

Studies on coccidian oocysts (Apicomplexa: Eucoccidiorida)

Estudos sobre oocistos de coccídios (Apicomplexa: Eucoccidiorida)

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Abstract

The oocysts of the coccidia are robust structures, frequently isolated from the feces or urine of their hosts, which provide resistance to mechanical damage and allow the parasites to survive and remain infective for prolonged periods. The diagnosis of coccidiosis, species description and systematics, are all dependent upon characterization of the oocyst. Therefore, this review aimed to provide a critical overview of the methodologies, advantages and limitations of the currently available morphological, morphometrical and molecular biology based approaches that may be utilized for characterization of these important structures. It has become apparent that no single methodology is sufficient to fully characterize these structures and the majority of researchers favor the use of combinational or polyphasic approaches.

Keywords: Morphology, morphometry, molecular studies, diagnostic tools, systematic, taxonomy.

Resumo

Os oocistos de coccídios são estruturas robustas, frequentemente isoladas das fezes ou urina de seus hospedeiros, os quais oferecem resistência a danos mecânicos e permitem que os parasitas sobrevivam e permaneçam infecciosos por períodos prolongados. O diagnóstico da coccidiose, descrição das espécies e sistemática são dependentes da caracterização do oocisto. Portanto, esta revisão teve como objetivo fornecer uma visão crítica das metodologias, vantagens e limitações dos métodos morfológicos, morfométricos e moleculares que podem ser utilizados para a caracterização destas estruturas importantes. Tornou-se evidente que nenhuma metodologia única é suficiente para caracterizar completamente essas estruturas e que a maioria das pesquisas favorecem o uso de metodologias combinadas ou polifásicas.

Palavras-chave: Morfologia, morfometria, estudos moleculares, ferramentas de diagnóstico, sistemática, taxonomia.

Introduction

The coccidian oocyst is a resistant structure that protects the sporozoites, which are the infective forms of coccidiosis. The oocyst is usually exogenous being released within the feces of the host, and for that reason morphological characterization of the oocyst has been used for diagnosis, species description and in systematic studies since the primordium of parasitology. Currently, other experimental approaches including quantification of oocysts per gram of feces (OoPG), host specificity, aspects of the life cycle, sites of infection, pathogenicity, antigenicity and nucleotide sequencing data are available and serve to complement the traditional morphological characterization of the coccidia, providing enhancements to diagnosis and species identification (DUSZYNSKI; WILBER, 1997; TENTER et al., 2002; BERTO et al., 2011a).

The current review aimed to detail the process of characterization of coccidia using parameters associated with the oocysts. Thus,

an overview of methods and guidelines for morphological, morphometrical and molecular studies is presented herein. It is important to note, that this review did not seek to alter the guidelines produced by Duszynski and Wilber (1997), rather it sought to emphasize some recent advances in the use of morphological characteristics and to present a concise synopsis on the applications of morphometrical and molecular methods used for the characterization of sporulated coccidian oocysts.

Taxonomic Review

Coccidia belong to the Apicomplexa, Conoidasida, along with gregarinas by having complete, hollow, truncated conoid. The gregarines are allocated in Eugregarinorida, parasitizing invertebrates with the mature gamonts being extracellular. All coccidia are allocated in Eucoccidiorida, usually they infect vertebrates and have intracellular gamonts. The main plesiomorphy of the gregarines in relation to the primitive coccidia (Adeleorina) is zygote, which corresponds to gametogony where gamonts develop

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closely, forming one or few microgametes (DUSZYNSKI et al., 1999; BARTA et al., 2012).

Cryptosporidium is a coccidium which has similar characteristics to gregarinas and to the higher coccidia. Barta et al. (2006) revealed the similarity between the feeding organelles and the epicytoplasmic localization of *Cryptosporidium* with gregarines, colpodellids and dinoflagellates; however, gametogony in *Cryptosporidium* does not occur by syzygy. These biological characteristics are supported by molecular phylogeny, with *Cryptosporidium* spp. having recently been shown to form a distinct clade between gregarinas and coccidia (BARTA et al., 2012).

Eucoccidiorida is subdivided into the families Adeleorina and Eimeriorina. Adeleorina comprises primitive coccidia which still develop syzygy. The main genera are *Adelea*, *Adelina*, *Klossia* and *Legerella* with monoxenous cycles in invertebrates; *Klossiella* in mammals, and the haemogregarines *Dactylosoma*, *Babesiosoma*, *Haemogregarina*, *Desseria*, *Cyrlia*, *Hepatoozon*, *Hemolivia* and *Karyolosus* with heteroxenous cycles (UPTON, 2000; BARTA et al., 2012). The oocysts of this group are polysporic and may be eliminated in the feces (e.g. *Adelina dimidiata*) or in urine (e.g. *Klossiella* spp.). Alternatively, they can be trapped in the biomass of the host necessitating predation to liberate the sporozoites (e.g. *Adelina palori*, *Hepatoozon* spp.), or may inoculate sporozoites in the intermediate host (e.g. *Haemogregarina* spp.) (KOPEČNÁ et al., 2006; BERTO et al., 2010a; BARTA et al., 2012).

Eimeriorina comprises higher coccidia which develop gametogony where gamonts develop separately and the microgametocyte produces numerous microgametes. Prior to detailed studies on the biology of coccidia and the advent of

molecular studies, the genera of Eimeriorina were separated according to the proportion of sporocysts and sporozoites per oocyst. Employing only those criteria, disporic tetrazoic oocysts were described as *Isospora* in various vertebrate and invertebrate hosts; a classification which disregarded some biological aspects that subsequently led to the establishment of the genus *Cystoisospora* and other tissue cyst-forming coccidia (Sarcocystidae). In the same way, tetrasporic dizoic oocysts were traditionally described as *Eimeria*, a practice which ignored biological characteristics which consequently led to the appointment of the genera *Calyptospora*, *Choleoeimeria* and *Acroeimeria* (LEVINE, 1985; SOULSBY, 1987; JIRKŮ et al., 2002; BARTA et al., 2012). The data presented in Table 1 differentiates the major validated genera of Eucoccidiorida according to the proportion of sporocysts and sporozoites per oocyst, groups, and additional aspects including sporocyst morphology, susceptible hosts, samples/ tissues for the recovery and identification of oocysts.

Recent phylogenetic systematic studies employing genotyping have served to confirm some of the assumptions of traditional taxonomy, but have also provided a basis for the resurrection of some taxa. As an example, the establishment of the *Calyptosporiidae* was supported by *Calyptospora* spp. which demonstrate distinct biology and morphology. Their life cycle is heteroxenous with sporozoites developing in basal cells of the intestinal mucosa of shrimps. The definitive hosts are fish, where tetrasporic dizoic oocysts develop in the liver. In addition, the sporocysts possess distinct characteristics such as suture, posterior extension and capitate/acapitate sporopodia (FOURNIE et al., 2000; ALBUQUERQUE; BRASIL-SATO, 2010; WHIPPS et al., 2012).

