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Challenges in the recognition of trematode species: Consideration of hypotheses in an inexact science

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Review Article

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Abstract

The description and delineation of trematode species is a major ongoing task. Across the field there has been, and currently still is, great variation in the standard of this work and in the sophistication of the proposal of taxonomic hypotheses. Although most species are relatively unambiguously distinct from their congeners, many are either morphologically very similar, including the major and rapidly growing component of cryptic species, or are highly variable morphologically despite little to no molecular variation for standard DNA markers. Here we review challenges in species delineation in the context provided to us by the historical literature, and the use of morphological, geographical, host, and molecular data. We observe that there are potential challenges associated with all these information sources. As a result, we encourage careful proposal of taxonomic hypotheses with consideration for underlying species concepts and frank acknowledgement of weaknesses or conflict in the data. It seems clear that there is no single source of data that provides a wholly reliable answer to our taxonomic challenges but that nuanced consideration of information from multiple sources (the 'integrated approach') provides the best possibility of developing hypotheses that will stand the test of time.

Introduction

At the inaugural 'Trematodes' meeting in Brisbane in September 2024, a workshop was conducted by Cribb and Achatz to consider approaches to, and challenges in, the recognition (or delineation) of trematode species (Neodermata: Trematoda). The goal of the workshop was not to be prescriptive to the approach or interpretation of species recognition, but, rather, to identify the issues that can arise and to encourage an open and nuanced discussion of the challenges that may lead to our taxonomic hypotheses being ultimately overturned. The review below, penned as a collective effort by a wide range of those attending, attempts to distil our understanding of the major challenges and best thinking in this field.

Background to the field

The problem

How many valid trematode species should be recognised? This is a known unknown. The most recent estimate of the number of described (nominal) species was 18,000 (Bray *et al.* 2008), while estimates of the true global number of trematode species (described and undescribed) have ranged from 24,000 (Poulin and Morand 2004) to over 181,000 (Carlson *et al.* 2020). The variation among these counts suggests that the true richness of this group is poorly understood. Certainly, many new species are described every year, and many existing species are imperfectly understood. The recognition and distinction of these species at the time of original description, or later, may be simple or very difficult. The reality of an apparent rate of error was revealed recently by an analysis by Poulin and Presswell (2024), which found that approximately 11% of trematode species' names have ended up being synonymised. Notably, this rate captures only the issue of mistakenly proposing new species; it tells us nothing about the proportion of recognised species that actually represents multiple species. The reasons for the first issue (synonymy) are many. Two names are sometimes proposed for the same species by workers unaware of each other's efforts. Sometimes it happens virtually simultaneously – for example, *Proalarioides tropidonotis* was described by Vidyarthi (1937) and then as *Travassosstomum natritis* by Bhalerao (1938). Sometimes, the descriptions are well separated in time; for example, *Dingularis anfracticirrus* was described as a new species (Jue Sue and Platt 1999) because the authors were likely unaware of the work of Nicoll (1914), who described the same species as *Aptorchis aequalis*. In both cases, the first-described species is now recognised as the senior synonym (or valid name). These rather common situations, and other pragmatic challenges such as description based on poor specimens, are not the focus of this work; several best-practice guides to 'doing taxonomy well' and proposing species have been published (Braby *et al.* 2024; Šlapeta 2013). Instead, we are concerned with the complex issues relating to how available evidence can be interpreted for recognition of species. As discussed below, there are many ways in which trematode taxonomists can be misled by morphological, ecological, and molecular data. Our goal is to encourage deeper consideration of the challenges inherent in our taxonomic hypotheses as applied to the Trematoda.

What use is a name?

The taxonomic level of 'species' is simultaneously one of the most fiercely debated and most broadly ignored issues in biology. The issue of what species concept we might use and how it affects our operations is considered separately below. What is largely beyond debate, however, is that the 'species' is a universal currency of biology. It is a critical concept that creates a basis for the generation of names that can be applied reliably to the organisms on which we work; reliable names underpin reproducible science. Importantly, species are, or should be, explicit testable hypotheses, as are species identifications, synonymies, and phylogenetic reconstructions.

The value of reliable species recognition is not limited to taxonomic and systematic studies. Reliable identification of species ensures that physiological, ecological, or evolutionary studies that purport to deal with a certain species do just that and that basic biological facts are not lost among inaccurate identifications. For example, ecological studies of parasites rely on accurate identification of organisms to infer impacts and interactions within an ecosystem. Results of such studies may look quite different depending on the identification. Accurate identification also fosters efforts to assess global patterns of biodiversity for often-neglected groups like trematode parasites (Carlson *et al.* 2020). Likewise, studies of local adaptation (e.g., Johnson *et al.* 2021) strongly rely on accurate species identification. This issue can have substantial and broadly felt impacts on species of economic, veterinary, or medical importance. Differentiation between closely related species may be critical for management of pathogens. For example, ranched southern bluefin tuna off South Australia were initially diagnosed as being infected by a single species of *Cardicola*, *C. forsteri*, a species that principally infects the hearts of the tuna (Cribb *et al.* 2000) and was implicated in significant losses for this high-value fish. The success of management interventions was initially based on surveys of tuna hearts (Aiken *et al.* 2006; Hayward *et al.* 2010). Later, however (Shirakashi *et al.* 2013), it emerged that a second species, *C. orientalis*, was also present, that it was concentrated in the gills rather than in the heart, and that it was responsible for more of the pathogenesis than *C. forsteri* (Aiken *et al.* 2015; Neumann *et al.* 2018; Polinski *et al.* 2013; Power *et al.* 2021; Power *et al.* 2023). Continued failure to recognise and distinguish the two species would have hampered effective monitoring and treatment of the disease they caused. Without correct identification, which is often muddied by the question of species recognition and an agreement on 'what is a species', the value of work is diminished or even negated.

Recognising the value of well-practiced taxonomic research as the basis of subsequent biological science has been advocated for repeatedly (Demoraes 1987; Dubois 2003; Khuroo *et al.* 2007; Mace 2004; Wheeler and Valdecasas 2007). Given the ubiquitous use of species names, in our case for trematodes, we have a responsibility to think carefully about the process of their application and to be as accurate as possible. It is our hope that this work will provide insights needed to help mitigate some of these challenges.

Species concepts for trematodes

If we are to recognise species, it follows that we should have a concept for what a species is. The *species problem* has infrequently involved considerations for parasites (although see Kunz 2002; Lymbery 1992; Thaenkham *et al.* 2022; Tibayrenc 2006). Numerous alternative, often overlapping yet sometimes incompatible species concepts were proposed in the latter half of last century [see reviews by de Queiroz (1998); Freudenstein *et al.* (2017); Luckow (1995); Mayden (1999); Wilkins (2018)]. In attempts to reconcile the various concepts, Mayden (1997, 1999) and de Queiroz (1998, 2005, 2007). Each proposed a version of an *integrated framework of species concepts* (sensu Naomi 2011) that emphasised the fundamental similarities between many proposals and recognised that much of the disagreement and confusion is resolved by distinguishing species delimitation criteria – that is, operational issues – from the theoretical species concept. Mayden (1997, 1999) organised the various concepts into a hierarchy and considered the *Evolutionary Species Concept* (ESC) (sensu Wiley 1978) to be the primary concept, whereas de Queiroz (1998, 2005, 2007) abstracted and united the fundamental theoretical similarities to arrive at the *General Lineage Concept* (GLC) or the *Unified Species Concept* (USC). Both the ESC and USC treat species as evolutionary lineages. Naomi

(2011) and Freudenstein *et al.* (2017) considered the USC to be an overly abstract reduction of the ESC, and thus, the ESC has emerged as a generally accepted universal theoretical concept.

However, two competing definitions of the ESC require consideration. The ESC sensu Wiley (1978) and advocated by Mayden (1997, 1999) and Naomi (2011) defines a species as '*a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate*'. In contrast, Freudenstein *et al.* (2017) argued that population lineages are necessary but insufficient for a theoretical species, emphasising the inherent phenotypic nature recognised in the meaning of biodiversity. They advocated revival for '*role*', which was included in the original definition of the ESC by Simpson (1951, 1961) and which Freudenstein *et al.* (2017) consider in a broad way, representing the totality of the phenotypic expression of individuals and the way they interact with the environment (e.g., the niche). Freudenstein *et al.* (2017) thereby arrived at the following simplification and restatement of the ESC: '*a species is a lineage or group of connected lineages with a distinct role*'.

