

Advances in Parasitology Volume 60

Cover illustration: *Echinobothrium elegans* from the blue-spotted ribbontail ray (*Taeniura lymma*) in Australia, a 'classical' hypothesis of tapeworm evolution proposed by Prof. Emeritus L. Euzet in 1959, and the molecular sequence data that now represent the basis of contemporary phylogenetic investigation. The emergence of molecular systematics at the end of the twentieth century provided a new class of data with which to revisit hypotheses based on interpretations of morphology and life history. The result has been a mixture of corroboration, upheaval and considerable insight into the correspondence between genetic divergence and taxonomic circumscription.

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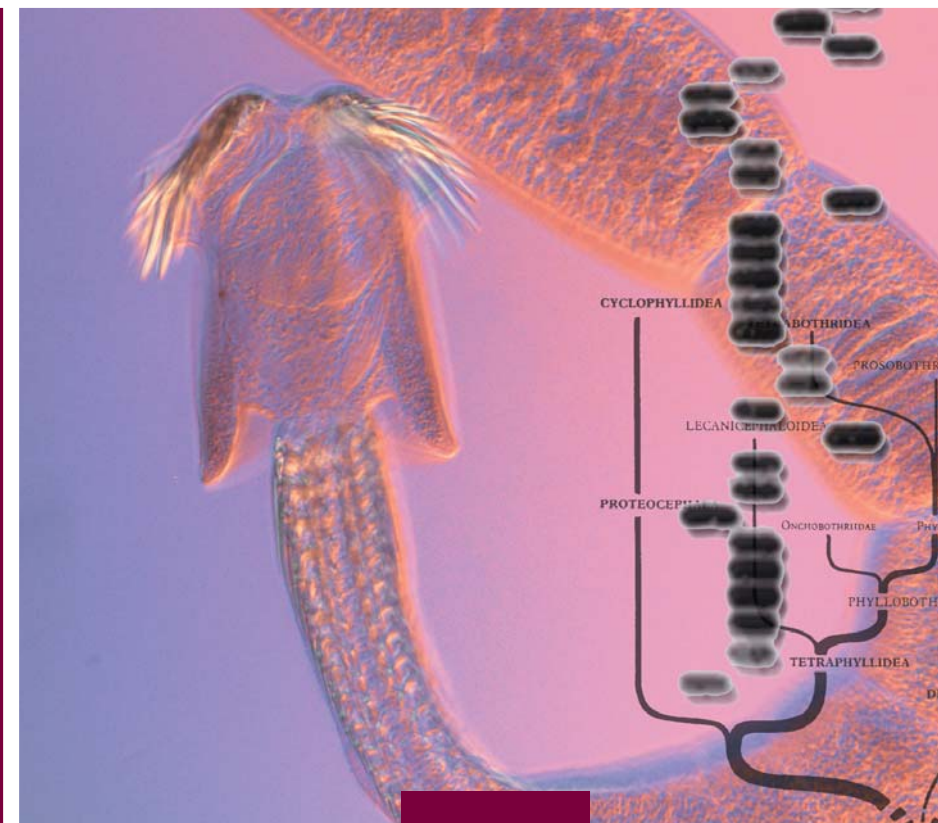
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Advances and Trends in the Molecular Systematics of the Parasitic Platyhelminthes

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ABSTRACT

The application of molecular systematics to the parasitic Platyhelminthes (Cestoda, Digenea and Monogenea) over the last decade has advanced our understanding of their interrelationships and evolution substantially. Here we review the current state of play and the early works that led to the molecular-based hypotheses that now predominate in the field; advances in their systematics, taxonomy, classification and phylogeny, as well as trends in species circumscription, molecular targets and analytical methods are discussed for each of the three major parasitic groups. A by-product of this effort has been an ever increasing number of parasitic flatworms characterized genetically, and the useful application of these data to the diagnosis of animal and human pathogens, and to the elucidation of life histories are presented. The final section considers future directions in the field, including taxon sampling, molecular targets of choice, and the current and future utility of mitochondrial and nuclear genomics in systematic study.

1. INTRODUCTION

From new developments in systematic theory and molecular biological techniques, notably the polymerase chain reaction, emerged the field of molecular phylogenetics and its effect has had an immediate and far-reaching impact on biology. Beyond simply coming to the fore of systematic biology, the field has facilitated the integration of comparative evolutionary thought throughout the sub-disciplines of biology, and our inferences are now richer for it. With regard to the Platyhelminthes, and particularly to the parasitic groups (the Neodermata), research over the past decade forms a significant body of

literature with the systematics of few major taxa remaining that have not been investigated at least preliminarily using molecular data. To some extent, however, studies have been concentrated at two extremes, aiming either to circumscribe major lineages and estimate their interrelationships, or to circumscribe species and strains, particularly in those of medical or economic importance. Fewer studies have addressed the systematics of intermediary taxonomic groups such as families and orders, although focus is increasingly moving in this direction.

This paper reviews the advances that have been made through molecular studies in our understanding of the systematics and evolution of the three obligately parasitic groups of flatworms: the Cestoda (tapeworms), Digenea (flukes) and Monogenea. In each of these taxa, new phylogenetic hypotheses, evolutionary scenarios and in some cases revised classifications have been proposed based on molecular data, mostly stemming from the ribosomal DNA (rDNA) genes. In attempting a comprehensive review of the literature that has led to these advances, we cannot give adequate attention to their many biological implications or the corresponding morphological arguments for or against the results based on molecules. The nature of the work and the many hypotheses that predate the field of molecular systematics make it equally difficult to avoid reference to morphology altogether, and at least some consideration of these issues is necessarily found in the text. In addition, it is not possible to provide comprehensive coverage of the many ways in which molecular data are being used for the diagnosis of medically important taxa, most studies of which do not bear directly on issues in systematics or evolution.

Ongoing technical advances in sequencing and associated laboratory procedures have led to an ever increasing expansion of available genetic data and ways in which such data may be applied to the study of systematics. The characterization and study of complete mitochondrial genomes, for example, are now readily possible and the characterization of nuclear genomes and their associated transcriptomes are being sought for a number of key platyhelminth taxa (e.g. *Schistosoma* and *Echinococcus*). The impact of genomic data has yet to make a tangible mark on the study of platyhelminth systematics, but may well introduce significant changes to the field in the near future. Work in

this area, as well as current practices in the analysis of molecular systematic data, are considered briefly in the final section.

1.1. DNA Taxonomy

Molecular data are being used not only to hypothesize ancestor–descendant relationships among living organisms in the pursuit of a comprehensive ‘tree of life’ (or perhaps ‘ring of life’; Rivera and Lake, 2004), but for the circumscription of our most basic of biological entities: species. Thus among the many species concepts that have been proposed and debated, typically with little or no direct influence over the practical, i.e. morphological, recognition of species, DNA-based taxonomy is quickly coming to the fore, and with it new criteria for delineating and testing species boundaries. A considerable number of the studies reviewed herein are based at least implicitly on such an approach, although the questions addressed and the results reported concern their contribution to the understanding of higher interrelationships, classification and related areas relevant to systematics broadly.

There is no doubt that species circumscription, as the foundation of practical systematic biology, has benefited immensely from the incorporation of molecular data. On the other hand, it could also be seen to have been made more complex because of the effectively continuous spectrum of variation available from the genome. Proponents of ‘DNA taxonomy’ argue for supremacy of their methods over traditional morphological species concepts citing reproducibility, efficiency and cost as being principal advantages (Tautz *et al.*, 2003), or as justified simply by the theoretical impossibility of describing all of Earth’s organisms using traditional methods (Blaxter and Floyd, 2003). In truth, however, both the theoretical and practical subjectivity of species boundaries remain as much (more so?) a problem for DNA-based taxonomy as they have been using traditional, morphologically based criteria, and this, along with many other factors (Lipscomb *et al.*, 2003; Seberg *et al.*, 2003), would suggest that a purely DNA-based approach to species circumscription makes sense only in the absence of other data (see also Spakulová, 2002, for a review of species-level issues and concepts in helminthology).

Whatever the long-term future and practice of DNA taxonomy may be, we already see that the combined application of morphological and molecular methods has become commonplace in the description of new species and will come to significantly improve our understanding of both genetic and morphological variability at the species boundary. Practical and theoretical considerations of the approach and its application to the parasitic platyhelminthes are not considered further here; a companion paper by Nolan and Cribb (this volume) provides an evaluation of these issues as applied to the Digenea.

1.2. The Platyhelminthes as Lophotrochozoan Bilaterians

Traditional views on the position of the Platyhelminthes within the animal kingdom held that they were a basal, 'primitive group' exemplifying the first appearance of bilateral symmetry among the triploblastic animals (e.g. Hyman, 1951). Results stemming from the analysis of 18S rDNA quickly overturned this and other deeply entrenched ideas in animal evolution (e.g. Halanych *et al.*, 1995; Aguinaldo *et al.*, 1997; Carranza *et al.*, 1997), and such a pivotal position in the evolution of the Bilateria is now postulated for the acoelomorph flatworms alone (Ruiz-Trillo *et al.*, 1999). The uniqueness of the acoelomorph flatworms is supported by multiple lines of evidence including both ribosomal and protein-coding genes (Ruiz-Trillo *et al.*, 1999, 2002; Jondelius *et al.*, 2002; Giribet, 2003; Telford *et al.*, 2003), Hox gene signatures (Cook *et al.*, 2004), and unique mitochondrial codon usage (Telford *et al.*, 2000). The revised Platyhelminthes (minus the acoelomorphs) in turn appears to be more derived and is grouped within the Lophotrochozoa, one of the three main branches of the Bilateria as inferred from 18S rDNA (Aguinaldo *et al.*, 1997) and other genes (e.g. Eernisse and Peterson, 2004; see Figure 1A). Although the interrelationships of the Lophotrochozoa remain contentious, the circumscription of a monophyletic Platyhelminthes and its new position in the animal kingdom are significant advances toward a better understanding of the origins and diversification of the phylum.

syncytial tegument. However, the interrelationships of the Neodermata have been and remain unsettled by the application of molecular data. Traditionally held views derived from Janicki (1920) and Bychowsky (1937) proposed that the presence of a ‘cercomer’ united the Monogenea with the Cestoda into the ‘Cercomeromorphae’, to the exclusion of the Trematoda. While this scenario has been supported by some molecular phylogenies based on 18S (e.g. Littlewood and Olson, 2001), others have suggested a closer relationship between the Cestoda and Digenea (e.g. Litvaitis and Rohde, 1999), including a recent study based on complete 18S and 28S rDNA (Figures 1B and C) that advocates rejecting the cercomer theory due to the questionable homology among such structures (Lockyer *et al.*, 2003a). The difficulty in resolving this basic three-taxon statement is further confounded by the likely paraphyly of the Monogenea (Mollaret *et al.*, 1997; Justine, 1998; Littlewood *et al.*, 1999b). Although supported by a number of morphological features (Boeger and Kritsky, 2001), the two main branches of the group, Monopisthocotylea and Polyopisthocotylea, show greater differences than similarities in their anatomy and biology (Justine, 1998; Euzet and Combes, 2003) and rate of sequence divergence (Olson and Littlewood, 2002). Indeed, it may be that the synapomorphies (shared *derived* characters) identified by Boeger and Kritsky (2001) are in fact symplesiomorphies (shared *ancestral* characters) simply retained in the more derived lineage of ‘monogeneans’ whose most recent ancestor is shared with either the Cestoda or Digenea (in which such features were lost). Thus other than support for the monophyly of the Neodermata itself, the interrelationships of the primary neodermatan lineages as estimated by molecular data remain controversial, precluding robust inferences regarding the condition of the ‘proto-typical’ parasitic flatworm.

