PLATYHELMINTH SYSTEMATICS AND THE EMERGENCE OF NEW CHARACTERS

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Summary:

Since the inclusion of molecular data in modern phylogenetic analyses, significant progress in resolving the origins and radiation of flatworms has been made, although some key problems remain. Here I review developments in the supply and use of systematic characters that provide the basis for diagnosis and phylogeny reconstruction, that in turn have driven systematic revisions and the interpretation of broader evolutionary patterns and processes; focus is placed on the parasitic taxa. Although useful tools have been refined to the point of becoming established systematic markers of broad utility, attention to the need for denser gene and taxon sampling is addressed in the light of unresolved questions and current trends in molecular systematics, from nucleotide to genome. Tradition and the nature of available comparative information tends to dictate the choice of systematic markers, but faced with incongruent phylogenies, the emergence of new technologies and the need for rapid species diagnosis, there is a pressing need to assess and standardize our choice of tools so they are fit for purpose, available to all and used widely. I present a brief review of existing and potential sources of phylogenetic characters and discuss their likely value in the context of the systematics and diagnostics of parasitic flatworms.

KEY WORDS : Platyhelminthes, systematics, molecular markers.

Resolving the interrelationships of flatworms has been of interest to a broad range of biologists for many years, but has received increased interest since molecular data were incorporated as a source of phylogenetic markers. Early estimates of metazoan relationships suggested that the Platyhelminthes might represent an early divergent lineage of the Bilateria, and for some of those pursuing an insight into the groundplan of the earliest triploblastic animal (*i.e.* the Urbilaterian), simple flatworms appeared to be not only suitable as models, but also possibly relictual in their possession of plesiomorphic features; see Ax (1996). Although the advent of modern character-based phylogenetic systematics recognized flatworms as a distinct, but difficult to place lineage within the protostomes, only the comprehensive assessments of morphology added much insight into the interrelationships of the

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major lineages within the phylum; principally Ehlers (1985), but see also Littlewood *et al.* (1999) and Zamparo *et al.* (2001). Ehlers' scheme of classification, which built on an accumulation of characters from a diversity of sources was the first to be based on cladistics. Subsequently, the explicit character set and the resulting phylogeny became the foundation for further character evaluation and phylogenetic testing.

From these early morphologically based phylogenetic estimates, it was clear that the numerous lineages of essentially free-living turbellarian flatworms were paraphyletic to a monophyletic clade of obligate parasites, including the Monogenea, the Trematoda (Aspidogastrea and Digenea) and Cestoda. From the outset, parasitism was recognised as a single evolutionary event and few authors argued with the membership of this monophyletic Neodermata (Blair *et al.*, 1996). However, interpreting the origins of parasitism, requires resolution of neodermatan interrelationships and the identification of the sister group to the Neodermata. Presently, the interrelationships of the Neodermata remain unresolved (Littlewood, 2006), although it seems likely that the Monogenea are paraphyletic and the Cestoda and Trematoda are sister taxa; see Lockyer *et al.* (2003) and citations therein. Each of these new hypotheses rejects previous morphological assessments, and although few morphological synapomorphies supported (Trematoda (Cestoda, Monogenea)) as suggested by Ehlers (1985), instability amongst molecular estimates have not promoted wide acceptance of any single molecular scenario. Meanwhile, the identity of the sister group to the Neodermata appears to be a large clade comprised of (Prolecithophora + Tricladida + (Genostomatidae + Fecampiida + Urastomidae)) (Littlewood *et al.*, 1999), a clade subsequently named the Adiaphanida by Norén & Jondelius (2002); this scenario has been established primarily with ribosomal genes and requires further sampling of genes (Fig. 1).