Table 1. Proportion of sporocysts and sporozoites from oocysts of coccidia (Apicomplexa: Eucoccidiorida) recovered from samples.

		Sporocysts per oocyst				
		0	1	2	4	Varied or many
Sporozoites per sporocyst	1					<i>Barroussia</i> ^{1,b}
	2			<i>Cyclospora</i> ^{IV,VI,2,a}	<i>Eimeria</i> ^{I,II,III,IV,V,VI,2,a} <i>Choleoeimeria</i> ^{IV,b,**} <i>Acroeimeria</i> ^{IV,b} <i>Calyptospora</i> ^{II,b,**}	<i>Adelea</i> ^{1,1,b} <i>Adelina</i> ^{1,1,b} <i>Pseudoklossia</i> ^{1,b,*} <i>Goussia</i> ^{II,III,b}
	4	<i>Cryptosporidium</i> ^{V,VI}		<i>Isospora</i> ^{III,IV,V,2,a} <i>Hyaloklossia</i> ^{III,3,b,*} <i>Nephroisospora</i> ^{VI,3,b,*} <i>Toxoplasma</i> ^{V,VI,3,b} <i>Cystoisospora</i> ^{V,VI,3,b} <i>Sarcocystis</i> ^{IV,V,VI,3,b} <i>Frenkelia</i> ^{V,VI,3,b} <i>Neospora</i> ^{VI,3,b} <i>Hammondia</i> ^{VI,3,b} <i>Besnoitia</i> ^{VI,3,b}		<i>Klossia</i> ^{1,1,b,*}
	8	<i>Tyzzeria</i> ^V <i>Pfeifferinella</i> ^{1,**}	<i>Caryospora</i> ^{IV,V,a,b}			
	Varied or many					<i>Klossiella</i> ^{VI,1,b,*} <i>Aggregata</i> ^{1,b,***}

Predominance of parasitism in invertebrates^I, fish^{II}, amphibians^{III}, reptiles^{IV}, birds^V and mammals^{VI}; Adeleid coccidia¹ (Adeleorina); Eimeriid coccidia² (Eimeriorina: Eimeriidae); and Tissue cyst-forming coccidia³ (Eimeriorina: Sarcocystidae); Presence of Stieda/substieda body^a or sutures in sporocyst^b; Oocysts in kidney*, liver** or mantle (Mollusca)***.

Traditionally, Eimeriidae brings together various genera with distinct characteristics (DUSZYNSKI et al., 1999). However, Jirků et al. (2002), based on morphology and molecular phylogeny, considered that all Eimeriidae present a complex of Stieda and substieda bodies. Based upon those affirmations, the genera *Tyzzeria*, *Pfeifferinella*, *Caryospora* of raptors, *Choleoeimeria*, *Acroeimeria*, *Goussia*, *Barroussia*, *Pseudoklossia* and *Aggregata* are no longer considered as members of the Eimeriidae, and have been included in other families.

Tyzzeria and *Pfeifferinella* have octozoic oocysts without sporocysts. *Tyzzeria* is a intestinal parasite of Anseriformes; while, *Pfeifferinella* is parasite of the digestive gland (liver) of Gastropoda. The presence of a 'vaginal tube' in macrogametes of some species of *Pfeifferinella* also clearly distinguishes it from *Tyzzeria* (WENYON, 1926; ALLEN, 1936; WACHA, 1980; FRITSCH, 1987; DUSZYNSKI et al., 1999; BERTO et al., 2007, 2008e; McALLISTER, 2013). Levine (1982) had considered the family Pfeifferinellidae, which could contain these two genera; however, Wacha (1980) demonstrated differences in macrogametes of *Pfeifferinella*, which contained 1 to 5 peripherally located nucleoli, whereas in *Tyzzeria* they showed a single, relatively large, centrally located nucleolus typical of macrogametes of Eimeriidae. These observations serve to complicate the elucidation of the taxonomic position of these genera.

Caryospora is the only traditional Eimeriidae with a facultative heteroxenous life cycle. Furthermore, the *Caryospora* spp. of reptiles show a complex of Stieda and substieda bodies, while the *Caryospora* spp. of raptors lack this feature. In this context, Barta et al. (2001) showed *Caryospora* and *Lankesterella* to be closely related based upon molecular phylogeny and in light of some common aspects of their life cycles. In rodents, *Caryospora* forms thin-walled oocysts in the facial dermis, which rupture releasing sporozoites that form the caryocyst. In *Lankesterella* sporozoites are also released from thin-walled oocysts, they invade blood cells that can subsequently be transported to other susceptible hosts via hematophagous invertebrates. Furthermore, these two genera were placed in the same clade by molecular phylogeny. These observations suggested that, the family Lankesterellidae could include the genera *Schellackia*, *Lankesterella*, *Lainsonia* and *Caryospora* (BARTA et al., 2001; JIRKŮ et al., 2002).

Choleoeimeria and *Acroeimeria* are tetrasporic dizoic parasites of reptiles; with *Choleoeimeria* parasitizing the biliary epithelial cells while *Acroeimeria* is an epicytoplasmic parasite of intestinal cells (PAPERNA; LAINSON, 1999; MODRÝ; JIRKŮ, 2006; AL-QURAIHY, 2011). *Goussia* has oocysts with variable numbers of dizoic sporocysts being reported from fish and amphibians. Morphological data in combination with molecular phylogeny highlighted the similarity of these genera to *Barroussia*, which are parasites of invertebrates. *Choleoeimeria*, *Acroeimeria*, *Goussia* and *Barroussia* each have a sporocyst wall composed of two plates joined by a longitudinal suture. The possession of this highly distinctive feature would provide the basis for the resurrection of Barroussiidae as proposed by Levine (1983), which would combine the four genera into a single family (JIRKŮ et al., 2002).

Pseudoklossia has oocysts with many dizoic sporocysts and generally occurs in the kidney of marine molluscs. This genus

was included in Aggregatidae along with *Aggregata* which is also a parasite of marine molluscs. However, *Aggregata* demonstrates polisporic polizoic oocysts that are trapped in the mantle and shows a heteroxenous life cycle, developing merogony in marine arthropods (WENYON, 1926; FRIEDMAN et al., 1995; MLADINEO; BOČINA, 2007; DUSZYNSKI et al., 1999).

The only genera classified in Eimeriidae, based upon their having a complex of Stieda and substieda bodies, and under some conditions a parastieda body as an excystation structure, are: *Eimeria*, *Isospora* and *Cyclospora* (JIRKŮ et al., 2002). *Eimeria* is the genus with the largest biodiversity in Eucoccidiorida, being reported from invertebrates and all classes of vertebrates. The species of Eimeriidae are mainly intestinal, although some species develop in tissues including the liver, spleen and lungs (e.g. *Isospora serini*; *Eimeria reichenowi*); renal tubules (e.g. *Eimeria truncata*); liver and bile ducts (e.g. *Cyclospora talpae*; *Eimeria stiedae*); and the uterus (e.g. *Eimeria neitzi*) (McCULLY et al., 1970; OWEN, 1970; BOX, 1981; ENTZEROTH et al., 1981; NOVILLA et al., 1981; GARDINER et al., 1998; ROSALES; MASCARO, 1999).