1.4. Abbreviations

The following abbreviations are used throughout the text: 18S, small subunit rDNA; 28S, large subunit nuclear rDNA; bp(s), base pair(s); *cox1*, cytochrome *c* oxidase subunit 1; gDNA, total genomic DNA; ITS1/ITS2, internal transcribed spacers 1 or 2; mtDNA, mitochondrial

DNA; *nad1*, nicotinamide adenine dinucleotide dehydrogenase subunit 1; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

2. MOLECULAR SYSTEMATICS OF THE CESTODA

The Cestoda comprises more than 5000 described species (Georgiev, 2003) including the aetiological agents of hydatidiosis and cysticercosis (*Echinococcus* and *Taenia*, respectively). As adults, they are enteric parasites of all classes of vertebrates and utilize arthropods as first intermediate hosts. They have been recognized since the time of ancient Greece (Grove, 1990) and their diversity has been well documented in the last century; systematic treatments include Yamaguti (1959), Schmidt (1986) and most recently Khalil *et al.* (1994), who recognized 14 major lineages at the Linnean rank of order. Nevertheless, the application of molecular data has challenged ideas based on morphology and host associations and has led to fundamental changes in our understanding of cestode relationships and evolution. Early studies in the molecular systematics of the Cestoda were reviewed by Mariaux and Olson (2001) and we have minimized repetition of their review here.

2.1. Interrelationships of the Major Lineages of Cestodes

Prior to molecular systematics, scenarios presented to explain the evolution of the cestodes were almost entirely lacking in agreement. Disagreement stemmed not only from the general pattern of evolution, but in the homology and evolutionary importance of morphological structures, and in the number and taxonomic composition of the major lineages themselves. The absence of segmentation in groups such as the Caryophyllidea, for example, could be justifiably argued as evidence of their 'primitive' condition or as having become secondarily lost; morphology and life history traits are themselves insufficient to arbitrate between the two interpretations. Unique

features of groups such as the Nippotaeniidea or Haplobothriidea only help to establish their dissimilarity to other groups; characters unambiguously uniting two or more groups to the exclusion of others are few and far between. Works by [Mariaux \(1998\)](#) and [Olson and Caira \(1999\)](#) were early attempts to overcome the limitations of morphology and examine the composition and relative positions of the major lineages of cestodes as had been defined in [Khalil *et al.* \(1994\)](#). Their studies (see [Figure 2](#)) differed in the representation of higher (ordinal) taxa and in the gene regions analyzed, making them more complementary than compatible, but together provided the initial evaluations of the major questions in cestode evolution. While limited in taxonomic representation and support from the data, a number of important general conclusions were consistent: the monozoic (Caryophyllidea and Spathebothriidea) and difossate (Diphylloidea, Pseudophyllidea and Trypanorhyncha) orders are basal to a clade of tetrafossate (four-part scoleces) groups, with the orders Cyclophyllidea, Nippotaeniidea and Tetrabothriidea forming a derived clade within the tetrafossate clade ([Figure 2](#)). In addition, the orders Pseudophyllidea and Tetracyphloidea were found to be paraphyletic, with the Diphyllobothriidae independent of the remaining 'Pseudophyllidea', and the Tetracyphloidea forming multiple lineages within the tetrafossate clade. The relative positions of the difossate lineages, as well as those within the tetrafossate clade were inconsistent between studies ([Mariaux, 1998](#); [Olson and Caira, 1999](#)) and among gene regions and methods of analysis ([Olson and Caira, 1999](#)).

Thus a reasonably solid and morphologically independent foundation for the group was forged and available for further testing. [Kodedová *et al.* \(2000\)](#), for instance, expanded the 18S data of [Olson and Caira \(1999\)](#) with additional taxa representing the Caryophyllidea, Proteocephalidea and Pseudophyllidea ([Figure 2](#)). Considered only from the interrelationships of the orders themselves, the improved taxon representation of these groups had the unfortunate effect of reducing the resolution (using parsimony analysis), although a derived tetrafossate clade was still supported. Similarly, [Hoberg *et al.* \(2001\)](#) combined morphological data with a few additional sequences added to the work of [Mariaux \(1998\)](#) and added further support of the conclusions described above. The same year, [Olson](#)

well as greater insight into the problems of paraphyly, but also showed how labile the internal nodes of the tree were. For this reason, no formal revision of the classification was made, although recommendations were mooted by the authors. [Figure 2](#) shows the positions of the Caryophyllidea (open circles) and Spathebothriidea (closed circles) as estimated in some of the works discussed above. In most of these, their positions are basal with respect to the fully segmented 'strobilate' groups, implying that they are primarily monozoic (non-segmented). However, strong and consistent support for their positions has not been obtained, and more derived positions are also recovered, albeit with an equal lack of support.

Below the class level, the interrelationships of few higher taxa (e.g. orders, families) have been examined even preliminarily using molecular data. A notable exception is the Proteocephalidea for which a concerted effort has been made. [de Chambrier *et al.* \(2004\)](#), for example, presented a comprehensive treatment, expanding the earlier work of [Zehnder and Mariaux \(1999\)](#). Their work ([de Chambrier *et al.*, 2004](#)) combined morphology with partial 28S data for 75 taxa and showed that a number of genera, including the highly speciose *Proteocephalus*, were paraphyletic and such taxa were formally amended by the authors. Unexpectedly, their work also revealed that neotropical species, which show the greatest present day diversity, were derived and therefore the neotropics could not be the centre of origin for the group as had been postulated previously. These works have been expanded further by [Hypsa *et al.* \(2005\)](#) who added ITS2 and partial (V4) 18S data to the sequences analyzed by [de Chambrier *et al.* \(2004\)](#), and also examined the utility of secondary structural characters (of ITS2). Considerable agreement is found between the two studies and the additional data helped primarily to resolve the more derived parts of the tree.

[Olson *et al.* \(1999\)](#) made a preliminary examination of the lineages that make up the 'Tetraphyllidea', parasites of sharks and rays, revealing clades specific to their hosts groups (i.e. sharks, cownose rays and diamond rays). This and other works (i.e. [Olson and Caira, 1999](#); [Olson *et al.*, 2001](#)) show that the Rhinebothriinae (sensu [Euzet, 1994](#)) appears as the most basal of the 'tetraphyllidean' lineages, and a recent analysis by [Caira *et al.* \(in press\)](#) has shown that this is followed

by a lineage representing the Cathetocephalidae. Beyond that results are less consistent, albeit the phyllobothriid (non-hooked) lineages generally appear basal to onchobothriid (hooked) lineages, the latter of which also includes the monophyletic Proteocephalidea.

Future works on the constituent cestode groups similar to that on the Proteocephalidea are needed, especially to resolve relationships among the difossate lineages Diphyllidea, 'Pseudophyllidea' and Trypanorhyncha, and to subdivide the 'Tetraphyllidea' into monophyletic lineages. Although a few works have addressed interrelationships in the Cyclophyllidea (e.g. [Mariaux, 1998](#); [von Nickisch-Roseneck *et al.*, 1999](#); [Foronda *et al.*, 2004](#)), no attempt at a comprehensive estimate has been made. It is in this group particularly, being more speciose than all other cestode orders combined and containing the most important cestode pathogens of man, that a large-scale analysis is needed. Ongoing work by the present authors and by many of our colleagues in cestodology is currently underway and aims to provide more comprehensive studies of a number of orders including the Cyclophyllidea, Lecaniccephalidea, Pseudophyllidea, Tetraphyllidea and Trypanorhyncha. In turn, such studies will provide a wealth of new data for re-examining the interrelationships of the orders themselves.

2.2. Inter- and Intraspecific Variation in the Cestodes

The Cyclophyllidea, especially the Taeniidae, contains the vast majority of cestode species reported from man ([Ashford and Crewe, 2003](#)) and are thus widely studied at the inter- and intraspecific levels. For example, molecular data have allowed the recognition of a new Asian species of taeniid from Man, *Taenia asiatica*, which occurs sympatrically alongside *T. saginata* and *T. solium* ([Eom *et al.*, 2002](#)), and PCR methods for their differentiation have been recently developed ([Gonzalez *et al.*, 2004](#)). Moreover, [Yamasaki *et al.* \(2002\)](#), using mitochondrial genes and two techniques capable of detecting single-base changes, found that Asian *T. solium* could be readily differentiated from American/African isolates, suggesting the possibility of yet another distinct Asian lineage. Variation in *cox1* was examined in seven species of *Taenia* and two species of *Echinococcus*, including 10 isolates

of *T. taeniaeformis* and six isolates of *E. multilocularis*, by Okamoto *et al.* (1995). High interspecific variation was found among the taeniid species and isolates of *T. taeniaeformis*, but no variation was found among those of *E. multilocularis*, regardless of host or location.

Kamenetzky *et al.* (2000) developed a technique to extract usable gDNA from both fertile and non-fertile hydatid cysts of *Echinococcus granulosus* and found that cyst fertility was not correlated with strain, as both types recovered from the intermediate host proved to be of the same genotype. Similarly, analysis of *nad1* sequences of *E. granulosus* cysts from domestic and wild animals as well as humans in Poland, Ukraine and Slovakia (Kędra *et al.*, 1999, 2000b; Tkach *et al.*, 2002b) has demonstrated that fertile and infertile cysts of the pig strain of *E. granulosus* may be found in both animals and humans. However, Obwaller *et al.* (2004) showed high levels of intraspecific strain differentiation in *E. granulosus* using *cox1* and *nad1* that corresponded largely to their host associations (e.g. sheep, horse, cattle, pig, etc.). Comparison of complete mitochondrial genomes (see Section 6.4.1) similarly link strain variation to host association (Le *et al.*, 2002b). Previously Kędra *et al.* (2000a, 2001) showed genetic variability in *nad1* sequences of *E. multilocularis* and *T. hydatigena*, from different geographic regions.

Genetic variation in *Mesocestoides*, primarily parasitic in canids, was studied by Crosbie *et al.* (2000) among isolates from domestic dogs and coyotes and showed a number of distinct genetic signatures using 18S and ITS2. *Hymenolepis nana* isolates from man and rodents in Western Australia could be differentiated using COI, albeit not with the more conserved ITS1 or paramyosin genes (Macnish *et al.*, 2002b). Attempts to infect mice with human isolates were unsuccessful (Macnish *et al.*, 2002a), further supporting the presence of two independent strains or species.

Fewer reports have dealt with cyclophyllidean species of wild animals. Genetic variation in species of the genus *Rodentolepis* (Hymenolepididae) from the Pyrenean mountains of Spain and France was examined using allozyme electrophoresis and morphometrics. Casanova *et al.* (2001) were able to discriminate between *R. straminea* and *R. microstoma* in murid rodents, and Santalla *et al.* (2002) examined intraspecific variation in *R. asymmetrica* from several species of voles,

finding minor morphometric, but no genetic variation. Another pair of studies provides extensive analysis of genetic and morphometric variation, as well as biogeography in anoplocephalid cestodes of Holarctic collared lemmings (*Dicrostonyx*): [Haukisalmi et al. \(2001\)](#) uncovered cryptic species of *Paranoplocephala*, and [Wickström et al. \(2001\)](#) showed cospeciation of *Andrya arctica* within the Holarctic region, but not in the host split between Eurasia and North America. Using *cox1* data, evidence for multiple cryptic species of anoplocephalids was found in *Paranoplocephala omphalodes* in voles ([Haukisalmi et al., 2004](#)), *P. arctica* in lemmings ([Wickström et al., 2003](#)) and in the genus *Progamotaenia* in Australian macropodid marsupials ([Hu et al., 2005](#)).

Outside the Cyclophyllidea, only a few members of the Pseudophyllidea are known from man ([Ashford and Crewe, 2003](#)) and all belong to the family Diphyllbothiidae, the only pseudophyllidean family infecting homeothermic tetrapods and now thought to represent an independent evolutionary lineage ([Mariaux, 1998](#); [Olson and Caira, 1999](#); [Olson et al., 2001](#); see below and [Figure 2](#)). The diphyllbothriid *Spirometra* is the aetiological agent of sparganosis, the result of infection with the plerocercoid stage of these canid/felid adult worms. Its extensive host and geographic ranges make it likely to be a complex of species, and [Zhu et al. \(2002\)](#) used SSCP to detect single-base variation in the *cox1* gene in populations stemming from different second intermediate hosts. Somewhat surprisingly, only two haplotypes were detected, differentiating specimens from non-amphibian and amphibian hosts in Australia.

