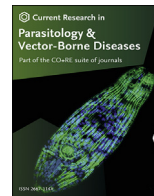


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## What lies behind the curtain: Cryptic diversity in helminth parasites of human and veterinary importance



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### ABSTRACT

Parasite cryptic species are morphologically indistinguishable but genetically distinct organisms, leading to taxa with unclear species boundaries. Speciation mechanisms such as cospeciation, host colonization, taxon pulse, and oscillation may lead to the emergence of cryptic species, influencing host-parasite interactions, parasite ecology, distribution, and biodiversity. The study of cryptic species diversity in helminth parasites of human and veterinary importance has gained relevance, since their distribution may affect clinical and epidemiological features such as pathogenicity, virulence, drug resistance and susceptibility, mortality, and morbidity, ultimately affecting patient management, course, and outcome of treatment. At the same time, the need for recognition of cryptic species diversity has implied a transition from morphological to molecular diagnostic methods, which are becoming more available and accessible in parasitology. Here, we discuss the general approaches for cryptic species delineation and summarize some examples found in nematodes, trematodes and cestodes of medical and veterinary importance, along with the clinical implications of their taxonomic status. Lastly, we highlight the need for the correct interpretation of molecular information, and the correct use of definitions when reporting or describing new cryptic species in parasitology, since molecular and morphological data should be integrated whenever possible.


### 1. Introduction

Parasites represent a large percentage of global biodiversity, distributed virtually in all vertebrate species and geographical areas of the world (Nadler & Pérez-Ponce de León, 2011). Parasites are a burden for approximately a billion humans suffering from neglected tropical diseases and billions of companion and production animals, as well as wildlife (Fenwick, 2012; Ondrejicka et al., 2014). Classification of parasites into nominal species is an essential task for biodiversity assessment, restoration ecology, conservation biology and for improving the understanding of host biogeography and parasite evolution. Nominal species are distinct genetic and morphological clusters separated from other taxa by speciation or separately evolving metapopulation lineages (De Queiroz, 2007; Shapiro et al., 2016) (Fig. 1). This evolutionary process in parasites is driven by multiple factors, including host colonization, taxon pulse, ecological fitting, host speciation, host population genetics, human intervention, landscape changes or parasite spillover (Hoberg & Brooks, 2008, 2010, 2013; Thompson, 2013).

Historically, taxonomists largely relied on morphological, ecological, evolutionary, phenetic, and phylogenetic features to classify organisms (i.e. taxa), or a combination of two or more of these features (Aldhebiani, 2018) (Fig. 1). Scientific advances, especially the use of molecular biology tools, allowed the reassessment of many of these nominal species, revealing that many in fact contained two or more distinct taxa. These separate entities are referred to as cryptic species (Knowlton, 1993), which are morphologically identical but genetically divergent (Daly et al., 2021). Cryptic species may be the result of host-parasite coevolutionary events, as well as host-independent events (Xavier et al., 2015). However, cryptic parasite diversity is much more complex, and several definitions have derived from this concept. For instance, cryptic species *sensu stricto* (s.s.) are delimited when there is no morphological differentiation of the cryptic organism when compared to reference specimens, but molecular analysis reveals significant divergence (Fig. 2). Cryptic species *sensu lato* (s.l.) are reported when molecular analyses reveal unexpectedly high genetic divergence when compared to other genetic sequences, while morphological differences have not yet been verified.

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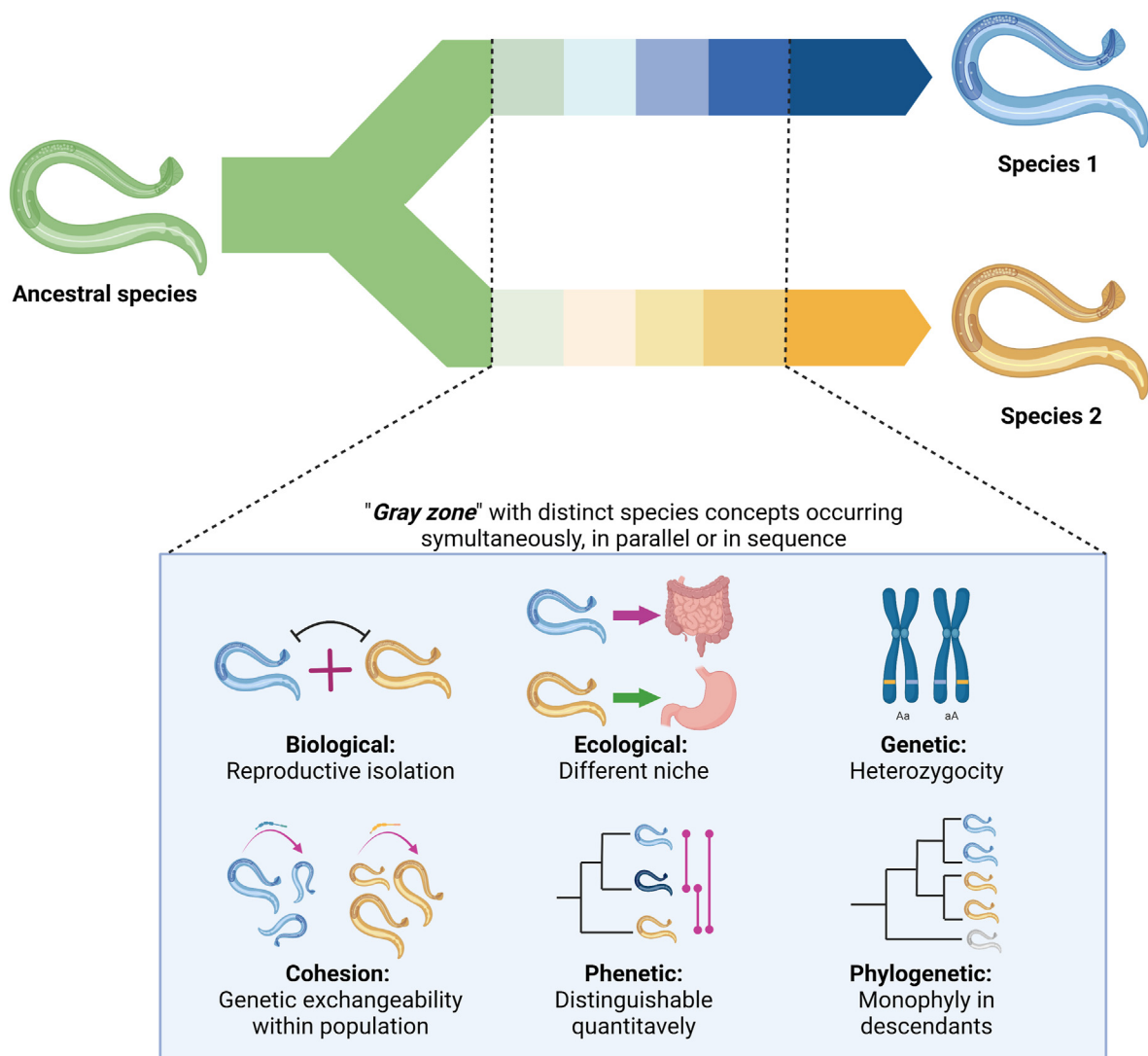
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**Fig. 1** Graphical representation of species concepts adapted from De Queiroz (2007), depicting an ancestor and its descendants sharing derived character states (Rosen, 1979; Donoghue, 1985; Mishler, 1985). One parasitic lineage (ancestral species indicated in green) originates two separate lineages (Species 1 and Species 2), where all three are reciprocally exclusive. The color gradient illustrates different species concepts (non-exhaustive: biological, ecological, genetic, cohesion, phenetic or phylogenetic) occurring simultaneously, in parallel or sequentially that lead to speciation. This figure was created using BioRender.com.

Furthermore, putative cryptic species *sensu stricto/sensu lato* may apply when genetic speciation is suggested by data, but there is lack of convincing evidence (Fig. 2). Additionally, cryptic genetically isolated units (CGIs) represent a category of cryptic species which are reproductively isolated but may potentially interbreed due to host range expansion, as well as the disappearance of a geographical barrier (Chenuil et al., 2019). However, definition of CGIs involves experimental data to test fertilization between the nominal and cryptic species, and fitness of their offspring, therefore, is a term less used for parasitic helminths due to their complex biology.

Delimitation of cryptic species of parasites has changed in the last decade to an almost all-molecular approach (Fišer et al., 2018) with implications in health sciences and clinical practice, since this knowledge may lead to a better understanding of parasite epidemiology, and implementation of diagnostics, control and prevention (Nadler & Pérez-Ponce de León, 2011). In addition, cryptic species can vary in pathogenicity, resulting in a different course of infection and outcome, as suggested for the protozoan *Tetratrichomonas gallinarum* (Cepicka et al., 2005; Nadler & Pérez-Ponce de León, 2011), but less understood for helminths. Furthermore, cryptic species may also be geographically segregated, leading to epidemiological implications for parasite control

and prevention, such as the case of the foodborne trematode *Opisthorchis viverrini* (Saijuntha et al., 2007; Nadler & Pérez-Ponce de León, 2011). Another relevant feature of cryptic species is how they are distributed among higher-level parasite taxa. In this sense, considering helminths, cryptic species are predominantly found in trematodes, followed by cestodes, and to a lesser extent in nematodes (Pérez-Ponce de León & Poulin, 2018). Lastly, other related features yet to be understood for helminth cryptic species are as follows: differences in virulence; applicability of molecular diagnostics for cryptic entities; drug susceptibility and resistance of the cryptic species; and their associated mortality and morbidity on host populations (Sithithaworn et al., 2015). Due to all these unsolved questions, parasitologists have advised the recognition of cryptic diversity as a medical priority (Sithithaworn et al., 2015; Pérez-Ponce de León & Nadler, 2016).

