Molecular approaches to trematode systematics: 'best practice' and implications for future study

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Received: 13 November 2015 / Accepted: 9 January 2016

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Abstract To date, morphological analysis has been the cornerstone to trematode systematics. However, since the late-1980s we have seen an increased integration of genetic data to overcome problems encountered when morphological data are considered in isolation. Here, we provide

advice regarding the ‘best molecular practice’ for trematode taxonomy and systematic studies, in an attempt to help unify the field and provide a solid foundation to underpin future work. Emphasis is placed on defining the study goals and recommendations are made regarding sample preservation, extraction methods, and the submission of molecular vouchers. We advocate generating sequence data from all parasite species/host species/geographic location combinations and stress the importance of selecting two independently evolving loci (one ribosomal and one mitochondrial marker). We recommend that loci should be chosen to provide genetic variation suitable to address the question at hand and for which sufficient ‘useful’ comparative sequence data already exist. Quality control of the molecular data *via* using proof-reading Taq polymerase, sequencing PCR amplicons using both forward and reverse primers, ensuring that a minimum of 85% overlap exists when constructing consensus sequences, and checking electropherograms by eye is stressed. We advise that all genetic results are best interpreted using a holistic biological approach, which considers morphology, host identity, collection locality, and ecology. Finally, we consider what advances next-generation sequencing holds for trematode taxonomy and systematics.

Introduction

Morphological data have been the cornerstone to the study of the Trematoda Rudolphi, 1808 for more than two centuries. Indeed, the morphological delineation of trematode species remains highly effective, with thousands of recognised species successfully described by morphology alone. However, the distinction between intraspecific phenotypic plasticity and interspecific variation can become somewhat obscured in trematode genera which experience a larger degree of morphological stasis or in taxa that belong to species-rich genera. Even more challenging when using morphological data in isolation is the identification and matching of the life-cycle stages, with larvae typically bearing little resemblance to adults and having few, indistinct morphological features for diagnosis. The advent of the polymerase chain reaction (PCR) (Saiki et al., 1988) and readily accessible DNA sequencing technologies have provided trematode taxonomists with an independent source of data to help overcome the limitations associated with morphological analyses. Indeed, since the late-1980s and early 1990s (e.g. Blair & McManus, 1989; Luton et al., 1992; Adlard et al., 1993), there has been a dramatic increase in the use of molecular data in trematode taxonomy and systematics. Genetic data provide researchers with a tremendous capacity to recognise species, illuminate phenotypic plasticity, link life-cycle stages, infer phylogeny, and allow an exploration of the geographical variation far beyond that which

can be inferred from morphological data alone (Nolan & Cribb, 2005). In 2010 a fundamental paper was published, in which the authors provided an exceptional framework for the collection and preservation of trematodes from fishes for morphological studies (Cribb & Bray, 2010). Given the now routine use of molecular data in studies of trematodes, it is rather surprising that there are not similar guidelines for 'best molecular practice'. Despite a number of reviews on the topic, which highlight theoretical and methodological issues or drawbacks of molecular methodologies (see Nolan & Cribb, 2005; Olson & Tkach, 2005; Pérez-Ponce de León & Nadler, 2010; Nadler & Pérez-Ponce de León, 2011; Poulin, 2011), there has been no attempt to unify the discipline. Here, in an effort to coalesce molecular methods used in studies of trematode taxa (i.e. species, genera, families), we carry out a bibliographic search of the leading parasitology journals from 2011 to 2015 and summarise recent trends as they apply to the Trematoda. We discuss several aspects of generating and employing molecular data in these studies, emphasise best practises for such methodologies, and consider what advances next-generation sequencing (NGS) holds for trematode systematics.

