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Invited Review

The conceptual basis for a new classification of the coccidia

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Abstract

At the joint meeting of the 8th International Coccidiosis Conference and the Annual Scientific Meeting of the Australian Society for Parasitology in Palm Cove, Australia, in July 2001, a Controversial Roundtable was held on 'New classification of coccidia'. The aim of this Roundtable was to stimulate and encourage discussion and debate on current classification schemes for the group of parasitic protozoa known as the eimeriid coccidia. In the past, such classifications have been based only on phenotypic characters such as morphology, ultrastructure, life cycles, and host specificity. However, over the past 10–15 years, molecular phylogenetic studies on taxa of the eimeriid coccidia have revealed that several of the families, subfamilies, and genera that have been erected based on non-molecular characters are paraphyletic. Therefore, this Roundtable was an important forum for initial discussions on how a new and more comprehensive classification of the eimeriid coccidia, which takes into consideration both phenotypic and molecular characters, can be devised. The stimulus came from invited speakers who gave introductions into selected areas of taxonomy and classification. Following these introductions, a more general discussion with the audience addressed potential steps that may be taken in future work. This review is the immediate outcome of the Roundtable. It describes advantages and disadvantages of the use of phenotypic or molecular characters as the base for taxonomic schemes for eimeriid coccidia. It gives specific examples for drawbacks of current classifications based only on phenotypic characters as well as potential pitfalls associated with the use of only molecular phylogenies. It addresses current controversies as well as rules of taxonomy and nomenclature relevant for the eimeriid coccidia. Finally, it recommends the establishment of an international group of scientists to meet on a regular basis, stimulate further discussions, and give direction on how the final goal, i.e. a proposal for a revised, and widely accepted, classification of the eimeriid coccidia, may be achieved.

Keywords: Coccidia; Classification; Taxonomy; Phylogeny; Ultrastructure; Ribosomal RNA

1. Introduction

The coccidia are a diverse group of parasitic protozoa. Some species of coccidia are homoxenous and strictly host specific, other species have complex heteroxenous life cycles that involve a broad range of different host species. Since their first description in the 19th century, coccidia have been found in almost every animal examined, including humans. Thus, while the oldest species and genera of coccidia have now been known for more than a century, others have been described and named only recently.

During the 19th and early 20th centuries, classifications of protozoa were based mainly on organelles of locomotion, but with increasing knowledge on their morphology, biology, life cycle, and host specificity, a broad range of phenotypic characters have been used to classify protozoa into different taxonomic groups (reviewed in Cox, 1991). Thus, several schemes for the classification of protozoa, including the coccidia, have been proposed during the second half of the 20th century. The first major reclassification was undertaken by an international 'Committee on Taxonomy and Taxonomic Problems' which was set up

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by the Society of Protozoologists in 1954 and published a revised classification of the phylum Protozoa 10 years later (Honigberg et al., 1964). This classification was based on phenotypic characters of about 48,000 species of protozoa recorded at the time and defined 140 taxa at suprafamilial levels. However, it incorporated only few ultrastructural data, which became available after the advent of electron microscopy in the 1950s and 1960s, and was thus confounded by incomplete knowledge on heteroxenous life cycles such as those of the tissue cyst-forming coccidia that were elucidated only in the 1970s (reviewed in Tenter and Johnson, 1997). Therefore, although the classification by Honigberg et al. (1964) was a major advance over the variety of traditional classification schemes that had been in use until the 1960s, it already appeared to be out of date by the end of the 1970s.

Consequently, in 1980 the 'Committee on Systematics and Evolution' of the Society of Protozoologists, again consisting of an international group of experts, published another revised classification of the protozoa that was based on knowledge of more than 65,000 species, of which more than half were extant and about 10,000 were parasitic, and incorporated information gained from ultrastructural data (Levine et al., 1980). This committee classified the Protozoa, then a subkingdom, into seven phyla, gave descriptions for 234 higher taxa down to the level of suborder, and gave examples of representative genera of each. The classification by Levine et al. (1980) was widely adopted by authors of zoological and biological textbooks, and most groups of protozoa defined in it were generally accepted by many protozoologists until the early 1990s. However, a major drawback of this classification was that only few molecular data from protozoa were available when it was produced. Over the past three decades, molecular data have been increasingly used to infer phylogenetic relationships among various protozoa. The results of such molecular phylogenetic studies supported the monophyly of some taxa defined by Levine et al. (1980), but several other taxa described in this classification have been found to be paraphyletic or even polyphyletic. This has led to the recently revised 'interim' classifications of unicellular organisms by Cavalier-Smith (1993) and Corliss (1994). However, these classifications vary greatly from each other. In fact, they probably represent two extremes with respect to the number of taxonomic categories that have been used in modern classifications of protozoa (Fig. 1), and neither has been widely accepted.

Thus, at the beginning of the 21st century, it appears appropriate to undertake another major revision of the classification of protozoa that, for the first time, will be based on both phenotypic and molecular characters. Such a classification should reflect the phylogeny of the taxonomic groups described in it as accurately as possible to ensure its stability for a reasonable number of years, and again should be produced by an international group of experts to encourage wide acceptance. Considering the number of new protozoan species described since 1980, which may now have exceeded well over 100,000, and the large amount of ultrastructural, biochemical, and molecular biological data that have been generated for a broad range of protozoa, such a revision will be an enormous task. A revised classification of the protozoa at higher taxonomic levels is currently being deliberated by another committee of the Society of Protozoologists and is hoped to become available within the next few years.

To contribute to the current efforts of producing a more accurate and stable classification of protozoa, a Controversial Roundtable on 'New classification of coccidia' was held during the joint meeting of the 8th International Coccidiosis Conference and the Annual Scientific Meeting of the Australian Society for Parasitology at Palm Cove, Australia, in July 2001. This Roundtable focussed on one group of coccidia, i.e. the eimeriid coccidia which are one of the largest groups of parasitic protozoa and comprise many species of veterinary and medical importance. While changes at higher taxonomic levels usually have no effect on applied areas of parasitology, taxonomic changes at subfamilial levels may change the names of the organisms involved and thus may be less acceptable to a wide community of general parasitologists, practitioners, and clinicians working in applied fields of veterinary and human medicine. However, although stability of nomenclature is greatly desired to aid communication and avoid confusion among protozoologists and non-protozoologists, an accurate classification of unicellular organisms that reflects their natural history also has impact on practical applications such as in the areas of differential diagnosis and drug development. It is also important for the elucidation of unknown life cycles and epidemiological investigations, in particular on emerging diseases. For example, the recognition of the human pathogen *Pneumocystis carinii*, previously classified with the protozoa, as a fungus increased knowledge and enabled new experiments to improve treatment of pneumocystic pneumonia (Edman et al., 1988).

The eimeriid coccidia are one of the more controversial groups of protozoa, and their taxonomy and classification have been debated for more than 50 years (reviewed in Cox, 1991, 1994; Tenter and Johnson, 1997). Therefore, the aim of the Roundtable held at Palm Cove was to initiate discussion on how a new and more comprehensive classification of eimeriid coccidia can be devised. The stimulus came from invited speakers who gave introductions into selected areas relevant to the taxonomy and classification of coccidia. Following these introductions to specific topics, a more general discussion with the audience, which consisted of more than 50 participants with interest in taxonomy, general biology, immunology, or molecular biology of coccidia as well as applied areas of coccidiosis, took place. This publication describes the immediate outcome of the Roundtable and subsequent discussions that continued over the following days of the conference. Individual authors have contributed various sections (taxonomy, Donald W. Duszynski; ultra-

structure, Heinz Mehlhorn; molecular characters, John R. Barta; *Cryptosporidium*, R.C. Andrew Thompson; phenetic/phylogenetic classifications, Ian Beveridge; future steps, David A. Morrison and Patricia A. Conrad) as well as contributions throughout the manuscript. It should be noted that this publication reflects the cooperative efforts of the authors, aided by the opinions of other participants of the Roundtable, and thus does not necessarily represent the views of any one single author. It is not complete and does not cover all aspects relevant to the taxonomy and classification of the coccidia. Rather, it should be taken as a stimulus for further discussion, preferably in conjunction with other conferences of interest to scientists and practitioners working on coccidia and the diseases caused by them.

