immunogenicity, increasing the challenge posed by *Eimeria*. Understanding the occurrence, diversity, and population structure of all ten *Eimeria* species that infect chickens is fundamental to their control.

Eimeria are commonly controlled using routine chemoprophylaxis or vaccination with varied formulations of live parasites (Lee et al. 2022).

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When combined with good husbandry these strategies can be highly effective, but limitations associated with public perception and widespread resistance for drugs, or relatively high production costs and inherently limited production capacity for live vaccines, now encourage development of novel tools for control (Shirley et al. 2005, Chapman and Jeffers, 2014). One option for control is development of sub-unit recombinant vaccines (Venkatas and Adeleke, 2019). However, the success and longevity of these vaccines largely depends on the extent of pre-existing genetic diversity and the structure of parasite populations in the field (Blake et al., 2015; Clark et al., 2016a, 2016b).

Attempts to define genetic diversity and population structure for *Eimeria* that infect chickens have centered on *E. tenella* (Blake et al., 2015; Vo et al., 2021; Alam et al., 2022), prompted by its widespread occurrence, pathogenicity and economic importance (Long et al. 1976, Gyorke et al. 2013). Recent development of a multi-locus sequence typing (MLST) panel for *E. maxima* has started to provide tools and data for a second *Eimeria* species (Carrisosa et al., 2022). Less attention has been given to *E. acervulina*, another prevalent and economically relevant species, despite its contribution to sub-clinical malabsorptive disorders that result in serious production losses in the poultry industry. *Eimeria acervulina* has been identified as the most frequently encountered cause of sub-clinical coccidiosis in commercial poultry (McDougald and Fitz-Coy, 2008). Despite sharing a broadly comparable life cycle, *E. acervulina* presents a biological profile that is distinct from *E. tenella* in terms of fecundity and patency (Michael, 1975, Long et al. 1976, McDougald and Jeffers, 1976), suggesting scope for a different population structure. Understanding differences between these two species can support optimization of species-specific control for *Eimeria*, recognizing differences in malabsorptive and haemorrhagic disease profiles (Blake et al. 2021).

Antigenic variation has been described for *E. acervulina*, with strains isolated from chickens in the UK or China found to exhibit differential escape from immunity induced by prior homologous or heterologous infection (Joyner, 1969, Wu et al. 2014). However, Random Amplified Polymorphic DNA (RAPD), single-locus sequence typing of common target loci, for example internal transcribed spacer (ITS) 1 and/or 2 and mitochondrial cytochrome c oxidase subunit I (COI) DNA, and comparison whole mitochondrial genomes, are the only molecular tools that have been used to describe intraspecific variations that exist between and among *E. acervulina* isolates (Bhaskaran et al., 2010; Clark et al., 2016b; Costa et al., 2001; Fatoba et al., 2020; Fernandez et al., 2003; Kumar et al., 2015; Morgan and Godwin, 2017; Nowzari et al., 2005; Schwarz et al., 2009). These studies have revealed considerable genetic variability that is notably higher than observed in *E. tenella* populations (Fernandez et al., 2003; Clark et al., 2016). However, while these molecular methods have proven useful in defining *E. acervulina* isolates, their narrow focus has limited interpretation of genome-wide diversity and species population structure.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) has become popular for accessible genotyping of apicomplexan parasites, using the differential band patterns produced after restriction enzyme digestion to define genetic differences that exist among DNA from individuals and populations. For example, PCR-RFLP has been used to genotype and differentiate *Toxoplasma gondii* populations isolated from several host species (Su et al., 2006; Burrells et al., 2016; Nzelu et al., 2021) and multiple locations (Dubey et al., 2007a, 2007b; Velmurugan et al., 2009). PCR-RFLP has also been developed to differentiate the piroplasms *Babesia* and *Theileria*, as well as *Cystoisospora* and *Sarcocystis* (Yang et al. 2002, Jefferies et al. 2007, Samarasinghe et al. 2008). PCR-RFLP is a reliable, sensitive, and affordable technology that is available to many laboratories, including those in resource-poor settings, requiring only a thermal cycler and gel electrophoresis equipment (Pegg et al., 2016). These advantages make comparative genetic analyses from various regions possible. For *Eimeria*, early studies focused on ITS-2 presented a PCR-RFLP to differentiate between *Eimeria* species, but diversity was limited within each species (Woods et al. 2000). More recently, a panel of PCR-RFLP assays targeting *E. tenella*-specific single nucleotide polymorphisms (SNPs) identified by comparison of next-generation sequenced genome assemblies was developed and used to define population structure in chickens from the UK and Republic of Ireland (Blake et al. 2015, Pegg et al. 2016). Currently, PCR-RFLPs suitable to sub-type other *Eimeria* species from chickens are not available.