Like *Spirometra*, *Ligula intestinalis* is more commonly encountered in the larval stage. The global ubiquity of *Ligula* plerocercoids in freshwater fishes and observed differences in pathological reactions of fish species also suggest the possibility of a large species complex. To this end, [Olson et al. \(2002\)](#) used the entire ITS region and partial 28S to examine *Ligula* populations in two different sympatric fish hosts in Northern Ireland. Within-host populations were identical, whereas consistent between-host differences were found in all gene regions (except the 5.8S which was invariant), with the ITS-2 showing the most variation. The same rDNA gene regions and sequences were used by [Luo et al. \(2003a\)](#) to examine the possible synonymy of

Ligula with *Digramma*. Their comparisons showed that intergeneric divergences were only marginally larger than intra-generic (0.5–2% vs. 0–1.2%) divergences. Confounding their judgement, however, were parsimony results that supported separated clades for each genus. The same year, Li and Liao (2003) provided additional evidence for the synonymy of *Ligula* and *Digramma*, finding no variation in the 28S and COI genes, and only 0.7 and 7.4% variation in the quickly evolving ITS1 and *nad1* genes, respectively. Subsequently, Logan *et al.* (2004) examined the interrelationships of the diphyllbothriid genera *Digramma*, *Diphyllbothrium*, *Ligula* and *Schistocephalus* using ITS2. Their results further corroborated those above (i.e. Li and Liao, 2003; Luo *et al.*, 2003a), and provided additional evidence for the likely species complex represented by *L. intestinalis*. Thus the long-standing question of the validity of *Digramma* appears to be answered in the negative.

Other studies on pseudophyllideans have targeted species in commercially important freshwater and marine fishes. For example, following a series of studies examining genetic variation in *Proteocephalus* (Král'ová, 1996; Král'ová and Spakulová, 1996; Král'ová *et al.*, 1997), Král'ová *et al.* (2001) used direct sequence comparison and PCR–RFLP to differentiate the salmonid cestodes *Eubothrium crassum* and *E. salvelini*, and more recently extended this to *E. rugosum* (Král'ová-Hromadová *et al.*, 2003). Snábel *et al.* (2004) approached the same problem via allozyme variation, showing *E. crassum* and *E. salvelini* to be distinct entities restricted to their respective fish hosts. In all cases, these species could be readily diagnosed by RFLP analysis of the ITS region. Much finer variation was studied by Luo *et al.* (2003b) in the fish cestode *Bothriocephalus acheilognathi* using microsatellite markers. Their work represents one of the few population genetics studies on cestodes (Wickström *et al.*, 2001; Snábel *et al.*, 2004) and follows an earlier, phylogenetically based study of the same species collected from the basin area of the Yangtze River in China (Luo *et al.*, 2002). Using eight microsatellite markers, they showed that heterozygosity was best accounted for by host species, rather than location, and genetic variation was higher than might be expected among sub-populations from sympatric host species, taken together as evidence of cryptic speciation (Luo *et al.*,

2003b). More recently the genus *Bothriocephalus* was shown to be paraphyletic based on ITS2 and 18S without the inclusion of a number of additional genera such as *Anantrum* and *Clestobothrium* (Skeríková *et al.*, 2004). Moreover, karyological variation in number and morphology has been recently documented in *Bothriocephalus* spp. by Petkeviciute (2003).

In the Proteocephalidea, Zehnder and de Chambrier (2000) and Zehnder *et al.* (2000) provided examples of the combined morphological and molecular approach to taxonomy. Similarly, Skeríková *et al.* (2001) showed the monophyly of the European species of *Proteocephalus*, a cosmopolitan genus previously shown to be non-natural (i.e. non-monophyletic, Zehnder and Mariaux, 1999; de Chambrier *et al.*, 2004), but found no evidence for host–parasite co-evolution. Rosas-Valdez *et al.* (2004) examined the monophyly of the subfamily Corallobothrinae using 28S and showed it to be a non-natural division of the Proteocephalidae, although the genera *Corallobothrium*, *Corallotaenia* and *Megathylacoides* formed a ‘North American’ clade with strong support.

3. MOLECULAR SYSTEMATICS OF THE DIGENEA

Comprising ~18 000 nominal species, the Digenea is by far the most speciose of the three main groups of parasitic Platyhelminthes, and may well be the largest group of internal metazoan parasites of animals (Cribb *et al.*, 2001). Despite a vast literature on the group extending back well into the eighteenth century, the classification and phylogeny of the Digenea remained unstable and the interrelationships of numerous digenean taxa, at both higher and lower taxonomic levels, unclear. Thus the origin and evolution of the digeneans and their remarkable diversity of life cycles have long been subjects of inquiry. Basic problems such as identifying the most ‘primitive’ extant digenean lineage have elicited heated debates without resolution (for instance, Gibson, 1987; Brooks, 1989; Brooks *et al.*, 1989; Pearson, 1992; Brooks and McLennan, 1993). At lower taxonomic levels, differentiating species and genera has been problematic in many families, and thus the families themselves lack clear morphological

boundaries. Studying digenean life history is especially challenging due to their complex sequence of ontogenetic stages, hosts and niches, and data on the ontogeny of most digenean groups are fragmentary or lacking entirely (Yamaguti, 1975). Thus the adoption of molecular techniques was especially welcomed by researchers working on their systematics, evolution and ecology.

Unlike the Cestoda, where considerable effort has focused on resolving the interrelationships of higher-level taxa (i.e. orders), studies of digeneans have generally concentrated on lower taxa and only in recent years have more comprehensive works appeared. This is explained in part by the extraordinary diversity of the digeneans, and thus the significantly greater time and resources required to generate a molecular database sufficient for analysis of the class as a whole. Nevertheless, data are now available for the major groups and most significant families, enabling both diagnostic and systematic research in the group to advance more rapidly.

3.1. Non-Sequence-Based Works

Publications based on DNA sequence data represent the mainstream of modern molecular systematics and thus form the majority of works covered here. However, a substantial number of works dealing with species differentiation and systematics were published before PCR and sequencing became routine techniques. By far the most commonly used non-PCR-related technique is alloenzyme electrophoresis (e.g. Andrews and Chilton, 1999), although other methods, such as thin layer chromatography and DNA hybridization, have also been applied to digeneans. The vast majority of these works has been devoted to differentiating among populations or species in medically important taxa such as the schistosomes (Fletcher *et al.*, 1980; Write and Ross, 1980; Viyanant and Upatham, 1985; Walker *et al.*, 1985, 1986, 1989a, b; Woodruff *et al.*, 1985; Yong *et al.*, 1985; Bobek *et al.*, 1991; Kaukas *et al.*, 1994; Webster *et al.*, 2003), paragonimids (Agatsuma, 1981; Agatsuma and Suzuki, 1981; Agatsuma and Habe, 1986; Agatsuma *et al.*, 1988, 1992), fasciolids (Blair and McManus, 1989) and opisthorchiids (Pauly *et al.*, 2003). Studies have also been

carried out on members of the genus *Echinostoma* (Bailey and Fried, 1977; Voltz *et al.*, 1988), *Halipegus* (Goater *et al.*, 1990) and recently Vilas and co-authors published a series of articles devoted to population and species-level genetic variability in the hemiurid genus *Lecithochirium* (Vilas *et al.*, 2000, 2002a–c, 2003a, b, 2004a, b).

The RAPD technique has been used in several studies on schistosomatids and diplostomids (Barral *et al.*, 1993; Kaukas *et al.*, 1994; Laskowski, 1996; Mone *et al.*, 2003) and liver flukes (Morozova *et al.*, 2002; Semyenova *et al.*, 2003), while RFLP analysis has been applied to studies of species differentiation in the Didymozoidae (Anderson and Barker, 1993) and the introduction of *Schistosoma mansoni* to America (Després *et al.*, 1993). These approaches have been largely superseded by direct sequence analysis and thus their application to the systematics of flatworms has become increasingly rare.

3.2. Interrelationships of the Major Lineages of Digeneans

All publications devoted to the molecular phylogenetics of the Platyhelminthes have included at least some representative digenean taxa (Baverstock *et al.*, 1991; Blair, 1993b; Blair and Barker, 1993; Rohde *et al.*, 1993, 1995; Blair *et al.*, 1998; Campos *et al.*, 1998; Littlewood *et al.*, 1998a, b, 1999a, b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001; Lockyer *et al.*, 2003a), but specifically these works do not address the interrelationships of the Digenea, and in recent years the accumulation of sequence data has enabled more comprehensive studies of digenean phylogeny. The first such analysis was that of Cribb *et al.* (2001) based on complete 18S and including a total of 75 digenean taxa representing 55 families (plus five aspidogastreaan outgroup taxa). Unlike the cestodes, for which comparisons at this taxonomic level using 18S reveal excessive variation in specific variable regions (e.g. V4 and V7), 18S sequences of distantly related digeneans show far less variability. The result is that analysis of 18S typically produces exceedingly short internodes and low nodal support. Nevertheless, the basic patterns revealed by Cribb *et al.* (2001) have been subsequently corroborated. For example, the

Diplostomata *sensu* Olson *et al.* (2003a), uniting a clade including the blood groups Schistosomatidae, Sanguinicolidae and Spirorchidae with the Clinostomidae, together with the Brachyolaimoidea and Diplostomoidea, forms one of the primary lineages in a basal split in the Digenea (Figure 3). This rejected the traditional trichotomy implied by their classification into three large groups and showed definitively the polyphyletic nature of the traditional grouping 'Echinostomida' (whereas the composition of the Plagiorchiida and Strigeida were, with exceptions, largely supported by molecular data). Re-examination and coding of morphological characters were also done by Cribb *et al.* (2001) and showed greater congruence with the results from molecules than had previous such analyses (e.g. Brooks *et al.*, 1985; Pearson, 1992), albeit considerable differences were found. Tkach *et al.* (2000a, b) presented phylogenies of the suborder Plagiorchiata based on 28S and demonstrated its derived phylogenetic position in relation to all other major digenean lineages. It was also found that the Rencolidae belongs to the most derived clade of digeneans, along with the Eucotyliidae whose systematic position was previously enigmatic. Although members of both families are parasitic in the kidneys of birds, their general morphology is so distinct that no one had previously hypothesized their close affinity.

The most comprehensive phylogenetic analysis of the Digenea to date was based on a combination of complete 18S and partial 28S and included 163 members of 77 families representing all major groups (Olson *et al.*, 2003a; Figure 3). As discussed above, their work recognized two major clades, the Diplostomida, corresponding to the Strigeida *sensu lato*, and the Plagiorchiida, comprising the traditional Plagiorchiida along with the members of the 'Echinostomida', the latter of which was found to be a polyphyletic taxon. The Diplostomida includes only one nominal superfamily, whereas the Plagiorchiida now comprises 13 superfamilies of which the Xiphidiata is the largest and most derived and members of which share the presence of a penetrating stylet in their cercariae. Due to the basal dichotomy, no one group is seen to occupy the most basal position in the tree. However, at the base of the Plagiorchiida two lineages were found, each of which had been hypothesized previously as the progenitors of the Digenea: the Transversotrematidae and Bivesiculidae. However,



Figure 3 Hypothesis of digenean interrelationships and revised classification based on Bayesian inference of 18S and 28S data by Olson *et al.* (2003a). In contrast to traditional digenean classification, their scheme recognizes two main lineages, the Diplostomida and Plagiorchiida, as indicated on the tree. Expanded resolution of the Schistosomatoidea and the genus *Schistosoma* represents a consensus of compatible trees found in the publications shown, all of which analysed a subset of the same data. Taxa found to be paraphyletic indicated in bold type.

support for their placement was weak. Within the Diplostomida, the position of the Clinostomidae within the Schistosomatoidea (comprising the blood dwelling groups Sanguinicolidae, Schistosomatidae and Spirorchidae) seems surprising, although it confirms existing

views based on shared features of their larval morphology (e.g. the presence of a dorsal cercarial body fin-fold).

Cribb *et al.* (2003) re-evaluated the evolution of digenean life cycles in light of the hypothesis presented in Olson *et al.* (2003a) and suggested that the two-host life cycle of blood flukes was the result of secondary abbreviation, whereas the active penetration of miracidia into snail hosts and infection of definitive (fish) hosts by the passive feeding on cercariae are plesiomorphic behaviours. No evidence of strict co-evolution between digeneans and their mollusc hosts was found. With respect to vertebrate parasitism, the recognition of the Diplostomida (all of which are parasitic in tetrapods except the Sanguinicolidae) forced the authors to conclude that the digeneans acquired vertebrate definitive hosts three separate times in their evolutionary history.