Species concepts applied to trematodes in the molecular age

Molecular data provide a study with independent information, which can help unravel phenotypic plasticity and cryptic speciation among trematode taxa. Remarkably, however, genetic data have still proven to be rather subjective, with authors interpreting genetic variation in different ways. This is largely due to the fact that trematode taxonomists spend almost no time discussing species concepts. Indeed, searching "Trematod*/Digenea*" + "species concept" in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) reveals a complete lack of reference to the topic. This philosophy becomes important when we examine the more subtle boundaries of where to recognise distinction between closely related and cryptic species. Outside of trematode systematics, endless debate has surrounded species concepts and their implications for estimates of biodiversity (e.g. Agapow et al., 2004; Poulin, 2011). Presently, the most widely accepted model for parasitic helminths, including the Trematoda, is the Linnaean or morphological species concept (see Mayden, 1997 for a review) where groups of individuals are classified together based on distinctive morphological characters rather than genetic traits. Trematode taxonomists employ the Linnaean concept as a surrogate of the biological species concept (Mayr, 1942), but to a large extent, they also use components of the biological species concept to delineate species. For example, different forms living in sympatry are considered distinct species, implicitly assuming mechanisms exist to prevent interbreeding. The phylogenetic

species concept (Rosen, 1979; Donoghue, 1985) is also applied, where a species may be recognised as groups of individuals that form reciprocally monophyletic clades (e.g. Locke et al., 2010; Miller et al., 2010; Cutmore et al., 2014; Zikmundová, et al. 2015). The most inclusive conceptualisation considers species as "*separately evolving metapopulation lineages*" (De Queiroz, 2007). This concept incorporates properties relevant in the Linnaean, biological, and phylogenetic concepts as lines of evidence (operational criteria) for assessing the delineation of species. This framework emphasises an integrative approach to taxonomy (Dayrat, 2005; Will et al., 2005) rather than one based on singular criteria, and has already been successfully applied to recognise many trematode species (e.g. Georgieva et al., 2013, Razo-Mendivil et al., 2013; Georgieva et al., 2014 in association with Faltýnková et al., 2015; Blasco-Costa et al., 2014 in association with Faltýnková et al., 2014; Herrmann et al., 2014; Diaz et al., 2015). This approach is not completely new to parasite taxonomists, since ecological data (e.g. host specificity, geographical distribution, life-cycles) have always been taken into account to distinguish taxa. Rather the novelty lays on its conceptualisation as the sum of distinct lines of evidence. We recommend the adoption of this model because it provides a holistic approach to studies of trematode diversity.

Trends in trematode systematics

To better understand the use of molecular data in recent trematode taxonomic and systematic investigations, we data-mined literature published in major parasitology journals (i.e. *Acta Parasitologica*, *Folia Parasitologica*, *Helminthologia*, *International Journal for Parasitology*, *Journal of Helminthology*, *Journal of Parasitology*, *Parasitology*, *Parasitology International*, *Parasitology Research*, *Systematic Parasitology*, and *Zootaxa*). Each study was categorised by the inclusion/exclusion of genetic data, the markers used, and the focus of the study (taxonomy/systematics [i.e. studies that resulted in a taxonomic or systematic act], diversity [e.g. barcoding studies or cryptic species detection without taxonomic actions], life-cycles, population variation, species diagnosis (identification), and phylogenetic inference). Data were compiled from 252 studies from 2011–2015 (May/July). This period was chosen as it coincides with the last major reviews on molecular methods in parasite systematics (see Pérez-Ponce de León & Nadler, 2010; Nadler & Pérez-Ponce de León, 2011; Poulin, 2011). Overall, three major trends were detected.

(i) Since 2012, the proportion of studies that do not incorporate molecular data has

declined and the proportion using one or two molecular markers has increased. The number of studies using three or more markers has remained mostly constant (see Fig. 1).

(ii) Of the taxonomic/systematic investigations in the past five years, 45% did not use genetic data (thus the Linnaean or biological species concepts were used to propose new taxa), and around 20 and 15%, respectively, used data from one or two markers. In contrast, over 50% of studies exploring taxon diversity and 35% of studies matching life-cycle stages used sequence data from at least two markers. Studies inferring phylogenetic relationships predominantly used data from one or two markers (see Fig. 2).