Here, we have developed and applied a robust, accessible, and cost-effective multi-locus PCR-RFLP approach to differentiate *E. acervulina* field isolates and explore the extent of genetic diversity that exists within and between African and European *E. acervulina* populations.

2. Materials and methods

2.1. Reference parasites – propagation, processing, and genomic DNA extraction

The Houghton (H) and Weybridge (W) reference isolates of *E. acervulina* (Peek and Landman, 2003; Reid et al., 2014) were maintained and propagated in specific pathogen-free Lohmann Valo chickens and purified as described elsewhere by Long et al. (1976) and MAFF (1986). Genomic DNA was extracted from purified sporulated oocysts using a QIAamp DNA Stool mini kit as recommended by the manufacturer (Qiagen, Hilden, Germany) after a mechanical disruption step with 0.4–0.6 mm glass beads (Kumar et al., 2014).

2.2. PCR-RFLP marker development

In the absence of validated *E. acervulina* species-specific SNPs, a panel of candidate loci were amplified and sequenced from the H and W strains. In total 13 loci were tested, prioritizing genomic regions containing reference genes and genes encoding putative vaccine candidates, surface antigens or other targets of possible biological significance (Table 1). Briefly, putative or confirmed coding sequences were identified in GenBank using the nucleotide search option (https://www.ncbi.nlm.nih.gov/nuccore) with the search terms “Eimeria”, “acervulina”, and the target gene name. Sequences were downloaded and used to identify homologous genomic sequences within the *E. acervulina* H strain genome sequence assembly for primer design using BLASTn in ToxoDB (Harb and Roos, 2020). Forward and reverse primers targeting 353–1348 bp genomic sequence centered on each locus were designed using Primer3 (Rozen and Skaletsky, 2000). Target amplicon size was set to 400 – 1000 bp for PCR-RFLP primer design with an optimal melting temperature (Tm) of 60 °C. The target amplicon size was expanded to 300 – 1400 bp when no suitable primers could be designed. All primers were synthesized by Sigma-Aldrich (UK). Primers were assessed for likely species-specificity by comparison with aligned equivalent sequences from the nine other *Eimeria* species identified by BLASTn using ToxoDB (recognised species) or local BLASTn in CLC Main Workbench v8.1 (new species, CLC Bio; CLC Bioinformatics, 2015).

2.2.1. Polymerase Chain Reaction (PCR)

Target loci were amplified from the H and W reference strains by PCR using MyTaq DNA polymerase (Bioline). Each PCR reaction contained 1 µl template DNA, 1 µl appropriate forward and reverse primers (10 µM stock), 12.5 µl MyTaq × 2 and 10.5 µl molecular grade water (Sigma). The primers used are shown in Table 1. PCR thermal cycling conditions were: 1 × initial denaturation at 95 ^{0}C for 1 min, 30 × (denaturation at 94 ^{0}C for 30 s, annealing at 58 ^{0}C for 30 s, extension at 72 ^{0}C for 1 min), and a final extension step at 72 ^{0}C for 7 min. To identify candidate SNP markers, PCR products were sequenced with the same primers used for their initial amplification (Eurofins Genomics). The sequences were assembled, annotated, and interrogated on CLC Main Workbench v8.1 (CLC Bio; CLC Bioinformatics, 2015) using BLASTn against the reference *E. acervulina* H strain genome sequence assembly (Reid et al. 2014).