The theoretical distinction between these definitions of the ESC is worth contemplation by taxonomists focused on parasites, because the host, which is both habitat and resource and therefore part of the niche, is often of critical consideration in delimiting parasite species. Should the *role* also form part of the theoretical concept? The answer might be found in our thinking concerning cryptic (i.e., morphologically indistinguishable) species. Many contemporary trematode taxonomists accept the recognition of strictly cryptic species, and indeed the Trematoda appears to include high cryptic richness (Pérez-Ponce de León and Poulin 2018; Poulin 2011). However, simultaneously, some trematode taxonomists have resisted, perhaps even rejected, purely molecular taxonomy, at least in some circumstances. For instance, geographically separated populations with substantial genetic differentiation but no detectable change in morphology or host species use – that is, with no distinction in 'role' – might not be universally recognised as different species (see below). Do such genetically distinct lineages qualify or have utility as distinct units of biodiversity? The definition of the ESC proposed by Freudenstein *et al.* (2017) also has the important implication of allowing for species to include paraphyletic assemblages of populations. We suspect most trematode taxonomists have operated with the assumption, at least implicitly, that species require monophyly. Theoretically, paraphyletic assemblages may occur in the temporal interim between speciation and coalescence of gene trees, or they might begin interbreeding again prior to the establishment of genetic barriers (Freudenstein *et al.* 2017). Whether such scenarios manifest in practice for trematode taxonomists is unclear, but detection will require consideration for the possibility.

Whereas study of the Trematoda has paid little explicit consideration to theoretical species concepts (Blasco-Costa *et al.* 2016; Cribb *et al.* 2021a), there has been substantial recent effort to develop and follow consistent operational criteria. These criteria reflect, in fact, various contingent biological properties that species may evolve post-divergence (de Queiroz 1998, 2007; Mayden 1997; Naomi 2011). In the now frequently applied *integrated approach*, as advocated by Blasco-Costa *et al.* (2016) (and see 'The path forward', below), these properties include the genetic, morphological, ecological (host, infection site, and geographical distribution), and behavioural (e.g., differential phototaxis); importantly, morphological distinctions may be apparent at only some life-cycle stages (Petkevičiūtė *et al.* 2023). Historically, such criteria related primarily to morphological information. Nowadays, morphology remains important but is heavily informed by genetic information, which has revealed how morphology can sometimes lead us astray; these issues are explored below. Most recently, Bray *et al.* (2022) proposed and applied a set of operational criteria for species delimitation which have since been explicitly applied in several subsequent investigations. These criteria are '*reciprocal monophyly in the most discriminating available molecular marker plus distinction in morphology or host distribution*'. These criteria imply an adopted theoretical concept partially consistent with the ESC sensu Freudenstein *et al.* (2017) because there is a requirement for *a distinction in morphology or host* – that is, phenotype or 'role' in addition to the necessary but insufficient condition of history (lineage). However, Bray *et al.* (2022) also explicitly disallowed species to include paraphyletic assemblages of populations and did not include geographic distinction as sufficient for delimitation in the absence of a distinction in host or morphology. Therefore, against these criteria, morphologically indistinguishable but genetically distinct lineages are only considered separate species where there are differences in hosts. The criteria of Bray *et al.* (2022) provide no solution to the problem of whether to distinguish cryptic species for forms that share the same host in sympatry.

Explicit declarations of the adopted species concept are rare in trematode taxonomy (e.g., Pérez-Ponce de Leon *et al.* 2016) and perhaps even redundant if the species problem can now be considered largely resolved with the emergence of a universal ESC. We would not insist that we collectively adopt a single species concept or set of delimitation criteria for study of the Trematoda. Nevertheless, it is important to recognise that defining species is a hypothesis-testing process. Articulating the concept and criteria facilitates that process for subsequent investigations, and neglecting to do so may lead to different conclusions (Braby *et al.* 2024), especially when dealing with cryptic diversity and other difficult cases (Bray *et al.* 2022; Pérez-Ponce de Leon *et al.* 2016).

The process of naming species

When we propose (describe and name) a trematode species, we follow the rules of the *International Code of Zoological Nomenclature* (ICZN). These rules are generally simple in outline and intent (available online at https://www.iczn.org). The intent is to promote stability in the scientific names of animals and to ensure that each taxon has a unique and universally used name. To help achieve this, we must provide at least a description that differentiates the species from other taxa, provide a proper binomial name, and publish the description in a widely accessible and permanent medium, and we *should* deposit type material in an appropriate collection (ICZN Recommendations 16C and 72F). Some of the complexity of the Code relates to dealing with the issues that arise when aspects of this process have broken down. The rules for the formal naming of a species somewhat obscures what is being done – the proposal of a scientific hypothesis that relates to the recognition of a species. Some scientific hypotheses seemingly pass beyond testing except for occasional major paradigm shifts. The hypotheses for trematode species recognition are not in this category; as mentioned above, 11% of the hypotheses that a new species was being named over the last 250 years are no longer supported, not to mention the reality that many of those same species actually comprise multiple cryptic species (Pérez-Ponce de León and Poulin 2018).

The complex life-cycle problem

The great majority of trematode species have been described based on sexual adults (= maritae) collected from vertebrate definitive hosts. However, this is not always the case. The digenean life-cycle is always complex, involving a succession of morphologically distinct stages and sexual and asexual reproduction (Galaktionov and Dobrovolskij 2013). The variety of their life-cycles is one of their most interesting attributes and therefore has been heavily studied since the trematode life-cycle was first understood by Steenstrup (1842). With few exceptions, we can distinguish eggs, miracidia, mother sporocysts and daughter parthenitae (rediae or daughter sporocysts, which each reproduce asexually), cercariae, often metacercariae, and the sexual adults. Among these stages, eggs, miracidia, mother sporocysts, and daughter parthenitae are generally considered difficult to work with or lacking morphological traits of much value in traditional taxonomy. In contrast, cercariae and metacercariae (especially those that infect a second intermediate host) are both relatively easily collected and may show sufficient morphological distinctiveness to allow recognition as distinct species. Cercariae usually possess dispersal adaptations (especially the tail) that may vary significantly between related forms (e.g., Watson 1984) although cercarial bodies may be less variable. The metacercaria is often much larger than the cercaria and may closely approach the morphology of the sexual adult, even sometimes becoming 'progenetic' (Poulin and Cribb 2002).

Cercariae and metacercariae may be encountered and studied without knowledge of their corresponding sexual adult stages. Hence, researchers have long described these stages – particularly cercariae – and provided them with a species name. Numerous species have been described and assigned to a valid genus on the basis of the metacercaria only [e.g., Chakrabarti (1968); Hutton (1954); Overstreet *et al.* (1992)]. Assignment of cercariae to a genus based on morphology is usually more challenging. Hence, researchers have historically described them and given them a formal, ICZN-regulated name such as the collective-group name *Cercaria* or some sort of informal, non-regulated name that may or may not have used *Cercaria* [see Hechinger (2023) for further details]. Use of provisional names has become by far the most common practice in the last several decades. However, Hechinger (2023) recently argued that trematode research would be fostered by returning to formally naming unidentified trematodes from first intermediate hosts; he proposed a new ICZN-regulated, collective-group naming scheme to provide the taxonomic precision lacking in *Cercaria*. The proposal is ambitious and creative and has already attracted some commentary (Pinto 2023). Whether or not this system should be adopted by our community is not the subject of this paper. Because most trematode alpha-taxonomy is based on the sexual adult, most of our comments and examples relate to that stage. However, we note that the fundamental issues concerning a theoretical species concept and the operational criteria for delimitation – that is, the use of morphological, host, geographical, and molecular data – apply equally to species recognition and delineation of the sexual adult and other life-cycle stages.

The 'Old-Literature' problem

Describing species today necessitates relating new findings to prior work. Early researchers had equipment and technology far inferior to that available now. Therefore, it is not surprising that they did not necessarily foresee the complexity of either the morphology of the animals on which they worked, or the richness of the overall fauna. For most of the 19th and early 20th centuries, almost all trematodes were classified based on gross morphology in large, catch-all genera like *Fasciola*, *Distoma* (later *Distomum*), and *Monostoma*. Many of these descriptions are brief passages that give only a limited understanding of the true morphology of the taxon they describe. Accompanying illustrations were sometimes magnificent (e.g., Looss 1899), but some were over-simplified, showing few of the informative features. Sometimes illustrations were lacking entirely. Standard specimen preparation often involved flattening (i.e., fixing or preparing specimens for mounting under weighted coverslip pressure). It is now widely, but not universally, thought that flattening is problematic because it distorts trematodes, alters relative positions of internal organs (often a key diagnostic feature in trematodes), and creates inconsistencies across specimen series and metrical errors (Cribb *et al.* 2021a; Cutmore *et al.* 2025; Huston *et al.* 2019; Ulmer 1952). However, it is worth noting that, to allow comparison with older flattened specimens, preparation of some flattened specimens is still useful. In addition, it is certainly the case that flattened specimens may be easier to interpret for some aspects of morphology. Problems with inconsistency of approach to preparation, when coupled with description from limited material, sometimes immature or in poor condition from hosts that have been dead for a long time, fundamentally undercut understanding of these taxa from the start.