3.3. Interrelationships of Genera and Families

Interestingly, molecular systematic studies aimed at these taxonomic levels tend to produce the most conclusive results; studies of higher taxa suffer from higher levels of homoplasy, whereas studies targeting inter- and intraspecific questions must deal with the subjectivity of delineating fine-scale genetic variation. Early publications were devoted to molecular variability among fasciolids and paragonimids (Blair, 1993a), and comparison of 18S sequences in gyliuchenids (Blair and Barker, 1993), leprocradiids and fellodistomids (Lumb *et al.*, 1993). Barker *et al.* (1993) used 18S sequences to test the phylogenetic hypothesis of Brooks *et al.* (1985) that *Heronimus* is the most basal extant digenean lineage. Although based on a very limited set of taxa, Barker *et al.* (1993) refuted this hypothesis and this result was corroborated a decade later by Olson *et al.* (2003a).

Anderson and Barker (1998) used ITS2 sequences to infer the phylogeny of members of the Didymozoidae, perhaps the most unusual and morphologically difficult group. Blair *et al.* (1998) studied phylogenetic relationships within the Hemiuroidea; a group with a complex taxonomic history and controversial views regarding its structure and content. No significant difference was found between

the phylogenies obtained based on a morphological matrix and analysis of sequences of the V4 domain of 18S (Neefs *et al.*, 1990), which showed the Hemiuroidea to be a monophyletic group comprising at least two primary lineages. Moreover, no support for the placement of the Azygiidae within the Hemiuroidea was found. Fernández *et al.* (1998a, b) published works on the molecular phylogeny of the families Campulidae and Nasitremitidae based on mtDNA and 18S sequences. In part, the molecular data supported the hypotheses that the Campulidae are phylogenetically closest to Acanthocolpidae and not to the Fasciolidae, indicating a host-switch from fish to mammals. Another host-switching event was hypothesized for the digenean parasites of marine mammals based on analysis of mitochondrial ND3 sequences of the genus *Lecithodesmus* (Fernández *et al.*, 2000). In this case the parasites were captured by one group of cetaceans from another.

Grabda-Kazubska *et al.* (1998) and Kostadinova *et al.* (2003) addressed the interrelationships of several echinostomatid taxa. In the latter paper, sequence data confirmed morphological identifications of specimens representing different stages of the life cycle and suggested that *Echinoparyphium aconiatum* probably does not belong to this genus. Both *nad1* and ITS data failed, however, to provide sufficient resolution of the species of *Echinostoma* used in the study. Analysis of the family Mesometridae by Jousson *et al.* (1998) revealed general trends in the evolution of this small group of digeneans parasitic in marine fish, such as the regression of the pharynx, a change in the body shape from elongated to subcircular, and the development of an accessory holdfast organ. Blair *et al.* (1999b) examined the paragonimid genera *Paragonimus*, *Euparagonimus* and *Pagumogonimus* using sequences of COI and ITS2, and showed that at least the type species of *Pagumogonimus*, *P. skrjabini*, clearly belongs to the genus *Paragonimus*, making the genus *Pagumogonimus* a junior synonym. In the same analysis, the position of *Euparagonimus cenocopiosus* was not stable and it appeared as either the sister taxon of *Paragonimus*, or was found within the latter genus (thus suggesting all three genera are synonymous). Hall *et al.* (1999) used the V4 region of the 18S to examine the family Fellodistomidae and showed that members of the group actually represent three separate families:

Fellodistomidae, Faustulidae and Tandanicolidae. Use of bivalve molluscs as intermediate hosts, which has been traditionally considered a robust synapomorphy for the members of Fellodistomidae, was instead considered by the authors as an indicator of multiple host-switching events within the radiation of the Digenea.

A series of molecular phylogenetic studies based on the partial 28S sequences by Tkach and co-authors was devoted to resolving the phylogenetic relationships and taxonomic status of a number of taxa traditionally circumscribed within the suborder Plagiorchiata. Tkach *et al.* (1999, 2001a) showed the close affinity of four genera (*Macrodero*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma*) from European snakes and their molecular results were corroborated by cercarial morphology and chaetotaxy, and the presence of an external seminal vesicle in adult worms. In subsequent publications, Tkach *et al.* (2001a, c, 2002a, 2003) studied the phylogenetic positions and interrelationships of the families Macroderoididae, Omphalometridae, Microphallidae, Lecithodendriidae, Prosthogonimidae and Pleurogenidae. Among other conclusions, it was shown that several genera (e.g. *Glypthelmins*, *Haplometra*) allocated to the Macroderoididae by previous authors, were not closely related and form independent lineages within the Plagiorchioidea. Alternatively, members of the Omphalometridae exhibited little sequence divergence despite the great differences in their size and body shape. Within the Microphalloidea, development of a seminal vesicle lying freely in parenchyma seems to have occurred independently at least twice. In turn, the Pleurogenidae has been found to be a family distinct from the Lecithodendriidae.

3.4. Inter- and Intraspecific Variation in the Digeneans

3.4.1. Systematics of *Schistosoma* and Related Blood Flukes

The Schistosomatidae is by far the best studied group of digeneans, and early studies on their molecular systematics have been summarized by Rollinson *et al.* (1997). Although the vast majority of effort in schistosome research concerns immunological, epidemiological

and other medical aspects of the species that infect human populations (i.e. *Schistosoma* species), significant advances in our understanding of the origins and diversification of these species and their close relatives in animal populations have been made in recent years (e.g. Combes *et al.*, 1992; Després *et al.*, 1992, 1993; Bowles *et al.*, 1995; Rollinson *et al.*, 1997; Snyder and Loker, 2000; Snyder *et al.*, 2001; Agatsuma, 2003; Lockyer *et al.*, 2003b; Morgan *et al.*, 2003; Snyder, 2004). Much of this work involves testing the validity of described species, and genetic analyses of isolates have also revealed new lineages. For example, ITS2 has been used to verify the species status of *S. hippopotami* (e.g. Després *et al.*, 1995), and genetic analysis of cercariae from its type locality (Lake Edward in western Uganda) has identified a third potential lineage in hippos (Morgan *et al.*, 2003). Population-level variability has been analysed in *S. japonicum* (Bowles *et al.*, 1993; van Herwerden *et al.*, 1998) and *S. mansoni* (Curtis *et al.*, 2002). In the latter work, microsatellite loci showed moderate genetic differentiation among *S. mansoni* populations within a single village in Brazil. Picard and Jousson (2001) characterized ITS sequences from cercariae causing swimmer's itch in Europe and although multiple sequences were found, some were identical to that of *Trichobilharzia regenti* recovered as adults from a mallard duck. Advances in the molecular systematics of European *Trichobilharzia* have been recently summarized by Dvorak *et al.* (2002) and Horak *et al.* (2002).

Snyder and Loker (2000) and Snyder *et al.* (2001) provided the first comprehensive phylogenetic assessment of the Schistosomatidae and were thus able to test its geographic origin, as well as the composition and interrelationships of genera and suprageneric lineages within the family. Their work first suggested an Asian origin of the genus *Schistosoma* and this was subsequently supported by Lockyer *et al.* (2003b; see Figure 3) through more extensive sampling, including 30 taxa and three genes (18S, 28S and COI), as well as morphological and biogeographic data. The avian schistosomes *Austroilharzia* and *Ornithobilharzia* were shown to form the sister lineages of the mammalian genus *Schistosoma*, whereas *Orientobilharzia* appears to be a junior synonym of *Schistosoma*. Other taxonomic changes included the synonymy of *T. ocellata* and *T. szidati*. Although a majority of

known schistosomatid taxa has been analyzed in the works above, the phylogenetic position of the only schistosome known from reptiles, *Gryphobilharzia* from Australian freshwater crocodiles, remains undetermined and has not been reported on since its original description by Platt *et al.* (1991). Recent collections (S. Snyder, pers. comm.) of specimens suitable for molecular analysis should mean that its phylogenetic position will be determined soon.

Snyder (2004) recently added 18S and partial 28S sequences of representatives of eight genera of spirorchiids from freshwater and marine turtles. His work confirmed the basal position of the spirorchiids in relation to the schistosomatids, corroborating Olson *et al.* (2003a) who had included only a single spirorchiid representative. The wide representation presented by Snyder (2004) showed that the Spirorchiidae was a paraphyletic taxon (Figure 3) that should be subdivided taxonomically. Spirorchiids of freshwater turtles were found to be basal to those of marine turtles, mirroring the evolution of their hosts for which the marine forms are thought to have evolved from freshwater ancestors.

3.4.2. Systematics of the Liver Flukes *Fasciola* and *Paragonimus*

Intraspecific variability and its use for species/population diagnostics in *Paragonimus* have been intensively studied using nuclear and mitochondrial markers (e.g. Agatsuma *et al.*, 1994b; Blair *et al.*, 1997a, b, 1999b; van Herwerden *et al.*, 1999, 2000; Iwagami *et al.*, 2000, 2003a, b; Agatsuma *et al.*, 2003; Cui *et al.*, 2003a; Park *et al.*, 2003) and early works were summarized in a paper by Blair *et al.* (1999b). Blair *et al.* (1997b) provided molecular evidence for the synonymy of three species of *Paragonimus*. Based on the comparison of ITS2 and COI sequences, they concluded that *P. iloktsuinensis* and *P. sadoensis* should be considered junior synonyms of *P. ohirai* despite differences in metacercarial morphology among these forms. Further works devoted to the molecular identification and differentiation among species of *Paragonimus* have been published by Ryu *et al.* (2000), Sugiyama *et al.* (2002) and Iwagami *et al.* (2003a, b). Several works dealing with the differentiation of nominal species of *Paragonimus* in

China and south-eastern Asia have been published (Cui *et al.*, 2003a, b; Chen *et al.*, 2004). Among other findings, it was concluded that *P. hokuoensis* and *P. szechuanensis* are genetically close to *P. skrjabini*, and the latter two species are most probably synonymous. van Herwerden *et al.* (1999) demonstrated that molecular markers should be used with caution, at least in some cases, as their study involving numerous clones of *P. westermani* and related species showed intra-individual variability among ITS1 sequences greater than that between individuals of the species complex(!) At the same time, sequence variation within individuals of *P. ohirai* was minimal. Similarly, van Herwerden *et al.* (2000) discovered at least two lineages of the mitochondrial *nad1* gene within individual worms that confounded the use of this gene for phylogenetic inference. Iwagami *et al.* (2000) summarized the state of molecular phylogeographic studies of *P. westermani* in Asia. Since then, several works on the subject have been published that address the possible geographic origin of triploid forms in *P. westermani* (Agatsuma *et al.*, 2003; Park *et al.*, 2003), variability among *P. skrjabini* from several provinces in China (Cui *et al.*, 2003a) and among *P. mexicanus* from Guatemala and Ecuador (Iwagami *et al.*, 2003a).

Due to the small number of species, fewer works have been devoted to the intrageneric systematics and phylogenetics of *Fasciola*. Adlard *et al.* (1993) and Agatsuma *et al.* (1994a) studied variability among populations, and Hashimoto *et al.* (1997) demonstrated that the Japanese species he examined should all be considered *F. gigantica*. Huang *et al.* (2004) compared ITS2 sequences from several samples of *Fasciola* in China and France. They were able to differentiate between *F. hepatica* and *F. gigantica* but found an intermediate genotype exhibiting sequences similar to both species and taken as evidence of heterogeneity in ITS2.

3.4.3. Systematics of Animal Flukes

Unlike the literature on species of medical and economic importance, molecular systematic literature on the majority of digeneans is less focused taxonomically and consists typically of isolated studies or

series of studies stemming from individual research groups. Thus, other than being united by resolving questions of taxonomic boundaries and patterns of evolution, there are few common themes to unite them. Among the first papers dealing with the molecular differentiation of digeneans of (non-human) animals was the work of [Luton *et al.* \(1992\)](#) on two species of *Dolichosaccus* in Australia. [Sorensen *et al.* \(1998\)](#) reported their results on the intraspecific variation in the ITS region of 37-collar-spined echinostomes from North America. [Jousson *et al.* \(2000\)](#) and [Jousson and Bartoli \(2001, 2002\)](#) investigated cryptic speciation in digeneans of the genera *Macvicaria*, *Monorchis* and *Cainocreadium* parasitic in marine fishes and found a pair of morphologically similar species in each case, prompting the description of a new species of *Monorchis*. In another investigation combining morphological, molecular and life cycle data, [Overstreet *et al.* \(2002\)](#) described a new species of *Bolbophorus* from the American white pelican and channel catfishes in the south-eastern United States. Their data set included sequences from a mixture of four nuclear and mitochondrial genes making their taxonomic conclusions well corroborated. [Levy *et al.* \(2002\)](#) and [Dzikowski \(2003\)](#) also addressed the problem of differentiating among *Bolbophorus* species, whose metacercariae cause significant losses to channel catfish farming in the Mississippi River delta. In these works, analysis of 18S as well as morphology were used to show that *B. damnificus*, *B. levantinus* and *B. confusus* were distinct species. [Platt and Tkach \(2003\)](#) used 18S, ITS and 28S in addition to morphological characters, to differentiate species of *Choanocotyle* from Australian turtles. Recently, [Dzikowski *et al.* \(2004b\)](#) used 18S and ITS to demonstrate the validity of two *Clinostomum* species, *C. marginatum* and *C. complanatum*, parasitic in aquatic birds in the Old and New Worlds, respectively.