Table 1
Summary of candidate *Eimeria acervulina* loci targeted and sequenced for development as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) markers. Eight genomic loci were taken forward for development of nine markers, as indicated in **bold**. Eten = no *E. acervulina* homologue available, *E. tenella* homologous sequence used as an alternative.

Target locus (abbreviation)	Reference sequence	ToxoDB reference[a]	Primer sequence (5′ – 3′)
Actin (Act)	XM_013396709	HG670679	F: GAGTTGGAAGGAACACGGGAAG R: TCGACGAGGAGATGAAGAATGC
Actin depolymerizing factor 1 (ActDepol)	XM_013391622	HG673508	F: TGTGTTCATTTTCTCGCGCTTT R: GCCATAGTGCCCTCCAACTTCT
Apical Membrane Antigen 1 (AMA1)	XM_013396429	HG670746	F: AAAAACCTTCACCGCACCAACT R: GGGAGTACGGCGTTTTATCAGG
Beta tubulin (Btub)	XM_013396319	HG670774	F: TATGTGAAAGGGGTGGTTGGTG R: AACTCGGACCTCAGGAAACTGG
Heat Shock Protein 90 (HSP90)	AY459442	HG671427	F: TTCCTTTAGCTGCATCGCTTTG R: GGGGCTCGTACTTGTCATCCTT
Immune Mapped Protein 1 (IMP1)	XM_013394891	HG671044	F: GCCTGCCTCTGTAGCGATTTCT R: GCCTCTGTGAAAGTGCACCAAT
Microneme protein 2 (MIC2)	KR063282	HG670307	F: TCCAACGAAGTTGCCAGATGAT R: AGAAGGTGAAACGGCTCCAAAG
Microneme protein 3 (MIC3)	KU359773	HG673221	F: ACGGATTCCTTCTTCTCCGATG R: CTCAGGTAGCCTCAGCGATTGA
Microneme protein 5 (MIC5a)	EF520719 (Eten)	HG670884	F: CGGAGAGACGATCTTCAGCAAA R: CTAAATGCCAGACGACCACAGG
Microneme protein 5 (MIC5b)	*Same as above*	*Same as above*	*Same as above* *Same as above*
Antigen MZ92/130 (MZ92/130)	M86628	HG670841	F: AACACTGGTACCCGCTTCAG R: TACGCACACGCTCTCAAAAC
Surface Antigen C-Type (SAGC)	XM_013394805	HG671062	F: ACTCGGTCACTACAGCGTCGTC R: TGGAGGCTCACAGAACTCAACC
Transhydrogenase (Transhyd)	L08392	HG670454	F: AGAACTTCAGCTTCCTCCCC R: CGATTTCTCCAGGCCACATG

[a] Source version(s): GCA_000499425.1, Nov 5, 2013, Data Set *Eimeria acervulina* Houghton Genome Sequence and Annotation (toxodb.org)

2.2.2. Restriction fragment length polymorphism (RFLP)

A PCR amplicon was selected for development as a PCR-RFLP if it contained a restriction endonuclease recognition site that was disrupted by a H or W strain-specific SNP; if it existed as a single copy per genome; and where the flanking region was suitable for primer design. Enzyme restriction sites were identified using the online tool NEBcutter (version 2.0, New England Biolabs; Vincze et al., 2003) and were set to represent 15 bp centred around each candidate SNP (Pegg et al., 2016).

PCR amplicons recovered for RFLP were subjected to restriction endonuclease digestion with the appropriate enzyme(s). Exactly 5 μl of PCR product was used as template and combined with 1 μl restriction enzyme, 1 μl appropriate 10 × Buffer (New England Biolabs) and molecular biology grade water to a final volume of 10 μl, followed by incubation for 60 min at the temperature required for each candidate enzyme.