The inaccurate nature of many early descriptions means that the species involved may be exceedingly difficult to recognise. This is problematic because the principle of priority that underpins the ICZN means that poorly described species cannot be simply ignored; such problematic species may be designated as *species inquirenda* or *species dubia* (https://www.iczn.org), but, if at all possible, we should relate putative new taxa to existing named species. This may be achievable, even in the face of poor descriptions, by examining type-specimens and other preserved material. Type-material can be crucial in such cases and can be unexpectedly discovered in collections even for species named long ago such as *Metagonimus romanicus* from 1914 [see Scholz *et al.* (2024)]. However, before ICZN regulations encouraged the accession of types in publicly accessible museum collections, many specimens were retained in private or institutional collections, and many species descriptions did not include information regarding where the specimens were stored or accession numbers. For example, of the 66 recognised species of the bucephalid genus *Rhipidocotyle*, the original descriptions for 38 (58% of the genus) either provided no accession information at all or named the institute but did not provide accession or catalogue numbers. This includes all species described before 1900 and over 75% of descriptions published before 1960; 100% of species described after the year 2000 had both institution and accession information provided. The type-series of at least two species were stated to be partly or wholly housed in personal collections. Lack of accession information and loss of some private collections renders type-specimens of many trematode species essentially untraceable. In some cases, type-specimens have been destroyed in war, fire, or other incidents [e.g., Velasquez (1958)]. A failure to accession specimens is a problem beyond species descriptions; many new reports of known species are also not substantiated by voucher specimens, nor accompanied by accession information. Finally, it is also often the case that, unfortunately, old type material is in poor condition and largely useless [e.g., Miyazaki (1981)].

Consideration of these old-literature problems deserves two qualifying observations. First, the upside of parasites being hidden and poorly studied is that, compared with many groups of free-living taxa, the old-literature problems are relatively mild and manageable. Approximately half of all recognised trematode species were proposed after 1970, three-quarters after 1940, and onefifth in the 21st century (based on species data in WoRMs). Second, the problems outlined above are not restricted to the past. Species continue to be characterised on the basis of limited and improperly handled material, inadequately described and insufficiently differentiated, and with little regard for biogeography and host-specificity (as considered below). Nevertheless, our taxonomic efforts face significant practical obstacles, even before we grapple with more conceptual problems considered below. Where there is difficulty in associating old names with what contemporary evidence suggests are recognisable species, we can only encourage the proposal of hypotheses that are argued explicitly (including by clearly acknowledging weaknesses in the case) and the use of names that plausibly respect the principle of priority.

The classical basis of trematode species recognition

Overview

Until the advent of molecular data, trematode species were mainly characterised based on three pillars of evidence — morphology, host identity, and geographical distribution (with the rare addition of life-cycle data). All sources of information remain important and are often highly informative. However, these sources of evidence are also all capable of causing serious misdirection. Here, we review the trustworthiness of each source of data.

Pillar 1: Morphology

Morphological difference has undoubtedly been the most widely used basis for distinction of trematode species. Just as the hosts of trematodes are typically distinguishable by their morphology, most putative trematode species are relatively easily differentiated. But it is often difficult to reliably distinguish congeners, especially in larger genera. The largest genera (*Allocreadium*, *Brachylecithum*, *Echinostoma*, *Lecithochirium*, *Phyllodistomum*, *Plagiorchis*, and *Stephanostomum* – each with over 100 nominal species) comprise many species typically constrained by their authors within a relatively narrow set of morphological features. Sometimes it seems that there is simply insufficient reliable morphological variation available (e.g., sucker ratio, distribution of the vitellarium, position of the gonads or genital pore, etc.) to allow the effective distinction of all the species; for all the above genera, it is undoubtedly the case that morphology alone has not allowed a reliable overall taxonomic hypothesis to emerge. For *Phyllodistomum*, the WoRMS database (WoRMS 2024) lists 127 species as presently valid. A further 50 are considered unrecognisable for a variety of reasons, including 14 that are junior subjective synonyms.

A major problem with morphology arises when species are (truly or operationally) morphologically indistinguishable – that is, cryptic species. Almost by definition, cryptic species are usually recognised from molecular data. We have found reports of cryptic trematode species from 23 families (Table 1); given the range of trematode and host taxa involved, it seems likely that cryptic species occur throughout the Trematoda. Pérez-Ponce de León and Poulin (2018) found that cryptic species are reported among trematodes more than in other helminth groups. An interesting point raised by a reviewer of this work is the issue of whether we can ever say a species is truly morphologically cryptic. Certainly, a morphological difference may well be found between species presently considered cryptic. In our view, however, the issue is that such a difference is likely to be so subtle that it may never be used and perhaps cannot be used other than by an expert (perhaps the expert who found the difference). Given the way that our field is embracing molecular approaches, it seems likely that the capacity to find reliable morphological differences is declining rather than improving. On that basis, we suspect that, inevitably, the trend will be to deal with taxonomically difficult combinations of species (whether they are truly cryptic or not) by way of molecular data. Arguably, we might refer to 'taxonomically difficult' rather than 'cryptic', but 'cryptic' is so entrenched that we do not see it disappearing as the term of choice.

Table 1. Trematode families for which combinations of cryptic species are recognised

Family	Reference
Aporocotylidae	Cutmore *et al.* (2021)
Aephnidiogenidae	Herrmann *et al.* (2014)
Allocreadiidae	Petkevičiūtė *et al.* (2023)
Bivesiculidae	Cribb *et al.* (2022)
Bunocotylidae	Duong *et al.* (2023)
Cyathocotylidae	Achatz *et al.* (2024)
Derogenidae	Bouguerche *et al.* (2023); Bouguerche *et al.* (2024); Krupenko *et al.* (2022)
Diplostomidae	Achatz *et al.* (2022c)
Echinostomatidae	Valadao *et al.* (2023)
Enenteridae	Huston *et al.* (2019)
Gorgoderidae	Rosas-Valdez *et al.* (2011)
Haplosplanchnidae	Atopkin *et al.* (2021)
Heterophyidae	Nakao *et al.* (2022)
Lepocreadiidae	Bray *et al.* (2022)
Megaperidae	Curran *et al.* (2013); Razo-Mendivil *et al.* (2010)
Monorchiidae	Jousson *et al.* (2000); Wee *et al.* (2022)
Notocotylidae	Gonchar and Galaktionov (2021)
Opecoelidae	Jousson and Bartoli (2000); Jousson *et al.* (2000); Martin *et al.* (2018)
Opisthorchiidae	Agustina *et al.* (2024)
Paragonimidae	Blair (2024)
Transversotrematidae	Cutmore *et al.* (2023)
Schistosomatidae	Ebbs *et al.* (2022)
Zoogonidae	Gilardoni *et al.* (2020)

The second problematic part of the value of morphology is where it is positively misleading in suggesting the presence of more species than actually exist. Misdirection can arise from host-related morphological variation (Blankespoor 1974; Cribb *et al.* 2022; Hildebrand *et al.* 2015; Presswell and Bennett 2019), from crowding effects (Swarnakumari and Madhavi 1992; Tkach and Bray 1995), from differences in handling, and probably from geographical variation. The last category is little reported for trematodes (e.g., Mateu *et al.* 2014), but the fact that many other animals vary noticeably over their ranges should lead us to expect the same for trematodes. However, even for such intensively studied groups such as birds, the interpretation of geographical variation remains difficult and contentious [for example, the cases of the wandering albatross (*Diomedea exulans*) complex (Burg and Croxall 2004; Penhallurick 2012; Robertson and Nunn 1998) and the rainbow lorikeet (*Trichoglossus haematodus*) complex (Braun *et al.* 2017;

Joseph *et al.* 2020)]. Both have been referred to as species complexes in recognition of the likely presence of cryptic species. We have barely begun to acknowledge the likelihood that this area is a problem for trematodes. Unfortunately, some of the few reports of geographical variation in trematode morphology (e.g., Kennedy 1980; Martorelli and Ivanov 1996) remain ambiguous in the absence of thorough molecular confirmation that only one species was actually under consideration.

Pillar 2: Host identity

Definitive host identity has long been a factor in considerations of whether parasite samples might relate to the same or different species. When done well, this can be reasonable, as it fits with the generally understood paradigm that parasitism leads to some level of host-specificity, creating barriers that allow for speciation to occur. However, there is so much variation in patterns of specificity for definitive hosts that it must be considered very carefully in species differentiation.

Variation in parasite host-specificity was neatly encapsulated by Euzet and Combes (1980) with the terms oioxenous (single hosts), stenoxenous (phylogenetically related hosts), and euryxenous (hosts not closely related but with overlapping ecology and/or physiology). There has since been significant proliferation in the complexity of characterisation of patterns of specificity (see Pojmanska and Niewiadomska 2012), but the oioxenous to euryxenous classification still captures the essence of the distinctions that are possible. All three forms of specificity are seen frequently for trematodes. Based on published records, oioxenous and stenoxenous patterns dominate, but euryxenous species are also common. Levels of specificity for definitive hosts vary between major trematode taxa with multiple species of Hemiuroidea being euryxenous and those of the various blood fluke lineages likely to be oioxenous. Table 2 lists cases where trematode species have proven (based on molecular data) to have narrower specificity for their definitive host than initially understood or, in contrast, to have unusually broad specificity.