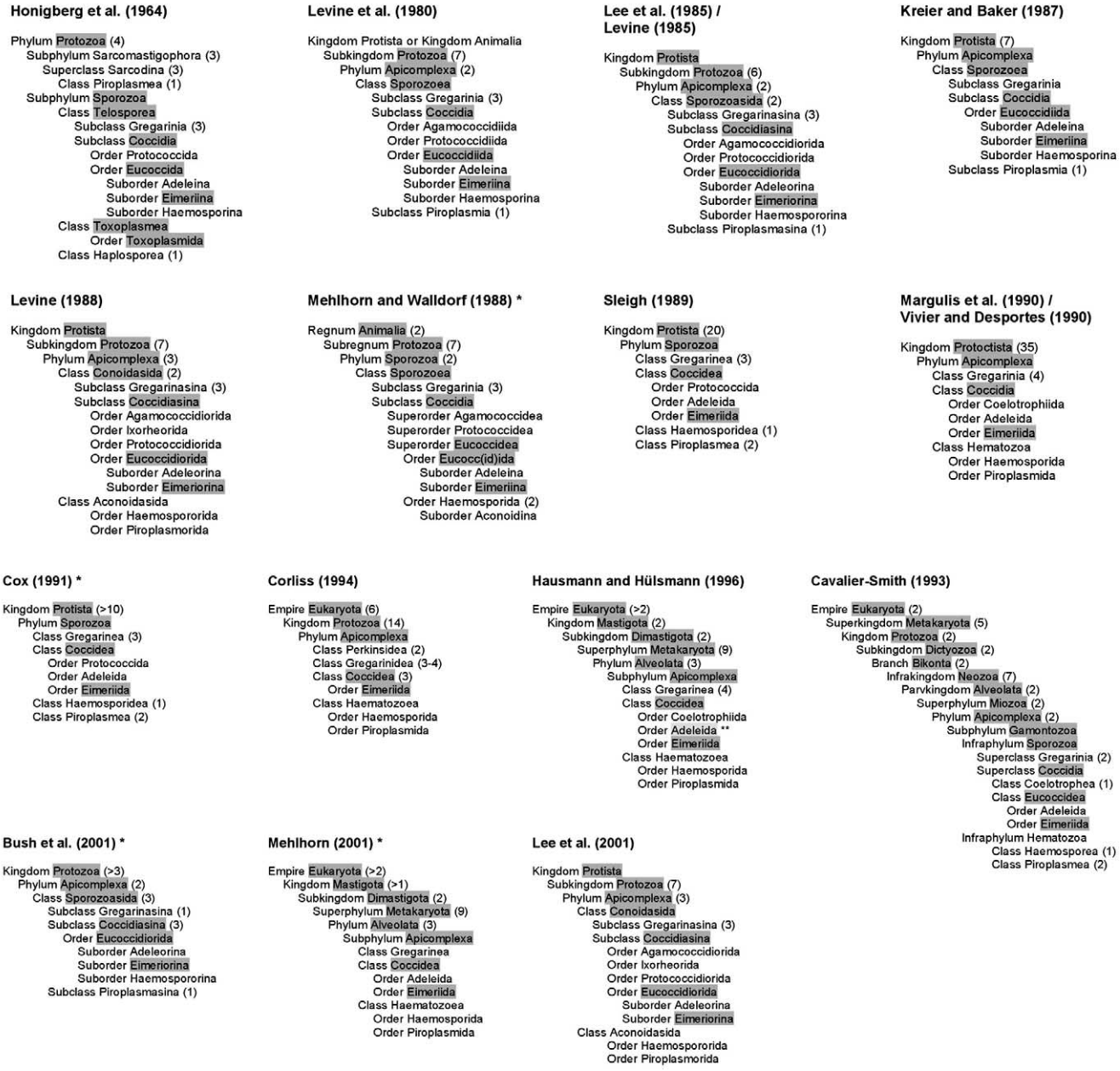


Fig. 1. Comparison of classifications of protozoa at suprafamilial levels proposed during the second half of the 20th century. Classifications have been based on phenotypic characters only, except for the classifications of Cavalier-Smith (1993) and Corliss (1994) who included information derived from phylogenetic reconstruction based on 18S rRNA gene sequences. Only those parts relevant to coccidia and their closest sister-taxa (gregarines, haemosporids, and piroplasms) are shown. Total numbers of taxa at the next lower hierarchic level, if not listed here, are given in brackets. Taxa containing eimeriid coccidia of medical and/or veterinary importance are shaded; *, only taxa containing parasitic protozoa were listed in the classification; ** in this classification the genus *Cryptosporidium* is classified with the adeleid coccidia. For cross-referencing and retrieval purposes, Margulis et al. (1990) is mentioned here in the text.

2. Traditional classifications of coccidia

The phylum Apicomplexa consists of a diverse group of parasitic protozoa that are characterised by an apical complex consisting of special organelles at the anterior end of their invasive life-cycle stages (Levine, 1970). This apical complex facilitates the entry of the parasites into their host cells (Soldati et al., 2001; Tomley et al., 2001). Except for the, still disputed, addition of the genus *Perkinsus* to this phylum, the whole group remains essentially identical to the phylum Sporozoa which was erected more than a century ago (Leuckart, 1879). The largest group of organisms in the phylum Apicomplexa are the coccidia which contain some of the most advanced sporozoa (reviewed in Levine, 1988; Cox, 1994; Lee et al., 2001). They are usually classified into this phylum as a class or subclass (Fig. 1) and are further divided into lower taxa based on phenotypic characters such as morphology and the pattern of their life cycles that alternate between asexual and sexual phases of reproduction (reviewed in Levine, 1985, 1988; Vivier and Desportes, 1990; Tenter and Johnson, 1997; Lee et al., 2001). While some authors include the haemosporids, the piroplasms, or both in the Coccidia (Honigberg et al., 1964; Levine et al., 1980; Levine, 1985; Kreier and Baker, 1987; Mehlhorn and Walldorf, 1988; Bush et al., 2001; Mehlhorn, 2001), the coccidia sensu stricto contain two subgroups, i.e. the adeleid and eimeriid coccidia (Cavalier-Smith, 1993; Cox, 1994).

The eimeriid coccidia (order Eimeriida or suborder Eimeri(or)ina, see Figs. 1 and 2) comprise members with a typical coccidian life cycle consisting of three phases: one or more generations of asexual multiplication by merogony, sexual reproduction by gamogony, and asexual reproduction by sporogony. However, unlike other coccidia, gamogony in the eimeriid coccidia is characterised by the independent development of macrogametes (female) and microgametes (male), with the latter being motile and often produced in large numbers. In addition, sporozoites are typically enclosed in sporocysts that form within oocysts which are passed into the environment as a resistant stage (Levine et al., 1980; Levine, 1985, 1988; Kreier and Baker, 1987; Cox, 1994; Hausmann and Hülsmann, 1996; Lee et al., 2001). Most species of eimeriid coccidia are homoxenous. However, the tissue cyst-forming coccidia are obligately or facultatively heteroxenous, with an asexual phase (merogony) of the life cycle leading to the formation of tissue cysts in various tissues of an intermediate host and the sexual phase (gamogony) to the formation of oocysts in the intestine of a definitive host (reviewed in Dubey, 1993; Tenter and Johnson, 1997). While the taxonomic position of eimeriid coccidia at suprafamilial levels is relatively consistent in traditional classifications of protozoa (Fig. 1), their classification into families, subfamilies, and genera has been inconsistent for many years (reviewed in Tenter and Johnson, 1997; Fig. 2). In fact, it has been considered 'one of the most controversial areas of parasitic protozoology' (Cox, 1991). Therefore, a stable classification of the eimeriid coccidia, which comprise many important parasites of humans and animals, at the subordinal level is urgently needed.

There are several pre-requisites to achieve this goal such as general agreement of the scientific community to follow the same rules of taxonomy and nomenclature, in particular when describing new species, consistency of nomenclature, agreement on what weight to assign to descriptions of individual stages of parasites with unknown life cycles, agreement on what weight to assign to molecular characters versus phenotypic characters, and which molecular characters to use for meaningful inference of phylogenetic relationships.

3. Current taxonomy and taxonomic problems of eimeriid coccidia

3.1. Defining the terms

Taxonomy is the most basic activity in biology because it involves the discovery, analysis of variation (quantitatively or qualitatively), naming (nomenclature), ordering (classification/systematics), and communication (publication) of the patterns of life-forms. It makes these life-forms (species) historical, temporal, and spatial entities that are the essential elements of biodiversity, i.e. the genealogical packages that store and transmit the information that leads to the interactions within complex ecosystems. Species are the atoms of biology, they comprise our periodic table.

Thus, taxonomy involves a number of important components, many of which have been done poorly or ignored by taxonomists of coccidia, even to the present day. Here, we briefly address the primary components of taxonomy (collection, identification, nomenclature, systematics) and comment on the status of these interrelated facets as they concern the coccidia.

3.1.1. Collection

Parasitologists are unique among their colleagues who study plants, animals, or microbes because they must collect not only the organism, but also the host(s) in/on which the organism resides. As a general rule, collections must include: (1) locality data, (2) reliably identified host specimen(s), if possible a statistically significant series, and (3) the parasite (in its different stages).

(1) Obligately intracellular protozoa such as the coccidia are associated intimately with their host taxon. Answers to important questions about both the host and its coccidia depend heavily on good locality data. Thus, data on the parasite should always include the collection locality, which is the locality of the individual host from which the type (parasite) specimen was obtained. Such data give valuable insight to the relationships among geography and host and parasite phylogeny that, if available, could

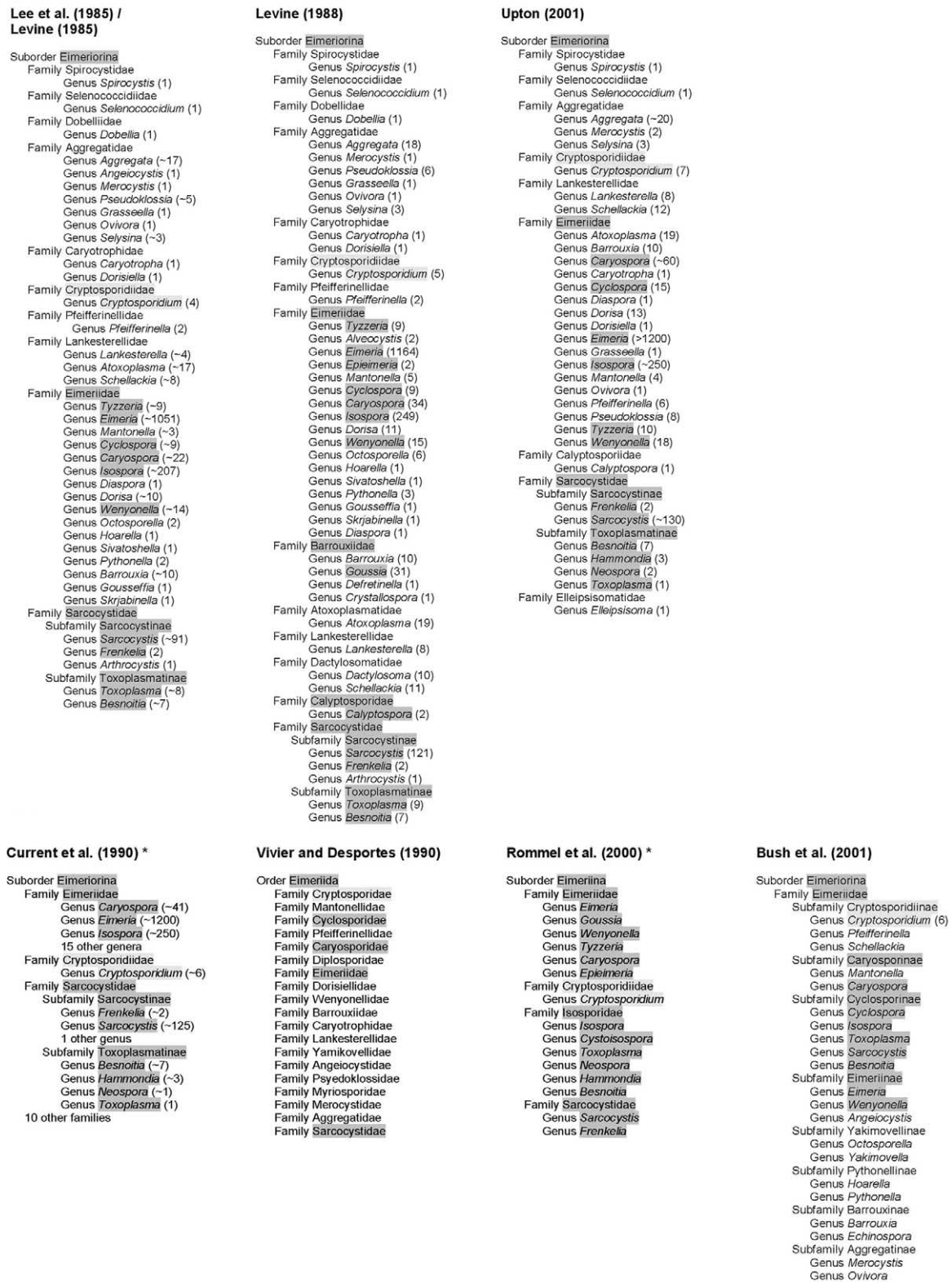


Fig. 2. Comparison of classifications of eimeriid coccidia at the subordinal level based on phenotypic characters. Numbers in brackets are the numbers of species in the respective genus. Taxa containing eimeriid coccidia of medical and/or veterinary importance are shaded in dark grey. The position of the genus *Cryptosporidium* is marked in light grey. *, Only coccidia of medical or veterinary importance were listed in the classification. For cross-referencing and retrieval purposes, Current et al. (1990), Rommel et al. (2000), Sleigh (1989) and Upton (2001) are mentioned here in the text.

revolutionise our understanding of the historical biogeography of these organisms. With good locality data, we can gain new perspectives on both the distribution and diversity of the coccidia in their host populations.