At about the same time cladistic analyses of major flatworm lineages were being assessed, the molecular revolution sparked and flamed the fires of disagreement. The first phylogenetic blaze, which all but smoulders today, concerns the acoelomorphs (Acoela and Nemertodermatida). Not only have they been removed
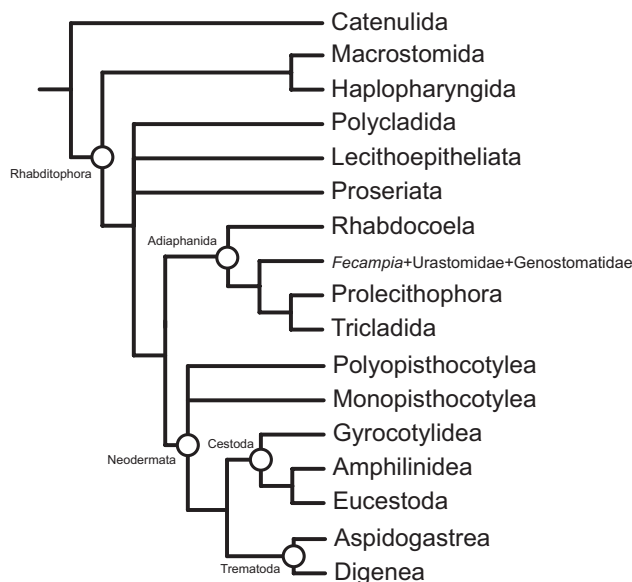
Fig 1. – Summary of major interrelationships amongst Platyhelminthes; drawn from various sources (*e.g.* see Table I)

from the Platyhelminthes *sensu stricto* (the phylum is now comprised of Catenulida + Rhabditophora), but their placement at or towards the base of the Bilateria from numerous sources of evidence (Baguñà *et al.*, 2008) has rejuvenated the possibility that these non-platyhelminth acoelomorph flatworms might yield an insight into the nature of the Urbilaterian (Heijnol & Martindale, 2008). Meanwhile, the remaining Platyhelminthes vie for a stable position within the Lophotrochozoa (*e.g.* see Jenner, 2004) and we still do not know the identity of the phylum's sister group (Giribet, 2008). Many authors, and especially parasitologists, still (falsely) view flatworms as a lineage within which ancestral bilaterian characteristics abound, or at least exist to the extent that they should be considered 'primitive' in some way. Instead, flatworms (and especially parasitic flatworms) are best viewed as an ancient, but relatively derived and specialized group of lophotrochozoans, with their lack of many key characters indicating losses rather than indicating 'primitive' bilaterian features.

Molecular data from the constituent lineages of flatworms has challenged the early phylogenetic estimates based on morphology, such that although the Catenulida are retained as the sister group to all other flatworms (the Rhabditophora), interrelationships within the "Turbellaria" and within the Neodermata remain somewhat unsettled.

Molecular data, principally derived from nuclear ribosomal RNA gene markers, consistently resolves the interrelationships of flatworm lineages as shown in Fig. 1; drawn from multiple sources with polytomies indicating the need for additional evidence. With the conclusion that *Udonella* is indeed a monogenean (Littlewood

et al., 1998), and neither it nor the Temnocephalida, nor the clade of Urastomidae + Genostomatidae, are sister to the Neodermata, the compelling scenario of a progressive move towards parasitism is denied; the inclusion of *Udonella* and Temnocephalida as potential sister groups to the Neodermata were suggested by Brooks and co-workers (summarised in Brooks & McLennan, 1993), and the Revertospermata clade (Fecampiida, Urastomidae + Genostomatidae and Neodermata) was suggested by Kornakova & Joffe (1999) based on sperm morphology. Although parasitic flatworms certainly emerged from ancestors that were free-living, the extant sister clade of the Neodermata is too species rich and too diverse in life habit for a progressive move towards parasitism to be inferred with much clarity. Notwithstanding these developments, the propensity for turbellarians from many lineages to show affinities towards parasitism still provides an insight into how neodermatans might have embarked on their unique obligate association with vertebrates and the many invertebrate taxa they use as intermediate hosts, albeit not from a phylogenetic perspective (Jennings, 1977). Recent reviews concerning the interrelationships of major flatworm groups with the principal sources and relevant references are shown in Table I; readers are encouraged to consult these for details of sampling, signal, phylogenetic resolution and unresolved issues.

CHARACTERS PAST, PRESENT AND FUTURE

In general, the growth of molecular data, and continued insight into the connections between genotype and phenotype, has provided a wealth of characters for metazoan phylogenetics, and congruence between phylogenetic estimates is emerging. However, the impact on the Platyhelminthes and across all taxonomic scales is only recently being realised. Moving from morphology to nucleotides, and more recently to the prospects offered by phylogenomics and evolutionary developmental genetics, some of the more significant developments concerning parasitic flatworm systematics are highlighted here, with an indication as to which questions they might be best suited to address.