In this review, we focus on speciation mechanisms previously described for helminth parasites, molecular approaches towards delimitation of cryptic helminth species, and the usefulness of various molecular markers and approaches for such a purpose (see Table 1). Finally, we discuss some of the most fascinating examples found in parasitic nematodes, trematodes and cestodes of human and veterinary importance that have implications for both public health and animal production systems.

**Table 1**  
Summary of cryptic diversity reported in the literature for nematodes, trematodes and cestodes of human and veterinary importance

Species	Host	Origin	Studied stage	Molecular markers employed <sup>a</sup>	Proposed status	Reference
Phylum Nematoda						
<i>Ascaris lumbricoides</i> , <i>Ascaris suum</i>	Humans	Kenia	Adults	Mitogenome (4 × ratio < 4), <i>cox1</i> (1–4 nucleotide differences), <i>nad4</i> , nuclear genome (1% polymorphism in major nuclear alleles)	CGIs	Easton et al. (2020)
<i>Toxocara cati</i> , <i>Toxocara malaysiensis</i> <sup>b</sup>	Cats	Malaysia	Adults	Mitogenome	Cryptic species	Jex et al. (2008)
<i>Toxascaris leonina</i> species complex (3 undescribed species)	Dogs, wolves, wild felids, red foxes	Poland	Adults	ITS1, <i>cox1</i> (1.7–6.58% K2P), <i>nad1</i>	Cryptic species complex	Fogt-Wyrwas et al. (2019)
<i>Strongyloides</i> spp. (undescribed species)	Philippine slow loris/humans and dogs	Malaysian Borneo/Australia, Cambodia, Japan, and Myanmar	Third-stage larvae	<i>cox1</i> /18S and <i>cox1</i>	Cryptic species <i>sensu lato</i>	Frias et al. (2018)/Beknazarova et al. (2019); Jaleta et al. (2017); Nagayasu et al. (2017)
<i>Trichinella chanchalensis</i>	Wolverine	Canada	Larval stage not specified	<i>cytb</i> , mitogenome, 15 SCNs	Cryptic species <i>sensu stricto</i>	Sharma et al. (2020)
<i>Dirofilaria</i> sp. “Thailand II”/ <i>Dirofilaria</i> sp. ( <i>D. immitis</i> -like) (2 undescribed species)	Carnivores/humans	Thailand	Adults	ITS1 (17%)/12S (5%), <i>cox1</i> (6%)	Cryptic species <i>sensu lato</i>	Yilmaz et al. (2016)
<i>Onchocerca</i> sp. (1 undescribed species)	Cervids	North America	Microfilariae and adult females	12S, 16S, <i>cox1</i>	Cryptic species <i>sensu lato</i>	McFrederick et al. (2013); Verocai et al. (2018)
<i>Oesophagostomum</i> sp. (undescribed species)	Human and non-human primates	Uganda	Eggs	ITS2: 2.9% Clade I vs <i>O. bifurcum</i> ; 0.6% Clade II vs <i>O. stephanostomum</i> ; Clade III 7.0–7.6% vs <i>O. bifurcum</i> and 6.4–7.0% vs <i>O. stephanostomum</i>	Cryptic species <i>sensu stricto</i>	Ghai et al. (2014)
<i>Teladorsagia boreoarticus</i> <sup>b</sup>	Sheep and goats	Holarctic	Adults	<i>nad4</i> (13%)	Cryptic species	Hoberg et al. (1999)
Class Trematoda						
<i>Opisthorchis viverrini</i>	Cyprinid fish (2nd intermediate hosts) and rodents	Thailand, Laos PDR	Adults	38 enzyme loci (MEE) (60%)	Cryptic species <i>sensu lato</i>	Saijuntha et al. (2007)
<i>Echinostoma</i> “ <i>revolutum</i> ” species complex (7 described species and 10 cryptic species-level lineages)	Gastropods (1st intermediate hosts), mammals and birds	Europe, North America, South America, Africa, Australia, New Zealand	Cercariae and adults	<i>nad1</i> (intraspecific divergence: 0–3.6%; interspecific divergence: 4.2–21.5%)	Cryptic species complex	Georgieva et al. (2014)
Class Cestoda						
<i>Echinococcus granulosus</i> (s.l.) species complex ( <i>E. granulosus</i> (s.s.), <i>Echinococcus equinus</i> , <i>Echinococcus ortleppi</i> , <i>Echinococcus canadensis</i> , <i>Echinococcus felidis</i> )	Sheep, dogs, dogs, reindeer/moose, and lion, respectively	Germany, UK, South Africa, Canada and South Africa, respectively	Eggs	<i>E. felidis</i> vs <i>E. granulosus</i> (s.s.), <i>E. equinus</i> , <i>E. ortleppi</i> and <i>E. canadensis</i> G6, G7 and G8, respectively: <i>cox1</i> (8.4%, 8.1%, 10.6%, 10.6%, 10.5%, 11.1%); <i>nad1</i> (17.1%, 16.7%, 18.4%, 17.9%, 17.9%, 19.3%); <i>cytb</i> (11.6%, 12.4%, 14.8%, 14.8%, 15.0%, 15.0%); mitochondrial <i>rnr</i> (6.6%, 7.6%, 8.9%, 9.2%, 9.2%, 8.6%); <i>efl-α</i> (1.4%, NA, 0.9%, 1.0%, 1.0%, 0.9%)	Species complex	Hüttner et al. (2008); Romig et al. (2015)
<i>Moniezia benedeni</i> , <i>Moniezia expansa</i>	Sheep and cattle	Australia	Adults	15 enzyme loci (MEE) (92% within <i>M. benedeni</i> , 33% within <i>M. expansa</i> )	Cryptic lineages within groups	Chilton et al. (2007)

Abbreviation: NA, not available.

<sup>a</sup> Genetic divergence in parentheses.

<sup>b</sup> Despite described as cryptic in the literature, the species possesses a valid morphological diagnosis.

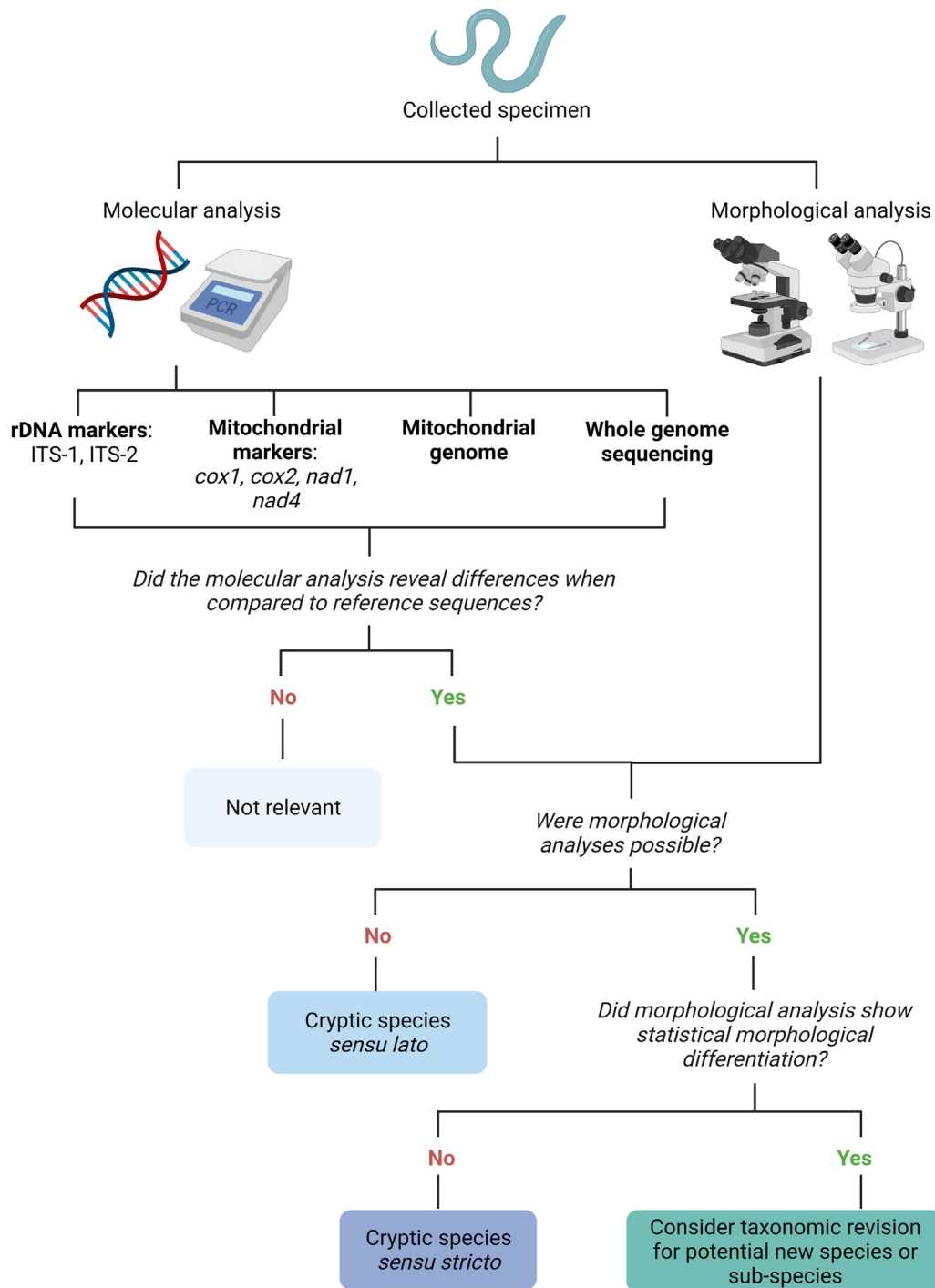


Fig. 2 Recommended general flowchart for cryptic species definition for a collected specimen. This figure was created using [BioRender.com](https://www.biorender.com).