(iii) The ribosomal RNA (rRNA) gene (18S-ITS1-5.8S-ITS2-28S) was mainly targeted (see Fig. 3), with the most variable markers (large subunit rDNA (LSU or 28S) and the internal transcribed spacers (ITS1 and ITS2) preferred. Of the mitochondrial genes analysed, the cytochrome *c* oxidase subunit 1 (*cox*1) was the most widely used, having higher relative importance in studies of diversity, species diagnosis and population variation. Remarkably, most taxonomy, diversity, life-cycle, and phylogenetic studies that generated mitochondrial DNA (mtDNA) sequence data also produced rDNA sequence data (data not shown). Other markers such as microsatellites (e.g. Reusch et al., 2004; Criscione et al., 2011) and 'inter-simple sequence repeats' (e.g. Robles-Pérez et al., 2015) were mainly applied to assess population variation and (rarely) to prospect for species diversity.

Clearly, the use of genetic data in trematode taxonomy and systematics has steadily increased since 2012 with researchers strongly reliant on just a handful of markers. We therefore consider it imperative to establish a steadfast framework for the use and integration of genetic data into studies of trematode diversity. This will underpin and enhance future investigations by ensuring that high quality data generated now can be reliably used in future studies.

'Best molecular practice in trematode systematics'

Study design

First and foremost, having a defined question or hypothesis in mind and understanding the scale of the study will help refine sampling strategy and focus the researcher on their choice of marker(s). This notion may seem unusual in trematode taxonomy, as most of the thousands of existing species were probably discovered as a consequence of a 'pure chance' finding (e.g. Nolan & Cribb, 2006). However, redundant sampling, in which multiple specimens of each species are collected from as many host-locality combinations as possible, should be the goal.

The number of specimens sequenced is particularly important if cryptic species are suspected (see Poulin, 2011). Generally, we recommend that three to five sequences of the most variable marker be generated for each host-parasite-locality (HPL) combination being reported.

Sample preservation

Collection methods and fixation strategies for preserving specimens for both molecular and morphological assessment have been discussed in-depth by Cribb & Bray (2010) and Justine et al. (2012). When preserving specimens for future molecular investigation, we strongly recommend using 95–100% ethanol. Concentrations < 90% may be sufficient to preserve material when targeting rDNA and mtDNA with hundreds of copies per cell; however, it may render samples unusable for future amplification of single copy markers or for the application of novel DNA technologies that require well-preserved, high quality DNA. Alternatively, aqueous, nontoxic, tissue storage reagents such as RNA*later*® (Ambion ™, Austin, USA) provide the opportunity to store samples at 4°C for weeks or at -20°C indefinitely, preserving both DNA and RNA, without jeopardising the quality or quantity of the molecular material obtained after subsequent isolation. Let us honour the advice of Dr. D. Timothy J. Littlewood (pers. comm., 30 Sept. 2015) "When you collect and fix material today, think of what you would like to do with it tomorrow".

Voucher specimens

Typically, when specimens are abundant, total genomic DNA (gDNA) has been extracted from whole individual worms and the subsequently generated sequence data submitted to a public database such as the GenBank (http://www.ncbi.nlm.nih.gov/genbank/). This now standard practice has led to the recommendation that a link between genetic data and morphological vouchers be established. Definitions for different types of vouchers have been proposed by Pleijel et al. (2008) and Astrin et al. (2013). We strongly recommend the deposition of morphological vouchers, at least one hologenophore (i.e. a piece of the organism used for the generation of molecular data, mounted in a preparation or preserved in a way that can be used for morphological identification) and one paragenophore (i.e. an individual organism collected from the same host, at the same time and place as the organism used for the generation of molecular data, and identified by the author as belonging to the same taxon) for descriptions of new species, together with additional molecular vouchers (specimens, or parts thereof, preserved in