(2) Frey et al. (1992) first recommended that the host from which the 'type' of a new parasite species is described be designated as the 'symbiotype' host. They argued that the accurate identification of a host is an important component in the taxonomic recognition of a new parasite species (especially for coccidia) and that curatorial management and safekeeping of the symbiotype host was desirable. Brooks (1993) concurred, emphasising that parasite evolutionary biology necessarily involves the host(s) of the parasite and, therefore, requires 'the best possible estimates of the host species involved', whether one is discussing host specificity in parasite evolution, host switching in parasite speciation, patterns or processes of parasite–host coevolution, or differentiation of evolutionary or ecological components of community evolution. To emphasise his point, Brooks (1993) noted that prior to the last quarter of the 20th century, herpetologists believed that only one species of leopard frog, *Rana pipiens*, ranged from near the Arctic Circle to Panama and that this species hosted dozens of parasite species of amazing diversity. However, by the beginning of the 21st century, herpetologists recognised that leopard frogs represent a clade of 27 (or more) extant and recently extinct species. With the exception of two studies by Brooks (1976, 1979), no other specimen of leopard frog is known to have been deposited in museum collections. Thus, there is no way to determine the specific identity of the ranid hosts reported in all other surveys to harbour helminth, arthropod, and protozoan parasites. Therefore, archiving hosts from which parasites are collected and described is critical to preserving their true identity in perpetuity.

(3) There are three critical elements when working with coccidia or other parasites once they have been collected from the host. First, they need to be isolated, handled, fixed, and stored properly. Second, as much qualitative and quantitative parasite data as possible should be recorded during observation to be able to write the most accurate, detailed description possible. Guidelines covering these techniques for homoxenous eimeriid coccidia have been provided by Duszynski and Wilber (1997). However, there are currently no such guidelines for heteroxenous coccidia, and a significant amount of the confusion that exists about the taxonomy and nomenclature of heteroxenous coccidia such as the tissue cyst-forming coccidia has come from incomplete species descriptions. Prior to the 1970s these were based either on the oocyst or sporocyst excreted by the definitive host, without knowledge of any intermediate host, or only on life-cycle stages found in an intermediate host, without knowledge of the definitive host (reviewed in Tenter and Johnson, 1997). Unfortunately, even today many authors describe and assign names to tissue cyst-forming coccidia on the basis of only individual stages found in an intermediate host, and the complete life cycles of many of these species are still unknown. Finally, the appropriate stage(s) of the parasite must be archived in an accredited museum from which they can be loaned to interested workers. Bandoni and Duszynski (1988) provided both philosophical and practical arguments why archiving type specimens of coccidia is important and suggested a template for archiving photomicrographs of sporulated oocysts of eimeriid coccidia. Duszynski (1999) reviewed the name-bearing 'types' for photomicrographs of protozoa and suggested that 'photosyntype' may be the best term to use when a series of several photomicrographs of different sporulated oocysts, representing the same new species, is submitted to a museum as part of the original publication and naming process. However, this is not sufficient for polyxenous coccidia which use a wide range of different host species, or for the tissue cyst-forming coccidia which often have a very similar oocyst morphology, but differ by their intermediate host specificity and the type and location of life-cycle stages that are formed in the intermediate host(s).

3.1.2. Identification

Identification is intimately entwined with collection. The locality, the host, and the parasite all must be identified accurately. Global Positioning Systems data, archiving the symbiotype host (Bandoni and Duszynski, 1988; Brooks, 1993), and photosyntypes of sporulated oocysts (Duszynski, 1999) are tools for accurate identification. Host-specificity issues dissolve without accurate host and parasite identifications. Therefore, it is important to note as many of the morphological details of sporulated oocysts as can be identified when describing eimeriid coccidia (Fig. 3). Life-cycle details and cross-transmission studies (Hnida and Duszynski, 1999a) can greatly aid one's decisions in making specific identifications of morphologically similar sporulated oocysts from closely related hosts, and are also essential for identification of the potential natural host range of polyxenous coccidia (Tenter and Johnson, 1997; Mugridge et al., 1999b; Tenter et al., 2000). Finally, if possible, pure samples of oocysts and/or tissue cysts should be preserved in absolute ethanol so that molecular data may be obtained later, i.e. when resources and new, more sophisticated methods are available, to support or refute previous identifications based solely on morphology (Hnida and Duszynski, 1999b). This will also enable phylogenetic relationships to be viewed in entirely new ways, in particular, where genotypes are associated with morphological characters. For example, it has recently been found that the presence or absence of a Stieda body or an oocyst residuum is associated with different genetic lineages within the current genera *Isospora* and *Eimeria* (Barta et al., 1997; Zhao and Duszynski, 2001a).

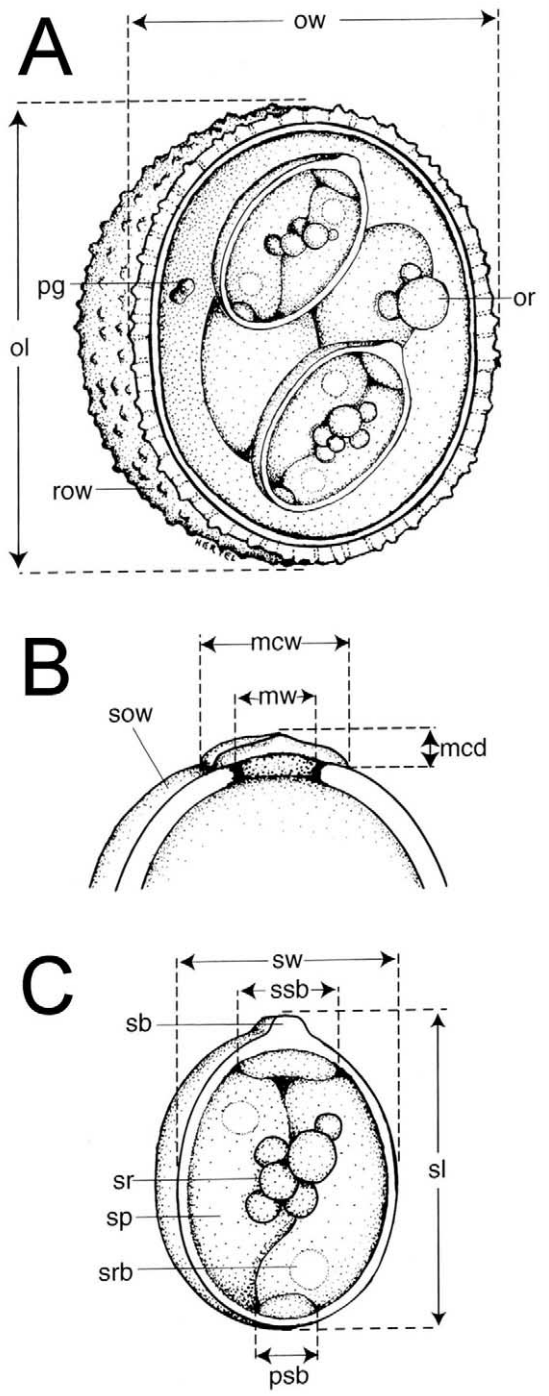


Fig. 3. Some morphological characters of oocysts of eimeriid coccidia. (A) Composite sporulated oocyst of an *Eimeria* species, drawn in optical cross-section: ol, length of the oocyst; or, residual body of oocyst; ow, width of the oocyst; pg, polar granule; row, rough outer wall. (B) The top of a hypothetical oocyst that has a micropyle, micropyle cap, and a smooth wall: mcd, depth (= height) of the micropyle cap; mcw, width of the micropyle cap; mw, width of the micropyle; sow, smooth outer wall. (C) Composite sporocyst of an eimerian-type oocyst: psb, parastieda body; sb, Stieda body; sl, length of the sporocyst; sp, sporozoite; sr, residual body of sporocyst; srb, sporozoite refractile body; ssb, substieda body; sw, width of the sporocyst. For further information and other characters see Duszynski and Wilber (1997).

3.1.3. Nomenclature

The relationship between naming an organism and the type specimen cannot be overemphasised. Names are important because they provide an unambiguous label for each species. The type specimen serves as the anchor for this name and, to some extent, it is the name (Mayr et al., 1953). Rules for guiding zoologists to arrive at names for taxa that are correct under given circumstances are presented in the International Code of Zoological Nomenclature (Ride et al., 1999; see Section 7).

3.1.4. Systematics

Initially, the 'ordering' of organisms into groups is based on their perceived similarities and differences, starting with species as the smallest unit. These are then classified into successively larger groups, i.e. species within genera within families within orders, etc. (Figs. 1 and 2). This 'nested hierarchy' implies a single branching tree with a common base that branches into ever finer divisions and thus embodies the causality of Darwinian evolution (Gould, 2000). The ordering of these groups changes as more rigorous and accurate methods become available and are used to infer more precisely the phylogenetic relationships among the different taxonomic groups. Most systematists strive to make classifications mirror evolutionary history by recognising only monophyletic groups as natural ones.

3.2. Where do we stand today?

Having defined the components of, and the connections among, taxonomy, nomenclature, and systematics, where do we stand in regard to our comprehension and interpretation of these relationships within the coccidia? Barraclough and Nee (2001) emphasised the critical importance of sampling to inferring meaningful species-level phylogenies from molecular data: "To obtain an accurate view of speciation in a higher group, nearly all the species from that group should be sampled. Missing species reduce the sample size of reconstructed speciation events available, and can introduce bias … in particular, the ability to consider the effects of other processes, such as extinction, on the observed patterns relies crucially on a very complete sample of species". It has been estimated that within the protists (unicellular algae, moulds, and protozoa), in general, we are ignorant of the number of species at least to the nearest order of magnitude (Kelly, 2000). Within the eimeriid coccidia, our ignorance may be even greater, as will be explained by five examples with which we are most familiar:

(1) Rodents comprise the most speciose group of mammals with 2,015 extant species in 443 genera and 29 families (Wilson and Reeder, 1993). However, only about 15% (300/2,015) of the species of rodents in 34% (150/443) of the genera and 52% (15/29) of the families have been examined for coccidia, with the description of more than 500 named species of coccidia that have been

classified into about 10 genera in two families (Eimeriidae and Sarcocystidae). These estimates do not include Cryptosporidiidae since molecular evidence suggests that these are not closely related to the eimeriid coccidia (see Section 6). Some rodent species are known to have at least five species of coccidia that are unique to them. If we estimate, conservatively, that each rodent species may be parasitised by three unique species of coccidia, then we currently know only about 8% (500/6,045) of the coccidian species that inhabit rodents. In addition, in most cases, we know only the name and a brief description of the sporulated oocyst, but nothing more.