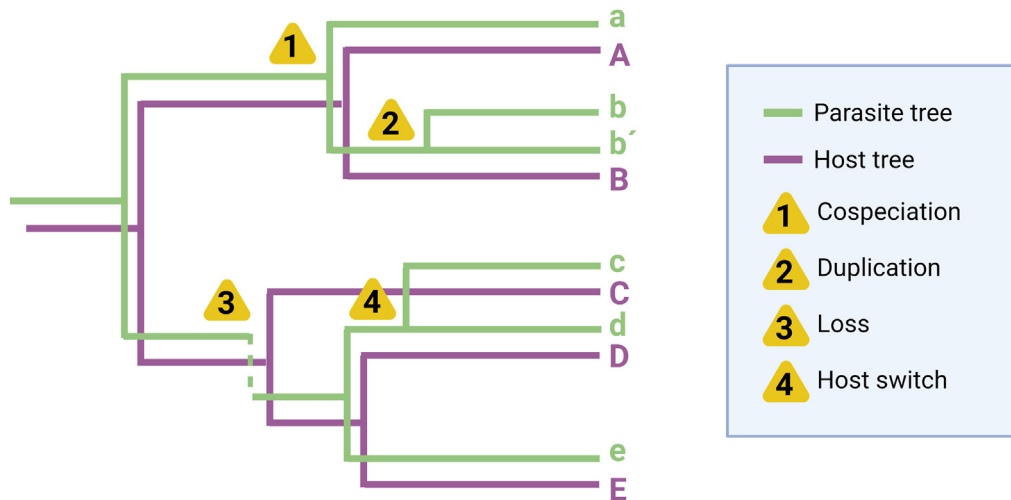
## 2. General speciation mechanisms in parasites

### 2.1. Coevolution, cophylogeny, cophylogenetic patterns and host-parasite interactions

Speciation refers to the process in which a species accumulates changes until it is considered a new taxonomic entity. This process is usually led by geographical, temporal, phenotypic, or phylogenetic changes and can be affected by climate and environmental alterations, biotic and geographical expansion, as well as host habitat invasion that leads to faunal mosaics (Hua & Wiens, 2013) (Fig. 1).

Cospeciation occurs when the speciation of a host or parasite takes place simultaneously to its counterpart, keeping their ecological relationship (Hayward et al., 2021). Nomenclature has been ever changing on this subject, and concepts can vary from one research group to another, but generally, main definitions on the subject can be separated as follows: (i) coevolution; (ii) cophylogeny; (iii) cophylogenetic events; and (iv) cospeciation processes.

Coevolution can be defined as the selective pressure of two species exerted on one another, resulting in a mutual evolutionary influence. In principle, the two species evolving are influenced by beneficial or unfavorable associations such as mutualism and parasitism, respectively



**Fig. 3** Graphical representation of host-parasite coevolutionary events. Overlapped parasite (in green) and host (in purple) phylogenetic trees depicting the following coevolutionary events: (1) cospéciation; (2) duplication; (3) loss; and (4) host switch. This figure was created using [BioRender.com](https://www.biorender.com).

(Hugot, 2006). Coevolutionary events can be studied using multiple tools, cophylogeny being the most widely used, which is described as the study of the phylogenetic congruence between two different organisms due to their long-standing interactions (Avino et al., 2019), through cophylogenetic signal. Phylogenetic congruence, explained by Fahrenholz's rule as "Parasite phylogeny mirrors that of its host" (Fahrenholz, 1913), could be defined as the extent to which each node and branch length in the parasite tree mirrors an associated taxon in the host tree (Blasco-Costa et al., 2021).

Coevolutionary events are predicted based on the branching of the parasite and host trees. These events based on phylogenetic relationships are mainly cospéciation, host switch, duplication, and loss (Fig. 3). Cospéciation or co-divergence, is the concomitant speciation of parasites and hosts (Brooks et al., 2015). Host switch, also known as host shift, horizontal transfer or host colonization, occurs when parasites speciate by occupying new hosts in a different ancestral lineage of its original host as a result of the parasite's low host specificity (De Vienne et al., 2013; Brooks et al., 2015). Two main conditions and stages are necessary for host switch speciation to occur, namely opportunity and compatibility (Araujo et al., 2015). Contrary to host switching, duplication happens when parasites speciate in the absence of a host change, meaning that parasite lineages duplicate within the same host (Johnson et al., 2003; Garamszegi, 2009). Finally, if the evolutionary fate of parasites and their hosts fail to compile with the processes mentioned above, they might suffer a loss, which can also be referred to as sorting or failure. If this happens repeatedly, a given parasite species may become extinct (Garamszegi, 2009). Interestingly, all these events are promoted by changes in the host-parasite interactions, also stated in the Red Queen Hypothesis. In biology, this hypothesis explains a dynamic set of antagonistic interactions of defence and invasion where the species that fails to catch up with its partner may become extinct (Rabajante et al., 2016).

The cophylospace is a tool for comparing different host-parasite systems and provides mechanistic explanations of cospéciation patterns (Russo et al., 2018; Blasco-Costa et al., 2021). As portrayed in these analyses, cospéciation may be exerted by one of the following events: (i) coevolution facilitated by mutual changes in host-parasite interactions; (ii) phylogenetic tracking in which speciation of either host or parasite is followed by speciation of its counterpart in an asymmetrical way; or (iii) vicariance where the detected phylogenetic congruence is due to geographical isolation, given ecological barriers to gene flow and dispersal as the result of host-parasite sympatry, as a counterpart to cospéciation (Althoff et al., 2014; Hoberg et al., 2017). Importantly, these mechanisms are not exclusionary, and a combination of each with different intensities is expected (Blasco-Costa et al., 2021).

## 2.2. Main drivers of parasite diversification: the Stockholm Paradigm

Even though cospéciation has been linked to parasite diversification, as mentioned previously, it is not the main generator of diversity. In turn, the Stockholm Paradigm has been proposed, which integrates macro- and microevolutionary dynamics, along with ecological and biogeographical information into faunal assembly and diversification. This paradigm is constituted by four main diversification drivers: (i) taxon pulse (TP); (ii) ecological fitting (EF); (iii) oscillation and (iv) geographic mosaics of coevolution (GMC) (Hoberg et al., 2017). It has been proposed that both EF and TP are primary mediators of macroevolutionary structure (i.e. evolution above the species level), followed by oscillation and GMC (Hoberg & Brooks, 2010).

Taxon pulse proposes that organism lineages suffer adaptive shifts through geographical or ecological spaces from their monophyletic beginning to their more derived end (Erwin, 1985). For this reason, TP describes the context for biotic mixing, or ecological collisions that increase the opportunity of host-parasite contact that are further explained by EF. In addition, TP explains the dynamics of episodic niche perturbation, stability and recurring host invasion that occur as a consequence of geographical isolation and geographical colonization (Halas et al., 2005; Lim, 2008; Hoberg & Brooks, 2010; Hoberg et al., 2017).

Ecological fitting (EF) initiates host switching or host colonization, either by resource tracking when the new host is similar to the original one, or by "sloppy fitness space", meaning that the new host offers novel resources (Agosta et al., 2010). Therefore, EF depends on the conditions explained above of opportunity, compatibility and conflict resolution, where opportunity is defined by expansion in TP (Janzen, 1985; Brooks & McLennan, 2002; Agosta et al., 2010; Hoberg & Brooks, 2010; Hoberg et al., 2017).

Oscillation explains that specialist parasites will eventually become generalists, and then these will produce new specialists (Araujo et al., 2015). This alternation of host range will depend on the use of resources with the ultimate narrowing of host range or ecological associations (Janz et al., 2006; Janz & Nylin, 2008; Hoberg & Brooks, 2010). Since oscillation largely defines host range, it may interact with EF, thereby determining host exploitation and colonization (Brooks & McLennan, 2002; Hoberg & Brooks, 2008; Hoberg et al., 2017).

The fourth and last driver is GMC, a framework describing coevolution between hosts and parasites in real populations and species (Brooks, 1979; Thompson, 2005; Hoberg et al., 2017). The GMC has been defined as a tripartite hypothesis, i.e. (i) different environmental conditions may lead to genetic variations of populations under those conditions, thus, selection may favor distinct evolutionary trajectories; (ii) there are



coevolutionary hot spots within communities where reciprocal coevolutionary selection occurs embedded in cold spots where selection is non-reciprocal; and (iii) trait remixing in populations facilitated by constant genomic alterations, gene flow and genetic drift, change the distribution of coevolving characters within and among populations over time. Finally, this hypothesis suggests that trait coevolution in hosts and parasites will be influenced by the favorable or unfavorable environmental conditions to which populations are exposed to, with few traits spreading to all populations of interacting species (Thompson, 1999).