2.2.3. Gel electrophoresis

Approximately 5 μl of each digested product was resolved by agarose gel electrophoresis using 1.5% (w/v) UltraPure agarose gel prepared in 1 × Tris-borate-EDTA buffer (TBE; all Sigma-Aldrich) with 0.01% (v/v) SafeView nucleic acid stain (NBS Biologicals). The gels were visualized on a U:Genius Gel Documentation System (Syngene).

2.3. Field sample analyses

Forty field samples previously found to be positive for *E. acervulina* were recruited into this study from broiler/layer flocks in Nigeria (n = 20), a broiler farm in Egypt (n = 1), broiler farms in the United Kingdom or Republic of Ireland (UK/RoI, n = 12), and broiler farms in other European countries (one each from France, Germany, Italy, Portugal, Ukraine, two from the Netherlands, n = 7 in total). These samples were obtained from small to medium scale commercial farms that did not employ the use of vaccines for coccidiosis control. Similar protocols described above for genomic DNA extraction, PCR amplification, and restriction endonuclease digestion of the reference strains were applied with minor modifications. To improve PCR sensitivity, the number of cycles for the denaturation, annealing, and extension steps were increased to 40 × . *Eimeria acervulina* H strain DNA and molecular biology grade water were used as template in positive and negative controls for each assay, respectively. To confirm identity, ~10% of amplified products for each SNP locus were selected at random and sequenced (GenBank accession numbers OQ509171 - OQ509236).

2.4. Data analyses

In total 42 *E. acervulina* samples were used for analysis, including 40 field isolates and both reference strains, creating a panel of 42. A SNP-genotype corresponding to either the H or W strain *E. acervulina* genetic type was manually assigned per sample/locus based on the band patterns obtained from PCR-RFLP. The presence of a single band indicated a SNP-type associated with restriction endonuclease recognition site disruption, while two (or three) bands indicated the occurrence of the SNP with maintenance of the site. Cases where both band profiles were obtained indicated a polyclonal population of haploid *E. acervulina* parasites, with the brightest band pattern recorded as the dominant genotype. Each sporulated oocyst is the product of sexual replication and can contain up to four distinct genome-wide genetic types following meiosis during sporocyst formation. Where results of endonuclease digestion were inconclusive, PCR amplicons were sequenced to determine the SNP-type *in-silico* as described above.

SNP genotypes were compiled in Microsoft Excel (version 2307 for Microsoft 365, Washington, USA) and combined to create SNP haplotypes for each sample. Where polyclonal infections were detected dominant and minority genotypes were assigned based on band intensity, pooling dominant and minority markers into separate haplotypes. The number of distinct SNP haplotypes detected was calculated using DnaSP (DNA Sequence Polymorphism version 5.10.01; Librado and Rozas, 2009). The program NETWORK version 10.2.0.0 (Bandelt et al., 1999) was used to produce a median joining phylogenetic network for PCR-RFLP SNP haplotypes. Fisher's exact test was used for statistical

comparison of haplotype occurrence between regions (GraphPad Prism, v 9.0.0). Where more than ten sequences were generated at a single locus all sequences were aligned using CLC Main Workbench v8.1 with the slow (very accurate) setting and exported to DnaSP to calculate nucleotide diversity (π).

3. Results

3.1. Selection and validation of PCR-RFLP markers

Nine SNP markers met the selection criteria for PCR-RFLP development based upon identification of isolated SNPs within the target H or W sequences and access to restriction digestion enzymes with suitable recognition sequences that would permit differentiation between the strains. The respective locus-specific primer pairs, restriction enzymes and conditions, and anticipated fragment profiles are presented in Tables 1 and 2. Using the *E. acervulina* H and W reference strains to test each PCR-RFLP assay yielded digested amplicons with expected sizes and distinct cut/non-cut band profiles (Table 2).