MORPHOLOGY

It is clear that distinguishing flatworms from one another, naming them and organizing them remains the stronghold of comparative morphology. Recent compilations of keys to the Cestoda (Khalil *et al.*, 1994) and Trematoda (Gibson *et al.*, 2002; Jones *et al.*, 2005; Bray *et al.*, 2008) demonstrate the wealth of characters available for differentiating genera; species definitions often depend on subtle variations between these same characters. It is perhaps symptomatic of the decline in

Taxonomic group	Source of data	Primary sources and recent reviews
Platyhelminthes		
	Morphology	Ehlers (1985), Brooks & McLennan (Brooks & McLennan, 1993), Littlewood (1999; 2001) Zamparo *et al.* (2001)
	Molecular	Littlewood *et al.* (1999; 2001), Lockyer *et al.* (2003)
Cestoda		
	Morphology	Hoberg *et al.* (2001), chapters in Littlewood & Bray (2001)
	Molecular	Olson *et al.* (2001), Olson & Tkach (2005), Waeschenbach *et al.* (2007)
Trematoda		
Aspidogastrea		
	Morphology	Rohde (2001)
Digenea		
	Morphology	Cribb *et al.* (2001)
	Molecular	Olson *et al.* (2003), Olson & Tkach (2005)
Monogenea		
	Morphology	Boeger & Kritsky (1993)
	Molecular	Olson & Littlewood (2002), Olson & Tkach (2005)

Table I. – Primary sources and recent reviews on phylogenetic estimates of major flatworm groups and their constituent lineages.

alpha taxonomy and revisionary systematics that a comparative volume for Monogenea is not forthcoming. Flatworm morphology, even if diverse, relies heavily on meristic differences in order to differentiate species. Although many of the same characters used for diagnosis across different taxonomic scales have provided the raw material for estimating phylogeny, it is clear that in order to resolve strictly bifurcating trees, especially to the species level, additional characters are needed; see chapters in Littlewood & Bray (2001) and sources listed in Table I. Moreover, the difficulty in identifying the many (morphologically similar, or even indistinguishable) ontogenetic forms of parasitic species has promoted the need for additional characters independent of morphology. Nevertheless, with morphology as the most readily understandable link with life history (transmission strategy, development, attachment, reproduction, and so on), an understanding of comparative anatomy remains critical in evaluating inferred evolutionary patterns as well as making biological sense of molecular phylogenies.

Light microscopy and the staining of particular features remains the mainstay of morphological assessments. Scanning and transmission electron microscopy provides further insights into key diagnostic features and recent developments in antibody-mediated staining (immunocytochemistry) combined with laser confocal microscopy have allowed organ systems such as neuromusculature to be scored with much greater precision for systematic characters (Reuter & Halton, 2001; Halton, 2004). No question in flatworm systematics or diagnostics can be addressed or answered satisfactorily without reference to morphology and morphology has become more important as gene function is beginning to be mapped.

MOLECULAR MARKERS – NUCLEOTIDES AND AMINO ACIDS

Molecular systematics remains a growing field in the study of parasitic flatworms. In combination with (or at least with reference to) morphology, new schemes for the interrelationships of all the major groups have been estimated (see Table I). As for many Metazoa, there are few phylogenies of flatworms that are not in conflict amongst multiple molecular markers or with morphology to some degree. However, overall, DNA-based systematics has provided greater resolution, whilst demonstrating that stability in nomenclature and taxonomy will depend heavily on its use. The prospect of a "DNA only taxonomy" for the flatworms seems highly unlikely in the short term, regardless of one's philosophical position (Dunn, 2003; Lipscomb *et al.*, 2003; Seberg *et al.*, 2003; Tautz *et al.*, 2003). Whilst it is entirely possible that short stretches of one, two or more genes will be able to differentiate within and between species (for diagnostics), and that these same genes may be able to provide robust phylogenetic estimates (for systematics), the identity of such genes remains elusive. The success of particular markers is discussed briefly below with reference to past successes and likely prospects for diagnostics and phylogenetics.