Host-specificity of digenean trematodes to their molluscan first-intermediate hosts is generally considered to be reliably high and is frequently far higher than that to definitive hosts (Wright 1960). Dawes (1946) summarised numerous examples of strict specificity (rarely beyond species of a single family) and stated that the few known (or suspected) exceptions did not invalidate the general principle. For example, of the 74 marine cercariae reported by Cable (1956, 1962, 1963) from 18 families of Caribbean marine molluscs, only three were reported from species of more than one mollusc family. The host distributions of the three exceptions have not been confirmed by molecular data. The thinking has not changed materially since these studies. There are certainly reports of trematode host distributions involving species of multiple molluscan families, but many of these have undoubtedly been made in error because cercariae may be morphologically very similar (Miura *et al.* 2005). Just a handful of cases convincingly support the recognition of infection incorporating multiple molluscan families. Hildebrand *et al.* (2019) showed that infections of *Lyperosomum petiolatum* (Dicrocoeliidae) occur in three families and two superfamilies of terrestrial gastropods. For the Notocotylidae, Gonchar and Galaktionov (2022) presented evidence that *Notocotylus atlanticus* infects truncatelloid gastropods belonging to separate families in North America and Europe. Wilke *et al.* (2000) claimed that two reported sympatric snail hosts for *Paragonimus skrjabini* in China belong to separate rissooidean families, but molecular confirmation of trematode identity is still lacking. For the Microphallidae, Galaktionov *et al.* (2012) reported multiple host families for species of *Microphallus.* Overall, significant distinction in the identity of the first intermediate host is far more

Table 2. Examples where trematode host-specificity for definitive hosts is now considered narrower than recognised in earlier work or exceptional within the family

Family	What happened	Reference
Bivesiculidae	*Bivesicula* spp. shared by Holocentridae, Muraenidae, and Serranidae yet absent from other seemingly suitable fishes.	Cribb *et al.* (2022)
Derogenidae	Apparent different levels of specificity of North Sea *Derogenes* species.	Bouguerche *et al.* (2023); Bouguerche *et al.* (2024); Krupenko *et al.* (2022)
Fellodistomidae	Three species of *Proctoeces* with specificity ranging from oioxenous to stenoxenous to euryxenous.	Wee *et al.* (2017)
Hemiuridae	Hemiurids overwhelmingly use fish definitive hosts, but *Lecithochirium*, *Plicatrium*, and *Tubulovesicula* each includes species in snakes.	Martin *et al.* (2023); Urabe *et al.* (2025)
Hirudinellidae	Molecular analyses demonstrated multiple species of *Hirudinella* with narrower specificity than previously accepted.	Calhoun *et al.* (2013)
Monorchiidae	*Monorchis parvus* in Mediterranean sparids recognised as two species with narrower host-specificity patterns than previously recognised.	Bartoli *et al.* (2000)
Opecoelidae	Of 15 opecoelids genetically characterised from the Great Barrier Reef, only *Trilobovarium parvvatis* routinely infects multiple fish families.	Martin *et al.* (2017a)
	Macvicaria crassigula in Mediterranean sparids recognised as two species with distinct host-specificity patterns.	Jousson *et al.* (2000)
	Host range for *Hamacreadium* reduced from 14 fish families to essentially two.	Martin *et al.* (2017b)
	Podocotyloides revised to recognise species from only one host family.	Martin *et al.* (2018)
Transversotrematidae	*Transversotrema licinum sensu lato* shown to comprise multiple species, most of which are stenoxenous.	Hunter and Cribb (2012); Cutmore *et al.* (2023)
	Transversotrema borboleta, T. chrysalis, and *T. polynesiae* all shared by the unrelated Chaetodontidae and Lutjanidae but absent from other seemingly suitable fishes.	Cribb *et al.* (2014); Cutmore *et al.* (2023)

likely to be an indicator of separate species than it is for sexual adult trematodes, but it is not a guarantee.

Host-specificity of digeneans in their second intermediate host is certainly less documented but has been reported in some species. For instance, *Posthodiplostomum minimum* and *Posthodiplostomum centrarchi* are morphologically similar species which most often parasitise various ardeid definitive hosts. These species are best separated based on their second intermediate hosts, cyprinid vs. centrarchid fishes (Achatz *et al.* 2021b; Locke *et al.* 2018). At the same time, some species that were previously separated based on second intermediate hosts (e.g., *Apatemon gracilis* and *Apatemon annuligerum*) have been demonstrated to be conspecific based on molecular data (Bell and Sommerville 2002). Certainly, knowledge of second intermediate hosts may be helpful for differentiation among closely related species. However, caution is required until molecular confirmations are possible.

In addition to the problem of misinterpreted specificity, many host species may harbour multiple trematode congeners (Table 3). This important phenomenon is so common that it should always be considered as a possible explanation of what might otherwise be interpreted as intraspecific variation.

Overall, host identity has the capacity to inform or mislead and must be used cautiously and thoughtfully as an indicator of trematode identity. Two aspects of host data are critical. First, self-evidently, the host identification should be reliable. Any interpretation of host-specificity for trematodes relies entirely on proper host identification, and the taxonomy of the hosts is also continuously evolving and changing. Any assessment of host-specificity requires an evaluation of the current status of the host species, particularly when host-specificity is discussed at the genus or species level. Second, but less obvious, is the context of 'evidence of absence'. For example, if all the available host species in a location have been sampled sufficiently and a particular trematode is found in only one of them (and regularly), then the information is far more powerful than a single record from a single host. Such nuances are often not addressed in publications in our field, but they should be.

Pillar 3: Geographical distribution

A separate workshop at *Trematodes 2024* considered problems and prospects in the study of trematode distributions and biogeography. A clear outcome of that workshop was that distributional data are seriously lacking for all but a handful of taxa. This point is also made by Poulin (2025) in his advocacy for more trematodes to be studied in greater depth; his analyses showed that most trematode species are reported from one locality and, in a 30-year span, never again! Frequently, geographical distribution is used in thinking on species differentiation, especially if the recorded localities of the compared species are considered significantly separated. This is reasonable given that few non-domesticated vertebrates or molluscs are genuinely cosmopolitan; why should their trematodes be any different? However, our underlying understanding of trematode distribution is arguably too weak to allow much use of geographical data in species differentiation.

Just as host specificity might be narrow (oioxenous), moderate (stenoxenous), or wide (euryxenous), geographic distributions of trematodes might be localised (single or closely connected sites), regional (e.g., continent wide), or some form of cosmopolitan. All three categories are represented in the literature for trematodes, but the available evidence is of variable quality because of two issues. First, sampling for trematodes over their potential range, specifically that of their known hosts, is typically insufficient. Thus, normally we have an absence of evidence rather than evidence of absence to allow inference of distributional limits. Second, we typically lack molecular data to corroborate morphology-based identifications. In this context, the nature of distributional patterns must be considered with great caution. The geographical distributions of vertebrates and more conspicuous invertebrates are typically supported by hundreds of records that allow the production of plausible distribution maps and the recognition of informative phenomena such as transition zones and disjunct distributions; such data are almost completely lacking for trematodes except for species of medical and veterinary importance.

There is surprisingly little positive evidence of highly limited or localised distributions. Certainly, we can presume highly restricted geographic ranges for trematodes that infect vertebrate species that are themselves highly restricted. The liolopid *Liolope copulans* presumably has a narrow distribution restricted by that of its definitive host, the Japanese giant salamander (Baba *et al.* 2011); probably many such inferences for trematodes of endemic hosts can be made. However, there are few explicitly documented cases of evidence of absence of trematode infections from susceptible host species. A few examples have been reported for trematodes of marine fishes. In the central and western Pacific, multiple species of *Neohexangitrema* that infect the acanthurid *Zebrasoma scopas* have been found with regionally segregated distributions (Cribb *et al.* 2025). On a smaller geographical scale, multiple species have been found on only the northern or southern GBR (e.g., Bray *et al.* 2014; Diaz *et al.* 2013; Huston *et al.* 2024). The overall frequency and explanation of such limited distributions is not understood.

Broad regional distributions, partly or largely consistent with all or parts of those of their definitive hosts, are relatively common. There is clear evidence for highly studied species infecting humans and their domesticated animals. Some species within genera such as *Clonorchis*, *Fasciola, Metagonimus*, *Paragonimus*, and *Schistosoma* are typically regionally widespread (Achatz *et al.* 2020; Achatz *et al.* 2022b; 2023a; Alves *et al.* 2020; Ebbs *et al.* 2016). Most are probably ultimately restricted by the distributions of their first intermediate hosts. Multiple trematode species of wild animals have also been demonstrated to have wide regional distributions. Widespread distributions in the tropical Indo-Pacific have been demonstrated for multiple fish trematode species (Huston *et al.* 2021; Magro *et al.* 2023; Pérez-Ponce de León *et al.* 2024; Wee *et al.* 2022). In the Atlantic, Vermaak *et al.* (2023a) demonstrated a range for *Proctoeces maculatus* from the Mediterranean to South Africa. For freshwater or terrestrial species, there is good evidence for wide regional distributions of multiples species [e.g., species of *Austrodiplostomum* (Sereno-Uribe *et al.* 2019), *Drepanocephalus* (Hernandez-Cruz *et al.* 2018; Kudlai *et al.* 2015), *Wardius* (Achatz *et al.* 2025), and see Locke *et al.* (2021) for further examples.] We predict that such broad but not cosmopolitan distributions are likely for most trematode species.