95–100% ethanol or a fixative that allows gDNA preservation; and/or extracted gDNA) to natural history collections that possess biobank repositories (e.g. DNA Bank Network, http://www.dnabank-network.org/ or Global Genome Biodiversity Network, http://ggbn.org/). Biobank repositories have policies to ensure the best curation and use of such vouchers. In publications, clear reference should be made to accession and voucher numbers to provide a connection for morphological vouchers from which sequences were generated (stating museum collection codes, preservation status, etc.) and additional molecular vouchers deposited in Biobank repositories. The deposition of morphological vouchers of the molecular samples cross-referenced with molecular vouchers will permit future taxonomic verification and the generation of new sequence data and/or the use of new molecular technologies for existing species. This issue is pertinent even if morphological characterisation is not the goal of the study (e.g. barcoding or molecular ecology studies), since GenBank is plagued by misidentified sequences (Valkiūnas et al., 2008; Georgieva et al., 2014). The scientific community is just starting to comprehend the importance of vouchering molecular samples. Despite this, authors are still rather idiosyncratically reluctant to share their specimens or make data publicly available unless specific journal policies enforce it. Thus, we urge authors, editors, and reviewers to consider the deposition of morphological vouchers of molecular samples and cross-referenced molecular vouchers a priority.

DNA extraction

A variety of methods are available for the extraction of high quality gDNA from single specimens, specimen fragments, and even cercariae or eggs (Nolan & Cribb, 2004; Cribb et al., 2011; O'Dwyer et al., 2014). If large numbers of samples are to be processed with little downstream application, we recommend the use of quick and inexpensive methods, such as Chelex and Proteinase K (e.g. Keeney et al., 2007) or guanidine thiocyanate lysis buffer (Tkach & Pawlowski, 1999). However, if DNA purity is essential for downstream processing (e.g. AFLP-PCR or NGS), classic phenol-chloroform (or similar) protocols (e.g. Sambrook & Russell, 2001) or commercial nucleic acid extraction kits should be used.

Choice of marker

The biggest issues when using molecular methods in trematode taxonomic studies are (i) which and how many markers should be used; and (ii) how should they be analysed? First, one must

consider ‘What are the disadvantages of the chosen gene(s)?’ As different genes evolve at dissimilar rates, selecting the optimal genetic marker requires careful consideration of the aim of the study. All genes accumulate mutations over time but some experience higher rates of genetic change (i.e. those unconstrained by function), while others accumulate fewer mutations (i.e. those encoding for particular biological functions). The challenge is to achieve a balance. A chosen gene must exhibit sufficient variation to discriminate closely related taxa but not so much as to hinder robust taxonomic/systematic/phylogenetic inferences. Typically, the most suitable markers are selected based on prior knowledge for the family of trematodes being studied and are those that exhibit > 70% but less than 100% similarity (Hillis & Dixon, 1991).

Ideally, molecular conclusions should be based on genetic data from independent loci. Unfortunately, in the case of trematodes it is restricted to two truly independent loci since all rDNA and all mtDNA markers are linked. The compromise between how many markers, from how many HPL combinations, and how many individual specimens for lower-level systematic or taxonomic studies [i.e. at the level of species (or within), intra-generic, or between closely related genera] could be prioritised as follows:

(i) Sequences of the most variable marker (allowing to detect intraspecific polymorphisms), Marker A, should be generated from three to five specimens from as many HPL combinations as possible. The more replicates in a study, the greater the confidence in the results. This is particularly important when the differences between/among species are few.

(ii) A second, more conserved gene (that displays interspecific variation only), Marker B, should be sequenced from those individuals with the greatest degree of sequence divergence at Marker A.

Studies focusing on higher-level systematics (at the suprageneric level) should prioritise markers spanning variation from the lowest taxonomic unit (e.g. genus) to higher ones (e.g. family) and obtain representative molecular data for all markers for each taxonomic unit included in the study.