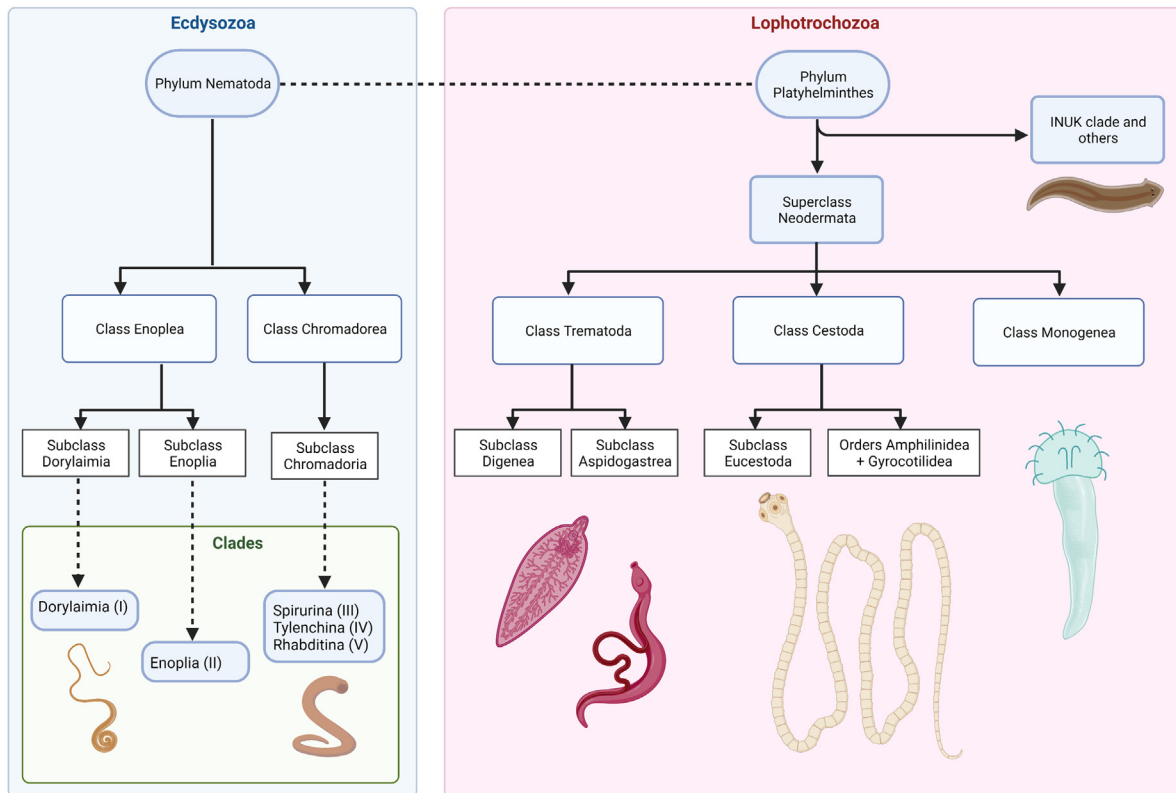
Other authors have suggested specific evolutionary processes to explain how cryptic species may have originated. Herein, it must be considered that cryptic species are the opposite of species resulting from adaptive radiation, given that in face of different ecological niches or barriers, these exhibit a reduced variation or disparity in phenotypic characteristics (e.g. morphology) (Struck & Cerca, 2019). These mechanisms may be referred to as recent divergence, convergence, parallelism, and stasis. Accordingly, recent divergence explains that speciation has occurred so recently that visible phenotypic changes have not yet taken place, excluding physiological, immunological, reproductive or behavioral changes (Knowlton, 1993; Struck & Cerca, 2019). Next, convergence and parallelism, while being antagonists in operation, result in the formation of cryptic species. The former implies the evolution of a derived character from different ancestral sets of traits given that these converge, while the latter implies the independent evolution of a character in different taxa from similar and shared ancestral set of traits (arising sets of parallel traits). Finally, stasis occurs when a specific phenotype is conserved during long time scales, even when environmental conditions fluctuate and highlight the potential deceleration of phenotypic evolution. These mechanisms demonstrate that while

phenotypic variation may be subtle, it is not absent in cryptic species (Struck & Cerca, 2019).

### 2.3. General classification of helminths according to phylogenetic analyses and cryptic species distribution

Taxonomy, systematics, and phylogeny in helminthology have transitioned from classic morphological approaches to the era of molecular data. As stated by Brooks (1985), clinical diagnosis in parasitology has focused on the study of unique traits found in organisms for separating taxa. This highlights the importance of unifying classification approaches in medical and veterinary helminthology, in order to determine zoonotic potential, implement control strategies, and study the spread of anthelmintic resistance (Betson & Stothard, 2016).

Helminths are classified into two main phyla: Nematoda and Platyhelminthes (Fig. 4). The phylum Nematoda represents free-living or parasitic pseudocoelomate organisms of animals and plants which are, in the vast majority, sexually dimorphic (Basyoni & Rizk, 2016). While nematodes were originally classified into classes Adenophorea and Chromadorea, phylogenetic approaches using the 18S rRNA gene (Blaxter et al., 1998; Kampfer et al., 1998; De Ley & Blaxter, 2002, 2004; Meldal et al., 2007; Holterman et al., 2009; Van Megen et al., 2009; Bik et al., 2010; Blaxter & Koutsovoulos, 2015) have resulted in a new reclassification (Fig. 4). Moreover, five clades have been proposed according to the 18S rDNA phylogeny of nematodes: (i) Dorylaimia; (ii) Enoplia; (iii) Spirurina; (iv) Tylenchina; and (v) Rhabditina. The greatest number of parasitic nematodes are members of the clade Rhabditina, followed by Dorylaimia and lastly, Enoplia, with only one known parasitic species of animals, namely *Ironus macrocephalum* (Blaxter &



**Fig. 4** Schematical phylogeny of helminths. The phylum Nematoda is composed of classes Enoplia and Chromadorea, which are subdivided into subclasses Dorylaimia, Enoplia and Chromadorea and their respective clades, as illustrated. Parasitic organisms in the phylum Platyhelminthes are represented by the superclass Neodermata, which is divided into classes Trematoda, Cestoda and Monogenea. Note that Cestodaria is not used to refer to Amphiliinidea + Gyrocotilidea since it is no longer a valid taxon. This figure was created using [BioRender.com](https://www.biorender.com).

Koutsovoulos, 2015). In addition, phylogenomic and transcriptomic analyses have rendered the same major nematode clades as obtained with the 18S-derived phylogeny by De Ley & Blaxter (2002, 2004), with the advantage of including a larger number of taxa leading to a better resolution of branching distances in the major Nematoda clades (Blaxter & Koutsovoulos, 2015; Smythe et al., 2019).

On the other hand, platyhelminths are an immensely diverse group of parasitic and free-living acoelomate and monoecious organisms (except for schistosomes) (Collins, 2017). This morphology-based classification has been confirmed with genomic data from 44 neodermatan platyhelminths and one free-living turbellarian worm, which has shown the same subdivision (Coghlan et al., 2019). According to phylogenetic analyses based on 18S and 28S rRNA genes, parasitic platyhelminths are classified in two major groups: Superclass Neodermata and the clade INUK (Carranza et al., 1997; Mollaret et al., 1997; Littlewood & Bray, 2001; Lockyer et al., 2003; Egger et al., 2015) (Fig. 4) that includes parasitic rhabditophoran platyhelminths of the genera *Ichthyophaga*, *Notentera*, *Urastoma* and *Kronborgia* (Littlewood et al., 1999a, b; Bagnù & Riutort, 2004). Superclass Neodermata is subdivided into three main classes: Trematoda, Cestoda and Monogenea. The first two contain parasites of human and veterinary importance, while the latter comprises mainly ectoparasites of fish (Lockyer et al., 2003; Bagnù & Riutort, 2004).

Parasites in the class Trematoda (flukes) and are subdivided in subclasses Digenea (involved in trematodiasis in humans and other vertebrate hosts) and Aspidogastrea (parasites of fish, reptiles, and bivalves) (Alves et al., 2015; Mehlhorn, 2016) (Fig. 4). Organisms belonging to the class Cestoda (tapeworms) are divided into the subclass Eucestoda, a monophyletic group composed of 17 orders, of which Cyclophyllidea is the most important in human and veterinary medicine; and a group composed of the orders Amphilinidea and Gyrocotylidea, formerly known as the subclass Cestodaria, for which there is no support of monophyly to date and is henceforth no longer considered a valid taxon (Caira et al., 2017).

As mentioned before in this review, the distribution of cryptic species in helminth species is uneven, given that the class Trematoda encompasses the greatest number of cryptic species reported, followed by the class Cestoda, and finally the phylum Nematoda (Pérez-Ponce de León & Poulin, 2018; Chan et al., 2022). This can be explained by a greater probability of somatic mutations occurring during the asexual reproduction of trematodes, to the subtler morphological differences exhibited in trematode species than in other taxa, and less specialized morphological studies (i.e. scanning electron micrographs) employed to describe such species, possibly leading to their initial misdiagnosis as cryptic species (Pérez-Ponce de León & Poulin, 2018).

### 3. Cryptic diversity in helminth parasites

#### 3.1. Elucidation of cryptic species through morphology, behavior or ecology

For many years, parasitologists have described, classified, and diagnosed species and higher taxa using solely morphological traits. However, this strategy has been proven problematic when cryptic entities have been recognized for a species (Pérez-Ponce de León & Nadler, 2010; Nadler & Pérez-Ponce de León, 2011). In the past, several non-morphological and non-molecular methodologies such as host ethology, parasite mating behavior, resource use, geographical distribution, biochemistry or natural history were widely employed for the detection of cryptic species (Nadler & Pérez-Ponce de León, 2011). For instance, *Ascaris suum* was originally described by Goeze (1782) as the roundworm of pigs (*Sus scrofa*), since it was found in this host for the first time, even though it did not exhibit morphological differences from *A. lumbricoides* (Goeze, 1782). The term cryptic species was used for the first time in helminthology in 1978 when differentiating *Parascaris equorum* from *P. univalens* using isoenzyme electrophoresis (Bullini et al., 1978). To date, PCR-based assays employing both mitochondrial and ribosomal genes are the most employed protocols for deciphering cryptic

entities in different fields, including helminthology (Nadler & Pérez-Ponce de León, 2011).