[Bell and Sommerville \(2002\)](#) compared ITS and COI sequences between metacercariae of *Apatemon annuligerum* and *A. gracilis* originating from different localities and fish hosts. The authors concluded that the species should be synonymized due to the lack of genetic variability, finding only a short repeat motif in ITS1. [Bell *et al.* \(2001\)](#) and [Bell and Sommerville \(2002\)](#) reached the same conclusion regarding synonymy of two strigeid species, *Ichthyocotylurus erraticus* and *I. pileatus*. [Bray *et al.* \(1999\)](#) examined two fellodistomid genera

from deep sea fishes, *Lepidapedon* and *Steringophorus*, and despite inconsistency among trees obtained using different DNA fragments, as well as apparent host-switching in the evolutionary history of genus *Steringophorus*, were able to conclude that the genera have most likely radiated in the deeper waters off the continental shelf.

Galazzo *et al.* (2002) showed that *Diplostomum* species from North America and Europe form two clades, and that species identified as *D. baeri* on both continents most likely represent distinct species. Niewiadomska and Laskowski (2002) attempted to differentiate among six species of *Diplostomum* using ITS and morphology, but found inconsistency among the phylogenetic analyses based on molecules as well as the morphology of different life cycle stages. Given the widespread and economically important nature of the genus, there is a clear need for additional sampling and greater taxonomic resolution. Tkach *et al.* (2000b) examined morphological and molecular differences among three species of the genus *Plagiorchis* parasitic in European bats. Molecular and morphological characteristics show reliable differences between the recently described *Plagiorchis muelleri* and the type species of this large cosmopolitan genus, *P. vesperitilionis*, which has now been re-described and its neotype established.

Echinostoma and related genera have been traditional models in digenean studies due to their ubiquity and impact on our health and economy; 16 species of the family Echinostomidae have been reported from man (Ashford and Crewe, 2003). Nevertheless, the taxonomy of echinostomids remains unclear, especially among species belonging to the 37-collar-spine group. Morgan and Blair (1995, 1998a, b, 2000) have published a series of works on the interrelationships of *Echinostoma* species using sequences of several mitochondrial and nuclear DNA regions. They demonstrated that Australian species are more diverse than was previously thought, analysed the relative merits of various DNA regions for phylogenetic inference and differentiation, and provided a useful review on the molecular biology of the group.

The common frog lung flukes (family Haematoloecidae) have also been the subject of a number of recent studies as their cosmopolitan distribution, and parasitism of some of the best studied vertebrates (frogs) makes them an interesting and convenient model for studies in phylogenetics and historical biogeography. Leon-Regagnon *et al.*

(1999) used partial ITS and 28S to examine the taxonomy and interrelationships of Mexican species of *Haematoloechus*, and Snyder and Tkach (2001) demonstrated the presence of three distinct evolutionary lineages within the Holarctic *Haematoloechus* of North America and Europe. The latter results confirmed the taxonomic validity of *Haematoloechus abbreviatus* and demonstrated that the presence or absence of extracecal uterine loops is labile, and therefore a poor character for the differentiation of genera. Two of three clades revealed by phylogenetic analysis were comprised of both European and North American species, indicating that lineages of *Haematoloechus* arose before the breakup of Laurasia and radiated after Eurasia and North America split. Later, Leon-Regagnon and colleagues (2001, 2002, 2003) used molecular markers to support the differentiation of two new species, *H. meridionalis* and *H. danbrooksi*, and presented an updated molecular phylogeny of the genus including several additional North American and two African species. Their study generally supported the conclusions of Snyder and Tkach (2001) and further revealed that African *Haematoloechus* appears to have evolved after the separation of Gondwana and Laurasia.

Growing concern regarding amphibian population declines and correlation of some of these events with limb deformities caused by metacercariae of the psilostomid digenean *Ribeiroia* has provoked interest in the taxonomy and systematic position of the genus. In the taxonomic part of their review of *Ribeiroia*, Johnson *et al.* (2004) used sequences of ITS2 to compare isolates from multiple localities in the USA, Puerto Rico, Guadeloupe and Kenya. Their results suggest the genus comprises three species, one of which (*R. marini*) was represented by two subspecies.

Casey *et al.* (2003) explored differences among green- and brown-banded sporocysts within the genus *Leucochloridium* occurring in Europe. They showed that each sporocyst colour morph is species specific (*L. paradoxum* or *L. variae*) with no intraspecific differences across samples from different European countries. Criscione and Blouin (2004) chose three species of digeneans parasitic as adults (*Deropagus aspina*, *Plagioporus shawi*) or metacercariae (*Nanophyetus salmincola*) in salmonids in the north-western United States as model taxa to investigate the effect of life cycle patterns on the distribution

of genetic variation within and among populations. The authors demonstrated that species with entirely aquatic life cycles had more structured populations with lower gene flow than did species with both aquatic and terrestrial phases in their life cycles, owing presumably to the higher dispersion ability of the latter. In the course of the same study, they were able to differentiate a genetically distinct form of *Deropegus aspina* that probably represents a cryptic species.

4. MOLECULAR SYSTEMATICS OF THE MONOGENEA

The Monogenea is the smallest of the three parasitic groups, encompassing less than half the diversity of the Cestoda and roughly one tenth that of the Digenea based on the number of described genera (Caira and Littlewood, 2001), although estimates suggest they may be far more diverse than appreciated at present (Whittington, 1998). They are primarily ectoparasites of fishes and, with notable exceptions, only rarely produce significant pathological effects to their hosts. Human beings are not hosts of monogeneans. Characteristically, they are skin parasites that erode the epidermis using proteolytic enzymes (Monopisthocotylea), or have become specialized to feed on blood (Polyopisthocotylea). However, a fascinating array of endoparasitic species have colonized the various internal cavities of their hosts that open to the exterior (Kearn, 1994). Perhaps their most fundamental distinction from the other parasitic flatworms is their reproduction, exhibiting both direct life cycles and viviparity. The best-known genus, *Gyrodactylus*, is responsible for enormous economic loss to fish farming, particularly in northern Europe, and has been the subject of intense study; almost half of the reports dealing with the molecular systematics of monogeneans concern this genus solely. From this work has evolved an implicitly DNA-based system of taxonomy now widely employed to help discriminate among the hundreds of described congeners. Outside the family Gyrodactylidae, the taxonomic focus of research has reflected both the varied interests of the workers themselves, as well as the need to establish a molecularly based ground plan for the class and its two constituent groups.

Recent multi-authored taxonomic keys for the Cestoda (Khalil *et al.*, 1994) and Digenea (Gibson *et al.*, 2002) have helped to guide molecular phylogenetic investigations by providing up-to-date working classifications, and thus circumscriptions of higher taxa (e.g. orders, families and genera). This has allowed greater consistency by ensuring that authors using the same taxonomic names are indeed describing the same entities when phylogenetic hypotheses are tested, whereas studies preceding such keys did not have this advantage. Unfortunately, such a key is neither available nor underway for the Monogenea.

4.1. Non-Monophyly of the Monogenea

Molecular data have not generally supported the monophyly of the Monogenea. From the earliest reports to the present (Baverstock *et al.*, 1991; Blair, 1993b; Mollaret *et al.*, 1997; Campos *et al.*, 1998; Littlewood *et al.*, 1999b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001), two monophyletic lineages representing the Monopisthocotylea and Polyopisthocotylea were shown to have evolved independently, with one or the other lineage sharing a more recent common ancestor with either the cestodes or trematodes. Relative positions of the two lineages and their relationship to the cestodes and trematodes vary relative to the data analysed, yielding little consensus among data sets (Justine, 1998; Lockyer *et al.*, 2003a). A notable exception was found by Lockyer *et al.* (2003a) who showed support for monophyly using the combination of complete 18S and 28S rDNA (Figure 1). However, results from independent analyses of the two genes and from different methods of analysis showed inconsistencies in relation to their monophyly. In general, molecular evidence against recognition of the 'Monogenea' has been a consistent finding despite the lack of support for alternate positions of the two independent lineages.

Few authors had previously questioned the validity of the Monogenea and the traditional classification can no doubt still be found in any current textbook of parasitology. However, significant differences in the morphology and ultrastructure, behaviour, nutrition and ecology of the Monopisthocotylea and Polyopisthocotylea have been

mooted previously (Justine, 1991, 1998; Euzet and Combes, 2003) and it is likely that most authors have, as expressed by Euzet and Combes (2003), simply chosen to emphasize the similarities rather than the differences between the two groups. Indeed, the weight of available evidence strongly favours paraphyly of the ‘Monogenea’ and thus now, in our opinion, the burden of proof is on the side supporting monophyly. See Justine (1998) for an excellent account of the molecular (and other) studies leading to this conclusion, and Euzet and Combes (2003) for detailed and compelling arguments based on fundamental differences in their biology.

4.2. Interrelationships of the Monopisthocotylea and Polyopisthocotylea

Only a handful of studies to date have sought to resolve the broader interrelationships of the Monopisthocotylea or Polyopisthocotylea using molecular data (e.g. Mollaret *et al.*, 2000a; Jovelin and Justine, 2001; Olson and Littlewood, 2002; Simková *et al.*, 2003), although numerous studies have addressed selected groups within these lineages (see below). Initial studies (e.g. Mollaret *et al.*, 1997) relied on partial (~500 bp) 28S data and this trend was continued by Mollaret *et al.* (2000a) and Jovelin and Justine (2001), the latter of whom also examined COI data but determined the gene to be saturated for such levels of comparison. Olson and Littlewood (2002) consolidated and augmented the different fragments of available 28S data (domains D1, D2, or both), as well as complete 18S data, in an attempt to provide more comprehensive estimates (see Figure 4). Their work illustrated the highly fragmentary nature of the available data, necessitating analysis of subsets of the taxa that taken together represented roughly half of the described families *sensu* Boeger and Kritsky (2001). Consistent patterns have emerged from these data that also provide independent support for inferences made on the basis of morphology (i.e. Boeger and Kritsky, 1993, 1997, 2001). For example, the general pattern of evolution in the Polyopisthocotylea reveals a basal split between radiations in tetrapods (i.e. Polystomatidae) and in fishes (Littlewood *et al.*, 1998b; Mollaret *et al.*, 2000a; Olson and Littlewood,