From the data-mined literature encompassing the last five years, the nuclear rRNA gene and the mitochondrial *cox*1 gene were shown to be the most popular choices for the molecular investigation of trematodes at low taxonomic levels, while rRNA gene (i.e. 28S and ITS) dominated phylogenetic studies in general. The major attractions to rDNA are undoubtedly the considerable volume of data already available in public databases for comparative purposes and its easy amplification with 'universal' primers that have been employed for over 25 years. It is clear to us that the more one or the same markers are used, the more valuable they become *in toto*. Hence, if a new gene that perfectly distinguished species every time (with no issues of variation) is identified in the future, would it be practical to switch to using it in isolation, and

abandon the makers that have been used to date? In our opinion, the answer is definitively no. However, this new marker should undoubtedly augment the existing molecular 'toolbox' for circumscribing trematode species.

Overall, we recommend the combined use of both ribosomal and mitochondrial genes, with the specific choice based on (i) what gene markers are already available online for the group in question; and (ii) what markers are informative to the specific questions being investigated. We suggest exploring variation at the intraspecific level with a mitochondrial gene and to use a nuclear variable region such as the ITS (the entire ITS1-5.8S-ITS2 region or a transcribed spacer in isolation) or 28S for corroborating interspecific variation for taxonomic and low level systematic studies. Unifying the choice of markers will be advantageous in two ways. First, it increases the available sequence data for all trematode taxa sequenced in future studies. Secondly, it is important that the existence of divergent lineages in a mitochondrial gene are corroborated by divergence at a nuclear locus, to clarify whether those mtDNA lineages reflect haplotype variation at the population level (especially if just few specimens of a species have been sequenced from distant geographic locations) or that independent historical lineages should be recognised as distinct species.

Ribosomal loci

Ribosomal RNA (rRNA) genes and the associated spacer regions consist of hundreds of tandemly repeated copies in the nuclear genome of eukaryotic cells. This region is highly informative for taxonomic investigations, because the entire repeat unit is thought to evolve in a concerted fashion (but see point v in the sequencing section below). New variants arising by random genetic drift or natural selection within a species become homogenised and fixed (Morgan & Blair, 1995), making sequence data representing an individual characteristic of an interbreeding group. This region is particularly useful in that it contains loci with differing rates of evolution, making it possible to answer a variety of systematic questions (Nolan & Cribb, 2005).

The small subunit rRNA gene (SSU or 18S) is amongst the slowest evolving gene regions in living organisms. We recommend its use to infer deep phylogenetic relationships (i.e. between families and above) (Hillis & Davis, 1986). The 28S rRNA gene shows a faster rate of evolution at the variable domains than 18S. However, it also possesses conserved regions, similar to those of 18S (Hillis & Dixon, 1991). Although both markers were initially used to define species/genus-level boundaries (e.g. Kaukas & Rollinson, 1997; León-Regagnon & Paredes, 2002), only the partial fragment (including D1-D4) of 28S rDNA is still used for this

purpose (e.g. Hildebrand et al., 2015; Pérez-Ponce de León et al., 2015). The two internal transcribed spacers, ITS1 and ITS2, have been extensively shown to be particularly effective, either separately or in combination, in the taxonomic investigation of trematode species (see Nolan & Cribb, 2005). Thus, we recommend the inclusion of at least one conserved region (18S or 28S rDNA) and one spacer (ITS1, ITS2, or the entire ITS1-5.8S-ITS2 region) for all new species descriptions. This will offer a comprehensive database for future delineative studies.

Mitochondrial loci

Mitochondrial DNA, located within each mitochondrial organelle of trematodes, is maternally inherited and forms a double-stranded circular structure that includes two ribosomal (*rrnS* and *rrnL*), 12 protein-coding (*atp8* is absent), and 22 transfer RNA (*trn*) genes (Le et al., 2002; but see Jannotti-Passos et al., 2001). The use of mtDNA markers holds several advantages over nuclear ones. First, the rate of mutation is higher than that observed for nuclear genes (Brown et al., 1979), which generates genetic variation at both population and species levels (Avise et al., 1987). Secondly, intraspecific nucleotide polymorphism is considered to be effectively neutral (Gerber et al., 2001). Thirdly, the effective population size (N_e) is one-quarter that of diploid nuclear loci in allogamous species, or one-half in hermaphroditic species that may reproduce by autogamy. Thus, haplotype frequencies can drift quickly, creating genetic differences between populations in a short time (Criscione & Blouin, 2005; Criscione et al., 2005). Finally, due to the absence of recombination, any two haplotypes in the population will 'coalesce' more rapidly into the most common recent ancestor so that monophyly will be achieved faster than for nuclear (autosomal) genes (Kingman, 1982a, b).