Finally, Sarcocystidae comprises coccidia with facultative heteroxenous life cycles, forming cysts in intermediate hosts. The morphology and location of the cysts, as well as some pathological features and susceptible host range, are crucial for differentiation of species (ODENING, 1998). Many genera are included in this family, including recently identified genera (*Nephroisospora*), resurrected genera (*Hyaloklossia*) and some genera considered as controversial because they are paraphyletic and/ or have biological and morphological similarities (*Toxoplasma*, *Neospora* and *Hammondia*) (ELLIS et al., 1999; FRENKEL; DUBEY, 2000; MEHLHORN; HEYDORN, 2000; MODRÝ et al., 2001; DUBEY et al., 2002; WÜNSCHMANN et al., 2010). Regarding the characteristics of oocysts, the main synapomorphy of this family is the sporocyst wall that is composed of four plates joined by sutures. As a result, the tissue cyst-forming coccidia do not present a complex of Stieda and substieda bodies (JIRKŮ et al., 2002). Indeed, it was this distinction that supported the relocation of mammalian *Isospora* to Sarcocystidae, and the resurrection of *Cystoisospora* (e.g. *Cystoisospora suis*, *Cystoisospora rivolta*, *Cystoisospora ohioensis*, *Cystoisospora belli*, etc.) (BARTA et al., 2005; SAMARASINGHE et al., 2008)

Morphological Studies

The main morphological features of sporulated oocysts of coccidia are presented in Figure 1. The drawing represents an *Eimeria*, which, like the other Eimeriidae, has a complex of Stieda and substieda bodies as well as additional features that may facilitate the identification and characterization of species. The other coccidia which have plates joined by sutures, posterior extensions, sporopodia or that lack sporocysts, can also be identified based upon their oocyst morphology, although this is considered to be more difficult. The sections which follow provide information in relation to the principal structures that should be observed and characterized.

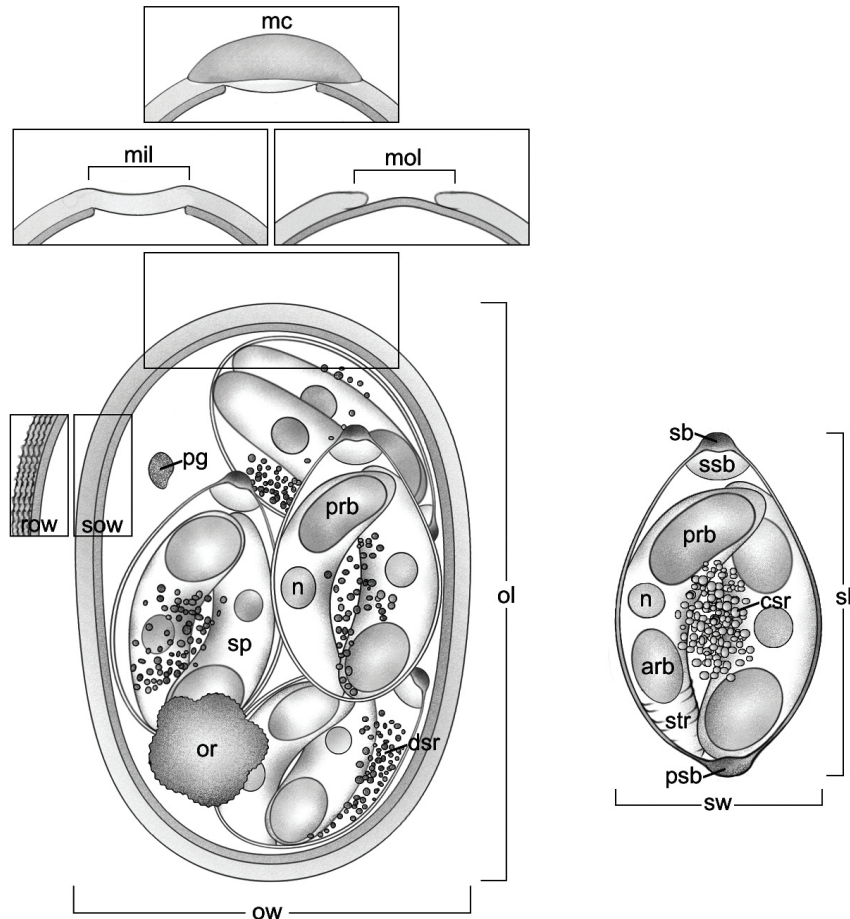


Figure 1. Line drawing of a sporulated oocyst (Apicomplexa: Eucoccidiorida: Eimeriidae) detailing the main structures that must be observed, measured and characterized, which are: (ow) oocyst width; (ol) oocyst length; (pg) polar granule; (or) oocyst residuum; (row) rough or (sow) smooth outer wall; micropyle in (mil) inner layer or (mol) outer layer; (mc) micropyle cap; (sw) sporocyst width; (sl) sporocyst length; (sb) Stieda body; (ssb) substieda body; (psb) parastieda body; (csr) compact or (dsr) diffuse sporocyst residuum; (sp) sporozoite; (prb) posterior and (arb) anterior refractile body of the sporozoite; (n) sporozoite nucleus; and (str), sporozoite striations.

Oocyst wall

The studies of Belli et al. (2006) and Mai et al. (2009) suggested that the basic structure of the oocyst wall is consistent across the coccidia. In both studies the authors convincingly demonstrated that the oocyst wall is comprised of two distinct layers, surrounded by an outer membrane, termed the outer veil, which is normally absent in mature oocysts isolated from feces. The bi-layer model is at odds with many earlier studies which considered oocysts with single-layered walls in their descriptions (LAINSON; SHAW, 1989; LOPES et al., 2014). Indeed, possession of a single layered wall forms a component of the guidelines for the preparation of species descriptions in the Eimeridae developed by Duszynski and Wilber (1997). Yet, it is currently believed that the single-layered walls described from some coccidia, were in fact double-layered with very thin and/ or fused layers, which hampered their correct observation by light microscopy (BELLI et al., 2006; MAI et al., 2009).

An additional important feature used in the description of the oocyst wall is the color. Many authors describe and differentiate species based on the color of the wall, characterizing them as

brown, yellowish, brownish, etc. (*e.g.* *Eimeria* spp. from swine) (DAUGSCHIES et al., 1999; BENNETT; HOBBS, 2011; ALFALEH et al., 2013), however in some cases it is pertinent to note that these colors may be artifactual, resulting from differences in the exposure time of the oocysts to preservatives (*e.g.* potassium dichromate), the light intensity or the choice of filter used for light microscopy (NOWELL; HIGGS, 1989). On the other hand, the difference in tonality between the two layers, considered as darker and lighter, can be an important feature in some cases (*e.g.* *Eimeria leuckarti*) (DE SOUZA et al., 2009).