#### 3.2. Detection of cryptic species with gene sequencing: mitochondrial vs ribosomal genes

Prospecting cryptic species using DNA markers must follow two basic criteria. First, the locus needs to evolve fast enough among individuals so genetic divergence can be quickly spotted even within populations with a low number of individuals. Secondly, the marker must have low intra-specific and intra-individual variability, in order to differentiate true events of speciation from normal intra-individual variability (Vilas et al., 2005).

Mitochondrial and ribosomal loci have been used for phylogeographic studies to identify cryptic species (Nadler & Pérez-Ponce de León, 2011). Mitochondrial DNA (mtDNA) genes such as the NADH dehydrogenase subunit 4 (*nad4*) and the cytochrome *c* oxidase subunit 1 (*cox1*) evolve rapidly and rarely recombine. These genes have proven to be excellent candidates to search for cryptic diversity because of their resolution power that reveals monophyly within very closely related species (Morgan & Blair, 1998; Nadler & Hudspeth, 2000; Blouin, 2002). Other mitochondrial genes, namely the 12S and 16S rRNA genes show an interspecific genetic difference comparable to that of *cox1* for the molecular identification of helminths (i.e. trematodes), and can discriminate closely related species (Chan et al., 2022). Additionally, these markers are suitable for discriminating possible cryptic species at initial molecular prospecting, given that their reported genetic divergence between different parasitic nematode genera ranges from approximately 10% to 20% (Blouin et al., 1998), and between 6.9% and 16.0% in congeneric species (Blouin, 2002). Furthermore, it has been observed that the maximum percentage of genetic divergence in an interbreeding population of the cattle parasitic nematode *Ostertagia ostertagi* is 6% when using *nad4* (Anderson et al., 1998; Blouin, 2002). However, intra-specific and intra-individual variation can range from 1% to 7% in members of the family Trichostrongylidae when using *nad4* (Blouin et al., 1998), which equals the degree of variation often found between closely related taxa. This situation may pose a difficulty when using mtDNA to identify cryptic species, since the discrimination of the intra-specific variability from the emergence of monophyly cannot be achieved, even when using partial *cox1* and *nad4* sequences (Blouin, 2002). For this reason, additional markers and larger DNA fragments are recommended to provide higher resolution regarding a species' taxonomic position. As for cestodes, mitochondrial markers such as *cox1* and *nad1* have been used to study cryptic diversity in the family Taeniidae, while multilocus enzyme electrophoresis (MEE) has been employed in the family Anoplocephalidae (Chilton et al., 2007; Lavikainen et al., 2010; Jia et al., 2012).

Another powerful mitochondria-associated tool is whole mitogenome sequencing, which has increased the resolution of unclear taxonomic relationships, even though only just over 200 nematode species have been sequenced to date (Kern et al., 2020; Roe et al., 2021). For instance, mitogenome sequences and morphological differences in reproductive structures were useful to distinguish the monogenean parasite *Benedenia seriola* from *B. humboldti* (Baeza et al., 2019). In addition, suborders Spirurina and Tylenchina have been proposed as monophyletic according to 18S rDNA phylogenies, whereas mitogenome information has separated them into several independent clusters (Kern et al., 2020). Still, discordances between mitogenome and nuclear gene phylogenies are observed, and the combination of several markers is recommended for a more robust interpretation of phylogenetic relationships.

Ribosomal loci like the internal transcribed spacers (ITS1 and ITS2) have been extensively used to distinguish between helminth species due to the high variability they hold, and the availability of universal primers targeting these regions (Blouin, 2002). For these reasons, ITS2 sequences have been useful for characterizing gastrointestinal Clade V nematode communities using deep amplicon sequencing in cattle, sheep and bison (Avramenko et al., 2015, 2017, 2018). Additionally, the presence of fixed

insertions and deletions (indels) in ITS sequences have made them appealing for diagnostics (Blouin, 2002). Nevertheless, exceptions have been detected for the carcinogenic nematode of canids *Spirocerca lupi*, where ITS1 intra-individual variation ranges from 0.37 to 2.85%, with each specimen showing two or more different copies of ITS1 (Rojas et al., 2018), an event that has also been reported in ITS2 of the nematodes *Varestrongylus alces*, *Varestrongylus* cf. *capreoli* and *Varestrongylus sagittatus* (Verocai et al., 2014) and *Trichuris* spp. (Callejón et al., 2012), the cestode *Echinococcus granulosus* (Blair & McManus, 1995), and the trematode *Paragonimus westermani* (Van Herwerden et al., 1999) as a result of incomplete rDNA homogenization.

Genetic divergence obtained using mtDNA markers has been compared to that obtained using the ITS loci. For instance, the *nad4* gene offers a better resolution when prospecting for cryptic species (Morgan & Blair, 1998; Blouin, 2002; Vilas et al., 2005) in nematodes (such as *Ancylostoma* spp. and *Haemonchus* spp.) than in trematodes (such as *Echinostoma* spp., *Fasciola* spp., *Schistosoma* spp.), and taeniid cestodes (*Echinococcus* and *Taenia*). Nonetheless, examples are found where neither ribosomal nor mitochondrial markers have been able to solve the cryptic species status. In these cases, whole genome sequencing (WGS) has offered a better resolution.

### 3.3. Advances of the whole genome sequencing

Whole genome sequencing (WGS) has improved the study of single copy nuclear genes (SCN). SCNs have not been routinely used for cryptic species prospecting, since their amplification from genomic DNA is challenging for some parasitic taxa, as described by Nadler & Pérez-Ponce de León (2011). Since 2007, when the genome of *Brugia malayi* was obtained for the first time in any helminth species, 81 parasitic nematodes and 31 flatworms have been sequenced to date (Ghedini et al., 2007; McVeigh, 2020). Furthermore, WGS has been applied to better understand the phylogenomics and phylogenetic relationship between the major parasitic nematodes of humans and pigs, *Ascaris lumbricoides* and *Ascaris suum*, respectively, which are morphologically indistinguishable from each other. In this case, WGS was necessary to elucidate the evolutionary history of both taxa (Easton et al., 2020). This illustrates that a wider look into a parasite's genetic composition can improve species resolution and reduce the uncertainty of using a limited number of genes. Overall, WGS techniques have expanded the available information for helminth phylogenetic reconstructions, simultaneously increasing the complexity for analysing large datasets (Maldonado et al., 2019; Easton et al., 2020).

### 3.4. Limitations of gene-based analyses: alternative methods for delimiting cryptic species

Cryptic species are usually delimited with the support of DNA differences. However, it is not advisable to delimit cryptic entities on a threshold of genetic divergence or a genetic yardstick alone. This is suggested based on the heterogenous relative rates of evolution in each species, since increased relative rates of gene change in a particular lineage may result in falsely elevated pairwise nucleotide distances (Nadler & Pérez-Ponce de León, 2011). Nevertheless, genetic thresholds may be useful for prospecting cryptic diversity in the same species, since high intraspecific genetic divergence is not expected (Blouin, 2002; Vilas et al., 2005).

Interpretation of molecular data alone, without analysing morphological characters when available (as opposed to cryptic species *sensu lato*) has occasionally led to the mislabelling of entities as cryptic. For instance, *Anisakis simplex*, *Anisakis pegreffii* and *Anisakis berlandi*, were resolved as new species based on allozyme electrophoresis and ITS, 12S and *cox1* sequence analyses, after being considered cryptic (Nascetti et al., 1986; Mattiucci et al., 2014). Another example is the report of a cryptic species within *Eucoleus*, based on a female adult collected from the bronchoalveolar lavage of a 12-week-old kitten (Calvani et al., 2021).

Herein, researchers found up to 15–19% divergence in the *cox1* sequence of this *Eucoleus* sp. isolate when compared to other available *Eucoleus* sequences; and a 14–23% divergence in mitochondrial protein coding gene sequences between the kitten isolate and *E. aerophilus*. However, differences in egg morphology found between the kitten isolate and *E. aerophilus* imply that the species is not strictly cryptic, even if adult morphology was not examined (Calvani et al., 2021). Moreover, the tapeworm of brown bears (*Ursus arctos*), *Taenia arctos*, was described as a nominal species with its own diagnosis after being originally reported as cryptic (Lavikainen et al., 2010; Haukisalmei et al., 2011). Similar evidence was reported for *Hydatigera taeniaeformis* (s.s.) and *H. kamiyai*, wherein both had been regarded as cryptic species of *H. taeniaeformis* (s.l.); however, rostellar hook analysis, deciphered their real identity (Jia et al., 2012; Lavikainen et al., 2016). These examples support the concept that classifying cryptic entities by their genetic divergence alone may not always serve as a yardstick in assessing cryptic diversity (Nadler & Pérez-Ponce de León, 2011).