(2) The Chiroptera (bats) are the second most speciose order of mammals with 925 species, 177 genera, and 17 families (Wilson and Reeder, 1993); yet, only 9% (86/925) of chiropteran species in 24% (43/177) of the genera and 59% (10/17) of the families have been examined for coccidia. What is even more surprising, given the ubiquitous nature of bats, is that only 2,114 individual bats have ever been examined for coccidia (Duszynski, 2002), and only 33 species of coccidia in three genera have been recorded. Only a few bat species have had large numbers (>30) of individuals examined and from some of these species at least two unique species of coccidia are known. Thus, if we assume that each bat species may be a host for two unique species of coccidia, we currently know less than 2% (33/1,850) of the coccidia harboured by bats on Earth.

(3) Insectivora (eg., shrews and moles) is the third largest order of mammals with 428 species, 66 genera, and seven families (Wilson and Reeder, 1993). However, only 9% (37/428) of the species of insectivores in 29% (19/66) of the genera and 57% (4/7) of the families have been examined for coccidia, and 75 species of coccidia in three genera of Eimeriidae have been found (Duszynski and Upton, 2000). The best studied genera of insectivores, *Sorex* and *Talpa*, have 10 and 11 coccidian species, respectively, that are unique to them. Assuming, again conservatively, that each species of insectivores may be a host for three unique species of coccidia, we know less than 6% (75/1,284) of the coccidia of insectivores.

(4) Primates, the fifth most speciose order of mammals, comprise 233 species, 60 genera, and 17 families (Wilson and Reeder, 1993). To date, only 8% (18/233) of primatial species in 29% (14/60) of the genera in 54% (7/17) of the families have been examined for coccidia, but only four of the 18 primate species examined were wild animals, while the other 14 species were humans, zoo, or captive breeding colony animals (Duszynski et al., 1999). To date, only 18 species of coccidia in only four genera in two families have been found in primates. Conservatively estimating that there may be two coccidia that are unique to each species of primates, we know less than 4% (18/466) of the coccidia in the mammals most closely related to ourselves.

(5) Finally, within the Reptilia, there are 2,973 species of snakes in 483 genera and 18 families (http://www.embl-heidelberg.de/~uetz/Reptiles.html). To date, less than 5% (138/2,973) of snake species in 15% (71/483) of the genera and 22% (4/18) of the families have been examined for coccidia, and 131 coccidian species have been described in seven genera in two families. Most snake species examined to date have three or more unique species of coccidia, often representing several genera. If we conservatively estimate that each snake species may be parasitised by three unique species of coccidia, we know less than 1.5% (131/8,919) of the coccidia of snakes (Duszynski and Upton, unpublished data).

If the above examples give a reasonable estimate of how little we know about the coccidia of vertebrates, then it is certainly premature to make sweeping changes within the taxonomy of the eimeriid coccidia when we know only about 4% (757/18,564 in the above examples) of the species that exist in this lineage of intracellular protozoan parasites. Imagine trying to do calculations in chemistry or physics knowing only 4% of the periodic table: any conclusions drawn would be so far-fetched and ignorant they would be laughable. Yet, we are trying to do taxonomic and systematic biology knowing only a fraction of the extant species. The eimeriid coccidia are only one of the many extant groups of parasites capable of living on or in animals and plants and we know as little about some of those groups as we do about the coccidia. Thus, what these numbers do tell us is that we are deeply ignorant about biodiversity, not only within the coccidia, but of life on Earth. In addition, most of what we believe we know about the relationships of the eimeriid coccidia among each other and to other groups of parasitic protozoa has been based only on phenotypic characters of the limited number of species that have been described so far, and only few molecular data have been collected for an even smaller number of species, i.e. mainly those of medical or veterinary importance.

4. Phenotypic characters used for classifications of eimeriid coccidia

Phenotypic characters traditionally used for the classification of eimeriid coccidia include the morphology of available parasite stages and host specificity (Table 1). One problem with the use of these characters for classification is that in most cases only the oocyst stage and the 'host' by which it was shed had been known when a new species was named. Such incomplete species descriptions are confounded by the fact that the 'host' described for the new species may not be its true natural host, because many animals may passage oocysts through their intestine without being a host for these parasites. For example, hunting dogs frequently shed oocysts of *Eimeria* in their faeces although no valid *Eimeria* species is known to infect canids. These oocysts may often originate from lagomorphs on

Table 1
Some phenotypic characters used for the classification of, and discrimination among, eimeriid coccidia at the subordinal level

Character	Character states
All eimeriid coccidia	
Oocyst[a]	
Number of sporocysts	0, 1, 2, 4, 8, 16, or >16
Size of sporulated oocyst	Several states[a]
Morphology of oocyst wall	Several states[a]
Micropyle	Present or absent
Micropyle cap	Present or absent
Polar granule(s)	Present or absent
Residual body of oocyst	Present or absent
Sporocyst[a]	
Number of sporozoites	1, 2, 3, 4, 8, 12, 16, or >16
Size of sporocyst	Several states[a]
Morphology of sporocyst wall	Several states[a]
Stieda body	Present or absent
Substieda body	Present or absent
Parastieda body	Present or absent
Residual body of sporocyst	Present or absent
Sporozoite[a]	
Refractile body(ies)	Present or absent, numbers, position
Life-cycle data	
Type of life cycle	Homoxenous or heteroxenous
Merogony	Intestinal or extraintestinal (several states[b])
Gamogony	Intestinal or extraintestinal (several states[b])
Extraintestinal dormozoites	Present or absent
Host data	
Specificity	Mammals, birds, reptiles, amphibia, fishes, invertebrates (several states[b])
Tissue cyst-forming coccidia only	
Definitive host	
Host specificity	Carnivores or omnivores (several states[b])
Degree of host specificity	Species-specific or variable (several states[b])
Intestinal phase of merogony	Present or absent
Extraintestinal stages	Present or absent
Location of zygote	Epithelium or lamina propria
Sporogony	Endogenous or exogenous
Period of patency	Days, weeks, or months
Intermediate host	
Host specificity	Herbivores or omnivores (several states[b])
Degree of host specificity	Species-specific or variable (several states[b])
Type of pre-cystic merogony	Endodyogeny or endopolygeny
Location of pre-cystic merogony	Vascular endothelial cells, hepatocytes, lymphoid cells, neural cells, or many types of host cells (several states[b])
Shape of merozoites	Elongate or spherical
Tissue cyst[c]	
Location of host nucleus	Outside of primary tissue cyst wall
Location of tissue cyst	Central nervous system, striated muscles, fibroblasts, or many types of host cells (several states[b])
Shape of tissue cyst	Spherical, subspherical, cylindrical, lobulated, long stretched or variable (several states[b])
Morphology of tissue cyst wall	Thin, thick, or variable (several states[b])
Ultrastructure of tissue cyst wall	Several states[b]
Number of zoites	Monozoic or polyzoic
Number of stages within tissue cyst	Monomorphic (only bradyzoites) or dimorphic (metrocytes and bradyzoites)
Septa within tissue cyst	Present or absent
Infectivity	Immediate or after weeks/months
Route of transmission	
Degree of heteroxeny	Obligatory or facultative
Oocyst infective for definitive host	Yes or no
Tissue cyst infective for intermediate host	Yes or no
Vertical transmission in intermediate host	Present or absent
Ultrastructure of tachyzoites in cell culture	
Location of rhoptries	Anterior and posterior to nucleus or anterior to nucleus only
Location of micronemes	Anterior and posterior to nucleus or anterior to nucleus only
Number of micronemes	Few or many (>100)

[a] For further information see Duszynski and Wilber (1997).
[b] The number of possible states is too large to be listed in this Table; for further information see Levine (1985, 1988), Levine and Baker (1987), Frenkel et al. (1987), Vivier and Desportes (1990), and references listed in Mugridge et al. (1999a,b).
[c] See Section 4.2.

which the dogs have been fed (Staub and Tenter, unpublished observation). Another example for such confounding factors is the description of the genus *Isospora* and its type species, *Isospora rara*, in *Limax cinereoniger* (Schneider, 1881). *Isospora rara* is the only species of *Isospora* ever described in an invertebrate (Levine, 1988). It is now believed to be a pseudoparasite, and the origin of the *Isospora* oocysts observed by Schneider (1881) in the gastropod is unclear. Thus, we have used an invalid species of *Isospora* as type species of the genus for more than 120 years. To avoid such confusion, it is essential that descriptions of new species are based on more characters than just the oocyst stage. Such characters should include the location and ultrastructure of developmental stages inside the host and data on the parasite's life cycle (Table 1), and identification of the correct host(s) of a new species should be confirmed by transmission experiments. However, such experiments have been carried out for only a small number of species to date.

4.1. Life cycles

The problem of naming new species based on only one stage of the parasite and the importance of transmission studies for species identification and elucidation of their correct natural host range has been highlighted by the discovery of heteroxenous life cycles for the tissue cyst-forming coccidia in the 1970s. Here, the history of *Toxoplasma gondii* may serve as an example.

Asexual stages of *Toxoplasma*-like parasites were first observed at the beginning of the 20th century in tissues of birds and mammals (reviewed in Tenter et al., 2000). In 1908, *T. gondii* merozoites (i.e. tachyzoites or endozoites) were comprehensively described in the spleen, liver, and blood of gondis, a species of North African rodents by Nicolle and Manceaux (1908). The authors first thought this parasite to resemble a species of *Leishmania* and assigned the name *Leishmania gondii* to it, but a more detailed study showed that it lacked a kinetoplast and, therefore, the generic name *Toxoplasma* (from the Greek toxon = arc, plasma = form) was proposed 1 year later (Nicolle and Manceaux, 1909). However, no relationship was recognised between *Toxoplasma* and the genus *Sarcocystis*, the tissue cysts of which had already been described 65 years earlier (Miescher, 1843). During the first half of the 20th century, several species of *Toxoplasma* were named in accordance with the host species in which they were detected (reviewed in Levine, 1977). It was not until the late 1930s that biological and immunological comparisons provided evidence that various isolates of animal and human origin were identical with *T. gondii*, but even then only asexual stages (merozoites and tissue cysts) of *T. gondii* were known and its classification remained uncertain (Fig. 1; reviewed in Tenter and Johnson, 1997; Tenter et al., 2000).

Evidence for the coccidian nature of *T. gondii* first came from electron microscopic studies carried out in the 1960s which revealed ultrastructural similarities between extraintestinal merozoites of *T. gondii* and intestinal merozoites of *Eimeria* species and, thus, indicated a coccidian-like life cycle for *T. gondii* (reviewed in Scholtyseck and Mehlhorn, 1973; Tenter et al., 2000). Finally, the heteroxenous life cycle of *T. gondii* was elucidated in the late 1960s after it had been found that the faeces of cats may contain an infectious stage of *T. gondii* which induces infection when ingested by intermediate hosts (Hutchison, 1965). This stage was eventually identified as an isosporan-type oocyst previously described as part of the *Isospora bigemina* complex, and in 1970, knowledge of the coccidian life cycle of *T. gondii* was completed by the discovery of sexual stages in the small intestine of cats (reviewed in Tenter and Johnson, 1997; Tenter et al., 2000).