The texture of the outer surface of the oocyst wall, which can vary from smooth (sow, Figure 1) to rough (row, Figure 1), is considered an important feature that, in some cases, allows differentiation between closely related *Eimeria* spp. (*e.g.* *Eimeria trinidadensis*; *Eimeria ichiloensis*; *Eimeria boliviensis*) (CASAS et al., 1995; ALBUQUERQUE et al., 2008). This feature is best observed, and consequently more accurately described, when the focus of the light microscope is placed on the outer surface of the oocyst wall, as described for *Eimeria philanderi* and *Eimeria caluromydis* by Lainson and Shaw (1989). In the case of *Isoospora* the texture of the outer surface of the oocyst wall is predominantly smooth,

which effectively negates the use of this characteristic for species differentiation (BERTO et al., 2011a, b). Finally, the presence of protruding structures on the outer surface of the oocyst, described as spines or conical projections, has been reported for some species (e.g. *Eimeria stylosa*; *Eimeria mitraria*) (McALLISTER; UPTON, 1989; ŠIROKÝ; MODRÝ, 2006)

Micropyle and micropyle cap

The micropyle can be defined as a discontinuity in one of the layers of the oocyst wall. This feature can be observed in the inner layer (mil, Figure 1) (e.g. *Eimeria bareillyi*; *Eimeria dorcadis*) (RAMIREZ et al., 2009a; MOHAMMED et al., 2012) or outer layer (mol, Figure 1) (e.g. *Eimeria leuckarti*) (de SOUZA et al., 2009). In some coccidian species (e.g. *Eimeria minasensis*), the micropyle appears to be covered by a cap, the micropyle cap (mc, Figure 1), that is considered to provide protection for discontinuous regions of the layers (SILVA; DIVINO-LIMA, 1998; ARSLAN et al., 2002; TURNER et al., 2012). These structures are common in *Eimeria*, but uncommon or absent in other genera. Interestingly, Wacha (1980), Fritsche (1987) and, more recently, McAllister (2013) reported micropyle in *Pfeifferinella gugleri*, however the morphological structure was not described in detailed in those studies.

Oocyst residuum and polar granule

The oocyst residuum (or, Figure 1) comprises a large structure within the oocysts, located between the sporocysts. This structure may be comprised of a regular and compact mass (e.g. *Eimeria heliophobii*; *Eimeria yamikamiae*) or it may be formed by an irregular mass of granules (e.g. *Eimeria stiedai*; *Eimeria media*; *Eimeria intestinalis*) (MODRÝ et al., 2005; EL-SHAHAWI et al., 2012).

The polar granule (pg, Figure 1) is an additional internal structure of the oocysts, also located between the sporocysts; however, this is smaller than the oocyst residuum and invariably dense (e.g. *Eimeria* spp. from Japanese quails). The format of this structure can be unique in many species, and may present a variety of characteristic shapes including splinter-like and comma-like (e.g. *Isoospora frontalis*) (BERTO et al., 2009d, 2013b).

Complex of Stieda and substieda bodies

The work of Grulet et al. (1982) revealed the importance of the Stieda (sb, Figure 1) and substieda (ssb, Figure 1) bodies for the accurate diagnosis and description of *Isoospora* spp. In that study, 12 new species were described from the domestic sparrow *Passer domesticus*, with variation in the Stieda and substieda bodies providing the basis for their differentiation. The utility of those structures to facilitate the identification of *Isoospora* has been clearly demonstrated via numerous descriptions of new species, where variation in the Stieda and substieda bodies were recorded as a key morphological trait (UPTON et al., 1985, 1988; McQUISTION; HOLMES, 1988; LAINSON; SHAW, 1989; UPTON et al., 1995; McQUISTION; CAPPARELLA, 1997; UPTON; WHITAKER,

2000; BERTO et al., 2008a-d, f, 2009a-f, 2010b, d, 2011b, c, 2013a; BALTHAZAR et al., 2009b; COELHO et al., 2011a, b, 2013; PEREIRA et al., 2011).

The size and shape of Stieda and substieda bodies in the sporocysts show a characteristic pattern for each *Isoospora* sp.; therefore, in the majority of cases the performance of a detailed description should be sufficient to arrive at a species level identification. Given the importance of this structure it is recommended that, whenever possible, variations observed by light microscopy for representatives of the same species must be characterized and drawn. Possible reasons for such variations include the position of the sporozoites within the sporocyst, or the position of the oocyst and sporocyst under the coverslip. In this context, these variations are likely to be subtle and may possibly be observed in sporocysts of a single oocyst. However, if these variations are evident in distinct oocysts, it is possible that the host contains two different species, as was described by Berto et al. (2011c) for *Isoospora coerebae* and *Isoospora cagasebi* from bananaquits *Coereba flaveola* (BERTO et al., 2008a-d, f, 2009a-f, 2010b, d, 2011b, c, 2013a; BALTHAZAR et al., 2009b; COELHO et al., 2011a, b, 2013; PEREIRA et al., 2011).

Figure 2 illustrates and provides suggestions for denominations for the main types and shapes of Stieda and substieda bodies that have previously been described in the literature. The nomenclature employed herein, is different from that used in some studies. However, it attempts to bring together the terminologies that are most widely employed for describing these structures. As such, it is hoped that this figure will serve as a guideline for classifying Stieda and substieda bodies, and that it may help to avoid discrepancies between future studies. Additionally, it should be noted that some features, including filaments; fine membranous cup-like formations; club-shaped projections; etc, have been reported in association with the Stieda body and these must be characterized (e.g. *Eimeria pangshurae*; *Eimeria arakanensis*; *Eimeria emydis*) (SEGADE et al., 2006; ŠIROKÝ; MODRÝ, 2010).

The importance of the substieda body has traditionally been associated with *Isoospora* spp.; however, recent studies have emphasized its potential value for the identification and diagnosis of *Eimeria* spp. (RAMIREZ et al., 2008a; BERTO et al., 2008g, 2009a, 2013b; HOFSTATTER; KAWAZOE, 2011; CHINCHILLA et al., 2013). In this context, the redescription of some species has been proposed based upon the observation of a substieda body in their sporocysts (BERTO et al., 2013b). Moreover, Berto et al. (2013b) proposed an algorithm, wherein the substieda body is one of the main characteristic features used in the formula, which enabled reliable identification of individual species of *Eimeria* during routine diagnosis of pathogens of the Japanese quail *Coturnix japonica*. In common with those workers, we consider this structure to be of immense importance for differential diagnosis and species identification of *Eimeria* in general.