3.2. PCR-RFLP profiles for African and European E. acervulina field isolates

A panel of 40 *E. acervulina* field samples collected from chickens reared in Africa and Europe (Clark et al. 2016a, 2016b) were characterized using the PCR-RFLP assays. The PCR-RFLP assays tested here were developed using *E. acervulina* strains isolated in the UK that were added to the panel. All field samples collected in the UK/RoI were successfully genotyped using the PCR-RFLP protocol at all nine loci. However, PCR amplicons were found to be too faint for RFLP genotyping for 2–15 samples from Nigeria per assay, requiring amplicon Sanger sequencing to determine RFLP-type *in silico*. No amplification was

Table 2
Summary of *Eimeria acervulina*-specific Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) marker characteristics, digest conditions and cut/uncut fragment patterns. Underlined = fragment unaffected by RFLP.

Target locus	Amplicon size (bp)	Restriction enzyme (digestion temp.)	Target site [SNP]	Digest fragments (bp)	Fragment Pattern H W
ActDepol	916	*Taq*α1 (65 °C)	TC[G/A]A	440/346/130	
HSP90	625	*Bcl*I (50 °C)	T[G/A]ATCA	445/180	
IMP1	353	*Pvu*II (37 °C)	CAGCT[G/A]	215/138	
MIC3	490	*Bpm*I (37 °C)	C[T/C]GAG	311/179	
MIC5a	948	*Nae*I (37 °C)	G[C/A][C/G]GG[C/T]	638/310	
MIC5b	948	*Bsa*I (37 °C)	[G/A]GTCTC	745/203	
MZ92/130	1499	*Bsa*I (37 °C)	[A/G]GTCTC(N)	896/603	
SAGc	1002	*Hpa*II (37 °C)	CC[G/C]G	529/473	
Transhyd	1348	*Sfc*I (37 °C)	CTG[G/C]AG	1159/189	

Table 3
Outcomes of PCR-RFLP analysis for *Eimeria acervulina* for all nine markers. Data presented as the number of samples genotyped by restriction digestion (true RFLP) / number of samples genotyped by sequencing and *in-silico* annotation of RFLP status / number of samples that failed with no amplification detected.

		No. markers defined: digestion/sequencing/fail (no amplification)								
Country	Total no. of samples	IMP1	SAGc	MIC5a	MIC5b	HSP90	MIC3	ActDepol	MZ92/130	Transhyd
Nigeria	20	17/3/0	0/13/7	16/4/0	16/4/0	18/2/0	18/2/0	5/15/0	0/0/20	2/13/5
Egypt	1	1/0/0	1/0/0	1/0/0	1/0/0	1/0/0	1/0/0	1/0/0	nd	nd
UK + RoI	14[a]	14/0/0	14/0/0[b]	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0[b]	14/0/0	14/0/0
Other Eur	7	7/0/0	7/0/0	7/0/0	7/0/0	7/0/0	7/0/0	7/0/0	nd	nd

nd = not done. UK = United Kingdom. RoI = Republic of Ireland. Eur = Europe. aIncluding the Houghton (H) and Weybridge (W) reference strains. bTen amplicons also sequenced for comparative analysis of nucleotide diversity.

achieved for MZ92/130 from any Nigerian isolate, with five and seven failures for Transhyd and SAGc from Nigerian samples, respectively. In response, these three assays were considered appropriate for national but not international sample genotyping and were not used to test the samples from Egypt or other European countries and the partial results were excluded from the subsequent haplotype analyses (Table 3). More than ten sequences were produced for the SAGc and ActDepol loci, with comparable nucleotide diversity in UK/RoI and Nigerian sequences (SAGc: UK/RoI $\pi = 0.0071$, Nigeria $\pi = 0.0064$; ActDepol: UK/RoI $\pi = 0.00073$, Nigeria $\pi = 0.00067$).