Thus, it was more than 60 years from the description of the first stage of the parasite until it was revealed that *T. gondii* is a tissue cyst-forming coccidium with a heteroxenous life cycle in which an asexual phase of development in tissues of various intermediate hosts is linked to a sexual phase of development in the intestine of feline definitive hosts. Since then, several other protozoa that had been assigned to the genus *Toxoplasma* during the first half of the 20th century, have either been synonymised with *T. gondii*, have been reclassified into other coccidian genera, or their descriptions superseded (Levine, 1977). Today, *T. gondii* is recognised as one of the more polyxenous parasites that can infect many types of host cells in probably all warm-blooded animals, including humans (Tenter et al., 2000). Thus, while some species of coccidia are strictly host-specific, others may use a broad range of hosts comprising several orders, and even classes, of animals. The elucidation of the life cycle of *T. gondii* significantly improved knowledge on the epidemiology of toxoplasmosis and, thus, enabled strategies for prevention and control of disease in risk groups. Thus, the history of this parasite highlights the importance of accurate description, life-cycle data, and correct classification of coccidian species for applied fields of medical and veterinary parasitology.

In the years following the elucidation of the life cycle of *T. gondii*, numerous experiments involving transmission of asexual stages of various other tissue cyst-forming protozoa to carnivores showed that long-known members of the genus *Isospora* were in fact developmental stages of species belonging to different genera of what are now recognised as the tissue cyst-forming coccidia (reviewed in Tenter and Johnson, 1997). It became evident that different stages of the same parasites had been associated with two or more different genera and thus had been given different names. These findings had great impact on the classification of coccidia. They led to the classification of the family Sarcocystidae within the eimeriid coccidia and to redefinitions of this family to include heteroxenous, tissue cyst-forming coccidia with an isosporan-type oocyst (Frenkel, 1977). Since the late 1970s, the family Sarcocystidae has been used in many, but not all (Fig. 2), classifications of eimeriid

coccidia. However, the number of lower taxa, i.e. subfamilies and genera, within this family, the validity of the family itself, its evolutionary history, and the value of heteroxeny as a character for classification of coccidia into different taxonomic groups have been heavily debated, even to the present day (Fig. 2; Barta, 1989; Cox, 1994; Tenter and Johnson, 1997; Frenkel and Dubey, 2000; Mehlhorn and Heydorn, 2000).

4.2. Morphology and ultrastructure

While it is greatly preferred that species descriptions should include data on the parasite's life cycle, a problem with this is that the complete life cycle is unknown for most coccidia that have been named to date and, as noted above, elucidation of the complete life cycle may be a tedious and time-consuming task. Therefore, in many cases, the designation of names to species of eimeriid coccidia has been based on morphological characters of individual stages seen with a light microscope and limited data on the host from which these stages have been obtained (Table 1).

If only such limited information is available for a given species, the comparison of ultrastructural characters may be useful for its classification into genera and families. The advent of electron microscopy in the second half of the 20th century enabled ultrastructural comparisons of a broad range of protozoa and in many cases confirmed, or refuted, previously assumed taxonomic relationships among them. The information derived from ultrastructural data has had great impact on current classifications of protozoa (see Section 1). Thus, while ultrastructure alone is not sufficient for species description, knowledge of distinct ultrastructural features may provide guidance for improved classification of coccidia with unknown life cycles as exemplified by the history of *T. gondii* described above (see Section 4.1), and as stated by Lee et al. (1985): "Had it been learned sooner that *Toxoplasma* belongs to the Apicomplexa (evidenced from electron microscopy), the effective chemotherapeutic combination of a low-molecular folic-reductase inhibitor of the pyrimethamine type, administered in synergistic combination with a sulfa drug, might have been used decades earlier (as developed for malaria), eliminating much misery".

Ultrastructural characters are particularly useful for species and genus determination of tissue cyst-forming coccidia. True coccidia are characterised by the occurrence, at least initially, of a parasitophorous vacuole that is limited by (mostly) one single membrane. As such the lack of this character excludes the genus *Cryptosporidium* from the coccidia sensu stricto. The mode of preservation or transformation of such a parasitophorous vacuole is genus-specific as are the structural features of the stages (i.e. sporozoites, merozoites, or bradyzoites) contained in the tissue cyst (Fig. 4; Mehlhorn and Frenkel, 1980; Mehlhorn, 2001).

4.2.1. Genus Cystoisospora

In *Cystoisospora* species, for example *Cystoisospora felis* or *Cystoisospora ohioensis*, the tissue cysts in intermediate hosts (e.g. cattle) represent a host cell with a parasitophorous vacuole, the limiting membrane of which has no infoldings. Inside this membrane a broad zone of dense material surrounds a typical, unchanged sporozoite which can be recognised by its refractile body (Mehlhorn and Dubey, 1983).

4.2.2. Genus Globidium

In the genus *Globidium* large tissue cysts occur e.g. in the abomasum of ruminants. The parasitised host cell is widely enlarged and contains a huge parasitophorous vacuole that is limited by a single membrane. This parasitised host cell is closely surrounded by a thick layer of host connective tissue, i.e. the secondary tissue cyst wall. The parasites inside the parasitophorous vacuole reproduce by repeated ectomerogonies. The final stages within the tissue cyst have a very similar appearance to eimerian merozoites.

4.2.3. Genus Besnoitia

The tissue cysts of the genus *Besnoitia*, for example those of *Besnoitia besnoiti* in cattle or *Besnoitia jellisoni* in mice, show a construction similar to that of *Globidium* species in that they cause the host cell to enlarge. However, the nucleus of this host cell divides into several nuclei, which then hypertrophy. The tissue cysts are also surrounded by a broad secondary tissue cyst wall of host connective tissue. The parasites inside the tissue cyst reproduce by repeated endodyogeny and show a unique ultrastructure, i.e. they possess enigmatic bodies.

4.2.4. Genera Toxoplasma, Hammondia, and Neospora

All tissue cysts of species within the genera *Toxoplasma*, *Hammondia*, and *Neospora* described so far are situated mainly in muscle or neural cells. They represent a non-septate parasitophorous vacuole, the limiting membrane of which shows many invaginations of varying lengths that reach into a zone of granular material, with similar appearance to that of *Cystoisospora*. Depending on the age and size of the tissue cyst this granular zone has diameters of 0.5–4.2 μm. The parasites inside the different tissue cysts cannot be distinguished among the three 'genera', either by ultrastructure or by size. They reproduce by continuous endodyogeny.

4.2.5. Genera Sarcocystis and Frenkelia

In the genera *Sarcocystis* and *Frenkelia*, tissue cysts are found in muscles, brain, or connective tissues and, depending on the species, are macroscopically visible or not. The tissue cysts are limited by a typical primary tissue cyst wall consisting of a single membrane that is fortified at regular distances by a dense layer. At the unfortified places, vesicle-like invaginations occur stretching into the granular ground substance that forms chamber-like hollows surrounding the

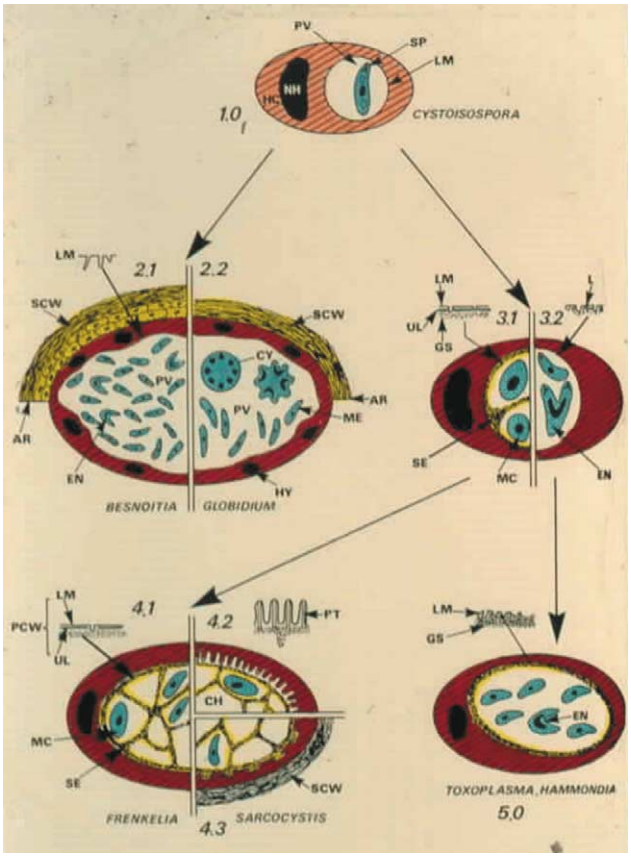


Fig. 4. Diagrammatic representation of the development of, and within, tissue cysts in different genera of the tissue cyst-forming coccidia (adapted from Mehlhorn and Frenkel, 1980; Mehlhorn, 2001). (1.0) The simplest tissue cyst formation: a sporozoite is included in a parasitophorous vacuole that is bounded by a single cell membrane. This is representative of monozoic tissue cysts of *Cystoisospora* in paratenic hosts. (2.1 and 2.2) In tissue cysts of *Besnoitia* and *Globidium* the original parasitophorous vacuole is enlarged and is filled by numerous parasites that reproduce by endodyogeny (2.1) or ectomerogony (2.2), respectively. Even in old tissue cysts the parasitophorous vacuole is bounded by a single, unthickened cell membrane. A secondary tissue cyst wall consisting of fibrillar material is always present. The host cell nucleus generally undergoes hypertrophy and hyperplasia. (3.1 and 3.2) Young tissue cysts of *Sarcocystis* and *Frenkelia* (3.1) contain spherical metrocytes in chamber-like spaces whereas young tissue cysts of *Toxoplasma* and *Hammondia* (3.2) contain slender parasites. All of these stages divide by endodyogeny. The membrane of the parasitophorous vacuole becomes thicker by underlying material thus forming a primary tissue cyst wall. (4.1 to 4.3) Mature tissue cysts of *Sarcocystis* and *Frenkelia* are characterised by the presence of typical septa that are formed by the ground substance. In *Frenkelia* and some species of *Sarcocystis* (4.1) the primary tissue cyst wall never forms long protrusions whereas in other species of *Sarcocystis* (4.2) typical protrusions are formed. In some species of *Sarcocystis* (4.3) such as *S. gigantea* a secondary tissue cyst wall surrounds the parasitised muscle fiber. (5.0) The primary tissue cyst wall of mature *Toxoplasma* and *Hammondia* tissue cysts remains smooth. The tissue cysts are tightly filled with bradyzoites; septa are absent. AR, artificially interrupted secondary tissue cyst wall; CH, chamber-like space filled with parasites; CY, cytomere; EN, endodyogeny; GS, ground substance; HC, host cell; HY, hypertrophic host cell nucleus; LM, limiting single membrane of parasitophorous vacuole; MC, metrocyte; ME, merozoite; NH, host cell nucleus; PCW, primary tissue cyst wall; PT, protrusion of primary tissue cyst wall; PV, parasitophorous vacuole; SCW, secondary tissue cyst wall; SE, septum formed by ground substance; SP, sporozoite; UL, underlying dense material.

parasites. In chambers of the periphery the parasites are large and ovoid. They are called metrocytes and develop by repeated endodyogeny into banana-shaped, infectious bradyzoites that fill the inner chambers of the tissue cyst. The bradyzoites are characterised by enormous numbers of closely packed micronemes which give them a unique appearance. The tissue cysts of some species, e.g. *Sarcocystis gigantea*, are additionally surrounded by a broad, whitish appearing secondary tissue cyst wall of host connective tissue. In some species of *Sarcocystis* the primary tissue cyst wall may produce typical protrusions (cauliflower-like, finger-like, quadratic, etc.). However, these cannot be used alone for species differentiation, since similar protrusions occur in several species (Mehlhorn and Heydorn, 1978).