Parastieda body

The parastieda body (psb, Figure 1) has been described infrequently in coccidia. Duszynski (1985) and Duszynski and Wilber (1997) observed and characterized this structure in *Eimeria parastiedica* as representing a substieda body located at the opposite

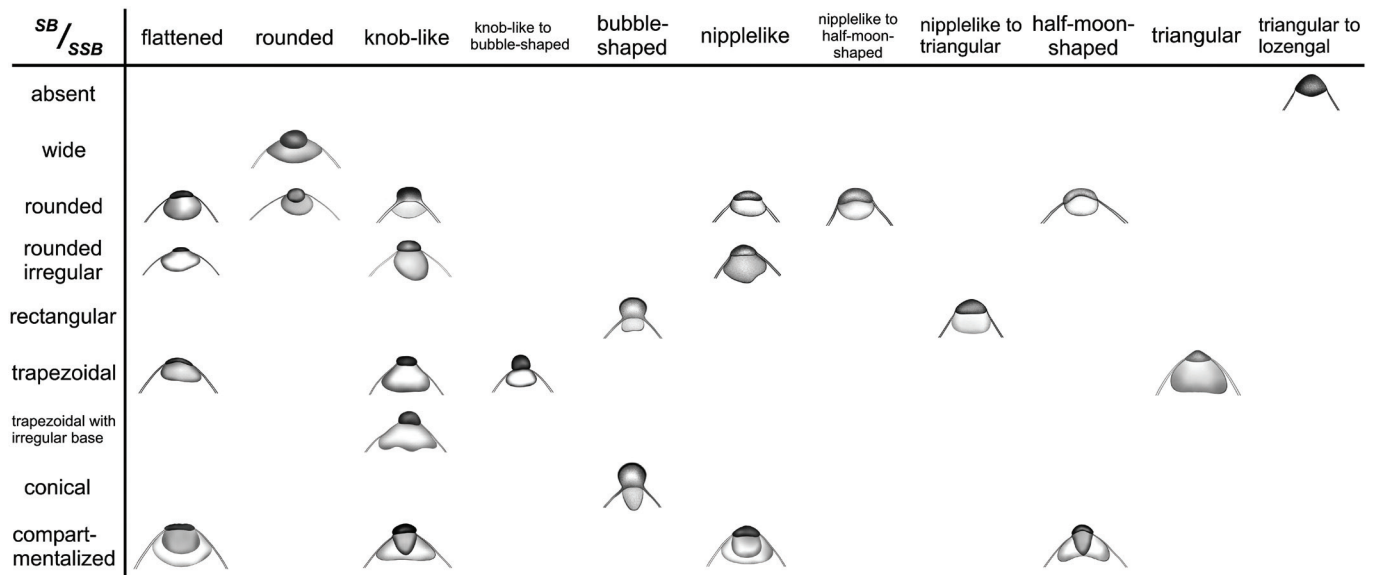


Figure 2. Line drawings of several complexes of Stieda and substieda bodies with suggestions of terms to be used for morphological description [adapted from Grulet et al. (1982), Berto et al. (2009a-c, e, f, 2010b, 2011b, c, 2013a, b), Balthazar et al. (2009b), Pereira et al. (2011) and Coelho et al. (2011a, b, 2013)].

end of the oocyst relative to the Stieda body. More recently, in a study of *Eimeria caviae*, Flausino et al. (2014) reported the presence of a parastieda body and considered it as a structure resembling an additional Stieda body, located at the opposite end of the oocyst, which served to complicate the localization of the anterior and posterior ends.

Sporocyst residuum

The sporocyst residuum can be quantified relatively in each coccidian species and a description of this structure is a component of most species descriptions. The structure may appear as being diffuse among sporozoites (dsr, Figure 1) (*e.g. Eimeria sicki*; *Isoospora teresopolensis*) or it can form a compact mass of granules (csr, Figure 1) (*e.g. Isoospora mionectesi*; *Isoospora chanchaioi*). In some cases, the compact mass may be surrounded by a membrane (*e.g. Isoospora paranaensis*); while in other species, a distinct pattern is observed as the granules develop a characteristic ring form (*e.g. Isoospora navarroi*) (SILVA et al., 2006; BERTO et al., 2009a, b, d, f, 2011a; LOPES et al., 2013).

Sporozoites

The structures associated with the sporozoite are the refractile bodies (rb, Figure 1), the nucleus (n, Figure 1) and striations (str, Figure 1). The refractile bodies may be unique to each sporozoite (*e.g. Isoospora marambaiensis*; *Eimeria bateri*), or may appear as a pair, one anterior (arb, Figure 1) and one posterior (prb, Figure 1) (*e.g. Isoospora trincaferri*; *Isoospora massardi*), and may be sub-spherical (*e.g. Isoospora trincaferri*) to elongated (*e.g. Isoospora marambaiensis*) in shape. The nucleus is generally smaller than the refractile bodies and is located in the center of the sporozoite (*e.g. Isoospora sayacae*; *Eimeria bateri*). In some cases, striations are

observed at the anterior end of the sporozoite (*e.g. Isoospora sayacae*) (BERTO et al., 2008d, 2009e, f, 2013a; LOPES et al., 2014)

Morphometrical Studies

All of the structures mentioned above can be measured for the characterization of oocysts of a coccidian species. However, the features which are most commonly taken into consideration are the length (ol, Figure 1) and width (ow, Figure 1) of the oocyst, and the length (ol, Figure 1) and width (ow, Figure 1) of the sporocyst. In addition, measurements of the height and width of Stieda and substieda bodies are extremely relevant for *Isoospora*, since these structures are prominent and highly discriminatory in this genus (DUSZYNSKI; WILBER, 1997; TENTER et al., 2002; BERTO et al., 2011a, b).

Shape-index (Length/ Width ratio)

The ratio of length over width (shape-index) is used to demonstrate the shape of the oocysts and sporocysts. In this ratio a value close to 1 denotes spheroid structures; therefore, spherical oocysts generally have shape-index equal to 1.0; sub-spherical oocysts have values between 1.0 to 1.1, and oocysts within the 'ellipsoidal complex' provide values higher than 1.1. The term 'ellipsoidal complex' was introduced because oocysts with shape-indexes higher than 1.1 may be ellipsoidal (*e.g. Eimeria bateri*; *Isoospora mionectesi*), ovoid (*e.g. Eimeria bovis*; *Isoospora sepetibensis*), pear-shaped (*e.g. Eimeria bareillyi*; *Eimeria anseris*), bottle-shaped (*e.g. Eimeria boschadis*; *Eimeria somateriae*), etc. Therefore, the oocysts and/ or sporocysts with shape-indexes greater than 1.1 should always be described with attention given to their shape (LEVINE, 1985; SOULSBY, 1987; ARSLAN et al., 2002; BERTO et al., 2009b, 2011b, 2013a, b; FLAUSINO et al., 2014)

Histograms

Statistical graphics can be produced targeting the morphometrics of the oocysts of a coccidian species in comparison with those of other species. The histograms plot the values of length, width and the shape-index of the oocysts, along with their frequencies within a sample (SAMPAIO, 2002; BERTO et al., 2011a, b, 2013b; HAUCK; HAFEZ, 2012; FLAUSINO et al., 2014).

This approach may serve to demonstrate tendencies and regularities in the distribution of the oocyst dimensions. Analysis of the data will reveal if the frequencies ascend and descend in the graph or if they simply ascend or descend linearly. Hence, histograms can confirm the presence of a single species in the oocyst sample; can determine the degree of polymorphism within that single species or can indicate the presence of multiple species. Figure 3, illustrates examples of regular (Figure 3a, c, e) and irregular (Figure 3b, d, f) histograms that suggest the presence of one (Figure 3a-d) or two species in the sample (Figure 3e, f) (BERTO et al., 2011a, b, 2013b; HAUCK; HAFEZ, 2012; FLAUSINO et al., 2014).