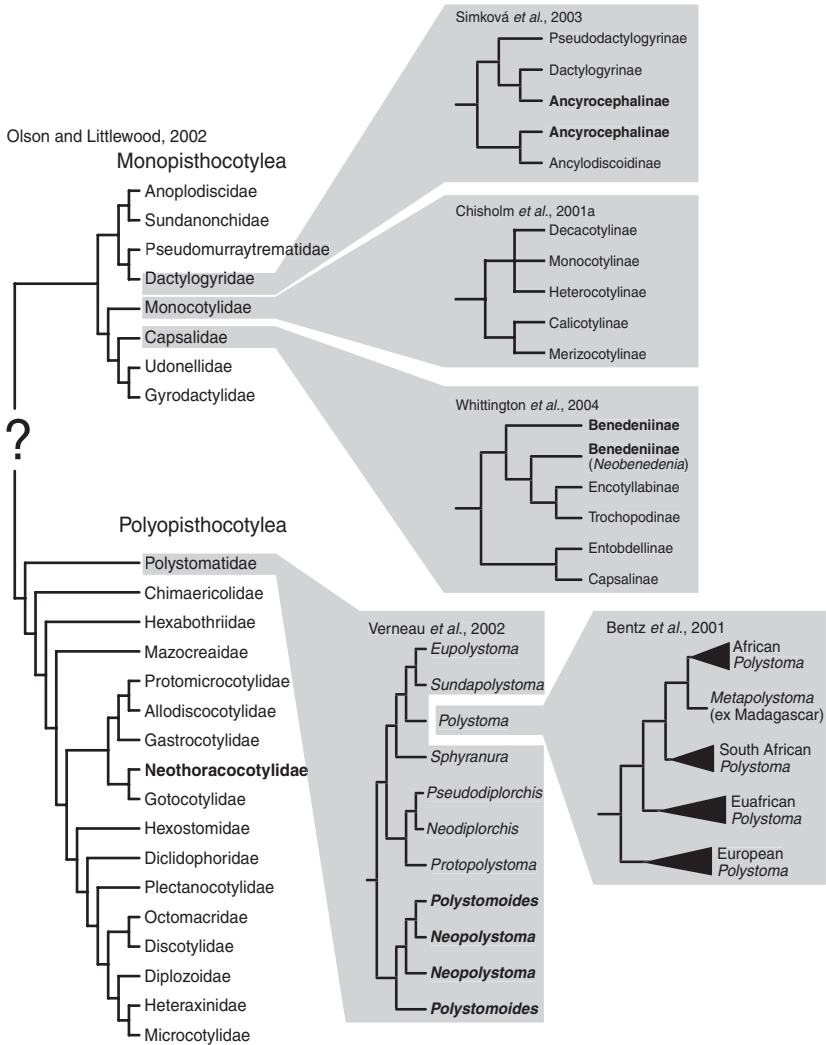


Figure 4 Phylogenetic hypotheses of the monopisthocotylean and polyopisthocotylean ‘monogeneans’ and a selection of available phylogenetic hypotheses for constituent groups. Taxa found to be paraphyletic indicated in bold type.

2002), with the latter clade showing a progressive series of lineages starting with polyopisthocotylean parasites of holocephalans (i.e. Chimaerocolidae), higher elasmobranchs (Hexabothriidae) and those of teleosts (Jovelin and Justine, 2001; see Figure 4).

Whereas the interrelationships of the Polyopisthocotylea may be described as largely pectinate (i.e. a hierarchy of nested clades), the Monopisthocotylea tends to show a bifurcating pattern (i.e. a series of splits). Thus, the Gyrocotylidae (plus *Udonella*, see below) is the sister group to the Capsalidae, which together form a sister group to the Monocotylidae, and these in turn form a sister group to the Dactylogyridae + Diplectanidae (Mollaret *et al.*, 2000a; Olson and Littlewood, 2002; Simková *et al.*, 2003). Beyond this, a more significant (and perhaps not entirely independent) difference is seen in the average divergence rate of the Monopisthocotylea, which is four times faster than that of the Polyopisthocotylea (as inferred from 18S rDNA; Olson and Littlewood, 2002). This increased divergence is also correlated with at least two cases of explosive radiation in the monopisthocotyleans, namely in the genera *Dactylogyrus* and *Gyrodactylus*. Such large differences in divergence rates can be problematic for parsimony and statistically based analyses alike and may be confounding our ability to resolve the 'Monogenea' as monophyletic using rDNA. However, given the many fundamental differences between the two groups, it is more likely a reflection of their independent evolutionary trajectories.

4.3. Systematics of Select Groups

A number of papers has used molecular data to examine the positions of poorly known or enigmatic taxa, some building on the comparative data resulting from the studies discussed in the preceding section and others necessarily generating data specific to the level of inference required. Mollaret *et al.* (2000b), for example, examined the positions of the genera *Sundanonchus*, *Thaparocleidus* and *Cichlidogyrus* and showed the validity of the Sundanonchidae. Justine *et al.* (2002) later added members of the Bothitrematidae and Neocalceostomatidae and found a strong association among *Bothitrema*, *Anoplodiscus* and *Sundanonchus*, each representing different families, whereas the position of the Neocalceostomatidae remained labile. Bentz *et al.* (2003) examined the position of *Euzetrema*, a monopisthocotylean genus unusually found in tetrapods, but found its position relative to the taxa parasitizing actinopterygian and chondrichthyan fishes equivocal

with regard to arbitrating between the possibilities of host capture or co-evolution. *Desdevises et al.* (2000) examined species-level genetic variation and host associations in the relatively large genus *Lamellogadus* and showed an interesting example of morphological variation in the absence of genetic variation. *Desdevises* (2001) later showed through molecular analysis that the monotypic genus *Furnestinia* was invalid and should be synonymized with *Lamellogadus*. *Matejusová et al.* (2001b) were able to differentiate genera and species of the unique Diplozoidae, but were subsequently unable to correlate differences in morphology in species of *Paradiplozoon* with variation in ITS2. Restriction digests of ITS2 were used subsequently to differentiate European species of diplozoids (*Matejusová et al.*, 2004).

Congruence between morphological and molecular estimates of phylogeny in the Monocotylidae (parasites of chondrichthyans; sharks, rays and chimaeras) was examined by *Chisholm et al.* (2001a, b; see Figure 4) whose revised classification (*Chisholm et al.*, 1995) was generally supported by partial 28S data. Within the family, comparison of *Calicotyle* species using 28S (*Chisholm et al.*, 2001c) suggested that species in the genus are not strictly host specific, although phylogenetic analysis showed that species clades reflected associations at least to the level of host genus. The commonly reported *C. kroyeri* was suggested to represent a convenient identity for members of the genus found in the cloaca of skates (*Raja* spp.), as divergences among species from different host species were in some cases greater than those among separate parasite species. Also using 28S, *Whittington et al.* (2004) provided a preliminary investigation of the interrelationships of the Capsalidae (see *Whittington*, 2004 for a recent review of the group) and showed that the genus *Neobenedenia* was closer to the *Encotyllabe* and *Trochopus* than to *Benedenia*, thus showing the Benedeniinae to be a paraphyletic subfamily (see Figure 4).

4.3.1. *Udonella* as a Monopisthocotylean Monogenean

An important question readily resolved through analysis of molecular data was the phylogenetic position of *Udonella*, a small genus of flatworm hyperparasites of caligid copepods that in turn parasitize

marine fishes. To some, the genus represented a potentially pivotal position in the evolution of the parasitic Platyhelminthes (e.g. the sister group to the Neodermata), whereas others considered it an aberrant monopisthocotylean whose phylogenetic position therefore had little bearing on the origin of parasitism in the phylum (Littlewood *et al.*, 1998b). Its position was first examined using rDNA by Littlewood *et al.* (1998b) who clearly showed its affinities to the Gyrocotylidae within the Monopisthocotylea, and this result has been supported by all subsequent analyses including members of both of these taxa (e.g. Olson and Littlewood, 2002; Simková *et al.*, 2003). Hypothesizing a position of *Udonella* outside the Monopisthocotylea (let alone outside the Neodermata, e.g. Zamparo *et al.*, 2001), in light of the data accumulated in recent years is simply unfounded.

4.3.2. Systematics of the Dactylogyridae

Simková *et al.* (2003, 2004) examined the position and speciation of the extraordinarily diverse Dactylogyridae. Their work (Simková *et al.*, 2003; Figure 4) showed that the group is recently derived within the Monopisthocotylea and tested the validity of the subdivisions of the family, rejecting the Ancyrocephalinae as paraphyletic. Subsequent work (Simková *et al.*, 2004) examined the intrageneric relationships of *Dactylogyrus*, suggesting that members of the Cyprininae were the original hosts and that the diversification of the genus was the result of sympatric speciation/separation on the gills. A very different situation regarding mode of speciation and niche specialization appears to be the case in the polystomes.

4.3.3. Systematics of the Polystomatidae

The Polystomatidae is the sole family of monogeneans to parasitize tetrapod hosts (primarily freshwater anurans and chelonians) and thus presents an interesting case in the evolution of the Polyopisthocotylea (Kearn, 1994), which has been addressed by a handful of molecular investigations. Verneau *et al.* (2002; see Figure 4) estimated

their interrelationships based on partial 18S using a large sampling of taxa and showed that the polystomes had strong affinities to their hosts groups, with those of chelonians forming a sister group to an anuran clade split between archaeobatrachian and neobatrachian hosts. According to the authors, molecular clock estimates of the nodes corresponded well with the divergence of the major host groups and suggested an origin of the Polystomatidae (*sensu stricto*) of ~353 mya. The position of the unique *Sphyramura* (a parasite of caudate amphibians) appeared embedded within the clade including anuran parasites, and thus did not support recognition of a separate family (i.e. Sphyranuridae). Their results agreed with the findings of Sinnappah *et al.* (2001) who postulated that *Sphyramura* was a neotenic genus restricted (partly) to neotenic hosts and in fact represented the pleisiomorphic condition of the group (a 'missing link'). The position of *Concinnocotyla*, a parasite of Australian lungfish, was also examined by Verneau *et al.* (2002) but it fell outside the group altogether. However, support for the positions of both of these genera has been weak and requires additional study. Within the speciose genus *Polystoma*, Bentz *et al.* (2001) showed a single colonization event of Africa from European stock (Figure 4). Embedded within the African clade of *Polystoma* species was the genus *Metapolystoma* from Madagascar, the validity of which was therefore rejected. Earlier, Littlewood *et al.* (1997) had studied speciation in *Polystoma* and related genera in order to test whether the species infecting different sites of the same host (e.g. oral cavity vs. urinary bladder, etc.) resulted from sympatric speciation. Analysis of 28S and COI strongly supported allopatric speciation and showed that site specificity was a stronger predictor of relatedness than was host specificity.

4.4. Systematics and Diagnostics in *Gyrodactylus*

Gyrodactylus is the most intensively studied group of monogeneans due to their economic importance in aquaculture, species richness and ubiquity. Members of the Gyrodactylidae, and particularly *Gyrodactylus*, create an enormous economic strain on fish farming in the UK, Scandinavia and elsewhere, and not surprisingly, much of the

work on gyrodactylids therefore involves differentiating pathogenic from non-pathogenic forms; a non-trivial task with over 400 described species in the genus (Harris *et al.*, 2004) and 50-fold more species predicted to be undescribed (Bakke *et al.*, 2002). Morphological diagnoses are based on subtle quantitative and qualitative differences of the sclerotized elements of the haptor, making it both difficult and potentially unreliable (but see the morphometric methods of Shinn *et al.*, 2001, 2004). Early efforts to characterize and differentiate gyrodactylids molecularly employed RFLP analysis of 5.8S and ITS sequences (Cunningham *et al.*, 1995b, c; Cunningham and Mo, 1997), although the 18S (Cunningham *et al.*, 1995a), the intergenic spacer region between ribosomal gene arrays (Collins and Cunningham, 2000), the 28S (Cunningham *et al.*, 2000; Matejusová and Cunningham, 2004), and more recently the *cox1* gene (Meinilä *et al.*, 2002, 2004) have also been investigated for their utility in discriminating among species of *Gyrodactylus*. Among these genes and gene regions, the ITS1–5.8S–ITS2 between the 18S and 28S genes has proven to be the target of choice for inter- and intraspecific diagnosis, particularly because of the number of comparative sequences now available (e.g. Cable *et al.*, 1999; Harris *et al.*, 1999; Harris and Cable, 2000; Zietara *et al.*, 2000; Bruno *et al.*, 2001; Matejusová *et al.*, 2001a, 2003; Huyse and Volckaert, 2002; Zietara *et al.*, 2002; Zietara and Lumme, 2002; Huyse *et al.*, 2003; Lindenstrøm *et al.*, 2003; Zietara and Lumme, 2003; Huyse and Malmberg, 2004; Meinilä *et al.*, 2004). Differences in the models of nucleotide substitution employed by various authors make genetic divergence estimates difficult to compare, but in general, variability in the region is highest in the ITS1, followed by ITS2 and 5.8S (the short 5.8S is often found to be invariant at the inter- and intra-specific levels, but has been shown to be useful for sub-generic diagnosis; Zietara *et al.*, 2002). In addition, minor geographic, as well as intra-specific variation has been shown through comparison of this region among disjunct populations (e.g. Matejusová *et al.*, 2001a).