4.2.6. Conclusions based on the characters of presence/absence of a parasitophorous vacuole and tissue cyst wall morphology

Ultrastructural comparison of eimeriid coccidia suggests that (1) *Cryptosporidium* is not a coccidian parasite; (2) tissue cysts of *Globidium* probably represent 'giant schizonts' of *Eimeria* species; (3) there is only one genus in the group of *Toxoplasma*, *Hammondia*, and *Neospora*; (4) *Frenkelia* and *Sarcocystis* belong to the same genus, as already proposed by Tadros and Laarman (1976).

4.3. Inference of phylogenetic relationships of coccidia based on phenotypic characters

For higher eukaryotes, information on their phylogenetic relationships can be derived from the comparison of homologous characters with fossil records that permit the placement of the organisms under study into evolutionary time frames and the construction of phylogenetic trees reflecting highly probable evolutionary histories. However, for soft-bodied protozoa such as the eimeriid coccidia, there are no fossil records and many intermediate forms that may have been useful for inference of phylogenetic relationships have been lost. Therefore, phylogenetic relationships of these parasites need to be inferred from comparisons of homologous characters in extant species.

Since the late 1960s, morphological/ultrastructural characters and life-cycle data have been the major characters used for the classification of eimeriid coccidia. However, while these characters are useful for species description, identification, and differentiation, a major problem with their use for reconstruction of phylogenetic relationships is to find those characters that are truly homologous among the species included in the analysis and, therefore, are phylogenetically informative (Barta, 1989). In addition, as described above, although many species of eimeriid coccidia have been named, their descriptions are often inadequate. In particular, there is a great lack of life-cycle data and the state of the characters listed in Table 1 is often unknown for a given species. Consequently, the phenotypic

characters currently used for the classification of eimeriid coccidia are limited in their phylogenetic information content, and a major drawback of such classifications is that they have been based almost exclusively on phenotypic characters (see Sections 1 and 2).

5. Inference of phylogenetic relationships of eimeriid coccidia based on molecular characters

Molecular characters can expand the range of evolutionarily informative characters that may be used for inferring phylogenetic relationships among different organisms (Sogin and Silberman, 1998). This is especially important for protozoan taxa in the phylum Apicomplexa because of the limited number of phenotypic characters that are suitable for evolutionary studies. Molecular characters can be reasonably assumed to be homologous in an evolutionary sense as well as having sufficient variability to generate character states for analysis (Barta, 1997). As products of the genome of the parasites, phenotypic characters are no less credible than molecular targets as potential characters for studies in systematics. However, there are relatively few phenotypic characters that can be used successfully for the eimeriid coccidia (see Section 4).

5.1. When are molecular characters best applied?

Molecular data are particularly useful for inferring both very ancient as well as relatively recent relationships (Olsen and Woese, 1993; Sogin and Silberman, 1998). Inference of extremely ancient relationships such as those among all eukaryotic taxa from phenotypic characters would be difficult because of the relatively limited number of features for which homology could be reasonably assigned. Furthermore, those characters that can be reasonably assumed to be homologous (e.g., the conoid in the Apicomplexa) may not demonstrate sufficient variation to be useful in a phylogenetic reconstruction. At the other end of the temporal scale, recent divergences such as among species and strains of eimeriid coccidia, may not be supported by any morphological variation whatsoever, but can often be readily detected using molecular characters.

5.2. 18S ribosomal RNA gene sequences for phylogenetic reconstruction

For eimeriid coccidia, the use of molecular characters for inferring relationships among a variety of taxa has relied almost exclusively on the 18S rRNA gene sequences (Barta et al., 1997, 2001; Jeffries et al., 1997; Morrison and Ellis, 1997; Pieniazek and Herwaldt, 1997; Tenter and Johnson, 1997; Carreno et al., 1998, 1999; Votypka et al., 1998; Carreno and Barta, 1999; Dolezel et al., 1999; Eberhard et al., 1999; Holmdahl et al., 1999; Jenkins et al., 1999; Lopez et al., 1999; Ellis et al., 2000; Franzen et al., 2000; Modry et al., 2001; Slapeta et al., 2001a,b; Yang et al., 2001; Zhao and Duszynski, 2001b). This reliance on a single gene has been highly informative but is clearly limited by the restrictive nature of the data (Olsen and Woese, 1993; Mugridge et al., 2000). The relationships inferred using these data must be considered evolutionary hypotheses of the 18S rRNA gene (a 'gene tree') and not an organismal phylogeny (Mugridge et al., 2000). Only analyses using multiple genes, preferably including both nuclear and organellar genes, could generate a molecular phylogeny for the eimeriid coccidia that could be considered an organismal evolutionary hypothesis. Notwithstanding this limitation, the monophyletic groupings suggested by analyses using the 18S rRNA gene have largely supported the major groupings of apicomplexan taxa recognised using morphological and life-cycle traits (Barta, 2001). These classical groups include: gregarines; *Cryptosporidium* species; eimeriid coccidia (and within these a clear distinction between the tissue cyst-forming coccidia, exemplified by *T. gondii* and various *Sarcocystis* species, and the monoxenous coccidia, exemplified by *Eimeria* species); haemosporids; piroplasms; and haemogregarines.

Despite this overall agreement, several inconsistencies have been noted between the relationships proposed among some apicomplexan parasites based on morphological characters and those based on 18S rRNA gene sequences. For example, the genus *Isospora* has been found to be paraphyletic (Carreno and Barta, 1999; Barta et al., 2001; Modry et al., 2001; Slapeta et al., 2001a). This recognition has led to a reconsideration of some morphological features, i.e. refractile bodies and oocyst structure, that may be helpful in dividing the coccidia into two major monophyletic clades (see Section 3.1). This ability to re-examine morphological characters, with the assistance of additional molecular characters, has been of considerable utility. In addition, *Cryptosporidium* species were found to be only distantly related to the coccidia but have been shown to have closer affinities to the gregarines that infect invertebrates (Carreno et al., 1999; Mathew et al., 2000; Barta et al., 2001). A re-analysis of ultrastructural, antigenic, and biological characters suggested that this proposed relationship might be correct (see Sections 4.2 and 6). However, while able to cluster apicomplexan taxa into a number of major groupings, the 18S rRNA sequences are largely unable to resolve relationships among closely related species within the eimeriid coccidia, and it is clear that other molecular targets will be required to elucidate these relationships.

5.3. Other molecular data for phylogenetic reconstruction

More recently, a few studies have used sequences of the nuclear 28S rRNA gene, the internal transcribed spacer (ITS) 1 region, or organellar sequences of the plastid to infer relationships among closely related species of eimeriid coccidia, i.e. within the genus *Eimeria* and the group of the tissue cyst-forming coccidia (Ellis et al., 1999, 2000; Hnida

and Duszynski, 1999b; Mugridge et al., 1999a,b, 2000; Zhao and Duszynski, 2001a,b; Zhao et al., 2001).

Phylogenetic reconstruction based on 28S rRNA gene sequences suggests that there are at least two lineages within the tissue cyst-forming coccidia (Fig. 5). One lineage includes the genera *Toxoplasma*, *Hammondia*, *Neospora*, and *Besnoitia*, with species in the genera *Toxoplasma*, *Hammondia*, and *Neospora* being very closely related, as already suggested from analyses of phenotypic characters (see Section 4.2), and the genus *Hammondia* being paraphyletic, as also shown by an analysis based on ITS 1 sequences (Ellis et al., 1999; Mugridge et al., 1999b, 2000). Another lineage includes species of the genera *Sarcocystis* and *Frenkelia* and suggests that either the genus *Sarcocystis* is paraphyletic or that *Frenkelia* should be synonymised with *Sarcocystis* (Mugridge et al., 1999a, 2000). This finding is consistent with reconstructions based on 18S rRNA gene sequences (Votypka et al., 1998; Dolezel et al., 1999; Jenkins et al., 1999) and earlier hypotheses based on phenotypic characters, i.e. that species of *Sarcocystis* and *Frenkelia* belong to the same genus (see Section 4.2; Tadros and Laarman, 1976; Cerna and Kolarova, 1978; Baker, 1987; Jakob et al., 1998; Odening, 1998).