The *cox*1 is a highly variable protein-coding gene in bilaterians and is widely used for molecular systematics of animals (Avise, 2000). Versatile primers, developed to amplify the barcode region (*c.*600 nt) of metazoans (Folmer et al., 1994), have recently been redesigned for trematodes (Moszczynska et al., 2009; Van Steenkiste et al., 2015) facilitating its use across a range of trematode families. Alternative primers amplifying a larger fragment covering the same region also exist for some trematode groups (e.g. Miura et al., 2005; Králová-Hromadová et al., 2008; Herrmann et al., 2014). Similar to ITS of the rDNA, *cox*1 is less suited to large phylogenetic reconstructions (especially if the barcoding fragment is used) or comparisons among distantly related taxa because of the likelihood of encountering substitution saturation (i.e. when multiple substitutions have occurred at the same nucleotide codon position, such that it is not possible to accurately estimate sequence divergence).

Other mtDNA markers have been used in studies of specific trematode families,

including the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) gene (e.g. Bray et al., 1999, 2009; Detwiler et al., 2010; Curran et al., 2013; Georgieva et al., 2014; Selbach et al., 2014), mitochondrial large subunit rRNA (*rrnL* or 16S rrn) gene (e.g. Li et al., 2010; Leung et al., 2009; Herrmann et al., 2014) and cytochrome *b* (*cytb*) gene (e.g. Zhao et al., 2012). We recommend these be used to complement the most widely used markers (e.g. ITS and *cox*1) mentioned above, rather than in isolation, due to the current lack of comparative data from mt markers in general for most trematode families.

Sequencing

Sanger sequencing, a method that selectively incorporates chain-terminating dideoxynucleotides by *Taq* polymerase during *in-vitro* DNA replication (Sanger et al., 1977), remains the preferred technology for most trematode studies using genetic data. Ensuring sequence quality is imperative since it is impossible to control it after the data are published. Thus, for the generation of reliable and publishable sequence data:

(i) Taq polymerase with 'proof-reading' activity should be chosen.

(ii) PCR amplicons must be sequenced using both forward and reverse primers.

(iii) Electropherograms must overlap by at least 85% when creating consensus sequences.

(iv) Electropherograms should be checked by eye. Automated base-calling programs can incorporate miscalled/artifactual bases (e.g. 'dye blobs') or overlook bases.

(v) Presence of double peaks at certain nucleotide positions in electropherograms should be reported in the sequences using the appropriate IUPAC code. Such intra-individual or intra-genomic sequence polymorphisms (e.g. in ribosomal gene regions), although generally not anticipated, should not be dismissed as uninformative noise. Ideally, cloning the PCR product and sequencing a minimum of 10 colonies containing the incorporated DNA fragment will provide an indication of the most frequent sequence (and nucleotide at the variable positions) among the various rDNA repeats in an individual. We suspect this phenomenon is more common than generally reported but evidence of this exists for only a few trematode taxa (e.g. Mone et al., 2012; Diaz et al., 2015).

As mentioned above, we advocate strongly for replication of sequencing for as many HPL combinations as possible, which also provides extra 'proof-reading' checks for ensuring the most reliable and highest quality DNA sequence data are published. In this context, we also recommend that authors provide links to the electropherogram data used to generate the published sequence data. Ideally, this will allow for detailed and transparent comparative analysis of putative intraspecific variation, signs of hybridisation and exploration of cryptic

species complexes that basic alphanumeric sequence data alone cannot provide. While this may not be practical in many circumstances, we suggest authors strive to make these data available to the wider community through databases for these types of data such as BOLD (www.barcodinglife.com) or as online supplementary data.