To a certain extent, the reliance on DNA for species discrimination has meant that an implicit system of DNA taxonomy based on ITS data has become the standard for species circumscription of gyrodactylids (e.g. Harris and Cable, 2000; Bruno *et al.*, 2001; Cunningham

et al., 2001; Lindenstrøm *et al.*, 2003; Zietara and Lumme, 2003; Huyse and Malmberg, 2004; Huyse *et al.*, 2004). While not explicit in the use of 'DNA taxonomy', the necessity and justification for such a system in *Gyrodactylus* (Zietara and Lumme, 2003) is effectively the same as that proposed by researchers studying other groups of organisms (e.g. Tautz *et al.*, 2003). Using DNA, both morphologically distinguishable, as well as 'cryptic' (Huyse and Volckaert, 2002; Zietara and Lumme, 2002) species have been described and variation in the ITS region is frequently guiding the re-examination of morphology, rather than being used simply to confirm morphological differences previously observed.

Species circumscription based on DNA has been widely applied to the salmonid parasite *Gyrodactylus salaris*, which has been a known cause of mortality in Atlantic salmon (*Salmo salar*) in Norway since the 1970s and remains a significant economic problem to the region (Mo, 1994). Distinguishing the variants of *G. salaris* and the documentation of the pathological differences among these strains or species and the various fish species they infect has been studied intensively in a number of laboratories. Sterud *et al.* (2002), for example, documented significant pathological differences between *G. salaris* and the nominal species *G. thymalli*, and Lindenstrøm *et al.* (2003) showed host preference in controlled infection experiments between *G. salaris* and a laboratory variant founded from a single individual. In both cases, morphology of the variants fell within ranges exhibited by *G. salaris sensu* Malmberg, 1957, and genetic distances based on the ITS region failed to show marked differences. Indeed, attempts to discriminate *G. salaris* from *G. thymalli* based on a variety of methods and gene regions have shown insufficient variation to support the specific status of *G. thymalli* (Cunningham, 1997; Cunningham *et al.*, 2003; Hansen *et al.*, 2003; Meinilä *et al.*, 2004). Using *cox1* data, for example, Hansen *et al.* (2003) found 12 haplotypes among 76 specimens of *G. salaris* and *G. thymalli* from 32 host populations. Phylogenetic analysis of the haplotypes showed no support for reciprocal monophyly of the two 'species'. Thus, by the standards established by these and other monogenan researchers, the use of DNA taxonomy in the case of *G. salaris/thymalli* may result in a species definition that fails to reflect significant differences in host

response and pathology, a situation that is unlikely to be readily accepted by workers whose concerns involve the recognition of the highly pathogenic strains (e.g. Sterud *et al.*, 2002). It remains to be seen if more sensitive methods will yield support for the recognition of *G. thymalli*.

A number of recent reports have built on and contributed to the growing number of characterized gyrodactylid sequences in order to address large-scale patterns of speciation in the genus, as well as the mechanisms that explain these patterns and account for their enormous radiation. In populations of both *G. salaris* as well as other species of *Gyrodactylus* studied, host-switching appears to be the predominant mode of speciation (Zietara and Lumme, 2002; Huyse *et al.*, 2003; Meinilä *et al.*, 2004), which in turn may be driving further speciation events (Zietara *et al.*, 2002). Host-switching between families (Zietara *et al.*, 2002) as well orders (Huyse *et al.*, 2003) of fishes has been shown, as have instances of sympatric speciation, although speciation through allopatry is more commonly implied by the patterns observed (Huyse *et al.*, 2003; Meinilä *et al.*, 2004). New phylogenies have also allowed a reassessment of the systematics and nomenclature of the Gyrodactylidae. For example, the well-established sub-generic divisions of the group based on features of the excretory system (Malmberg, 1970) is rejected by both 5.8S (Zietara *et al.*, 2002; Huyse *et al.*, 2003) and ITS (Huyse *et al.*, 2003; Matejusová *et al.*, 2003) data, although such nomenclatural problems are hardly surprising in a species group of this size. Taxonomic issues aside, the studies above demonstrate that *Gyrodactylus* may be used as an interesting model system for studying speciation in animal systems generally.

5. BEYOND SYSTEMATICS: MOLECULAR DIAGNOSTICS

The complexity and variation of life history strategies in cestodes and digeneans remain among the most compelling and intriguing aspects of the biology of these parasites. Elucidating their life cycles was a cornerstone of the field for much of the early part of the twentieth

century, by which time the basic ontogenetic and host sequences had been worked out for most major groups. Since then, new information on life cycles diminished as parasitologists turned first to experimental, and later molecular questions and approaches. Today, it would be difficult to justify the labour and expense involved in a trial-and-error approach to life cycle elucidation using *in vivo* systems or *in vitro* cultivation (e.g. Chambers *et al.*, 2000). However, the accumulation of molecular data, or ‘barcodes’ (Besansky *et al.*, 2003; Hebert *et al.*, 2003; Moritz and Cicero, 2004), of adult sequences has enabled a direct and efficient means of identifying larval ontogenetic stages and thus inferring complete life cycles (see also McManus and Bowles, 1996 for an early review including other approaches to molecular diagnostics). Coupled with phylogenetic analysis, broader affinities may be discerned even in cases where exact matches are not attainable through direct sequence comparison. ‘Barcoding’ and its use in diagnostics is a new and somewhat contentious field, if only because it may be confounded in practice by issues of ‘DNA taxonomy’ (see Section 1.1, also Moritz and Cicero, 2004). Although its application to the parasitic Platyhelminthes is limited at present, in the coming years we anticipate the method to produce a rapid acceleration in studies of host associations and life cycles.

Precise identification of larval flatworm parasites is generally not possible on the basis of comparative morphology alone. At present, our knowledge of host utilization and specificity is restricted largely to the definitive host, and fully elucidated life cycles are exceptionally few in comparison to the number of adult species described. In the Cestoda, at the family level, and at the ordinal level in some groups, a single complete life cycle is unknown (Beveridge, 2001), and in the Digenea, perhaps half of the groups remain in a similar state of ignorance (Cribb *et al.*, 2003). Obviously, the utility of molecular diagnostics is directly proportional to the number of species for which sequence data have been characterized, and workers interested in diagnosing cestode or digenean parasites should concentrate on the 18S and 28S genes in order to maximize the number of species for which comparative sequences are currently available (e.g. Mariaux, 1998; Olson *et al.*, 1999, 2001, 2003a; Tkach *et al.*, 2001b; Lockyer *et al.*, 2003b; de Chambrier *et al.*, 2004). The extraordinary growth of

publicly available genetic repositories and linked databases dedicated to biodiversity (e.g. the Global Biodiversity Information Facility, www.gbif.org; Consortium for the Barcode of Life, barcoding.si.edu) will rapidly increase our understanding of helminth life history, host associations and trophic interactions by making identifications simple and efficient, and thus making large-scale biotic survey and inventory of both larval and adult helminths feasible for the first time.

5.1. Ecological Diagnostics and Life Cycle Studies

5.1.1. *Cestoda*

In the Cestoda, [Brickle *et al.* \(2001\)](#) identified larval tetraphyllidean parasites of an important commercial squid fishery using partial 28S sequences and found that they differed from the adult species infecting the local skate population, the only elasmobranchs endemic to the Falkland Islands. These data were used subsequently by [Agustí *et al.* \(2005\)](#) who examined the larval cestodes of dolphins in the Mediterranean. Like [Brickle *et al.* \(2001\)](#), they found larval worm sequences exceedingly similar to that of *Clistobothrium montaukensis*, a tetraphyllidean cestode of lamniform sharks, suggesting that members of this genus are widespread both in terms of geography and intermediate host range. [Dezfuli *et al.* \(2002\)](#) used partial sequencing of 18S to match procercooids with adults of the spathebothriid *Cyathocephalus truncatus*, and to discriminate between these procercooids and cysticercooids of *Microsomacanthus pachycephala* (Hymenolepididae) in co-infected amphipods (*Echinogammarus stammeri*) in Northern Italy. They stressed the importance of using such data where co-infection of hosts may confound the identities (albeit larval discrimination between caryophyllidean and cyclophyllidean cestodes could be readily done on the basis of morphology). [Reyda and Olson \(2003\)](#) used partial 28S rDNA data to verify the identity of proteocephalidean tapeworms encysted within the parenchyma of adult tetraphyllidean worms in freshwater rays in Peru. Although a handful of similar reports of cestode–cestode hyperparasitism in South America had been published previously, molecular

data provided the first independent evidence that proteocephali-dean 'hyperparasites' could infect other groups of cestodes. Moreover, thanks to a large reference database stemming from previous works on the phylogeny of the Proteocephalidea (Zehnder and Mariaux, 1999), a far more precise identification of the cysts was made than would have been possible based on their morphology alone.

5.1.2. Digenea

The complexity of digenean life cycles makes their elucidation via molecular diagnostics of even greater utility than for the cestodes, and the earliest and most abundant of such works have been in the Digenea. Life cycles in the digeneans of marine animals have been especially difficult to elucidate due to the expense and difficulties of the animal husbandry required. With the accumulation of sequence data on adult forms, identification of cercariae found in snails and metacercariae from second intermediate or paratenic hosts is now as easy for marine species as it is for freshwater or terrestrial (save perhaps the collection of specimens themselves). For example, Cribb *et al.* (1998) demonstrated a three-host life cycle in the unusual Bivesiculidae, and Anderson (1999) used DNA to identify metacercariae of *Indodidymozoon pearsoni*, a member of the Didymozoidae. Schulenburg and Wägele (1998) examined digenean metacercaria in the isopod *Cyathura carinata* using ITS1 and 18S, but were unable to identify these precisely due to a lack of available comparative data at the time. Jousson *et al.* (1998, 1999), Jousson and Bartoli (2000), Bartoli *et al.* (2000) and Bartoli and Jousson (2003) demonstrated the life cycles of a number of marine digeneans belonging to the families Monorchidae and Opecoeloidae. Mone *et al.* (2003) detected larval stages of *S. mansoni* for the first time in Oman using RAPD. Recently, Hertel *et al.* (2003, 2004) used tandemly repeated DNA sequences characterized in *Echinostoma* and *Schistosoma* in order to differentiate among congeneric species. Similarly, Hust *et al.* (2004) compared ITS region sequences of cercariae belonging to two microphallid species (*Maritrema subdolum* and an undetermined species)

and designed species-specific primers enabling their reliable differentiation. Dzikowski *et al.* (2004a) identified different life cycle stages of several species belonging to the family Heterophyidae and outlined their phylogenetic position using newly obtained and previously published sequences of 18S. Their work resolved the life cycle of a widely distributed heterophyid, *Pygidiopsis genata*. The works on the molecular and morphological differentiation among species of *Bolbophorus* discussed previously (i.e. Levy *et al.*, 2002; Overstreet *et al.*, 2002; Dzikowski *et al.*, 2003), also included comparative analysis of DNA sequences obtained from both adult and larval stages of the parasites. Galazzo *et al.* (2002) identified metacercarial stages of the genus *Diplostomum*, a genus most commonly encountered as metacercariae, and as a result, rarely identified to species. Donald *et al.* (2004) obtained ITS2 sequences from digenean larvae recovered from topshells (Mollusca: Trochidae) and found that they represented a mixture of species whose taxonomic positions could not be discerned due to a lack of comparative data. Fortunately, such reports can be readily re-investigated once the appropriate data become available.

5.2. Clinical Diagnostics

In clinical situations, identification of helminths may be problematic despite recognition of disease agents and the numerous methods that continue to be developed for diagnosing species of medical and economic importance (see review by Ito, 2002). Under some circumstances, conditions may be present that result in aberrant parasite development and/or unrecognized pathology, and that create opportunity for rare or unknown zoonotic diseases to establish. Such was the case in an early report of a ‘possible mutated sparganum’ (Conner *et al.*, 1976) in a Hodgkin’s patient being treated with a regime of immunosuppressive drugs. A more recent case involving an AIDS patient (Santamaría-Frías *et al.*, 1996) was similarly difficult to diagnosis due to a metastatic disease process that showed no morphological indication of having resulted from a metazoan parasite. Both cases were subsequently re-investigated using molecular sequencing techniques (Olson *et al.*, 2003b), demonstrating that the common, and

generally benign human dwarf tapeworm, *H. nana*, was capable of producing lethal pathological consequences in the case of the AIDS patient, and that the Hodgkin's patient's disease was the result not of an infection with a sparganum, but with a cyclophyllidean cestode *not* belonging to one of the well-known genera previously reported from man. These applications show the immediate utility of genetically characterized animal helminths, and the need for such data in identifying the aetiological agents of emerging diseases. We necessarily spend the greatest resources on those species known to be a threat to our health and economy, but it is difficult to predict which parasite species may become the cause of zoonotic disease when conditions for such infections become favourable. Thus, the broader our knowledge and characterization of helminth biodiversity, the more likely we will be to identify the agents of opportunistic, rare and emerging disease.