The final category of distributions, cosmopolitan, is perhaps as poorly documented as for localised distributions. Certainly, there is far less evidence for such distributions than for regional ones. Here, we arbitrarily consider cosmopolitan to mean occurrence in both the Atlantic and the Indo-West Pacific oceans for marine trematodes and in both the old and new worlds for terrestrial and freshwater trematodes. For marine trematodes, there is evidence of cosmopolitan distributions for some Accacoeliidae (Louvard *et al.* 2024), Aporocotylidae (Aiken *et al.* 2007), Hapalotrematidae (Corner *et al.* 2022), and Haplosplanchnidae (Pérez-Ponce de León *et al.* 2024). Perhaps unsurprisingly, the definitive hosts involved

Table 3. Trematode families where two congeners infect single definitive host species

Trematode family/genus	Host	Reference
Acanthocolpidae: *Stephanostomum*	Pisc.: Carangidae: *Caranx sexfasciatus*	Bray and Cribb (2003)
Allocreadiidae: *Wallinia*	Pisc.: Characidae: *Astyanax aeneus*	Hernández-Mena *et al.* (2019)
Alloglossidiidae: *Alloglossidium*	Pisc.: Ictaluridae: *Ameiurus melas* Annelida: Haemopidae: *Haemopis grandis*	Kasl *et al.* (2018); Tkach and Mills (2011); Tkach et al. (2013)
Aporocotylidae: *Psettarium*	Pisc.: Tetraodontidae: *Arothron hispidus*	Yong *et al.* (2018)
Bivesiculidae: *Bivesicula*	Pisc.: Serranidae: *Epinephelus fasciatus*	Cribb *et al.* (2022)
Bunocotylidae: *Hysterolecitha*	Pisc.: Pomacentridae: *Abudefduf bengalensis*	Duong *et al.* (2023)
Brachycladiidae: *Nasitrema*	Mamm.: Phocoenidae: *Neophocaena asiaeorientalis*	Kim *et al.* (2023)
Brachycladiidae: *Orthosplanchnus*	Mamm.: Phocidae: *Erignathus barbatus*	Price (1932)
Bucephalidae: *Prosorhynchus*	Pisc.: Serranidae: *Plectropomus leopardus*	Bott *et al.* (2013)
Choanocotylidae: *Choanocotyle*	Rept: Chelidae: *Chelodina oblonga*	Platt and Tkach (2003)
Clinostomidae: *Clinostomum*	Aves: Ardeidae: *Ardea alba, Tigrisoma mexicanum*	Pérez-Ponce de León *et al.* (2016)
Cryptogonimidae: *Retrovarium*	Pisc.: Lutjanidae: *Symphorus nematophorus*	Miller and Cribb (2007)
Cyathocotylidae: *Gogatea*	Rept: Acrochordidae: *Acrochordus arafurae*	Achatz *et al.* (2024)
Dicrocoeliidae: *Anenterotrema*, *Metadelphis*	Mamm.: Phyllostomidae: *Phyllostomus discolor*; *Lonchophylla robusta*	Fernandes *et al.* (2021); Tkach *et al.* (2018)
Didymozoidae: *Koellikoerioides*	Pisc.: Scombridae: *Neothunnus macropterus*	Yamaguti (1970)
Diplostomidae: *Cardiocephaloides Proterodiplostomum*; *Pseudoneodiplostomum*, *Dungalabatrema*, *Uvulifer*	Pisc.: Clinidae: *Clinus superciliosus* Rept.: Crocodylidae: *Caiman jacare*, *Crocodylus niloticus*; *Crocodylus johnstoni* Aves: Alcedinidae: *Megaceryle alcyon*	Achatz *et al.* (2019); Achatz *et al.* (2022a); Tkach *et al.* (2020); Vermaak *et al.* (2021)
Echinostomatidae: *Rhopalias*	Mamm.: Didelphidae: *Didelphis marsupialis*	Lopez-Caballero *et al.* (2019)
Emprostiotrematidae: *Emprostiotrema*	Pisc.: Siganidae: *Siganus argenteus*	Huston *et al.* (2024)
Enenteridae: *Enenterum*	Pisc.: Kyphosidae: *Kyphosus bigibbus*	Bray and Cribb (2002)
Faustulidae: *Paradiscogaster*	Pisc.: Chaetodontidae: *Chaetodon aureofasciatus*	Bray *et al.* (1994)
Fellodistomidae: *Fellodistomum*	Pisc.: Anarhichadidae: *Anarhichas lupus*	Krupenko *et al.* (2020)
Fellodistomidae: *Symmetrovesicula*	Pisc.: Chaetodontidae: *Chaetodon lineolatus*	Downie *et al.* (2011)
Gorgoderidae: *Phyllodistomum*	Pisc.: Sinipercidae: *Siniperca chuatsi*	Long and Wai (1958)
Haploporidae: *Hapladena*	Pisc.: Acanthuridae: *Naso unicornis*	Machida and Uchida (1990)
Haplosplanchnidae: *Schikhobalotrema*	Pisc.: Labridae: *Sparisoma chrysopterum*	Nahhas and Cable (1964)
Hasstilesiidae: *Strzeleckia*	Mamm.: Dasyuridae *Antechinus swainsonii*	Cribb and Spratt (1991)
Heterophyidae: *Scaphanocephalus*	Ave.: Pandionidae: *Pandion haliaetus*	Locke *et al.* (2024)
Lecithasteridae: *Quadrifoliovarium*	Pisc. Acanthuridae: *Naso annulatus*	Chambers and Cribb (2006)
Lecithodendriidae: *Ochoterenatrema*	Mamm.: Molossidae: *Mollosus molossus* Mamm. Vespertilionidae: *Myotis diminutus*	Tkach *et al.* (2024)
Lepidapedidae: *Doorochen*	Pisc.: Labridae: *Choerodon graphicus*	Bray *et al.* (2023)
Lepocreadiidae: *Neohypocreadium*	Pisc.: Chaetodontidae: *Chaetodon auriga*	Machida and Uchida (1987)
Megaperidae: *Blendiella*	Pisc.: Balistidae: *Balistapus undulatus*	Magro *et al.* (2023)
Microphallidae: *Microphallus*	Aves: Anatidae: *Somateria mollissima*	Galaktionov *et al.* (2012)
Microscaphidiidae: *Microscaphidium*	Rep.: Cheloniidae: *Chelonia mydas*	Blair (1986)
Monorchiidae: *Hurleytrematoides; Sinistroporomonorchis*	Pisc.: Chaetodontidae: *Chaetodon auriga*; Mugilidae: *Mugil curema*	McNamara and Cribb (2011); Andrade-Gomez et al. (2023)
Opecoelidae: *Pseudoplagioporus*; *Coitocaecum*	Pisc.: Lethrinidae: *Lethrinus nebulosus*; Clinidae: *Clinus superciliosus*	Martin *et al.* (2019); Vermaak *et al.* (2023)
Plagiorchiidae: *Plagiorchis*	Mamm.: Vespertilionidae: *Myotis daubentoni*	Tkach *et al.* (2000)

(*Continued*)

Table 3. (*Continued*)

Trematode family/genus	Host	Reference
Pleurogenidae: *Parabascus*	Mamm.: Vespertilionidae: *Myotis daubentoni*	Tkach *et al.* (2003)
Psilostomidae: *Neopsilotrema*	Aves: Anatidae: *Aythya affinis*	Achatz *et al.* (2021a); Kudlai *et al.* (2016)
Renicolidae: *Renicola*	Aves: Anatidae: *Somateria mollissima*	Galaktionov *et al.* (2024b)
Spirorchiidae: *Neospirorchis*	Rep.: Cheloniidae: *Chelonia mydas*	Corner *et al.* (2023)
Spirorchiidae: *Uterotrema*	Rep.: Emydidae: *Emydura krefftii*	Platt and Blair (1996)
Telorchiidae: *Dolichosaccus*	Amph: Bufonidae: *Rhinella marina*	Barton (1994); Luton *et al.* (1992)
Transversotrematidae: *Transversotrema*	Pisc.: Lutjanidae: *Lutjanus gibbus*	Cutmore *et al.* (2023)
Zoogonidae: *Overstreetia*	Pisc.: Atherinidae: *Atherinomorus lacunosus*	Bray and Justine (2014)

are all widespread taxa with high vagility – ocean sunfish, tuna, turtles, and belonid fishes. Cosmopolitan distributions have also been reported for terrestrial and freshwater trematodes. These distributions typically involve highly vagile or migratory bird such as ducks, herons, shorebirds, and seabirds (e.g., Gonchar and Galaktionov 2020; Locke *et al.* 2021; Ebbs *et al.* 2025). Ornela Beltrame *et al.* (2020) reported evidence of *Fasciola hepatica* in pre-Hispanic coprolites from South America, suggesting a long-standing cosmopolitan distribution for that species.