There are alternatives to gene sequencing for detecting cryptic diversity, such as MEE, allozyme electrophoresis, restriction fragment length polymorphism (RFLPs), single strand conformational polymorphism (SSCPs), amplified fragment length polymorphism (AFLPs), randomly amplified polymorphic DNA (RAPDs) and microsatellites, some of which may be coupled with PCR (RFLPs, RAPDs) (Nadler & Pérez-Ponce de León, 2011). Each strategy has its advantages and disadvantages. For example, MEE may not be suitable in studies detecting genetic variation, since electrophoretic patterns depend only on factors altering electrophoretic mobility, such as charge and protein conformation (Nadler, 1990; Andrews & Chilton, 1999). In addition, computational resources for species delimitation, like the Bayesian Phylogenetics and Phylogeography (BPP) program have been used (Yang, 2015). This software, previously applied to the study of *Baylisascaris* phylogenetics and systematics, integrates independent evolutionary histories of different loci through the multispecies coalescent model (MSC), considering the population size of ancestral and modern species, and species divergence times (Camp et al., 2018). Other studies dealing with cryptic diversity of trematodes, such as *Stegodexamene anguillae* and *Phyllodistomum* have also employed a BPP approach to delimit cryptic diversity (Herrmann et al., 2014; Pinacho-Pinacho et al., 2021).

## 4. Examples of cryptic diversity in parasites of human and veterinary importance

In this section we discuss some of the most relevant examples of cryptic diversity found among helminths of human and veterinary importance, due to their impact in public health or production systems. These are listed in higher-level taxonomic order below.

### 4.1. Phylum Nematoda: *Ascaris* spp.

Ascariasis is a widespread geohelminthiasis caused by the roundworms *A. lumbricoides* and *A. suum* in humans and pigs, respectively. These two ascarids are morphologically indistinguishable from one another, and molecular markers have failed to resolve their phylogenetic relationship. The lack of resolution between these two species of *Ascaris* has been a cause of debate for decades (Da Silva Alves et al., 2016).

Four hypotheses have been proposed to correctly assess the taxonomic status of *A. suum* and *A. lumbricoides* and explain their evolutionary history: (i) *A. lumbricoides* and *A. suum* are not monophyletic and are thus two separate and valid species, where speciation occurred before the domestication of pigs by humans from a common ancestor (Leles et al., 2012); (ii) following pig domestication, *A. suum* infected humans and derived to *A. lumbricoides* by host-switch or host colonization and the latter persisted as a species (Leles et al., 2012; Zhou et al., 2020); (iii) after pig domestication, *A. lumbricoides* from humans switched to pigs and derived to *A. suum* (Leles et al., 2012); and (iv) these two nominal species of ascarids are in fact conspecific. Previously, it has been



suggested that human and pig ascarids share a common recent ancestor with *Parascaris equorum*, an ascarid of equids (Nadler & Hudspeth, 2000) based on morphology, mitochondrial and nuclear genes, indicating that one did not originate from the other (Leles et al., 2012), but rather that they comprise a sister group, deriving from their most common recent ancestor, along with *P. equorum*. However, further research is needed to determine the support associated to each of the mentioned hypotheses.

The low genetic divergence in *A. lumbricoides* and *A. suum* mitogenomes and paleoparasitological evidence (i.e. the close contact between humans and pigs, before and during their domestication, facilitating host switch or colonization, cross-infections and hybridization events) (Leles et al., 2012) has provided further evidence to the hypothesis that both ascarids are conspecific. However, other studies have pointed towards derivation of one ascarid following infection with the other ascarid species. In this regard, hybrid genotypes of *A. lumbricoides* and *A. suum* have been recognized circulating among human and pig populations in the USA, Thailand, Lao PDR, Myanmar, Guatemala and China suggesting anthrozoootic transmission to pigs has occurred in the past (Criscione et al., 2007; Jesudoss Chelladurai et al., 2017; Sadaow et al., 2018).

A high-quality *Ascaris* reference genome was constructed in 2020 from a single representative female worm collected from a human in Kenya, assumed to be infected by *A. lumbricoides* due to the lack of pig husbandry in their village (Easton et al., 2020). This study revealed that *A. suum* and *A. lumbricoides* are an inter-breeding and cross-infecting genetic complex after analysing 68 mitogenomes from *Ascaris* specimens collected from humans and the resemblance of the *cox1* and *nad4* haplotypes to other *A. suum* and *A. lumbricoides* sequences. Remarkably, the same study confirmed the presence of three genetic clades based on the genetic diversity at the whole genome, nuclear and mitochondrial level: A (mainly pig-derived specimens); B (mainly human-derived specimens); and C (pig-derived specimens from Europe and Asia) (Cavallero et al., 2013; Easton et al., 2020).

*Ascaris* hybrid populations may behave as CGIs, since these groups have been reproductively isolated, and are able to interbreed, as a consequence of host range expansion (Chenuil et al., 2019). In this sense, *A. suum* was originally described as a separate species from *A. lumbricoides* based on host specificity, and it was believed that *A. lumbricoides* solely parasitized humans (Goeze, 1782). Interestingly, experimental cross-infections of pig and human *Ascaris* have been demonstrated in both hosts, indicating the ability of this parasite to infect several types of hosts. Furthermore, ascariasis in humans is considered a zoonotic disease in Denmark, where prevalence of this parasite is low in humans and thus, pigs may be the main source of infection to humans (Anderson, 1995; Nejsun et al., 2005). This confirms the findings of Easton et al. (2020) that *Ascaris* can cross-infect pigs and humans and interbreed, and highlights the possible transfer of anthelmintic resistance genes from one *Ascaris* population in humans or pigs to another.

#### 4.2. Phylum Nematoda: *Toxocara* spp.

Species of the genus *Toxocara* are intestinal parasites of mammals, with some species holding zoonotic importance. Canids serve as definitive hosts of *T. canis*, whereas felids are the definitive hosts of *Toxocara cati* and *Toxocara malaysiensis*. Definitive hosts harbour adults in the small intestine with the consequent excretion of their eggs in feces (Fialho & Corrêa, 2016; Chen et al., 2018). On the other hand, adult development is arrested in humans and a strong inflammatory response is released against migrating larval stages (Fialho & Corrêa, 2016) leading to covert toxocarosis or visceral, neural, ocular *larva migrans* (Chen et al., 2018).

Cryptic diversity in the genus has been regarded since the description of the *Toxocara* sp. variant found in cats in Malaysia (Rohde, 1962; Lee et al., 1993) which could not be morphologically discriminated from *T. canis* (Zhu et al., 1998). ITS1, ITS2 and 5.8S ribosomal markers and

restriction analysis (PCR-RFLP) were used for comparative analysis of this Malaysian *Toxocara* isolate from *T. canis* and *T. cati*. The results of Zhu et al. (1998) initially indicated that the Malaysian *Toxocara* sp. isolates were closer to *T. cati* than to *T. canis* when all molecular markers were considered, contrary to the initial morphological diagnosis. This led to the report of *Toxocara* cf. *canis* for the Malaysian specimens, which were later formally described as *Toxocara malaysiensis* based on morphological traits of lips, cervical alae, spicule length and female reproductive system (Gibbons et al., 2001). Furthermore, research on the *T. canis* mitogenome suggested that *T. malaysiensis* and *T. cati* represented cryptic species (Jex et al., 2008). Nonetheless, a closer look might aid in evaluating cryptic species in the genus since *T. malaysiensis* has unique morphological characters that distinguish it from the other species of *Toxocara* (Gibbons et al., 2001).

#### 4.3. Phylum Nematoda: *Toxascaris leonina*

*Toxascaris* is a monotypic nematode genus, with *Ta. leonina* as its only representative. This parasite commonly infects a large variety of definitive canid and felid hosts, such as dogs, cats, red foxes, tigers, lions and wolves worldwide, where they develop into adults in the small intestine potentially causing disease in young animals (Okulewicz et al., 2002, 2012). Additionally, humans can potentially become infected with larval stages of *Ta. leonina* and develop visceral, ocular or cerebral *larva migrans* (Robertson & Thompson, 2002; Okulewicz et al., 2012).

Several molecular studies have demonstrated the presence of cryptic species among *Ta. leonina* isolates, with apparent species delimitation according to host (Song et al., 2015; Li et al., 2018; Fogt-Wyrwas et al., 2019; Jin et al., 2019; Xie et al., 2020). In a preliminary study, *cox1* was employed to determine the genetic relationship between *Ta. leonina* from dogs and cheetahs, revealing a difference of 6.8% between collected specimens from different hosts (Li et al., 2018). Another study found a difference of 9.0% and 10.8% in the *nad1* and *nad4* genes, respectively, between worms obtained from canid and felid hosts from South China (Song et al., 2015). A subsequent study obtained a 7.2% divergence in the coding regions of *Ta. leonina* mitogenomes isolated from cheetahs and dogs, suggesting the presence of cryptic species (Jin et al., 2019). Importantly, those specimens were morphologically identified as *Ta. leonina*, and it was suggested that further detailed morphological analyses of this species from different hosts should be undertaken. Additionally, ITS1, *cox1* and *nad1* sequence analysis of 35 *Ta. leonina* collected from red foxes (*Vulpes vulpes*), domestic dogs (*Canis lupus familiaris*), grey wolves (*C. lupus*), tigers (*Panthera tigris amoyensis*, *P. t. altaica*, *P. t. corbetti*), lions (*P. leo spelaea*), and the Eurasian lynx (*Lynx lynx*) found three *Ta. leonina* subclades clustered according to the host phylogenetic groups: (i) dogs and wolves; (ii) wild felids; and (iii) red foxes (Fogt-Wyrwas et al., 2019). Therefore, *Ta. leonina* from dogs and wolves clustered together (Kimura 2-parameter (K2P) barcode gap distance > 1.7%), while those from wild felids (K2P barcode gap distance of 3%) and red foxes (K2P barcode gap distance of 6.58%) formed two separate groups, suggesting the existence of three cryptic species within the complex. Interestingly, this research also pointed out that gene flow among wild felid isolates could be low (Fogt-Wyrwas et al., 2019) as supported by complete ITS sequences and partial *cox2* and *nad1* analyses (Xie et al., 2020).