• Ribosomal genes

The nuclear ribosomal array, comprised of small subunit (18S), large subunit (28S) and various internal transcribed spacer (ITS) and intergenic spacer (IGS) regions, continues to provide a wealth of nucleotide markers since first used over 20 years ago (Blair, 2006). It would be fair to say that 18S and 28S rRNA genes provide the bedrock of molecular systematics for the parasitic platyhelminths, having been used extensively for revealing interrelationships within and between families and across the phylum (see Table I). Most recent evidence (Lockyer *et al.*, 2003; Waeschenbach *et al.*, 2007) confirms that a combination of complete 18S and 28S rDNA provides added resolution where the more popular complete 18S alone, or the D1-D3 variable regions of 28S alone (or these 18S and 28S fragments

in combination), fail to provide stability across the tree, particularly amongst the deeper nodes (Olson & Caira, 1999; Olson & Littlewood, 2002; Olson *et al.*, 2003). Faster rates of evolution and higher variability in the ITSs and IGSs has provided diagnostic markers for species (Blair, 2006), although as might be expected these have been tested mostly on parasites of medical or economic importance. Additionally, the ITS region has proved useful as a diagnostic region in the identification of larval forms (*e.g.* Nolan & Cribb, 2005). Nuclear ribosomal markers will likely be of continued utility long into the future, and the notion that partial 28S rDNA might provide useful barcodes for species diagnostics (Sonnenberg *et al.*, 2007), has not gone unnoticed amongst flatworm workers (Brickle *et al.*, 2001). There remains considerable scope for the continued use of nuclear ribosomal markers for species diagnostics and phylogenetic assessments whether they are required to work between species, or between major flatworm lineages.

• Other nuclear markers

There is a general paucity of ubiquitous molecular markers for systematic studies of flatworms and in particular from non-ribosomal nuclear markers. Genome projects (Table II) have yielded many homologous

Major Groups (as classified by GenBank)	prots	core nucs	mt DNA	Genome projects	nuc ESTs[F]
"Turbellaria"					
all except Acoelomorpha	564	193,615	2	x1[A]	95,649
Cestoda					
Amphilinidea		11			
Caryophyllidae		1			
Caryophyllidea	1	19			
Cathetocephalidea		5			
Cyclophyllidea	2,485	3,436	9	x2[B]	35,927
Diphyllidea	1	18			
Gyrocotylidea		7			
Haplobothriidea	1	5			
Lecanicephalidea	1	7			
Litobothriidea	2	6			
Nippotaeniidea	1	10			
Proteocephalidea	21	326			
Pseudophyllidea	186	468	2		
Spathebothriidea		57			
Tetrabothriidea	1	13			
Tetraphyllidea	6	306			
Trypanorhyncha	13	171			
Trematoda					
Aspidogastrea					
Aspidogastridae		32			
Multicalycidae		5			
Rugogastridae	1	4			
Digenea					
Azygiida	157	277			
Diplodiscidae		2			
Echinostomida	331	556	1	x1[C]	358
Faustulidae		11			
Gastrodiscidae		1			
Gymnophallidae	1	3			
Neodiplostomidae	1	2			
Opisthorchiida	432	743		x1[D]	6,745
Plagiorchiida	612	1,280	1		505
Pronocephalidae		1			
Strigeidida	11,598	12,499	6	x3[E]	256,390
Tandanicolidae		5			