The histogram of shape-index (Figure 3h), is provided in an attempt to demonstrate the tendency of shape of the oocysts of a species. For example, a given species may have sub-spherical to ellipsoidal oocysts with shape-indexes ranging from 1.0 to 1.3, but the histogram may insert 95% of the oocysts in class 1.0 to 1.1 demonstrating a tendency towards a sub-spherical shape, with a limited number of the oocysts being ellipsoidal. The counter example may also occur whereby most of the oocysts would be inserted in the class of 1.1 to 1.3, revealing another species with the same range of shape-indexes, but demonstrating a different tendency (BERTO et al., 2011b).

Additionally, histograms can be produced to identify species with no morphological differences, but with possible morphometrical tendencies. In this way, Cardozo et al. (2013) compared *Cryptosporidium* oocysts recovered from mussels with oocysts of *Cryptosporidium hominis*.

More recently, some researchers have produced histograms of length and width superimposed in the graph as shown in Figure 3g. This method has the advantage of providing more detailed observations, permitting confirmation of the shape-index by observing the proximity between the more frequent classes and their respective values (BERTO et al., 2013b; FLAUSINO et al., 2014).

Linear regression

Linear regression analyses of plot measurements of width on length of oocysts (Figure 4), is a long established statistical method that can be used to relate measurements of oocysts of the same or different species and hosts (NORTON; JOYNER, 1981; MEIRELES; FIGUEIREDO, 1992; HASSUM et al., 2007; COSENDEY et al., 2008; BERTO et al. 2008g, h, 2011a, 2013b; HAUCK; HAFEZ, 2012; FLAUSINO et al., 2014).

In this method the measurements of width are arranged in the Y axis and the measurements of length in the X axis. In the graphs, the line regression, the datapoints, the R^2 (coefficient of

determination) value and the coefficient of the regression line must be presented. The R^2 value provides most of the meaningful observations derived from the linear regression. Thus, when R^2 values are higher than 0.5 the data points are distributed close to the regression line on the graph, which serves to demonstrate slight variations of width over length and, therefore, a clear pattern can be established for this species. In contrast, R^2 value lower than 0.5 will result in a distribution of data points distant from the regression line, indicating polymorphism within the oocyst sample (SAMPAIO, 2002; HASSUM et al., 2007; BERTO et al., 2008g, h, 2011a, b, 2013b; HAUCK; HAFEZ, 2012; FLAUSINO et al., 2014).

In this context, polymorphism consists of low values for the proportionality of width on length, and does not necessarily infer the presence of more than one species among oocysts measured, or failure in the measurement process. It is important to note, that oocyst polymorphism has been described previously by several authors for a variety of coccidia, and is considered to be related to factors including: (1) stress; (2) nutrition; (3) immunity of the host; (4) the infecting dose; (5) the timing of oocysts discharge during the patent period; (6) substance in which and period that the oocysts were stored; and (7) phenotypic plasticity, described as the ability of a coccidium to adopt different phenotypes in response to its environment (DUSZYNSKI, 1971; CATCHPOLE et al., 1975; JOYNER, 1982; FAYER, 1980; GOMEZ et al., 1982; PARKER; DUSZYNSKI, 1986; GARDNER; DUSZYNSKI, 1990; BERTO et al., 2008h, 2011b; RAMIREZ et al., 2009a, b; FLAUSINO et al., 2014).

An additional phenomenon worthy of mention is the increased possibility of polymorphism in oocysts which demonstrate shape-index values higher than 1.1, and which belong to the 'ellipsoidal complex'. It should be remembered, that the oocyst is a three-dimensional form which is measured two-dimensionally, under an optical microscope. The ellipsoidal oocyst, depending on its position under the cover slip (*i.e.* the angle of observation) may appear as spherical to ellipsoidal, while the spherical oocyst, regardless of the position under cover slip will always have the same dimensions. That being the case, the ellipsoidal oocysts must be measured only when positioned lengthwise. To meet this prerequisite, the coverslip may be gently maneuvered to move the oocyst such that it will reveal its true position and shape (BERTO et al., 2011b).

The primary use of linear regression is to evaluate the measurements of oocysts of a single species. However, it may also be of value in situations where different host-species shed oocysts of the same coccidian-species or when a host-species shed oocysts of different coccidian-species. In such cases, the linear regressions must be performed for each host/coccidium, with the data being superimposed within the graph. Here, the relative positions of the lines on the same graph can provide some valuable results. In the case of studies reporting a new host, regression lines which are close to or that overlap the line(s) produced by analysis of oocysts recovered from a previously established host(s) will provide support for the definition of the new host. Alternatively, in studies comparing oocysts of different species comparison of regression lines can reveal a variety of differential features: (1) ranges, in accordance with the length of the regression lines; (2) size, here larger oocysts will be recorded higher and more to the right in the