It is currently unknown which *Ta. leonina* cryptic species may infect humans, as well as their differences in pathogenicity, clinical course of infection and diagnosis. This is an issue that should be addressed in the future, since epidemiological research suggests that *Ta. leonina* is a potentially emerging zoonotic pathogen due to its close contact with humans, dogs and cats (Xie et al., 2019). Monitoring for cryptic species in human and animal cases is especially important considering the greater chance of morphology-based misdiagnosis associated with the genus, which is unlikely to be monotypic (Gasser, 2006; Chen et al., 2012; Fogt-Wyrwas et al., 2019; Xie et al., 2019).

#### 4.4. Phylum Nematoda: *Strongyloides* spp.

*Strongyloides* is a speciose genus composed of over 50 valid species, that dwell between free-living and parasitic generations. The infective L3 larvae penetrate the host skin, migrate, and establish themselves in the small intestine mucosa, with only parthenogenetic females being parasitic. Most species of this genus are host-specific and have been reported in humans, non-human primates (NHP), various mammals, birds, amphibians and reptiles (Thamsborg et al., 2017; Jaleta & Lok, 2019). A few exceptions are *S. stercoralis* and *S. fuelleborni*, which have a wide host range (Thamsborg et al., 2017).

There is evidence of cryptic diversity in the genus, in isolates from NHP, humans and dogs. One study depicting the diversity of *Strongyloides* spp. in various NHP from Malaysian Borneo detected cryptic diversity in L3 obtained from fresh fecal samples of one Philippine slow loris (*Nycticebus menagensis*) (Frias et al., 2018). Through *cox1* analysis, the sequences obtained from these animals clustered into two haplotypes apart from *S. stercoralis* or any other *Strongyloides* spp. sequences, possibly constituting a cryptic subpopulation of the former, with pairwise distances suggesting that the loris-associated clade may be distinct from those found in free and captive NHP, dogs, and humans. In addition, *S. fuelleborni* was also found in slow loris from Borneo, separated from isolates of other geographical regions.

These findings have epidemiological implications, since the host range of *Strongyloides* isolates belonging to the loris clade remains unknown and there is no information regarding whether this host may promote persistent parasite populations through autoinfection or if these parasites are better adapted to the free-living cycle (Frias et al., 2018). Favorable conditions for zoonotic transmission of this clade could be an emerging issue given the close phylogenetic relationship of the loris cluster to *S. stercoralis*, the potential parasite spillover facilitated by human activity, and that lorises are a popular target for wildlife trade, leading to an increased strongyloidiasis burden (Thompson, 2013). Additionally, evidence of cryptic and potentially zoonotic *Strongyloides* strains have been reported from dogs in Australia, and from humans and dogs in rural Cambodia, Japan, and Myanmar, on account of 18S rDNA and *cox1* phylogenies (Jaleta et al., 2017; Nagayasu et al., 2017; Barratt et al., 2019; Beknazarova et al., 2019). These studies detected existing zoonotic and canine-specific cryptic *Strongyloides* lineages, emphasizing the importance of studying cryptic diversity among different hosts to further comprehend the extent of species distribution (Jaleta & Lok, 2019). Lastly, variation in pathogenicity and virulence in cryptic strains should be assessed, being of the utmost importance in parasites such as *S. stercoralis* and *S. fuelleborni*, considering their wide host range (Hasegawa et al., 2010; Frias et al., 2018; Wulcan et al., 2019).

#### 4.5. Phylum Nematoda: *Trichinella* spp.

Species within the genus *Trichinella* are divided into two clades, namely the encapsulated (T1, T2, T3, T5, T6, T7, T8, T9, T12 and T13) and non-encapsulated (T4, T10, T11) clades; depending on whether they possess a collagen capsule arranging the nurse cell in the host's muscle. Out of these 13 genotypes, 10 have been recognized and named as species, and three remain formally undescribed (i.e. T6, T8 and T9) (Pozio & Zarlenga, 2013; Korhonen et al., 2016; Zarlenga et al., 2020). Most species in the genus *Trichinella* are zoonotic nematodes of mammals, while *T. pseudospiralis* (T4) may parasitize avian hosts, and *T. papuae* (T10) and *T. zimbabwensis* (T11) can parasitize reptiles as well as homeothermic hosts (Sharma et al., 2020; Zarlenga et al., 2020). Trichinellosis occurs after ingestion of raw or undercooked meat, containing the infective first-stage larvae (L1) (Gottstein et al., 2009). The larval stages develop into adults and sexually reproduce in the small intestine of the definitive host, with the new-born larvae reaching the muscle later on, finally becoming encapsulated depending on their genotype (Zarlenga et al., 2020).

A novel PCR-RFLP-based assay targeting the *cytb* gene detected the presence of a new cryptic species, *T. chanchalensis* (T13), in the frozen tissue of wolverines (*Gulo gulo*) from Canada, in single infections and coinfections with *T. nativa* (T2) and T6. Additionally, morphometric analysis of 25 specimens was unable to differentiate T13 when compared to other species, implying that *T. chanchalensis* is a cryptic species *sensu stricto*; supported by phylogenetic inference based on whole mitogenome and 15 SCNs (Sharma et al., 2020).

*Trichinella chanchalensis* has proven to be a freeze-resistant species, as well as T2 and T6. This may pose a relevant epidemiological implication, since freezing is an acceptable post-harvest treatment for *T. spiralis* inactivation in domestic pig meat (Noeckler et al., 2019; Johne et al., 2020). Moreover, naturally occurring wild hybrids of T2 and T6, and those of *T. spiralis* and *T. britovi* (T3) have already been reported (Dunams-Morel et al., 2012; Franssen et al., 2015). The existence of such hybrids supports the possibility of transferring genes conferring freeze-resistance (i.e. from T2, T6, T13) into freeze-susceptible genotypes, which may result in parasites that are better adapted to meat preservation conditions and may successfully infect their hosts (Zarlenga et al., 2020). These findings highlight the importance of accurately detecting cryptic species, since their biological features may change the infective potential of parasites.

#### 4.6. Phylum Nematoda: *Dirofilaria* spp.

Species of *Dirofilaria* are vector-borne pathogens of carnivores, with many species proven to be zoonotic. *Dirofilaria immitis* is distributed worldwide and leads to the heartworm disease in canids, whereas *Dirofilaria repens* produces subcutaneous dirofilariasis and is mostly restricted to the Old World, with few cases reported in the Americas (Dantas-Torres & Otranto, 2013). *Dirofilaria immitis* and *D. repens* can infect humans and produce nodular lesions in lungs, subcutaneous tissues or eyes (Antolová et al., 2015).

A high genetic diversity has been observed in *D. repens* isolates from Asia, as opposed to specimens collected from Europe (Yilmaz et al., 2016). Four complete mitochondrial genomes of specimens identified as *D. repens* from Europe ( $n = 3$ ) and India ( $n = 1$ ), and 46 *D. repens* mitochondrial genome fragments from adults and microfilariae of blood samples from Italy, Vietnam, Thailand, and India were analyzed. The three European mitogenomes were identified as *D. repens*, while the Indian mitogenome was tentatively referred to as "*Dirofilaria hongkongensis*", an agent of subcutaneous and subconjunctival human and animal dirofilariasis based on *cox1* analysis. Moreover, the authors demonstrated the presence of a possible cryptic species detected in blood samples from cats, namely *Dirofilaria* sp. "Thailand II", which could also belong to very divergent isolates referred to as "*D. hongkongensis*". *Dirofilaria repens*-like parasites have been identified as *D. hongkongensis* based on ITS1 sequences (To et al., 2012). In addition, *D. hongkongensis* specimens collected from humans and canids have shown ITS2 sequences identical to *D. repens*, adding further evidence for the existence of cryptic diversity (Suzuki et al., 2015; Liesner et al., 2016; Yilmaz et al., 2016).

In addition, the existence of cryptic diversity was suggested for a *Dirofilaria* specimen recovered from a 16-year-old Brazilian male suffering ocular dirofilariasis. In this case, a 5% and 6% genetic difference was found in 12S rDNA and *cox1* genes, respectively, in comparison with publicly available sequences of other species within the genus (Otranto et al., 2011). Interestingly, the specimen was morphologically similar to *D. immitis* and *D. spectans*, therefore, this could represent an undescribed cryptic species *sensu lato*. These findings underline the rich species diversity in the genus with no molecular data matching morphologically identical specimens and increase the interest for molecular prospecting of cryptic species in dirofilarial vectors such as *Culex* spp. in the Americas and their possible roles in contributing to hybridization.

#### 4.7. Phylum Nematoda: *Onchocerca* spp.

*Onchocerca volvulus*, the agent of river blindness, produces ocular and subcutaneous infections in humans, endemic in several African countries, Yemen and the Amazonian focus straddling Venezuela and Brazil (Milton et al., 2020). The congeneric *Onchocerca lupi* induces ocular lesions in dogs, cats and humans. *Onchocerca volvulus* is transmitted by arthropod vectors of the family Simuliidae (Basáñez et al., 2009), while the vector intermediate host of *O. lupi* is still unknown (Rojas et al., 2021). It has been suggested that domestication of *Onchocerca* vertebrate hosts may have guided the parasite's speciation into different taxa, since host switch events may have occurred between the host families Bovidae, Canidae and humans with recent speciation into *O. lupi*, *Onchocerca gutturosa*, *Onchocerca lienalis*, *Onchocerca ochengi* and *O. volvulus* (Lefoulon et al., 2015).