Major Groups (as classified by GenBank)	prots	core nucs	mt DNA	Genome projects	nuc ESTs[F]
Monogenea					
Monopisthocotylea					
Ancylodiscoididae		9			
Ancyrocephalidae		31			
Anoplodiscidae		4			
Bothitrematidae		1			
Calceostomatidae		1			
Capsalidae	5	38			
Cichlidogyridae		23			
Dactylogyridae	50	211			
Diplectanidae		60			
Gyrodactylidae	256	632	2		
Hexabothriidae		3			
Microbothriidae		2			
Monocotylidae		44			
Neocalceostomatidae		1			
Pseudodactylogyridae		12			
Pseudomurraytrematidae		2			
Sundanonchidae		2			
Tetraonchidae		2			
Udonellidae		4			
Polyopisthocotylea					
Allodiscocotylidae		2			
Axinidae		4			
Chimaericolidae	1	2			
Diclidophoridae	3	26			
Diplozoidae	1	34			
Discocotylidae	1	4			
Gastrocotylidae	2	9			
Gotocotylidae	2	8			
Heteraxinidae		1			
Hexostomatidae		8			
Mazocraeidae		6			
Microcotylidae	16	34			
Neothoracocotylidae		4			
Octomacridae		2			
Plectanocotylidae	2	8			
Polystomatidae	21	146			
Pyragraphoridae	1	2			
Sphyranuridae		1			

[A]*Schmidtea mediterranea*; [B]*Echinococcus granulosus, Taenia solium*; [C]*Fasciola hepatica*; [D]*Clonorchis sinensis*; [E]*Schistosoma japonicum, S. mansoni, S. haematobium*; [F]most EST projects concentrate on the same species elected for genome projects.

Table II. – Nucleotide, protein, mt genome (mtDNA) and genomic (number of on-going genome projects and Expressed Sequence Tags) effort for parasitic flatworms; data from GenBank (December, 2007).

(usually protein-coding) markers that have been used for phylogenomic studies focussing on metazoan-wide systematic assessments (see below), but few have been tested extensively for the flatworms or within the Neodermata. The problem, it seems, is that although nuclear ribosomal genes have frequently provided meaningful phylogenies, few other genes have been tested, and of those few have managed this in isolation, or at least to the extent that they have attracted widespread use. For example, elongation factor 1-alpha has had mixed success within the phylum (Littlewood *et al.*, 2001) and for the Cestoda (Olson & Caira, 1999), and now remains largely untested and unused; myosin heavy chain type II and histone H3 remain candidates worthy of further study.

It is high time that candidate genes were gleaned from the genome and EST-based studies, and tested with systematics in mind. Whether this should be done sooner making use of currently available data from the derived and disparately related exemplars to hand (Table II), or later when more comparative genomic information becomes available for more taxa, remains unclear. However, even a preliminary assessment seems timely (see also section below on characters from genomes). Additional nuclear genes are required to provide independent estimates of phylogeny across all taxonomic scales.

• Mitochondrial (mt) genes and mt genomes

With 12 protein-coding genes, 22 tRNAs and two ribosomal genes (*rrnS* and *rrnL*; also known as 12S and 16S respectively), mt genomes appear to offer a wealth of homologous markers available for systematics and diagnostics. However, few genes have been tested extensively, largely because PCR primers have only been available for all but the most conserved genes, such as *cox1*, *rrnS* and *cytb*; this contrasts with the nuclear ribosomal genes for which there are well established primers (see references listed above). Mitochondrial genes have been used singly and largely as a supplement to the nuclear ribosomal genes. Most studies have concentrated on restricted taxonomic sampling for one or two genes for which conserved primers are available. The reluctance to use mt genes for deeper level phylogenetics, reflects the view that mt genes evolve considerably more rapidly than nuclear ribosomal markers and are therefore better used for resolving younger clades and more recent radiations. Nevertheless, we have found some considerable success in resolving deeper level phylogenies of the Cestoda by employing lengths of mtDNA that span multiple genes (Waeschenbach & Littlewood, unpublished), and it is clear that estimates of deeper level phylogenies are well within the scope of mtDNA; see also Hardman & Hardman (2006).

To date, over 20 mt genomes have been fully characterized for a diversity of parasitic flatworms (Table II). Rhabditophoran flatworms exhibit two changes to the invertebrate mitochondrial genetic code (Telford *et al.*, 2000), yielding mt genome level apomorphies for the group. Unique gene orders amongst species of *Schistosoma* (Digenea: Schistosomatidae) have provided evidence of shared ancestry (Littlewood *et al.*, 2006) and have allowed many new additional mt genes and mtDNA fragments to be amplified for other parasitic, usually closely-related flatworms, albeit not without technical problems (Littlewood *et al.*, unpublished). Unique gene orders amongst turbellarians (Ruiz-Trillo *et al.*, 2004; Littlewood *et al.*, 2006) and some monogeneans (Park *et al.*, 2007b) suggest that mt genome arrangement may yet provide additional phylogenetic markers, but in the meantime analyses of concatenated mt genes are beginning to provide an insight into deeper relationships (Park *et al.*, 2007a; Park *et al.*, 2007b).