For Nigeria, 20 samples were tested. Ten samples (50%) contained more than one PCR-RFLP genotype, creating a panel of 30 haplotypes from six PCR-RFLPs in total when faint secondary fragments were scored as minority genotypes (Table 3). Combined, 14 distinct haplotypes were detected, including 10 that were specific to Nigeria and four that were detected in at least one other country (Table 4). For the UK/RoI, compared together due to their close geographical proximity, 14 samples were tested from which 10 showed profiles indicative of polyclonal infection. Here, seven distinct haplotypes were detected of which five were region specific (Table 4). Seven samples collected from other European countries were included, all of which showed polyclonal PCR-RFLP haplotype profiles. Here, 60% of distinct haplotypes were shared with at least one other region (Table 4). Sequencing a subset of PCR amplicons confirmed identity at each SNP locus. The nucleotide sequence data are available from GenBank under the accession numbers OQ509171 - OQ509236. Combined, 25 unique haplotypes were detected with six found in more than one region and evidence of polyclonal infection in 64% of samples (Table 4, Fig. 1). Statistical comparison of the number of haplotypes detected in Nigeria and the UK/RoI found no significant difference ($p = 0.77$, Fisher's exact test).

3.3. Delineation of optimal PCR-RFLP markers by region

The Houghton (H) genotype was most common for most markers in all regions except Egypt, where only one sample was assessed. All PCR-RFLP markers were polymorphic and able to differentiate between samples in every region apart from HSP90 in 'Other Europe', where all genotypes were of the W type. Marker discriminatory power did vary between regions. Two markers (IMP1 and HSP90) were most discriminatory within samples collected from Nigeria, while HSP90 and MIC3 were most informative in the UK/RoI (Table 5). ActDepol, MIC5b and SAGc were equally discriminatory between samples collected in Other Europe, highlighting variation between regions.

4. Discussion

Remarkably little is known about genome-wide genetic diversity and population structure for *Eimeria* species parasites. As immunoprotective antigens such as Apical Membrane Antigen 1 (AMA1) and Immune-Mapped Protein 1 (IMP1) have become candidates for use in sub-unit or recombinant anticoccidial vaccines, population-level analyses are increasingly relevant to predict future efficacy and longevity (Blake et al., 2011; Clark et al., 2016). Knowledge defining the occurrence of genetic diversity, frequency of polyclonal infection and consequences of hybridization can be used to inform vaccine deployment, but tools for *Eimeria* are scarce. Protocols for high-throughput genotyping, next-generation sequencing and quantification of cross-fertilisation are gradually becoming available (Blake et al. 2015, Aunin et al. 2021, Liu et al. 2023), but cost and technical challenges can remain limiting. One popular alternative has been PCR-RFLP, a low-resource technique for genotyping (Hashim and Al-Shuhaib, 2019). Here, we describe a multi-locus PCR-RFLP panel for *E. acervulina* and its application to assess genetic diversity in parasite populations sampled from chickens reared in Africa and Europe.

Application of six PCR-RFLP assays to genotype 40 *E. acervulina* field samples and two reference strains identified 25 distinct haplotypes. Using PCR-RFLP fragment intensity to identify dominant and minority haplotypes within polyclonal infections is likely to have resulted in an underestimate of genetic diversity, suggesting that the true haplotype occurrence might be higher. The high genetic variability described in this study agrees with previous reports that used RAPD markers to differentiate *E. acervulina* strains (Procunier et al., 1993; Costa et al., 2001; Fernandez et al., 2003; Nowzari et al., 2005). Using ITS-1 and − 2 sequences, Clark et al. (2016a), (2016b) reported a fixation index (F_{ST}) of 0.04 for *E. acervulina* compared to 0.13 for *E. tenella*, indicating signatures of more regular interbreeding between genotypes for the former. Similarly, genetic diversity was found to be more restricted in British and Irish *E. tenella* populations assessed using PCR-RFLP than for *E. acervulina* described here (4 vs 7 haplotypes detected in 27 and 14 samples, respectively; Pegg et al., 2016). The relatively higher level polymorphism observed in *E. acervulina* might be attributed to marked characteristics in its biology. *Eimeria acervulina* has a faster generation time (shorter pre-patent period) and higher fecundity than *E. tenella*, potentially increasing opportunities for coinfection, genetic recombination and genome evolution. Finding a higher level of polyclonal *E. acervulina* infection here compared to *E. tenella* in the UK/RoI supports this hypothesis (Pegg et al. 2016). Increasing the number of samples and genomic coverage will be required to test this further.