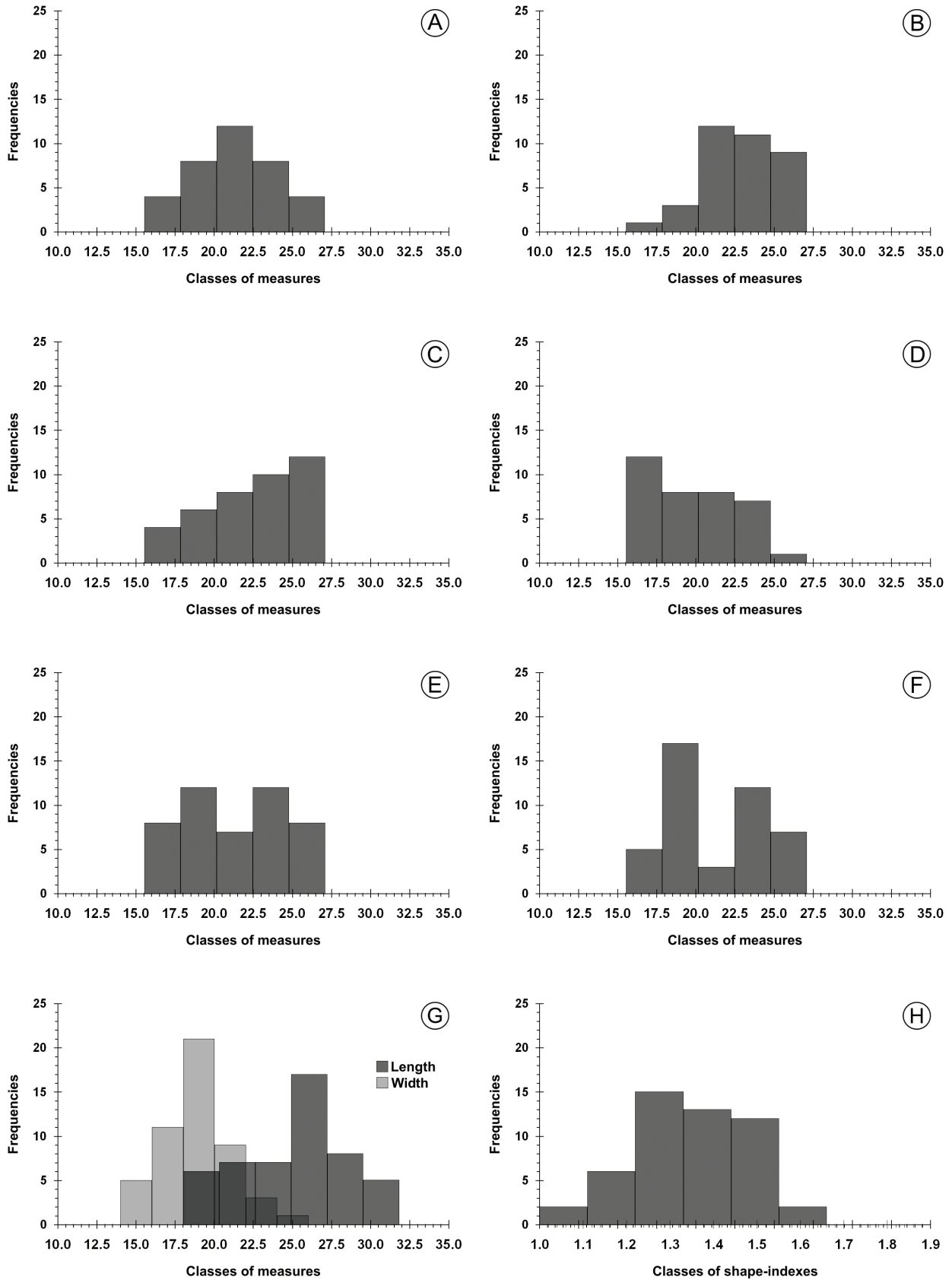


Figure 3. Examples of histograms which demonstrate (A, C, E) regular and (B, D, F) irregular distribution and suggest (A, B, C, D) one or (E, F) two coccidian-species. Histograms of length and width can be superimposed in the graph providing more detailed observations, as in histogram (G) that reveals tendencies in the dimensions 24.7-27.2 × 18.0-20.0, which is evidenced in (H) a histogram of shape-index that has higher frequency in the classes of 1.22 to 1.55 [adapted from Berto et al. (2013b)].

graph; and (3) shape, sub-spherical oocysts generate regression lines with a greater gradient of slope (Figure 4) (HASSUM et al., 2007; BERTO et al., 2008g, h, 2011b).

Finally, linear regression provides a basis by which to evaluate whether one or more methods (e.g. collection medium, preservative, staining methods) alter the morphometry of oocysts (RAMIREZ et al., 2008b)

Mean comparison tests

It is recommended that Analysis of variance (ANOVA), Student's t-test or other mean comparison tests should be used to compare measurements of length, width and shape-index of oocysts and sporocysts. In some cases, these methods may be used to compare mean measurements of specific structures considered relevant to identification, such as the Stieda and substieda bodies (BERTO et al., 2011c). The mean comparison tests may also be utilized to compare additional, quantitative parameters determined for the specific oocysts under investigation. As an example, Berto et al. (2013b) employed ANOVA to compare the values of an algorithm developed for the identification of coccidian parasites of Japanese quails. In general, these types of mean comparison tests are used for comparing coccidian species recovered from

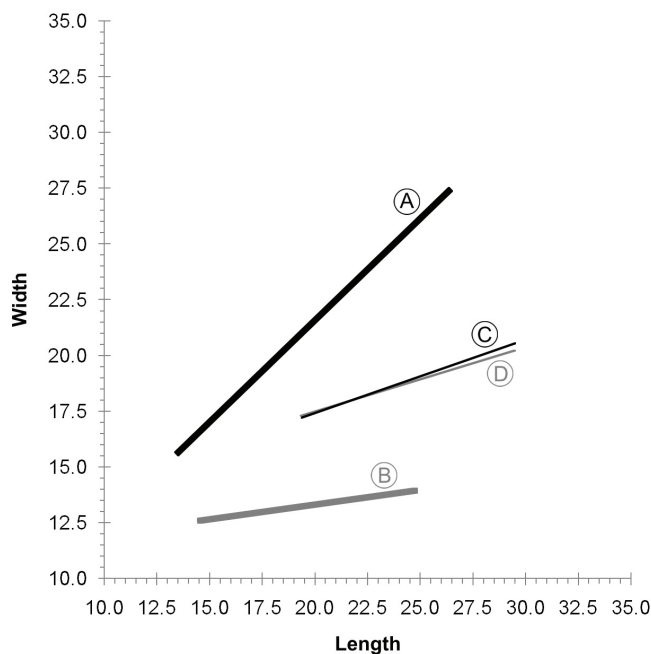


Figure 4. Example of linear regressions superimposed in a single graph. The thick and black regression line (A) represents a coccidian-species with larger oocysts, higher range and sub-spherical shape as indicated by its superior position, increased line length and slope. The thick and gray regression line (B) represents a coccidian-species with smaller oocysts, lower range and an ellipsoidal shape as inferred by its inferior position, lower line length and slope. The thin regression lines (C, D) may represent the same coccidian-species or possibly different coccidian-species with similar length and width of oocysts. In this type of situation, it would be necessary to examine additional morphological and morphometric parameters in order to differentiate the coccidia.

the same host/family or alternatively they may be employed for comparing the same coccidian species recovered from different host species (GOMEZ et al., 1982; BALTHAZAR et al., 2009a; BERTO et al., 2008g, h, 2010c, 2011a, b, 2013b)

Molecular biology

An analysis of recent literature dealing with the detection, systematics and taxonomy of the coccidia reveals that, in common with numerous other areas of parasitology and microbiology, the application of molecular methods has steadily increased since the mid 90's and is now a firmly established practice, particularly with regard to the coccidian parasites of production animals (CARVALHO et al., 2011; CHAPMAN et al., 2013). The principal molecular method employed is the polymerase chain reaction (PCR), which can be used to amplify species specific DNA sequences for diagnostic purposes (OGEDENGBE et al., 2011a), or to amplify genus or family level molecular markers, with a "universal" distribution; including the nuclear 18S rDNA locus (MORRISON et al., 2004), the internal transcribed spacer (ITS) regions (LEW et al., 2003; MOTRIUK-SMITH et al., 2011) or the gene (cox-1, COI), encoding the mitochondrial cytochrome c oxidase subunit I (OGEDENGBE et al., 2011b). Comparative nucleotide sequence analysis of the markers, involving the determination of nucleotide similarity to sequences derived from known species which are deposited in public databanks e.g. GenBank, can generate genus or species level identification of the parasites, lend support to the establishment of new species and may provide the basis for molecular phylogenetic studies.