As additional mt data accumulate, more primers become available with which to amplify and characterise entire mt genomes, or large fragments from them. Also recently, the field of comparative mitogenomics has allowed for tailored PCR primer design, in which regions encapsulating the greatest nucleotide variation can be targeted (*e.g. Schistosoma*, Zarowiecki *et al.*, 2007), or diagnostic markers can be developed (*e.g. Diphyllobothrium*, Kim *et al.*, 2007). With complete mt genomes to hand for many of the most important human flatworm pathogens, reliance on a few established mt markers (*e.g. cox1*) for molecular ecology and diagnostics is currently under review (Littlewood & Zarowiecki, ongoing). Nevertheless, and in spite of recent advances in characterizing entire mt genomes of parasitic helminths (Hu *et al.*, 2007), comparative mitogenomics is not likely to reach mainstream systematics except in cases where pathogens of biomedical or economic importance drive the need for more accurate diagnosis within and between species, or the need for variable mt markers in their own right (*e.g.* for population genetics) promotes the use of comparative mitogenomics. High throughput methods of sequencing mt genomes, however, may make this valuable source of data more easily available.

PHYLOGENOMICS AND CHARACTERS FROM GENOMES

Table II indicates the number of on-going genome projects concerning flatworms. Not surprisingly these include the genomes of some of the most significant of parasitic flatworms affecting humans. The "turbellarian" example comes from a model organism (*Schmidtea mediterranea*) used extensively in regeneration studies. From these genome studies, and other EST projects, there now exist gene markers used extensively for broader phylogenomic assessments of metazoan interrela-

tionships, based on concatenated gene sets (*e.g.* Lartillot & Philippe, 2008), but as mentioned, few of these same markers have been targeted as an additional source of nuclear markers for resolving further the systematics of the flatworms themselves. As additional taxa are sampled, the variability of genes and genomic characters across the phylum can be assessed and, if suitably variable, tested for information content by sampling flatworm lineages more densely.

As indicated for mt genomes, comparative genomics offers a wealth of possible phylogenetic markers. Boore (2006) provides an excellent review of the nature and potential wealth of characters emerging from genomic data in general. Although the genomes of *Schmidtea* and *Schistosoma* are poised to provide a wealth of potential markers for flatworms (*e.g.* see Johnston, 2006), it will require complementary data from a greater diversity of flatworms before markers designed *in silico* can be pursued with confidence. Currently, with the advances in high throughput sequencing technology (Mitchelson, 2007), it might seem more appropriate to address the need for taxonomic coverage before undertaking what will certainly be a difficult bioinformatic task.

Just as genomes inform phylogenies, we have yet to reach the stage of sampling of genomes where phylogenies inform much about flatworm genomes. Littlewood *et al.*, (2006) mapped mitogenomic characters onto a molecular phylogeny for which entire mt genomes were available, to highlight possible genomic apomorphies (*e.g.* unique gene orders, shared tRNA secondary structures etc.), but until more data become available convincing molecular synapomorphies will likely be restricted to indels from nucleotide sequences (*e.g.* in 18S rDNA defining various clades including the Neodermata, Joffe & Kornakova, 2001) or amino acids. Frustratingly, there are few automated methods available for detecting patterns of variation in genome-level characters. Molecular systematists must learn to treat their data (alignments, secondary structures, presence/absence of features, etc.) as a comparative morphologist would view a collection of whole animals; *i.e.* search for patterns across all levels of organisation.