When Sanger sequencing mitochondrial genes, an added confounding factor may be the amplification and sequencing of pseudogenes. The presence of nuclear mitochondrial paralogs (pseudogenes or numts, an abbreviation for "nuclear mitochondrial DNA"; Richly & Leister, 2004) can be co-amplified with the mitochondrial locus, or even be the only sequence amplified in some cases. If these are not recognised it can lead to an overestimation of the number of species (Song et al., 2008). Pseudogene sequences can be discarded *a posteriori* by identifying the presence of stop codons or an excess of non-synonymous mutations, but some numts may remain undetected. The best solution is to avoid amplifying numts through methods such as pre-PCR dilution (Calvignac et al., 2011).

Interpretation

DNA taxonomy should not be considered a substitute for morphology-based interpretations. Rather, genetic sequence data are best utilised when augmenting total biological information (i.e. host, locality, ecology, and morphology). Theoretically, distinguishing species in sympatry is generally uncomplicated. However, it is in allopatry where smallish sequence differences turn up that problems may occur. Our recommended approach to this is that if the differences in allopatry are as great as those seen between comparable combinations of species in sympatry, then specimens likely represent different species (e.g. *Transversotrema polynesiae* Cribb, Adlard, Bray, Sasal & Cutmore, 2014; see Cribb et al., 2014). On the other hand, if the allopatric differences are less than those seen in sympatry, then it is geographical (or inter-host) variation (e.g. allopatric populations of *Transversotrema gigantica* Hunter, Ingram, Adlard, Bray & Cribb, 2010 off Heron and Ningaloo Islands; see Hunter et al., 2010). Unfortunately, there is no general genetic yardstick (i.e. a set level of base differences) that can be used to delineate species. The degree of genetic divergence will depend on the taxonomic group, the gene region analysed, and the geographical relationships of the samples, to name just a few considerations. Thus, the independent source of data that molecular information brings to an investigation should be interpreted in a holistic biological and ecological context as it has (generally) since the late-1980s.

The next step

Revolutionary advances in NGS technologies offer opportunities to explore many aspects linked to the fundamental biology of parasites on a scale that was previously unimaginable. Sequencing whole mitochondrial genomes is possibly the next step beyond traditional DNA barcoding. Comparative mitogenomic studies of closely related species may allow the discovery of more informative mitochondrial genes and better diagnostic markers, and have the potential to provide better nodal support for phylogenetic relationship inference. Recent studies on the phylogenetic signal and genetic variability of mitochondrial genes for species of *Diplostomum* von Nordmann, 1832, *Fasciola* Linnaeus, 1758 and *Schistosoma* Weinland, 1858 suggest that *cox*1 is not the most suitable marker for accurate species identification (Zarowlecki et al., 2007; Liu et al., 2014; Brabec et al., 2015). Instead, *cox*3, *nad*4, *nad*5, or *nad*6 have been identified as better markers for species diagnosis. Sequencing the mitochondrial genomes of key trematode taxa has the potential to provide new insights into taxonomy, systematics and evolutionary history, not only within selected families but also across the Trematoda as a whole.

Comparative nuclear genomics also offer the promise of finding additional independent loci that may capture genetic variation at different scales to be used in combination with already well-established ribosomal and mitochondrial genes. Introns of single-copy genes are ideal for fine-scale studies (population or intraspecific level variation) since they have little structural constraint whereas neighbouring exons are typically conserved, making it possible to design PCR primers. Such markers, often called EPIC (exon-primed intron-crossing) can be defined with increasing ease now that complete genome sequences are available for several trematode species and other closely related platyhelminths. However, the expertise to make the most of these new datasets may lie beyond the grasp of most trematode researchers. Moving forward would require, perhaps more than ever, collaborative projects and consortiums between geneticists, bioinformaticians, and parasitologists. Certainly, such combined efforts would advance the field of molecular systematics in this group and provide stronger molecular tools that may help compensate for the shortage of professional taxonomists (Brooks & Hoberg, 2001).