The performance of these methods is not technically demanding, which has served to make them widely accessible. Yet, in contrast, it has become apparent that interpretation of the growing body of molecular data available for these parasites is not a straightforward process. A thought provoking overview of the complexities and shortcomings of molecular characterisation of the coccidia was recently produced by Chapman et al. (2013). Therein, the authors drew attention to the potential pitfalls of molecular characterization based on analysis of a single molecular marker, and provided topical examples as to why caution should be exercised when employing 18S rDNA sequences for identification or in systematic studies. In this context, recent reports of independently evolving paralogous copies of 18S rDNA in the nuclear genome of the chicken coccidium *Eimeria mitis* (VRBA et al., 2011) and in the turkey coccidium *E. meleagridis* (EL-SHERRY et al., 2013), have served to question the reliability of using this sequence as a species level molecular marker for coccidia. In the case of *E. mitis*, the paralogous copies of 18S rRNA showed 1.3-1.7% sequence divergence from the previously identified 18S rDNA sequences of this species. However, these novel sequences were shown to be 99.3% to 99.5% similar to published sequences submitted under the name *Eimeria mivati*, a species described as morphologically indistinguishable from *E. mitis*. Vrba et al. (2011), concluded that *E. mivati* and *E. mitis* are almost certainly the same species and that the appearance of two species, based upon the observed differences in 18S rDNA sequences, most likely occurred as a consequence of inadequate sampling. The levels of intra-specific

sequence divergence, among paralogous copies of *E. meleagridis* 18S rDNA, was even higher (approximately 2.6%) than the values recorded for *E. mitis*. The implications of such intraspecies variation for species identification, becomes apparent when compared to the level of the interspecific sequence divergence (1.1%) that is present between some well-recognized species such as *Eimeria tenella* and *Eimeria necatrix* (EL-SHERRY et al., 2013). It is important to note that the sequence variation was observed in DNA which had been extracted from single-oocyst-derived lines of the *Eimeria* species. Clearly the use of such lines, or alternatively of single isolated oocysts as reported by Dolnik et al. (2009), is to be highly recommended in order to provide the maximal level of confidence in the molecular data.

In an attempt to counterbalance the emerging problems associated with use of 18S rDNA as a marker, a tendency has begun to emerge among researchers to employ the mitochondrial COI sequence as a complementary, or even as an alternative marker for species level identification (SCHWARZ et al., 2009; OGEDENGBE et al., 2011b; CHAPMAN et al., 2013; EL-SHERRY et al., 2013). The utility of this marker has been firmly established in many areas of biology, and is at the heart of the Barcode of Life Project (iBOL.org), which aims to develop a molecular barcode-based identification system for animals, including parasites (RATNASINGHAM; HEBERT, 2007; OGEDENGBE et al., 2011b). The major disadvantage associated with the use of this marker is the fact that, at present, there exists a lack of coverage for coccidian COI sequences in public databases; however it is anticipated that this situation will improve as the advantages of this marker become established.

Continuing reductions in the costs associated with molecular based methods, particularly nucleotide sequencing, coupled with advances in the field of bioinformatics will likely stimulate the adoption of multi-maker, or even whole genome, sequencing as means through which to arrive at definitive identifications of coccidia (CHAPMAN et al., 2013). Sequencing of the complete mitochondrial genomes, comprising 3 genes for proteins (cox1, cox3, and cytb), 12 gene fragments for the large subunit (LSU) rRNA, and 7 gene fragments for the small subunit (SSU) rRNA, of six *Eimeria* species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. tenella* and *E. praecox*) isolated from chickens was recently reported (LIN et al., 2011). The genomes which were approximately 6200 bp in size, showed similar organization and A+T contents. Subsequent phylogenetic analyses performed using concatenated nucleotide sequences of 2 protein-coding genes (cytb and cox1) of the 6 genomes with three different computational algorithms (Bayesian analysis, maximum parsimony and maximum likelihood), all revealed distinct groups with high statistical support, indicating that the six *Eimeria* spp. represent six distinct but closely-related species.

The application of molecular techniques to the study of well established and socio-economically important coccidian species has answered some old questions, but at the same time it has raised some new ones. Molecular methods seem set to play a growing role in the study of all coccidia, they hold the potential to produce highly precise insights into the biology of these parasites, particularly when used in combination with morphological and morphometric data as a component of a polyphasic approach.

Diagnostic Tools

In addition to PCR based diagnostics, dichotomous key and algorithms represent two tools that are especially useful for the differentiation and diagnosis of coccidia (SCOTT; DUSZYNSKI, 1997; DAUGSCHIES et al., 1999; HASSUM et al., 2007; BERTO et al., 2013b).

Dichotomous key

Dichotomous keys are compatible with data generated from the morphological characterization of coccidian-species. In this context, the presence, absence, shape, color, location, *etc.* of structures of the oocyst and sporocyst, which are qualitative data, support the preparation of dichotomies that may suggest a species (SCOTT; DUSZYNSKI, 1997; BERTO et al., 2010d). Although not widely adopted, the use of dichotomous keys is both recommended and encouraged in situations where differentiation of species is based almost exclusively upon morphological data.

Scott and Duszynski (1997) developed a dichotomous key for *Eimeria* spp. described from New World bats. Subsequently, Berto et al. (2010d) developed a dichotomous key for *Isospora* spp. described in tanagers in Central and South America, which are sympatric with most New World tanagers (BERTO; LOPES, 2013).

Algorithms

In contrast to the dichotomous keys, algorithms are most compatible with morphometrical data produced from coccidian-species. Thus, size, proportions and characters that generate quantitative data, provide the basis for the development of a formula that results in a value which should fluctuate within a range suggestive of a species. It is important to note that qualitative data may also be inserted into algorithms; however, to do so, they must first be converted into a numerical format (DAUGSCHIES et al., 1999; HASSUM et al., 2007; BERTO et al., 2013b).

The complexity of the algorithm is directly proportional to the number of associated coccidian-species. As a result, hosts which can be infected by multiple coccidian-species may require more than one complex formula. In this context, Dausgchies et al. (1999) implemented different algorithms for differentiation of porcine *Eimeria* spp. dividing seven species into groups. In contrast, in the study of Berto et al. (2013b) the three species identified in Japanese quails could be diagnosed using a single algorithm.

Conclusion

As demonstrated in this review there are several procedures, approaches and tools that can be employed for the identification and characterization of a coccidian-species based on its oocysts.

Clearly the choice of which method(s) to employ is the responsibility of the scientist undertaking the research. In this context, it was not our intention to establish rigid guidelines for the description of all coccidian-species or to suggest that any given method is of greater or lesser value than another method; however

when appropriate, for example for the establishment of new species, we consider that if it is practical to do so, oocysts should be characterized as completely as possible through a combination of techniques, for the objectives of facilitating identification and to form a solid basis for future studies.

Finally, it would be fair to affirm that establishing the characteristics of the oocysts is a pre-requisite of any study of the coccidia. Clearly, the application of histological methods that reveal details of the biology and pathogenicity, methods for experimental infection that confirm host susceptibility and determine parasite load, serological and molecular methods which uncover inter- and intra-specific differences and provide taxonomic adjustments must all be considered as highly relevant. However, such methods should be viewed as serving to complement the fundamental characterization of a coccidian-species based on its exogenous forms: the oocyst.

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