No significant difference was noted in the level of *E. acervulina* haplotype diversity between Nigeria and the UK/RoI. This contrasted with the North/South geographical divide observed previously for *E. tenella* (Blake et al., 2015; Pegg et al., 2016), where haplotype diversity between Nigeria and the UK/RoI was significantly different ($p = 0.01$, Fisher's exact test). Future PCR-RFLP development for *E. acervulina* would benefit from including Nigerian (or other) sequences in SNP identification to support application with samples from different continents. However, while a comparable level of genetic diversity may define *E. acervulina* populations in the different regions sampled here, mixing between regions appeared to have been limited (Fig. 1). Only six haplotypes were detected in more than one region.

A panel of nine PCR-RFLP assays were developed here and used to produce haplotypes from *E. acervulina* samples collected in the UK/RoI. However, three (MZ92/130, SAGc and Transhyd) failed partially or completely when applied to samples from Nigeria. Results for these three SNP markers were excluded from the haplotype analyses described here. Possible explanations include the occurrence of polymorphisms

Table 4
Summary of PCR-RFLP genotyping for *Eimeria acervulina* isolates from Africa and Europe. Six markers that yielded complete profiles were used for the analyses, including IMP1, MIC5 a and b, HSP90, MIC3, and ActDepol. The markers MZ92/130, SAGc and Transhyd were excluded due to incomplete representation within the sample dataset.

Country	No.	Total no.	SNP haplotypes			
	samples	haplotypes detected	No. distinct haplotypes	Country-specific	Cross-country	Poly-clonal infection (%)
Nigeria	20	30	14	10	4	10 (50)
Egypt	1	1	1	0	1	0 (0)
UK + RoI	14[a]	24	7	5	2	10 (71)
Other Eur	7	14	10	4	6	7 (100)
Total	42	69		19	6	27

na = not applicable. UK = United Kingdom. RoI = Republic of Ireland. Eur = Europe. aIncluding the Houghton (H) and Weybridge (W) reference strains.

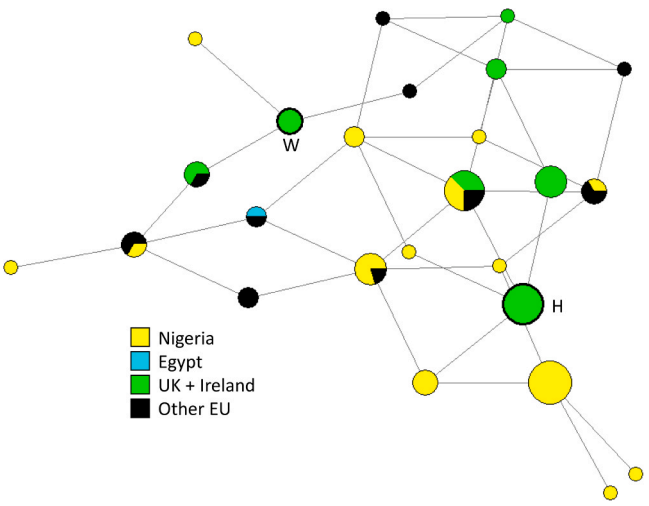

Fig. 1. Median-joining phylogenetic NETWORK of African and European *Eimeria acervulina* SNP haplotype diversity assessed using six PCR-RFLP markers. Each node represents a unique haplotype, and node size indicates frequency of haplotype occurrence. Nodes shown outlined in bold represent the Houghton (H) and Weybridge (W) reference SNP haplotypes, as indicated.

Table 5
Dominant SNP-type detected for *Eimeria acervulina* per region. The Houghton and Weybridge genotypes are represented as 'H' and 'W' respectively.