5.4. Conclusions drawn from inference of phylogenetic relationships of eimeriid coccidia based on molecular characters

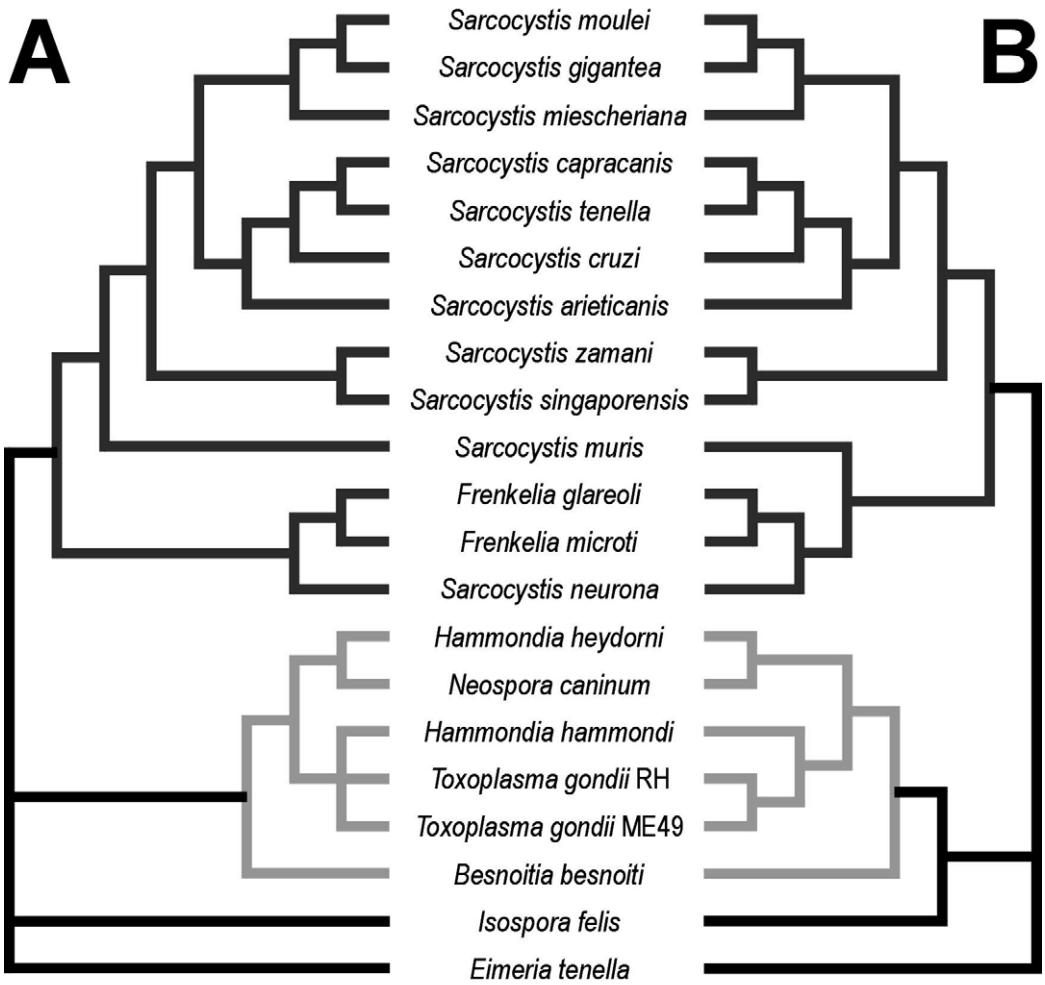
The molecular phylogenetic reconstructions generated to date call for revisions of the current families Eimeriidae and Sarcocystidae as well as some of the genera that have been placed into these families (Tenter and Johnson, 1997; Carreno et al., 1998; Carreno and Barta, 1999; Franzen et al., 2000; Barta et al., 2001; Modry et al., 2001). However, at present these reconstructions not only suffer from being based mainly on a single gene, i.e. the 18S rRNA gene, but they are also incredibly biased in their sampling of the biological diversity within this group of parasites. The vast majority of eimeriid coccidia for which there are 18S rRNA gene sequences are found within a small number of genera, i.e. *Besnoitia*, *Caryospora*, *Cyclospora*, *Eimeria*, *Frenkelia*, *Hammondia*, *Isospora*, *Lankesterella*, *Neospora*, *Sarcocystis*, and *Toxoplasma*. Other phylogenetically informative, molecular data are available for even fewer genera, i.e. mainly those of medical and/or veterinary interest as pathogens. Although understandable, this lack of sampling may very well be biasing the relationships that are proposed because of insufficient sampling of intermediate taxa. Many phylogenetic methods can suffer from so-called 'long branch attraction' that may affect the monophyletic groupings suggested by analysis of 18S rRNA or other gene sequences. More intensive and systematic sampling of the biological diversity found within the phylum Apicomplexa is required at both the phenotypic and molecular level.

Once completed, such a combined dataset including phenotypic characters and genetic data from multiple nuclear and organellar genes should have the power to resolve a relatively unambiguous organismal phylogeny for parasites in the phylum Apicomplexa such as the eimeriid coccidia. This phylogeny can then be used to erect a robust taxonomic framework that reflects the evolutionary history of the parasites and that will be widely accepted in the scientific and medical communities.

6. The special case of *Cryptosporidium*

Recent molecular epidemiological investigations in which *Cryptosporidium* isolates have been characterised from various host species in different endemic regions support a revision of the species-level taxonomy of *Cryptosporidium* (Morgan et al., 1999; Thompson et al., 2000; Xiao et al., 2000). Ten species are currently recognised of which the status of four was confirmed within the last few years on the basis of their genetic distinctness. In addition, at least eight genotypes of *Cryptosporidium parvum* have been well characterised and most probably represent host-adapted species, some of which were recognised taxonomically many years ago. The 'cattle' genotype has the widest host range and is the only form of zoonotic significance to immunologically competent humans. However, the rigid host specificity of the so-called 'human' genotype of *C. parvum* may be in doubt following its recent identification in patent infections in dugongs (*Dugong dugon*) (Morgan et al., 2001). Clearly, the species-level taxonomy of *Cryptosporidium* is in need of revision, particularly since an increasing number of novel genotypes are being described from various animal species (Morgan et al., 2001; Perz and Le Blancq, 2000).

In addition, the affinity of the genus *Cryptosporidium* with the eimeriid coccidia is being increasingly questioned (see Sections 3.2, 4.2, and 5.2). Species of *Cryptosporidium* are consistently placed separately from taxa of the eimeriid coccidia in molecular studies, and the most recent phylogenetic analysis based on sequences of the 18S rRNA gene by Carreno et al. (1999) demonstrated a close affinity with the gregarines (see Section 5.2). This is supported by the atypical development of *Cryptosporidium*, and in particular its association with the host cell. The ability to maintain the life cycle of *Cryptosporidium* in vitro (Hijjawi et al., 2001) has enabled comprehensive studies on the development of *Cryptosporidium*. These have identified additional gregarine-like developmental stages in its life cycle (Hijjawi et al., 2002), which further supports a re-appraisal of the classification of *Cryptosporidium* as a coccidium. If *Cryptosporidium* is a gregarine, this would explain why it is insensitive to anti-coccidial drugs as well as the frequent occurrence of false-positive results with environmental samples when antibody-based methods are used, which may cross-react with other gregarine stages. This, again, highlights that correct classifications of parasitic protozoa are not merely



Subfamily Sarcocystinae

Phenotypic characters:
development in intermediate host by endopolygeny and endodyogeny,
two types of reproductive stages (metrocytes and bradyzoites) in tissue cyst,
gamogony in subepithelial intestinal tissues of definitive host,
sporogony endogenous

Subfamily Toxoplasmatinae

Phenotypic characters:
development in intermediate host by endodyogeny,
endopolygeny preceeding gamogony in definitive host,
gamogony in intestinal epithelium of definitive host,
sporogony exogenous

Fig. 5. Phylogenetic relationships of eimeriid coccidia inferred from 28S rRNA gene sequences. The trees were generated using (A) maximum-parsimony and (B) neighbour-joining analyses of a secondary-structure alignment of complete 28S rRNA gene sequences (Mugridge, 2002).

of scientific interest, but are of considerable importance for applied fields of parasitology and epidemiology.

7. Transfering from phenetic to phylogenetic classifications

Current controversies in coccidian taxonomy are not unique. Although apparently diverse, they are, arguably, one expression of a phenomenon that is occurring in many branches of parasitology (and other disciplines), in particular in helminthology. If this type of 'controversy' is in fact a more general phenomenon, then examination of the broader issues are likely to prove informative. Generalisations are invariably extremely dangerous, but looking back over 30 years, there has been a significant shift in attitudes towards classification of parasite taxa. Thirty years ago, phenetic classifications were the norm and in retrospect appear to have been remarkably stable. Phylogenetic classifications were viewed as something of an unattainable dream. Two changes have occurred in the intervening decades. Firstly, there has been a fairly general adoption of cladistic methods for phylogenetic analysis. The second change has been the increasing availability of molecular data. This change has probably been even more significant in the case of protozoa, for the purely practical reasons that detailed morphological and life-cycle data are intrinsically more difficult to elucidate in protozoa than in metazoa. The adoption of cladistic methods has been much more rapid by molecular workers than by morphological taxonomists, thereby accelerating the shift towards the realisation of the previously unattainable dream, a phylogenetic classification.

While these advances are entirely laudable, they inevitably lead to tensions in classifications. Probably more so than in parasitic metazoa, molecular data have resulted in massive upheavals in the way in which relationships between major taxa of the protozoa have been understood (Sogin and Silberman, 1998). It has always been accepted that classifications are hypotheses subject to continual revision, but in the era of phenetic classifications, the changes were probably less dramatic or less rapid. Now, new phylogenies appear almost on a weekly basis. While the generation of novel hypotheses is stimulating and exciting for those with a prime interest in the evolution of the parasitic protozoa, if each hypothesis was translated into a new classification, it could be a severe irritation to workers in applied fields who require some stability of nomenclature for their research. Nevertheless, classifications that reflect the phylogenetic relationships of parasitic protozoa as accurately as possible are important for both the scientific and medical communities, as highlighted in the examples described above (see Sections 1, 4.1, and 6). Thus, how can this conflict be solved?

7.1. Lessons from other parasites

The tension between existing, largely phenetic classifications, and novel phylogenetic classifications exists in other fields of parasitology and it is interesting to observe that while it may have proved to be 'controversial' in some parasitic groups, in others the tensions are being managed in a fashion which avoids overt controversy.

There appear to be several means of coping with such tensions. The most obvious approach is to use the time-honoured phenetic classification as the null hypothesis and demand that sufficient evidence be adduced to overturn the null hypothesis before the classification can be altered (Baverstock, 1987). A specific example would be in the cestodes, in which a phenetic classification (Khalil et al., 1994) which is manifestly inadequate from a phylogenetic perspective, is universally accepted and provides some stability while phylogenetic relationships are elucidated (Hoberg et al., 2000). The latter process is ongoing and is unlikely to be finalised in the near future. Presumably when some agreement is reached, there will be a consensual move to a new classification.

A second example would be the cyathostome nematode parasites of equids, in which a phenetic classification is universally accepted, to the level of species (Lichtenfels et al., 1998). Meanwhile, phylogenetic possibilities are being explored using molecular techniques (Hung et al., 2000; McDonnell et al., 2000), with the hope that the classification may be changed once the currently conflicting phylogenetic hypotheses are resolved. Again, it is hoped that the resulting classification will be achieved by consensus.

In these instances, a stable phenetic classification is retained as an interim hypothesis. The approach follows the paradigm utilised by Baverstock (1987) in dealing with conflicts between morphological and molecular data in which the conventional morphological hypothesis becomes the null hypothesis which must be overturned by convincing molecular data before it can be rejected. However, an important intermediary step is a phylogenetic classification based on phenotypic characters. Often phenetic classifications based on phenotypic characters are compared with molecular phylogenetic classifications. On occasions, a phylogenetic analysis of the morphological data will produce a tree congruent with the molecular data (Chilton et al., 1997). Phylogenetic analyses of morphological data are available for the cestodes as well as a 'total evidence' phylogeny based on a combination of morphological and molecular data (Hoberg et al., 2000). In the case of the equid nematodes, a similar process is underway (J.R. Lichtenfels, personal communication). For the eimeriid coccidia, it appears that various molecular hypotheses currently exist, but the seminal phylogenetic study of Barta (1989) based on morphological and life-cycle data has not been expanded to encompass all current genera (e.g. *Hammondia*). Consequently, there appear to have been only few attempts to combine molecular and phenotypic characters for phylogenetic analyses of apicomplexan protozoa (Mathew et al., 2000), currently a favoured approach with platyhelminth phylogeneticists (Littlewood and Bray, 2001).

7.2. The issue of stability of nomenclature versus the principle of priority

The second issue concerns nomenclature. If every new phylogenetic hypothesis was converted into a taxonomic system, instability of nomenclature may become entrenched. When phylogeneticists feel impelled to change the classification of a group to conform to the tree that they have generated, they do need to be cognisant of the provisions of the International Code of Zoological Nomenclature (Ride et al., 1999) for the stability of names. There is an important provision for the conservation of names based on usage. The Preamble to the fourth edition contains a significant opening paragraph (P.3) including the sentence that "the object of the Code is to provide stability and universality in the scientific names of animals", that "all its provisions and recommendations are subservient to these ends" but "none restricts the freedom of taxonomic thought or action". This would appear to be quite an enabling statement. However, in the Introduction to the third edition, David Ride wrote [p. xiv (4)] "Nomenclatural rules are tools that are designed to provide the maximum stability compatible with taxonomic freedom. Accordingly, they must also enable the Principle of Priority to be set aside in particular cases when the application of the principle would be destructive of stability or universality or would cause confusion" (Ride, 1985).