From the range of patterns reported above, it is arguably unwise to be much influenced in trematode identification by geographical distribution. A regional distribution broadly consistent with that of known hosts is perhaps generally plausible, but wider and narrower distributions occur. Certainly, new geographical or host records should be thoroughly documented, and voucher specimens (including material suitable for molecular analysis) should be deposited in internationally recognised collections. Without this underpinning, the records lose their future utility, especially for ecological studies where the mere listing of parasite names in association with specific hosts and localities is insufficient.

Classical approaches: The challenges in summary

The combination of good morphological, host data, and geographical data is undoubtedly powerful. Prior to the arrival of the molecular revolution in our field, these data enabled the establishment of a sound basis for trematode taxonomy. It is evident, however, that there are numerous taxonomic challenges that the classical approach struggled to resolve. It is for this reason that the use of DNA sequencing (first Sanger and now various high-throughput sequencing [HTS] platforms) has enabled a quantum leap in our capacity to resolve the sorts of challenges inherent to the classical approaches.

Molecular data

Typical applications and problems

Following early work involving allozyme analysis (e.g., Agatsuma and Suzuki 1980; Bray and Rollinson 1985; Goater *et al.* 1990) and prior to the recent avalanche of data from genome-wide sequencing analyses that continues to grow (Coghlan *et al.* 2019; Locke *et al.* 2018; Locke *et al.* 2021), molecular work on the identification of trematodes has focused on a handful of markers, especially nuclear ribosomal (rDNA/rRNA) and mitochondrial (mt) genes (Thaenkham *et al.* 2022). These have enabled tremendous progress. The advantages of molecular data (essentially DNA sequence data for the purpose of this publication) are numerous. The data are researcher-independent (assuming that the sequences are accurate), independent of host/habitat/crowding/age/life-cycle stage, character-rich, have low ambiguity, and are ever-cheaper to generate. The premise of the use of molecular data for species recognition is a simple one. We expect that, for our marker or markers of choice, specimens of the same species will show little to no sequence variation, and separate species will show greater consistent differences. Differences and similarities among sequences being evaluated are typically visualised as a phylogenetic tree or genetic distance matrices. Phylogenetic trees can identify clades that can be treated as operational taxonomic units (OTUs) or candidate species. Relative levels of support for clades in the trees can be assessed based on a range of statistical models. However, the researcher is still required to interpret the pattern obtained and especially resolve conflicts between markers (Blasco-Costa *et al.* 2016).

Three genes/regions have been used most widely in the molecular distinction of trematode species (Blasco-Costa *et al.* 2016; Thaenkham *et al.* 2022). These are the nuclear 28S rRNA gene (usually partial), complete or partial nuclear internal transcribed spacer (ITS) rDNA region (typically with some flanking regions, whether it is the whole region or only ITS1 or ITS2), and mitochondrial *cox*1 fragments of variable length. A significant issue in the analysis of *cox*1 sequences is that different authors have used different fragments so that not all data is usefully comparable (see Corner *et al.* 2023). As is typical for eukaryotes, nuclear ribosomal genes occur in tandem clusters, each cluster containing the 18S, 5.8S, and 28S genes separated by various spacers including ITS1 and ITS2 (Blair 2006; Nolan and Cribb 2005). The ribosomal gene tandem array's chromosomal position is known for two trematodes. It is on chromosome 4 in *Paragonimus ohirai* (see Hirai 1988) and on chromosome 3 in *Schistosoma mansoni* (Buddenborg *et al.* 2021). Different portions of the ribosomal cluster accumulate changes at different rates. For example, the 28S rRNA gene contains regions that are extremely conserved (differing little across great phylogenetic distances) and regions that may vary among closely related species. The ITS regions have the most variable sequences and thus are generally more suitable for assisting species delimitation. Indeed, there can be intra-individual variation in the spacer regions, usually in the form of variation in numbers of short repeats in individual copies of the spacer (usually ITS1) (Blasco-Costa *et al.* 2016). This has been reported, for example, for species of *Paragonimus* (van Herwerden *et al.* 1999). There can also be interspecific variation in the numbers of such ITS1 repeats, for example, between members of the choanocotylid genus *Aptorchis* (Tkach and Snyder 2008). Although these repeats were responsible for most of the sequence length difference, they were not considered as evidence of interspecific divergence. Similar findings for ITS1 have been noted between *Schistosoma* species (Kane and Rollinson 1994; van Herwerden *et al.* 1998) and species of *Dolichosaccus* (see Luton *et al.* 1992). Overall, variable repeat regions in ribosomal markers may be problematic to handle but appear to have little taxonomic significance and occur relatively sporadically.

Mitochondrial genomes are small, circular, maternally inherited and haploid, and contain 12–13 protein-coding genes, two ribosomal RNA genes, and 22 transfer RNA genes (Thaenkham *et al.* 2022). There is also a variable non-coding region. Many mitochondria occur in an individual cell. Thus, there are many copies of the mitochondrial (mt) genome per cell, facilitating molecular study. Mutation rates are generally high in this type of genome, making mt sequence data, such as the *cox*1 gene, a widely used 'barcode' resource for distinguishing species and for some aspects of population genetics. Variation might occur between individuals at a single locality, or across a broad geographic range. This can be seen for *Paragonimus westermani* (see Devi *et al.* 2013) and monorchiids of butterflyfishes (see McNamara *et al.* 2014).

Overall, the commonly used markers behave broadly as required; this is precisely why they have become popular. Typical findings are that rDNA sequences for accepted species vary little or not at all in sympatry and marginally over geographic range (Cribb *et al.* 2021b; Magro *et al.* 2023). Between accepted species, there is typically consistent distinction for these markers, though this distinction may be slight (e.g., Trieu *et al.* 2015). *Cox*1 sequences routinely vary far more than the ribosomal markers [occasionally less – for example, Bray *et al.* (2022)] both within and between species. Within accepted species, some intraspecific variation in *cox*1 is common in sympatry, and much greater variation occurs with increasing geographic separation (e.g., Bray *et al.* 2022). Such patterns are typically easily interpreted, especially when *cox*1 and rDNA markers are used together and as part of an integrated approach, as recommend by Blasco-Costa *et al.* (2016). However, there are now several examples of cases where interpretation of patterns in *cox*1 and rDNA markers are not straightforward, and these can be summarised into three categories.

First, *cox*1 is nonrecombinant, which means that signal of past separation between lineages is retained even when those lineages subsequently fuse (e.g. Bray *et al.* 2022). This lineage history is useful for biogeographical and phylogeographical investigation but can confuse species recognition by overestimating diversity when considered in isolation, and may potentially lead to alternative interpretations depending on whether the assumed theoretical species concepts requires monophyly of population lineages or allows paraphyly (see species recognition, above). A consequence of the frequency of and high level of *cox*1 variation is that their meaningful interpretation requires substantially greater numbers of replicate sequences, which has time and cost implications.

Second, in multiple studies, either 28S or ITS sequences (or both) are identical between closely related species, whereas the *cox*1 sequences show substantial variation. For the lepidapedid genus *Doorochen*, two pairs of species, *D. secundum* + *D. uberis* and *D. spissum* + *D. zdzitowieckii*, each have identical ITS2 rDNA sequences but partial *cox*1 distinctions of 12.4–13.0% and 5.9–6.8%, respectively, which aligned with host and morphological distinctions (Bray *et al.* 2023). For two aporocotylids, *Phthinomita munozae* and *P. poulini*, ITS2 sequences were identical but partial *cox*1 sequences differed at 7.0–8.6%. The two species are also morphologically distinguishable and infect different fish families; one is found exclusively in mullids and the other only in labrids (Cutmore *et al.* 2021). For two lecithodendriids, *Ochoterenatrema fraternum* and *O. piriforme*, there were no differences in the partial 28S gene but 8.9% divergence in partial *cox*1 gene sequences that was consistent with their status as separate species (Tkach *et al.* 2024). For two opecoelids, *Coitocaecum capense* and *Coitocaecum* sp., 28S and ITS2 sequences did not differentiate these morphologically distinct species, but *cox*1 sequences differed by 14.9% (Vermaak *et al.* 2023b). In all these cases, ribosomal data failed to distinguish species that were morphologically and ecologically distinct and clearly distinguished by *cox*1 sequence data. Table 4 provides examples of further cases. Thus, if a study is based only on ribosomal markers, we may miss important diversity. The trematode literature has many studies where specimens are interpreted as single species based on identical ITS or 28S sequences; the developing evidence suggests that a non-trivial fraction of these may obscure unrecognised species-level richness.