Target locus	Dominant SNP type (% samples)			
	Nigeria	Egypt	UK+ RoI	Other Eur
No. included	30	1	24	14
ActDepol	H (73.3)	W ([b])	H (79.2)	H (57.1)[a]
HSP90	H (56.6)	W ([b])	H (58.3)	W (100.0)
IMP1	H/W (50.0)[a]	H ([b])	H (79.2)	H (64.3)
MIC3	H (96.7)	H ([b])	H (58.3)[a]	H (78.6)
MIC5a	H (86.7)	H ([b])	H (83.3)	H (64.3)
MIC5b	W (73.3)	W ([b])	H (83.3)	W (57.1)[a]
MZ92/130	*	*	W (63.2)	*
SAGC	*	W ([b])	H (70.8)	W (57.1)[a]
Transhyd	*	*	H (68.4)	*

UK = United Kingdom. RoI = Republic of Ireland. Eur = Europe.
* Dominant SNP type could not be determined because of incomplete profiles.
[a] Most discriminatory marker in each region.
[b] No percentage given as a single sample was represented.

within one or more primer binding sites. The primers used for amplification in this study were designed using the Houghton (H) and Weybridge (W) reference strains, both of which originated from the UK. Unknown sequence diversity in primer binding sites from field isolates collected in other regions risks false negative results. Based on these results these three assays can be considered appropriate for analysis of *E. acervulina* collected in the UK/RoI, but should be validated further for use in other countries. Differences in *E. acervulina* DNA concentration per sample could be another reason for different results between countries using these three assays, since all the target loci are thought to exist in single copies per genome based upon BLASTn comparison with the reference genome sequence assembly. The sensitivity of an RFLP assay is affected by low concentration of template amplicons and the limited capability of agarose gels to separate molecules (Hashim and Al-Shuhaib, 2019).

All nine PCR-RFLPs described here were informative, with both cut and uncut genotypes detected for all. Consideration of genotype ratios at each locus revealed that IMP1 and HSP90 were highly discriminatory for the Nigerian isolates, while MIC3 and HSP90 were most discriminatory in the UK/RoI, although HSP90 was non-polymorphic in the other European countries sample set. Combined, these findings support the use of six of these genetic loci as markers for future genetic diversity studies. The remaining three appear to need some refinement, for example re-designing primers to be used in the PCR phase of PCR-RFLP.

5. Conclusions

This study revealed that *E. acervulina* populations are highly polymorphic in Africa and Europe, with polyclonal infections found to be common. While some of the most dominant haplotypes detected were region-specific, increased sampling depth can be expected to yield more haplotypes and possibly greater overlap between regions. There is no evidence in support of different region-specific occurrence of genetic diversity, unlike previous descriptions for the closely related *E. tenella*. High genetic diversity and regular opportunities for polyclonal infection indicate that genetic recombination might be common, with potential to influence the longevity of efficacy for future sub-unit vaccines.

Animal Welfare Statement

This study was conducted in strict accordance with the Animals (Scientific Procedures) Act 1986, an Act of Parliament of the United Kingdom, under project license assigned by the United Kingdom Home Office. All procedures were approved by the Animal Welfare Ethical Review Body (AWERB) of the Royal Veterinary College.

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CRediT authorship contribution statement

Jatau Isa D: Writing – review & editing, Supervision. **Blake Damer P:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Evans Laura:** Writing – review & editing, Methodology, Investigation. **Formoy Claire:** Writing – review & editing, Methodology, Investigation. **Idowu**

Emmanuel T: Supervision. **Akinsanya Bamidele:** Supervision. **Adeyemi Oluwayomi:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Quill Alexandra:** Writing – review & editing, Methodology, Investigation. **Morikone Margeen:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Damer Blake reports financial support was provided by Biotechnology and Biological Sciences Research Council. Oluwayomi Adeyemi reports financial support was provided by Tertiary Education Trust Fund (TETFUND) of Nigeria.

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