6. FUTURE DIRECTIONS

6.1. Taxonomic Considerations

Early molecular phylogenetic studies of the parasitic flatworms, as in other taxa, have tended to address questions from the bottom up; that is, from the basal divergences among major lineages to subsequent investigations of divergences in more restricted and recent clades. The result is that we have a phylogenetic framework, based on one or more genes, for the phylum, the Neodermata, and for each of the major neodermatan groups. While these hypotheses require further corroboration, effort is increasingly needed to fill out the branches of the trees by providing significantly greater representation of the diversity encompassed by the constituent groups. In the Cestoda, only the order Proteocephalidea has received such comprehensive treatment (e.g. Zehnder and Mariaux, 1999; de Chambrier *et al.*, 2004), although molecular phylogenetic investigations of other orders are presently underway. Priority areas must be seen to include the Cyclophyllidea, which not only contains the most important cestode pathogens of man, but also a greater diversity than all other cestode orders combined. Moreover, the diversity of life cycles

(including the transition to terrestrial cycles), host associations and biogeographic patterns provide a large number of interesting questions yet to be addressed in a molecular phylogenetic context. Other priority areas include the circumscription of a monophyletic Tetraphyllidea, resolving interrelationships among difossate groups, and corroborating the positions of basal taxa.

Although the recent analysis by Olson *et al.* (2003a) included representatives of 77 digenean families, a large number of omissions remain; according to Gibson *et al.* (2002) the Digenea comprises 140 families. Among unattended taxa there are many smaller families with unknown phylogenetic affinities, including the Mesotretidae, Rhytidodidae and Urotrematidae, as well as families crucial to the full elucidation of digenean evolution such as the Allocreadiidae, Gymnophallidae, Liolopidae, Paramphistomidae and Ptychogonimidae. The basal clade formed by the superfamilies Brachylaimoidea, Diplostomoidea and Schistosomatoidea merits further support in order to verify existing hypotheses on the evolution of their life cycles. Also needed in future investigations are detailed studies of the internal phylogenies of families, clarification of their taxonomic content, and the allocation of numerous genera with presently unclear affinities. The Aspidogastrea (see Figure 1) is routinely confirmed as the sister group to the Digenea, but has otherwise yet to be examined in detail with molecular tools. Although small in number, they are equal in age to the Digenea and have left an interesting suite of host associations that should be investigated in a molecular phylogenetic context.

Work on monogeneans remains dominated by studies on *Gyrodactylus*, and unusually, the broader interrelationships of the group have been arguably studied more intensively with morphology than molecules (e.g. Boeger and Kritsky, 1993, 1997, 2001). While a number of groups outside of the Gyrodactylidae have also been studied in some detail (see Section 4.3), few comprehensive treatments have been published and the work of Olson and Littlewood (2002) illustrates the fragmented nature of the available molecular data for the group as a whole. A more coordinated effort within the monogenean research community would help to establish the phylogenetic groundplan of the group(s) and thus link together the more restricted clades (e.g. monocotylids, polystomatids, etc.) in which interrelationships have

been studied. Equally important is firmly establishing the relative positions of the monopisthocotylean and polyopisthocotylean lineages relative to the cestode and digenean clades, a problem that has yet to yield to analyses of rDNA. It also remains to be seen to what extent a formal DNA-based taxonomy will be adopted in the genus *Gyrodactylus* and whether or not such criteria can be extended to other groups of monogeneans.

6.2. Analytical Considerations

Ancient divergences, rapid diversification and convergent evolution are all potential sources of error that confound phylogenetic estimation. Being cumulative, these effects become more pronounced with the age of divergence. Thus, future studies, particularly at higher taxonomic levels, should aim not only to increase the number of taxa and gene loci, but to become increasingly mindful that multiple substitutions in the sequence data must be compensated for. Statistical methods in phylogenetic reconstruction such as maximum likelihood and Bayesian inference necessarily incorporate various models of nucleotide or amino acid substitution and thereby allow for more realistic treatment of the data (Huelsenbeck *et al.*, 2001; Holder and Lewis, 2003). Fortunately, Bayesian analysis has proven computationally feasible even with large datasets (e.g. Olson *et al.*, 2003a). Statistical methods are also available for choosing an appropriate model based on the data to be analysed (Posada and Crandall, 1998; Posada and Buckley, 2004), with the selection criterion being the minimization of the number of estimated parameters. Incorporating complex substitution cost matrices in parsimony analysis is significantly more difficult, whereas unweighted parsimony makes no attempt to account for multiple substitutions and its greatest utility is in providing a least-change estimation. Given advances in statistical methods, distance analyses (e.g. neighbour joining, minimum evolution), despite the ability to incorporate substitution models into pair wise distance estimates, are phenetic and should be avoided when the estimation of phylogeny, rather than overall genetic similarity, is sought. The development and refinement of analytical techniques in molecular

systematics continues rapidly and reviews on current practices abound (e.g. Lewis, 2001; Whelan *et al.*, 2001; Holder and Lewis, 2003).

6.3. Molecular Targets

Ribosomal data have provided the basis for molecular phylogenetic investigations of animals, protists and prokaryotes since some of the earliest works in the field (botanists have favoured the *rbcl* (rubisco large subunit) gene from the chloroplast genome); the current basis of animal interrelationships rests largely on 18S data alone (e.g. Aguinaldo *et al.*, 1997). As in other taxonomic groups and as this review shows, 18S, and later 28S, have been widely employed in studies of the parasitic platyhelminths and now provide the most diverse reference database of molecular characters. The ITS regions have been used widely as well in cases requiring high levels of variation, albeit the highly variable regions of the 18S and 28S genes may be equally suited to such analyses. The mitochondrial genes tend to be faster evolving than those of the nuclear genome (Simon *et al.*, 1994) and, with exceptions, are generally not useful for studies of the parasitic platyhelminths due to the presumed antiquity of the group. Reliance on ribosomal and mitochondrial genes in light of questions left unresolved has led eventually to a search for additional nuclear protein-coding markers and a corresponding weariness of the utility of rDNA. However, comparative analysis of additional such genes has failed to produce alternative markers with wide utility, and thus the rDNA genes have, for better and for worse, continued to be the most popular markers for making phylogenetic inferences at a variety of taxonomic levels. New markers, and perhaps new classes of data, will surely come to light as the emerging field of genomics matures.

6.4. Genomics

6.4.1. Mitochondrial Genomes

Technical advances in PCR, cloning and automated sequencing technology have made large-scale sequencing projects feasible even in

modestly sized and funded laboratories. No complete genome of a platyhelminth has been characterized to date, but a multi-national effort has been underway for some time that will make *S. mansoni* the first flatworm for which this is achieved (see http://www.nhm.ac.uk/hosted_sites/schisto/). On the other hand, mitochondrial genomes (Figure 5), which average ~14 000 bp in flatworms (Le *et al.*, 2002a), have been completed for a handful of cestodes and digeneans, but a wider representation will be published in the near future (D.T.J. Littlewood, pers. comm.). Characterized mitochondrial genomes in the cestodes include *E. granulosus* (Le *et al.*, 2002b), *H. diminuta* (von Nickisch-Roseneck *et al.*, 2001; see Figure 5), *T. crassiceps* (Le *et al.*, 2000) and *T. solium* (Nakao *et al.*, 2003). In the Digenea, a number of shistosome species (Blair *et al.*, 1999a; Le *et al.*, 2001b), *Fasciola hepatica* (Le *et al.*, 2001a) and *Paragonimus westermani* (Le *et al.*, 2000) have all been completed (see recent review by McManus *et al.*, 2004). Although largely similar to other metazoan mitochondrial genomes, the *atp8* gene is missing (Le *et al.*, 2002a) and minor differences are known to exist in their genetic code (Nakao *et al.*, 2000; Telford *et al.*, 2000). These data will have many uses, especially in diagnostics and strain differentiation. The phylogenetic utility of large-scale changes such as gene order seems dubious (Le *et al.*, 2000) but it remains to be seen if the large number of potentially conserved amino acid changes will help elucidate early divergences in platyhelminth evolution.

6.4.2. Nuclear Genomes and Transcriptomes

There can be little doubt that in due course the characterization and, more importantly, understanding of nuclear genomes, as well as their expressed products (transcriptomes), will have a profound impact on our concept of species, and thus on systematics. In the short term, a seemingly limitless number of new targets will be revealed that may be useful for phylogenetic studies (e.g. Philippe *et al.*, 2004), and access to a suite of targets will allow for the generation of datasets better tailored to the question at hand. In the longer term, it is easy to see genomics affecting the practice of systematics more profoundly, in

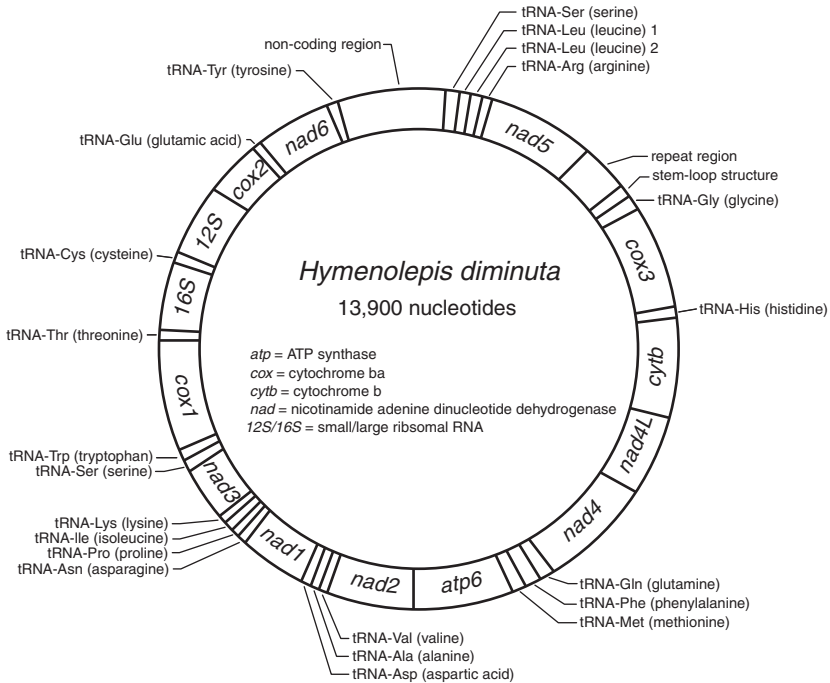


Figure 5 Mitochondrial genome of *H. diminuta*, adapted from von Nickisch-Rosenegk *et al.* (2001). Mitochondrial gene order in *H. diminuta* appears to be typical of mtDNA in the parasitic Platyhelminthes generally. (Note: All genes are encoded on the same strand and the direction of transcription is clockwise.)

a manner similar to the advent of molecular systematics which required the development and adoption of new analytical and theoretical tools, and which stands to change the practice of systematics more profoundly still through DNA-based taxonomy. At present, however, the impact of genomics on the field of systematics, and certainly on platyhelminth systematics, is marginal. Instead, we see phylogenetic techniques being used to better understand the genome (Hardison, 2004), rather than platyhelminth genomes being used to understand organismal relationships. Among the parasitic Platyhelminthes, *Schistosoma* is the only taxon for which a complete genome project has been initiated, although EST (expressed sequence tag) projects aiming to characterize site or stage-specific transcripts are

ongoing for a number of medically important digeneans. In the cestodes and monogeneans, no genome project has been initiated, but a large-scale EST project for *Echinococcus* is underway (see www.sanger.ac.uk/Projects/Helminths). The full fruits of these endeavours, and the ways in which they will contribute to our understanding of platyhelminth evolution is yet on the horizon.

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ERRATA

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Page 200, line 15. "Gyrocotylidae" should read "Gyrodactylidae"

Page 202, section 4.3.1., line 11. *Ibid.*