Third, a consequence of the discriminating power of *cox*1 sequences is that they may show substantial variation when analysed over geographic range. The extent to which this may be problematic appears to relate to the vagility of the hosts concerned. For parasites of highly vagile birds, multiple studies have shown little effect on sequence variation from geographic distance (e.g., Juhasova et al. 2025; Locke *et al.* 2015a; Locke *et al.* 2015b). In contrast, multiple trematodes of marine fishes (for which seemingly none of the infected hosts have high vagility), incorporate substantial and consistent variation in *cox*1 sequences over range (Bray *et al.* 2018; Bray *et al.* 2022; Huston *et al.* 2021; McNamara *et al.* 2014). In some cases, the levels of distinction are as great as between what are considered good species on the basis of morphological distinction (e.g., Cutmore *et al.* 2023; McNamara *et al.* 2014).

Table 4. Combinations of congeners interpreted as distinct species but with identical ITS2 or 28S rDNA sequences

Taxon	Reference
Acanthocolpidae: *Neophasis*	Kremnev *et al.* (2021)
Aporocotylidae: *Phthinomita*	Cutmore *et al.* (2021)
Bivesiculidae: *Bivesicula*	Cribb *et al.* (2022)
Diplostomidae: *Alaria*, *Crassiphiala*, *Diplostomum*, *Neodiplostomum*, *Posthodiplostomum*	Achatz *et al.* (2021b); Achatz *et al.* (2022b); Achatz *et al.* (2022c); Achatz *et al.* (2023b); Young M.A. *et al.* (in press)
Cyathocotylidae: *Gogatea*	Achatz *et al.* (2024)
Lecithasteridae: *Lecithaster*	Krupenko *et al.* (under review)
Lepidapedidae: *Doorochen*	Bray *et al.* (2023)
Opecoelidae: *Coitocaecum*; *Macvicaria*	Vermaak *et al.* (2023); Vermaak *et al.* (in prep.)
Opisthorchiidae: *Metorchis*	Besprozvannykh *et al.* (2019)
Schistosomatidae: *Schistosoma curassoni*, *S. intercalatum* and *S. bovis* (N.B. Later studies found slight (1 or 2 bp) differences between these species.)	Després *et al.* (1992)
Schistosomatidae: *Trichobilharzia franki* complex	Jouet *et al.* (2015)
Transversotrematidae: *Transversotrema*	Cutmore *et al.* (2023)

The interpretation of the status of these populations presently tends to be partly subjective. Although we are unaware of any circumstance where *cox*1 sequences do not differ materially between accepted species, there is also no completely reliable 'barcode gap' that allows the differential recognition of populations and species. McNamara *et al.* (2014) showed clear overlap between levels of intra- and interspecific variation for *cox*1 sequences of morphospecies of the monorchiid genus *Hurleytrematoides* in Indo-Pacific butterflyfishes. Their interpretations may not survive future analysis, but there was no strong basis to inform a different species-level hypothesis.

The combination of patterns of variability means that ITS and 28S sequence data cannot be relied upon to distinguish species in isolation and that there is often ambiguity in the interpretation of *cox*1 sequences. Nadler and Pérez-Ponce de León (2011) discussed in further detail the barcoding gap in parasitic organisms and argued that the use of single-locus DNA barcodes and the 'barcoding gap' are insufficient approaches to completely reliably delimit species and because of that, other sources of information, including other genes, are required. Importantly, as demonstrated by studies comparing complete mitochondrial genomes (e.g., Suleman et al. 2020), the genes most often used for trematode species differentiation (*cox*1, *nad*1) are not even the fastest mutating. Some other mt genes, such as *nad*3, *nad*5, and *atp*6, may be additional, or better, markers for species discrimination. In the best-case scenario, especially with the availability and increased accessibility of the HTS technologies, it is becoming desirable to utilise more genes (e.g., complete mt genomes or concatenated protein-coding mt genes) for this purpose.

Automated species delimitation

To assist in the process of species delimitation, algorithms using DNA sequences have been developed and applied extensively in the last two decades. These approaches seek an objective basis to improve our capacity to distinguish species and estimate species-level richness (Magoga *et al.* 2021; Rannala and Yang 2020). A popular method is the single-locus Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.* 2012). This was developed largely in response to the accumulation of 'DNA barcode' sequences as part of the 'Barcode of Life' initiative (https://ibol.org/). The usual barcoding region for animals is a portion of the mt *cox*1 gene. This method partitions data under the assumption that differences within a species are less than differences between species. ABGD is based on genetic distances computed from a single locus rather than an explicit species concept and requires an a priori specification of an intraspecific distance threshold. Puillandre *et al.* (2021) later introduced ASAP (Assemble Species by Automatic Partitioning), which is more user-friendly than ABGD, assisting the choice of priors and ultimate delimitation, which in ABGD are left to the user. Another method used for delimitation estimates based on a single locus is the Generalised Mixed Yule-coalescent (GMYC), which requires an ultrametric estimate of the gene tree (Fujisawa and Barraclough 2013). Similarly, Poisson Tree Processes (PTP) (Zhang *et al.* 2013) requires a phylogeny (not necessarily ultrametric) as input but overcomes some of the limitations of other methods. BPP (Bayesian Phylogenetics and Phylogeography) uses a Bayesian framework and the multispecies coalescent model to delimit species based on data from multiple loci. Since it estimates phylogeny in the process, no user-specified tree is required (Yang 2015). Despite the promise of automated species-delimitation algorithms, user interpretation is still required, disagreement between methods is frequent, and performance can vary (e.g., Luo *et al.* 2018). In a review of such methods, Carstens *et al.* (2013) recommended using as many species-delimitation methods as possible and preferring results that are congruent between methods. Pérez-Ponce de León *et al.* (2016) used the ABGD algorithm to analyse *cox*1 and ITS sequences of *Clinostomum* species from Central America. They interpreted the output of ABGD in the light of other data, mainly phylogenetic trees, and suggested that other lines of evidence (e.g., evidence of absence of gene flow, host association and biogeography) might also be used to support or modify the results from automated species-delimitation tools.

These methods have had few applications to date in studies on trematodes: only 15 reports have used at least one automated species-delimitation method to discriminate multiple species (Table 5). A problem that might affect studies on trematodes is sampling density (Ahrens *et al.* 2016; Phillips *et al.* 2019). Often, only one or a few sequences are available for a putative trematode species, and these may not reflect the true genetic diversity, especially over the geographic or host distribution. However, as sampling density increases and new DNA-sequencing approaches yield floods of data, species delimitation using algorithmic tools is likely to find broader use.

Table 5. Trematodes studied through single- and multiple-locus automated species delimitation methods

Taxon	Species delimitation method (s)	Reference
Allocreadiidae: *Margotrema*	GMYC	Martinez-Aquino *et al.* (2013)
Azygiidae: *Azygia*	ABGD, GMYC, bPTP	Vainutis *et al.* (2023)
Clinostomidae: *Clinostomum*	ABGD, Species Tree Ancestral Reconstruction	Pérez-Ponce de León *et al.* (2016)
Clinostomidae: *Clinostomum*	ABGD, Barcode Index Numbers (BINs)	Locke *et al.* (2015b)
Diplostomidae: *Diplostomum*	BPP	Blasco-Costa *et al.* (2014)
Diplostomidae: *Diplostomum*	ABGD, Barcode Index Numbers (BINs)	Locke *et al.* (2015a)
Diplostomidae: *Cotylurus*	GMYC	Pyrka *et al.* (2022)
Echinostomatidae: *Echinostoma*	Automatic partitioning (ASAP), GMYC, bPTP	Chomchoei *et al.* (2022)
Gorgoderidae: *Phyllodistomum*	BPP	Pinacho-Pinacho *et al.* (2021)
Gymnophallidae: *Parvatrema*	ASAP	Galaktionov *et al.* (2024a)
Lepocreadiidae: *Stegodexamene*	BPP	Herrmann *et al.* (2014)
Leucochloridiidae: *Leucochloridium*	ABGD, ASAP, bPTP, mPTP	Fernandez *et al.* (2024)
Notocotylidae: *Notocotylus*	ASAP	Gonchar and Galaktionov (2022)
Opecoelidae: *Podocotyle*	ASAP	Krupenko *et al.* (2024)
Renicolidae: *Renicola*	ASAP	Galaktionov *et al.* (2023)

Effective molecular sampling

An effective molecular sampling effort will be based on a survey of the range of the parasite's possible hosts and geographical distribution. Resulting samples should be studied by parallel or iterative morphological and molecular analysis. The latter might involve the sequencing of (possibly) multiple markers in sufficient depth to identify or distinguish species, establish host-specificity, characterise populations, enable life-cycle matching, and underpin phylogenetic analysis. How many morphological and molecular samples are needed to achieve all this is debatable and typically pragmatically limited by availability of material and funding. However, the importance of increasing sequencing effort emerges from meta-analyses that find sequencing effort (but not number of different markers, or marker type) to be predictive of cryptic diversity encountered (Blasco-Costa and Locke 2017; Pérez-Ponce de León and Poulin 2018; Poulin 2011). From these observations, it is difficult to derive practical advice except the obvious point that the more comprehensive the study, the better and more informative the results will be. It is sobering that cryptic species can occur over range, between hosts, and even in complete sympatry (same host, same locality). Their discovery, by definition, therefore, requires the sequencing of as many individuals as possible from as many host/parasite/locality combinations as possible. There is no reason to expect that co-occurring cryptic species will be present at equal prevalences and intensities. The burden of sequencing to identify all the cryptic diversity among trematodes is thus truly intimidating.