Gene expression patterns, evo-devo and back to phenotype

The use of evolutionary developmental biology, in which patterns of gene presence/absence and expression are mapped and compared, brings molecular tools firmly back into the fold of whole organism biology, and completes the spectrum of character evolution from nucleotide to phenotype. Led by genome and large-scale EST projects, maps of gene expression (*e.g.* Hox and ParaHox, see Olson, 2007 and citations therein) and tools such as RNAi in which gene function can

be ascertained are emerging, in spite of the difficulty in handling parasites with complex life cycles (Aboobaker & Blaxter, 2004; Skelly, 2006). Although progress has been made in this area for various turbellarians (in particular triclads, polyclads, and macrostomids for which there exist extensive EST libraries) the field is in its infancy for neodermatans. As they develop, these tools promise to provide enormous insight into the development and ontogeny of parasitic worms and their (biochemical and developmental) interactions with their abiotic and biotic (especially host) environments.

TAXONOMIC SCALE AND TOOL SELECTION FOR PHYLOGENY

Morphology is well established as providing the language for species description and diagnosis. However, molecular data have tended to supplement morphology across a diversity of taxonomic and evolutionary scales. Increasingly, molecular markers are being incorporated as part of species descriptions and revisions, often with a molecular-based phylogeny demonstrating the uniqueness of a new taxon, and/or its relationship amongst established taxa (*e.g.* Bray *et al.*, 2007)

As the density and breadth of taxonomic sampling increases, the need for markers that provide phylogenetic resolution across multiple time scales increases. Given that in combination mt and nuclear genomes can provide us with these tools, it seems imperative to sample taxa more broadly so that existing and candidate genes can be formally assessed and incorporated routinely as aids in species description, diagnosis and phylogenetic analysis. No single gene will fit this range of purposes, and it is clear that some sort of standard needs to be established. However, I can see no reason why partial 28S rDNA should not be a prime candidate, along with another, perhaps mitochondrial protein coding marker, at least as a basis to test such a scheme more formally. Amongst flatworms, a combination of complete 18S and partial or complete 28S rDNA provides resolution for phylogenies from phylum to species, but there is no consensus as to which mt gene or mt genomic fragment works reliably and consistently for all taxa. Given the increasing availability of mt genomes for parasitic flatworms, an empirical test of popular (*e.g.* *cox1*, *cytb*) and other possibly more variable genes within and between major flatworm lineages (to species level) would seem to be of prime importance. Comparative mitogenomics is already revealing patterns of nucleotide variation that may guide the choice of genes to be tested (Zarowiecki *et al.*, 2007; Huyse *et al.*, 2008).

MOLECULAR DIAGNOSTICS, BARCODES AND CRYPTIC SPECIES

Regardless of choice of gene, it is clear that the value of molecular data increases if morphological voucher specimens are available, especially in the context of DNA barcoding (Moritz & Cicero, 2004), and better still if tissue samples are accepted more widely as museum vouchers so they are available for further testing of candidate markers; see also (Petersen *et al.*, 2007). Archival tissue collections, whether frozen or fixed, are expensive to maintain and curate, but it seems short sighted for modern collections not to be made with tissue collection, storage and molecular analyses firmly in mind. Retrieving DNA from formalin-fixed tissues is a long way short of being reliable, predictable or justified, and modern collecting cannot rely on future developments to address these issues when suitable fixatives (ethanol) or stabilizing fluids (*e.g.* RNAlater; Ambion, Inc.) can easily be taken into the field.

Use of partial *cox1* as a marker capable of diagnosing individual species, has become widely popular, particularly those promoting DNA barcoding as a means of molecular diagnostics (Hebert *et al.*, 2003). However, there has been some criticism of this gene, in its inability to distinguish hybrids (*e.g.* in *Schistosoma*, Webster *et al.*, 2006) or pathogenic strains and lineages amongst closely related taxa (*e.g.* in *Gyrodactylus*, Hansen *et al.*, 2007). With the momentum offered by the wealth of existing data and laboratory protocols for characterizing partial (D1-D3) 28S rRNA genes, it is difficult to promote *cox1* as an alternative marker, at least not without extensive additional testing.

Meanwhile, molecular markers aimed at differentiating species, resolving species-level phylogenies or as used in molecular ecology, are frequently revealing cryptic species (Criscione *et al.*, 2005). Parasitic flatworms are themselves most often cryptic and it seems that only when the use of broadly accepted, widely used markers becomes routine, will the extent of parasite diversity, life history complexity, host use and importance of parasitic flatworms be fully understood.

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