Acknowledgements We thank Associate Professor Thomas Cribb for organising the workshop ‘The biodiversity of trematodes of fishes’ at the 9th International Symposium on Fish Parasites and sharing his opinion on this topic. We are also grateful to two anonymous reviewers and the Editor-in-Chief for their very constructive comments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Figure legends

Fig. 1 Five-year trends in the use of genetic data in 252 investigations of the Trematoda published in 11 major parasitology journals from 2011 to 2015

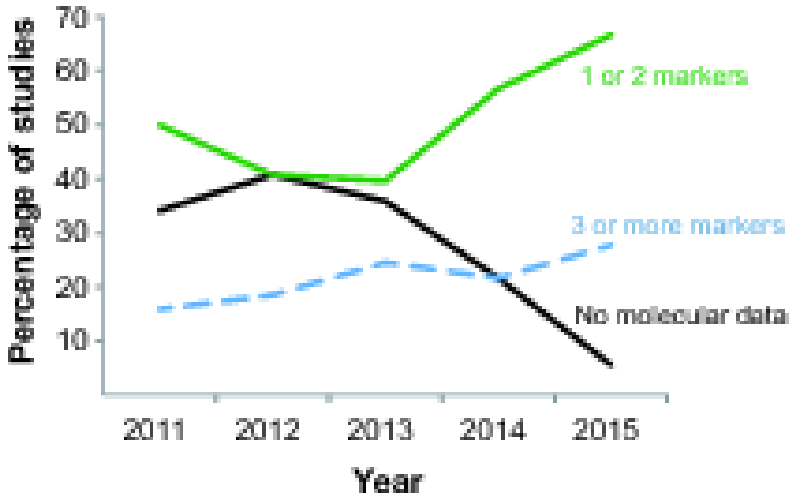

Fig. 2 The percentage of studies on taxonomy/systematics, diversity, life-cycles, population variation, species diagnosis (identification), and phylogenetic inference investigations of trematodes taxa using 1, 2, 3, 4, 5 or > 5 molecular markers

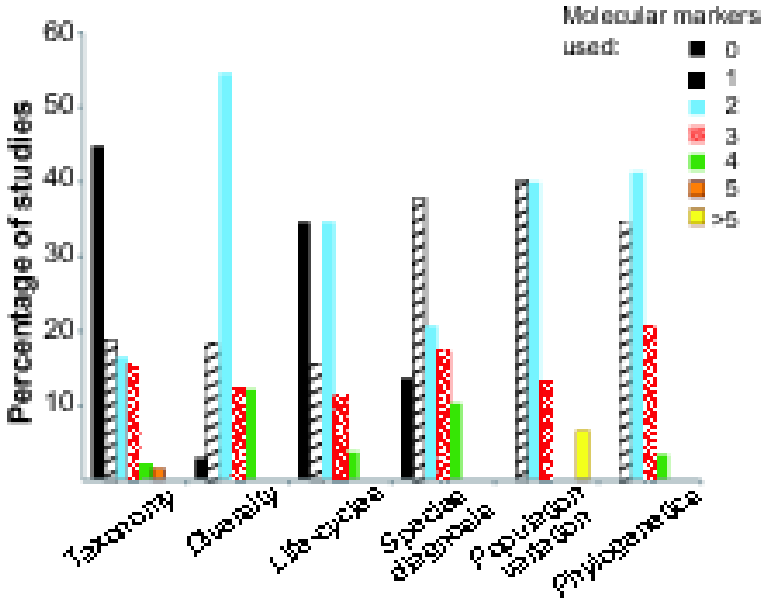

Fig. 3 The percentage of studies on taxonomy/systematics, diversity, life-cycles, population variation, species diagnosis (identification), and phylogenetic inference investigations using each of the most common genetic markers. *Abbreviations*: 18S, small subunit ribosomal RNA gene; ITS, internal transcribed spacers 1 or 2, or the entire ITS1-5.8S-ITS2 region; 28S, large subunit ribosomal RNA gene; mt, mitochondrial; *cox*1, cytochrome *c* oxidase subunit 1 gene