Molecular approaches: Additional confounding factors

Some problems with molecular data and analyses have already been mentioned above. Intra-individual sequence variation can be a problem and can arise in various ways. The presence of genetically different mitochondrial populations in an individual is termed 'heteroplasmy'. Intra-individual variation in an mt gene in several *Paragonimus* species is likely an example of this (van Herwerden *et al.* 2000), and it has also been detected in *Clonorchis sinensis* (see Kinkar *et al.* 2020). The significance of this for species delimitation in trematodes is unclear. Another cause of intra-individual variation in mt sequences is the presence of 'numts' (nuclear copies of mitochondrial genes). Primers used to amplify mt genes might also inadvertently amplify numts. These are often non-functional pseudogenes and are subject to mutational pressures different from those that act on their functional mitochondrial ancestors. Numts often have mutations (insertions/deletions and premature stop codons) that cause loss of their function. The extent to which numts occur in trematodes is not clear, but they have been demonstrated in cestodes (e.g., Brabec *et al.* 2012).

Numts are examples of paralogous genes: slightly diverged copies of the same ancestral gene, which have arisen through gene-duplication events in the past and now reside in different places in the genome. The nuclear genome itself contains many gene families, often containing numerous members derived by serial duplication of an ancestral gene. Paralogous genes can resemble each other enough that PCR with the same primers may, by chance, amplify one version from one sample and a paralogue from another sample, creating a false impression of genetic diversity. Phylogenies constructed using paralogous genes will produce a gene tree (showing the history of duplication events) rather than a species tree. Nuclear paralogues have been detected in several flatworms, including trematodes (Bae *et al.* 2016; Kim and Bae 2017; Labbunruang *et al.* 2016), but not in any of the genetic markers commonly used for species recognition. It seems that paralogous genes have not yet been positively identified as having caused misdirection in trematode taxonomy, but the problem may yet arise.

Another phenomenon that can cause problems in species recognition is hybridisation. Examples of recognised trematode hybrids are scarce and mainly limited to a few of the most intensively studied taxa, such as *Schistosoma* spp. (Agniwo *et al.* 2024; Ajakaye *et al.* 2024; Berger *et al.* 2022; Léger and Webster 2017), *Paragonimus* spp. (Doanh *et al.* 2013), and *Fasciola* spp. (Nguyen *et al.* 2018; Nukeri *et al.* 2022) (but see Caffara *et al.* 2019). Virtually nothing is known about the countless other trematode species not associated with human disease, but there is little doubt that other trematodes may hybridise when coinfection occurs within definitive hosts. As discussed above, the presence of multiple trematode congeners in individual vertebrate species is common and presumably establishes a basis for potential hybridisation and introgression (Platt *et al.* 2019).

Hybridisation may account for some of the extensive morphological variation detected in some trematode species across their distributions in regions with multiple congeners that utilise the same hosts. When it comes to DNA sequences, mixed sites in, for example, ribosomal ITS chromatograms (i.e., sites with two clear peaks at positions diagnostic of two species) may suggest the presence of a hybrid (Boon *et al.* 2018). In such cases, the mitochondrial genome and one of the nuclear ITS copies will have come from the maternal parental species. If only mitochondrial markers are used for species identification, then the frequency of hybridisation may be under-estimated. Researchers should remain aware of the possibility of hybrids, or of other sources of tree incongruence (e.g., incomplete lineage sorting, deep coalescence) when drawing taxonomic conclusions. To that end, a combination of nuclear and mitochondrial markers for species differentiation is essential, as is the use of as many such markers as possible and dedicated tools (e.g., phylogenetic networks) to allow detection of hybridisation from multiple gene trees (see Yu *et al.* 2012). Full characterisation of hybridisation is likely to require heavy sequencing replication (to detect hybrids and non-hybrids) and parallel study of hologenophore specimens to assess the morphological implications.

Molecular approaches in summary: The risks

Despite the remarkable progress that molecular data have enabled, the actual and potential challenges outlined above require vigilance. We think it unlikely that there is a single marker/gene/locus that can function as a magic bullet for the distinction of species (i.e., reliable distinction between species but lacking ambiguous intraspecific variation) or a level of variability that can be universally considered as a strict threshold (yardstick of a sort) for all trematode taxa. After all, like other organisms, trematodes evolve and speciate. The process is a continuum, and each species at any point of time is at a different stage of the process. This results in a variety of situations and ranges of variability between and across various trematode groups.

The current synthesis

The path forward

Although morphology of adult stages has been, and remains, the most important and commonly used basis for separation and description of species, trematodologists have always used a variety of data/character sources in delineation of species, including hosts, distribution, life histories, and morphology of larval stages. This general idea is in line with the term 'integrative taxonomy' as coined by Dayrat (2005) as an approach to delimit the units of life's diversity from multiple and complementary perspectives, including phylogeography, comparative morphology, population genetics, ecology, development, and behaviour. The idea is a relatively simple but compelling one – to use all the available data in formulating taxonomic hypotheses (Dayrat 2005; Schlick-Steiner *et al.* 2010). Arguably, trematode taxonomy has often been significantly 'integrative', given our instinctual considerations of morphology in the context of parasitological data in the form of host identity and geographical distribution. In practice, the modern idea of 'integrative' approach for our field really implies that we should add molecular data to the mix. Such combined studies are clearly generating the best work and enabling the strongest hypotheses presently possible. However, it must be remembered that the burden associated with the integrated approach is that frequently the data sources will conflict. As discussed above, every source of data presently used has the capacity to be either uninformative or misleading. Where all the evidence agrees, the integrative approach allows for the most compelling and least controversial hypotheses. It is where some elements of the evidence conflict that we are forced to propose hypotheses that are open to debate and require the accretion of further evidence. Conflict may be as mild as the issue of finding two congeners in a single host species or a surprisingly wide host distribution. More problematic are circumstances where the data from different molecular markers conflict or where the levels of distinction are marginal. In such circumstances, it remains our role to balance the competing sources of evidence and propose the best possible hypothesis.

We note that there may be situations where it is quite reasonable that we feel unable to assign formal names to lineages within an evolving complex. The parasites in question may not be a single homogeneous population, but the various subpopulations may seem insufficiently diverged to justify recognition as multiple distinct species. What should we do in such situations? It seems legitimate in such circumstances to refer to a species complex (complex species?), perhaps flagged by the use of 'sensu lato', which indicates that we do not know and need more data. Recognition of cryptic species will increasingly be the end-product of the process (if there is an endpoint), or a marker of progress so far in an ongoing process.

Principles

Our goal above has been to explore the issues that we face as we grapple with the recognition of trematode species. As a distillation, we recommend that we should acknowledge and bear in mind the following principles as we go about our work.

1. We must understand and acknowledge that our conclusions (especially difficult ones) are hypotheses which may well be rejected. This is not problematic because (a) when the facts change, our interpretations can change too, and (b) differing interpretations are to be encouraged.
2. We must appreciate that the available evidence relating to previous species records is likely to be inadequate relative to what is now achievable; we are engaged in the art of the possible and filling the gaps retrospectively.
3. Our work should consider the basis of our interpretation, and we should consider including an indication of our theoretical species concept and our operational criteria for species delimitation against that concept in difficult cases. We must understand that no species concept is beyond critique, nor can any set of operational criteria be usefully imposed to deal effectively with all sets of circumstances.
4. We should understand that every source of information is potentially problematic and capable of misleading towards either over- or under-estimation of richness.
5. We should accept that the probability of a reliable hypothesis, and its practicality, are enhanced by the amount and variety of data considered. Thus, the 'integrative' approach is recommended. A molecules-only hypothesis will be a barren one, and a morphology-only one will probably have errors. Hypotheses that consider molecular and morphological data in the context of biology, ecology, and biogeography of the parasites will give the most satisfying interpretations.
6. We should consider the use of one or more of the available species delimitation methods, using molecular data, to further validate our species hypothesis, although we acknowledge that these methods need further evaluation for use on trematode taxa.
7. We must expect (perhaps hope for) continued change in the nature of available data and its interpretation (especially molecular data). But we should not expect the arrival of a magic bullet able to resolve all our taxonomic challenges. We must always rely on careful thinking and evaluation of the lines of evidence when evaluating alternative hypotheses.
8. We should understand that good taxonomy of parasites is critical information for colleagues working in related fields. At the same time as the parasite is collected, information on the host needs to be collected, including material to enable molecular sequences to ensure accurate host identification. Information on infection of hosts by parasites, including absence of infection, is also important to collect and report, especially in the face of changing host and geographic distributions.

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