The current debate concerning the paraphyly of the genera *Sarcocystis*, *Isospora*, and *Hammondia* (see Sections 5.2 and 5.3), for example, has important ramifications not only for these genera, but also for the continued use of the generic names *Frenkelia*, *Toxoplasma*, and *Neospora*. Some phylogenetic studies suggest that, in order to render the genus *Sarcocystis* monophyletic, *Frenkelia* should be treated as a synonym of *Sarcocystis* (Votypka et al., 1998; Mugridge et al., 1999b, 2000). This proposition has caused little, if any, comments from workers in applied fields, and the recent inclusion of *Sarcocystis buteonis* (syn. *Frenkelia microti*) and *Sarcocystis glareoli* (syn. *Frenkelia glareoli*) in a list of 189 named species of *Sarcocystis* (Odening, 1998) has almost gone unnoticed. However, similar propositions to place the only five species in the genera *Toxoplasma*, *Hammondia*, and *Neospora* into one genus, with or without the mammalian *Isospora* species, have caused significant debate in the scientific and medical communities (Levine, 1977; Overdulve, 1978; Kreier and Baker, 1987; Kogut, 1990; Mehlhorn and Heydorn, 2000). Were this action to be undertaken, a submission could well be made for conservation of the names *Toxoplasma* and *Neospora* on the basis of their usage. Such a case would have to be considered very strong given the medical significance of toxoplasmosis and the veterinary significance of both toxoplasmosis and neosporosis. How do phylogeneticists avoid these problems? The first possibility has already been discussed and involves using the existing classification as the null hypothesis and having to find sufficient evidence to overturn it. The second very simple possibility is, if e.g. *Toxoplasma* truly lies within the genus *Isospora* from a phylogenetic perspective, to retain the name *Toxoplasma* as a subgeneric name, thereby retaining its use in applied studies. This stratagem, while perhaps at odds with the purity of phylogenetic ideals, accommodates very simply the necessity of, at the same time, providing a classification that has utility for workers in applied fields.

7.3. Characters values

How valuable are particular morphological or life-cycle characters in the protozoa? This seems to be a continuing cause of concern in the taxonomy of the eimeriid coccidia. However, it appears only rarely to have been addressed, as other parasitologists have tackled it, by developing a robust tree, molecular or phenotypic, and then mapping the characters in question onto the tree to test how reliable they are. Barta (1989) used the method successfully to show that heteroxenous life cycles in the coccidia have evolved independently, but it has not apparently been more widely employed to closely related questions such as that of obligate versus facultative heteroxeny (Frenkel and Dubey, 2000). This method, while still open to criticism, removes many of the subjective elements that are evident in controversies over the use of characters. Rather than the debate being about phenotypic as opposed to molecular characters, it becomes a question of testing the value of each character within a given tree and removing characters that consistently appear to be homoplasious. The reduced character set will then hopefully be quite robust. This procedure leads on to the use of 'total evidence' approaches which are currently in use amongst platyhelminthologists (Littlewood and Bray, 2001).

7.4. The importance of dialogue

The third and most important feature for achieving consensus is dialogue. Written contributions may inflame rather than illuminate contentious issues, whereas face to face discussions of a problem are more likely to lead to consensus and resolution. There are strong precedents for such fora to enable participants to discuss potentially controversial issues in taxonomy (e.g. the Workshops on Cestode Systematics held every 3–4 years; and Workshops on the Systematics of the Cyathostomes). The spirit of the Code of Zoological Nomenclature relies ultimately upon agreement and cooperation between scientists to maintain nomenclatural stability, rather than on coercion.

8. How can a new and more comprehensive classification of the eimeriid coccidia be devised?

Widely adopted classifications are neither forced upon scientists as a group nor are they ratified by formal consensus. Such classifications are usually sanctioned because a

majority of people who have considered the evidence in their favour have found it convincing. It is therefore the acquisition of convincing evidence that is the key to a stable classification, and if taxonomy is to reflect phylogeny, as is expected in most fields, then it is phylogenetic evidence that is the crucial evidence. In recent years, phylogenetic evidence provided by molecular characters has led many scientists to question traditional classifications and to propose changes that are often quite dramatic.

It is therefore instructive to consider the important criteria that have historically been shown need to be met before radical taxonomic changes are successfully made based on non-traditional data. There seem to be five significant criteria. These are not prescriptions for what must happen, but merely observations about what has happened in practice for the successful re-evaluation of traditional classifications, followed by their wide acceptance.

8.1. Criterion 1: phylogenetic reconstruction

If taxonomy is to reflect phylogeny then we need to reconstruct the phylogeny of the eimeriid coccidia under study. A cladogram from a single molecular sequence such as that of the 18S rRNA gene (see Section 5.2), represents only the phylogeny of that one gene, which is not necessarily the phylogeny of the coccidia. Thus, there needs to be concordance between phylogenies derived from several molecular sequences, preferably at least one of them nuclear and one organellar such as mitochondrial or plastid. Only concordance will be accepted as evidence of organismal phylogeny rather than gene phylogeny. Phylogenetic trees for morphological, life-cycle, and molecular characters have been produced for only a small subset of the known species of eimeriid coccidia (see Sections 3.2, 5.4, and 7). Not all of the phylogenetic trees generated to date, inadequate as they are, show concordance, and the conflicts are presumably the result of different gene histories or the lack of intermediate taxa.

8.2. Criterion 2: re-interpretation of phenotypic characters

The homologies previously proposed for any contradictory phenotypic characters need to be capable of re-interpretation so that they agree with the new molecular phylogeny (see Sections 3.1 and 5.2). Any such re-interpretation might involve the realisation that some characters are functionally or developmentally correlated, rather than being independently informative phylogenetically. The phenotypic data might also have been over-simplified by combining several independent characters into one complex character. Alternatively, the data set may have been incomplete or inaccurate. Failure of any re-interpretation, or disagreement about the re-interpretation, greatly weakens the evidence provided by gene sequences, as does conflict among molecular data sets.

8.3. Criterion 3: sampling of adequate taxa

Taxon sampling needs to be adequate in order to provide a convincing case for particular taxonomic boundaries. Showing that a taxonomic problem such as a paraphyletic group, exists is easy, even with a small sample size, but revealing the appropriate solution takes much more effort. Taxon sampling for the eimeriid coccidia is woeful for most molecular sequences (see Section 5.4), and a lot more pertinent data need to be collected before any reliable phylogenies, and thus stable classifications, are likely to emerge. There are now several thousand named species of eimeriid coccidia from which to choose exemplar taxa for phylogenetic analysis, but the ones chosen to date have usually been based on their veterinary or medical importance (see Sections 3.2 and 5.4).

8.4. Criterion 4: collation of pertinent molecular data

There needs to be a widespread base of people actively collating pertinent molecular data for the chosen gene sequences. Without this base, taxon sampling will be inadequate, in the sense that data will not be available for the critical exemplar taxa. This leads to uncertainties about taxon boundaries, and concordance of several gene sequences cannot be demonstrated. At the moment, the collection of data for the eimeriid coccidia can best be described as haphazard, which is unlikely to be of much practical value. In most cases the data have been collected from species that are readily available and can easily be grown, either in vitro or in vivo, and not with the needs of a phylogenetic analysis in mind. This means that the critical taxa, of which sufficient material for molecular analyses may be more difficult to obtain, have not necessarily been the focus of data collection, as they must be if taxonomic problems are to be resolved. However, fortuitously available data are unlikely to be of value for phylogenetic analysis, except by random chance.

8.5. Criterion 5: establishment of an informal working group on phylogeny and systematics of coccidia

Some relatively small informal group of people needs to take responsibility for the compilation and analysis of the necessary data, rather than continuing with often contradictory taxonomic proposals based on ad hoc analyses of fortuitously available data. A formal group could lead to the perception of autocracy, and examples of successful informal groups in the area of parasitology include those who are participating in the regular workshops on cestode and cyathostome systematics (see Section 7.4), as well as the Angiosperm Phylogeny Group (Angiosperm Phylogeny Group, 1998; Chase et al., 2000) and the Grass Phylogeny Working Group (2000, 2001), both of which are actively engaged in re-assessing the higher-level taxonomy of specific plant taxa. There is no formal membership of these groups, as they were created as collaborations among

those people active in the relevant areas and who were willing to be proactive in resolving the relevant taxonomic questions.

9. Conclusions

Overall, the Controversial Roundtable held at the joint meeting of the 8th International Coccidiosis Conference and the Annual Scientific Meeting of the Australian Society for Parasitology was an important forum for first discussions on how a new and widely accepted classification of the eimeriid coccidia, which takes into consideration both phenotypic and molecular characters, can be devised. The discussions during the Roundtable and thereafter revealed a diversity of concerns regarding the classification of eimeriid coccidia. Topics ranged from the need for a broad reconsideration of the useful characters for classification of these parasites to the need to obtain prompt and complete access to sequence submissions in international nucleotide sequence databases such as GenBank, DDBJ, or EMBL. Individual concerns varied from how best to approach a major revaluation of the taxonomy of apicomplexan parasites at the subordinal level to how best to determine when a newly recognised parasite isolate is sufficiently distinct to warrant its classification as a new species. The conservative opinion purporting that far more parasites, as well as relevant information, both molecular and phenotypic, about each parasite, be collected before any taxonomic decisions and/or revisions be undertaken was in marked contrast to the opinion expressed by others that taxonomy is never perfect and should be dynamic.

In the course of the lively group discussion that took place, there emerged some consensus as to the collective needs and considerable commonality of purpose of the participants at this Roundtable. There was recognition of the need for a more clear and universal understanding by non-taxonomists of the rules and provisions of the International Code of Zoological Nomenclature, as it applies to parasitic protozoa. The importance of more extensive collection, careful description, and critical differentiation of eimeriid coccidia, as well as the necessity for the appropriate deposition of type specimens for future molecular and morphological comparisons, was acknowledged. Above all, there was a recognised need for further open discussion and the development of a consensus of opinion on which specific characters, both phenotypic and molecular, and approach, both philosophical and practical, are required to resolve some of the existing controversies regarding the classification of eimeriid coccidia.

A new classification of the eimeriid coccidia will require commitment and co-operation on the part of interested, informed coccidiologists. This should be undertaken via an inclusive process, which may require a series of workshops and discussions held in conjunction with well-attended international conferences. The workshops may be designed so as to focus on specific topics or general areas of interest relevant to the taxonomy and classification of eimeriid coccidia. Finally, there was agreement that these workshops should be well publicised to encourage broad participation and the information and outcome of each should be published in a timely fashion in an international journal.

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