Cryptic diversity has been described in *Onchocerca* spp. associated with wild animals, especially hosts of the family Cervidae in the Nearctic (McFrederick et al., 2013; Verocai et al., 2018; Kulpa et al., 2021). Adult worms and microfilariae collected from the moose *Alces alces* have been molecularly identified as *O. cervipedis*, which was for decades assumed to represent the only species infecting North American cervids (Verocai et al., 2012; McFrederick et al., 2013). However, microfilariae obtained from the white-tailed deer *Odocoileus virginianus* analyzed using 12S, 16S and *cox1* mitochondrial genes, did not cluster with *O. cervipedis* obtained from moose. This has suggested that the *Onchocerca* isolates collected from deer could represent a different or cryptic species of *O. cervipedis*, a matter that will remain unsolved until morphological analysis is completed (McFrederick et al., 2013). Additionally, a molecular surveillance of *Onchocerca* spp. in 1434 blackflies from California using the *nad5* gene revealed an uncharacterized *Onchocerca* sp. infecting *Simulium tescorum* and *S. vittatum* (s.l.) (presumably *S. tribulatum*) (Verocai et al., 2018). *Onchocerca* sp. sequences from simuliids grouped together with the *Onchocerca* sp. previously found in the white-tailed deer discussed above. Moreover, partial *cox1* sequences exhibited a difference greater than the intraspecific variation for this marker. Although no morphological analyses have been performed, both organisms may represent the same cryptic species *sensu lato*.

Host switch leading to parasite speciation has been suggested before for the genus *Onchocerca* (Lefoulon et al., 2015). Thus, it is imperative that cryptic diversity is assessed throughout the genus and its hosts, since this information may provide a better understanding of onchocercid genetic distribution, a feature that may aid in further comprehending onchocerciasis epidemiology and pathophysiology.

#### 4.8. Phylum Nematoda: *Oesophagostomum* spp.

Species of the genus *Oesophagostomum* are parasitic strongylids of cattle, ruminants, pigs (Stewart & Gasbarre, 1989) and non-human primates (Polderman et al., 1991). Infection with these nematodes results in large economic losses in the production animal industry (Rashid et al., 2019). *Oesophagostomum* spp. induce the formation of nodules in the digestive subserosa of their hosts and can parasitize humans from endemic regions of West Africa, such as Togo and Ghana (Polderman et al., 1991; Polderman & Blotkamp, 1995).

*Oesophagostomum* diversity in Ugandan human and non-human primates is large, due to the existence of three cryptic clades according to ITS2 loci, that are morphologically indistinguishable. Clade 1 was 97.1–100% similar to *Oesophagostomum bifurcum*, while Clade 2 sequences from other primate species were 99.4% identical to *Oesophagostomum stephanostomum* and Clade 3 contained sequences from humans and other primates 92.4–93.0% and 93.0–93.6% similar to *O. bifurcum* and *O. stephanostomum*, respectively, with no similarity to any other *Oesophagostomum* spp. reference sequence (Ghai et al., 2014). In this study, the need for morphological analysis of L3-larvae and adult was highlighted to accurately confirm that sequences in Clade 3 belong to a cryptic lineage. Importantly, analysis of mitochondrial markers might

render a greater resolution on Clade 3's phylogenetic relationships to other *Oesophagostomum* spp. since the study of Ghai et al. (2014) was based on ITS2 analysis alone.

Implications of cryptic diversity within the genus *Oesophagostomum* are yet to be assessed. It has been reported that *Oesophagostomum* spp. induce a weak immune response with a late Th2 response in hosts and a different cytokine profile when compared to the conventional Th2 profile expected in helminthiasis, resulting in immune system modulation and evasion (Roepstorff et al., 2011). It is not known if cryptic *Oesophagostomum* lineages present antigenic differences and whether these could derive in isolates with different immunogenicity, ultimately contributing to determine the fate of the clinical outcome.

#### 4.9. Phylum Nematoda: *Teladorsagia* spp.

*Teladorsagia circumcincta* is a trimorphic nematode species (i.e. a morphospecies containing three morphotypes) that infects caprines and cervids in the Holarctic, with cryptic diversity in the genus proposed in the past (Hoberg et al., 2001). Moreover, *T. circumcincta* has been suggested as a complex of cryptic species, composed of two strains irrespective of their morphology, one occurring in goats and the other in both sheep and goats (Wyrobisz et al., 2016; Wyrobisz-Papiewska et al., 2018). In a morphometric and molecular analysis conducted by Hoberg et al. (1999), *Teladorsagia boreoarcticus* was described as a dimorphic species which was later found to be trimorphic (Wyrobisz et al., 2016). Its description was based on integrated morphological characters, including the synophe, esophageal valve, spicules, gubernaculum and bursa, and molecular characterization of the *nad4* gene, showing a 13% divergence when compared to sequences of other *Teladorsagia* spp. Therefore, *T. boreoarcticus* represented a separate nominal species on the grounds of a valid morphological diagnosis, in a genus permeated of cryptic diversity, instead of a cryptic species.

#### 4.10. Class Trematoda: *Opisthorchis viverrini*

*Opisthorchis viverrini* is a parasitic trematode of humans, endemic in Southeast Asia and a type 1 carcinogen. This parasite infects the liver and is responsible for opisthorchiasis-associated cholangiocarcinoma in chronic infections (Saijuntha et al., 2007; Khuntikeo et al., 2018).

Cryptic diversity has been suggested in *O. viverrini* isolates from cyprinid fish in Thailand and Lao PDR using MEE (Saijuntha et al., 2007). Analysis of 32 enzyme loci showed that 19 loci were different between *O. viverrini* isolates, with separation into two major groups and at least one species with differences in more than 30% of the loci. Additionally, MEE analysis of the gastropod first intermediate host, *Bithynia siamensis goniomphalos*, isolates revealed 17% fixed genetic variation, separating them into two major genetic groups, consistent with the division seen in *O. viverrini* fish isolates, which suggests host-parasite cospeciation (Saijuntha et al., 2007). These results indicate that different *O. viverrini* (s.l.) populations or species exist in Thailand and Lao PDR which may influence epidemiological changes in prevalence and morbidity, as well as in opisthorchiid control, prevention and treatment effectiveness (Saijuntha et al., 2007).

Overall, the identification of cryptic diversity and cryptic species within trematodes is key to understand the risks associated with the spread and extent of foodborne trematodiasis such as opisthorchiasis (Pérez-Ponce de León & Nadler, 2016). Aquacultural activities and fish commerce may be facilitators for parasite spillover and may enhance human contact with these pathogens. This may ultimately lead to the spread of cryptic species (or origin thereof) outside areas of endemicity (Thompson, 2013; Pérez-Ponce de León & Nadler, 2016).

#### 4.11. Class Trematoda: *Echinostoma revolutum*

Echinostomiasis is an intestinal food-borne parasitic infection caused by *Echinostoma* spp. in humans and animals associated with ingestion of



raw molluscs and fish (Sah et al., 2018). Seventeen species and species-level genetic lineages have been delineated using *nad1* mitochondrial gene within the *Echinostoma* “*revolutum*” species complex with cryptic lineages radiating in North America and in central and northern Europe (Georgieva et al., 2014). Morphological and molecular analysis of the *nad1* gene of *Echinostoma* spp. cercariae collected from snail intermediate hosts in Germany and Iceland revealed the occurrence of a novel cryptic species *sensu stricto* in the “*revolutum*” group, namely *Echinostoma* sp. IG (p-distance range 17.2–21.6%) (Georgieva et al., 2013).

#### 4.12. Class Cestoda: *Echinococcus granulosus*

*Echinococcus* spp. belong to the family Taeniidae and use carnivores of the families Canidae, Felidae and Hyaenidae as their definitive hosts (Romig et al., 2017). These cestodes can also parasitize humans due to their wide intermediate host range (Romig et al., 2017). For instance, *Echinococcus granulosus* (s.l.) induces cystic echinococcosis, *Echinococcus multilocularis* leads to alveolar echinococcosis and *E. oligarthra* and *E. vogeli* produce polycystic echinococcosis (Wen et al., 2019).

In the past, it was believed that *E. granulosus* consisted of a single species with an enormous genotypic and phenotypic variation. However, mitochondrial gene and whole mitogenome analyses have confirmed that *E. granulosus* is not a single species comprising strains or variants, but rather a cryptic species complex, with species exhibiting fascinating differences in their life-cycle and epidemiological settings (Romig et al., 2017). Altogether, the species complex is comprised by *E. granulosus* (s.s.) (strains G1, G2 and G3), *E. equinus* (G4, horse strain), *E. ortleppi* (G5, cattle strain), *E. canadensis* (G6 or camel strain, G7 or pig strain, G8 or cervid strain along with G10, and G9 now considered as a G7 micro-variant) and *E. felidis* (formerly considered the lion strain) (Romig et al., 2015). Importantly, *E. granulosus* (s.s.) is the main species parasitizing humans in cystic echinococcosis but it has been shown that *E. canadensis* can also infect humans and cause disease to a lesser extent than the former species (Romig et al., 2015). To date, whole genome sequences from *E. granulosus*, *E. multilocularis* and *E. oligarthra* have been published, allowing further genomic comparisons and analyses of the species complex (Maldonado et al., 2019).

The correct identification of cryptic *Echinococcus* diversity might aid in determining which epidemiological factors are key for disease control and prevention, which becomes highly relevant due to the diverse range of intermediate hosts that may change transmission dynamics (Sithithaworn et al., 2015). This species complex also exhibits differences in factors such as antigenicity, chemotherapeutic sensitivity, and pathology, further illustrating the relevance of a correct diagnosis and research regarding cryptic diversity (McManus, 2013).

#### 4.13. Class Cestoda: *Moniezia* spp.

*Moniezia* is a member of the family Anoplocephalidae that utilizes ruminants, pigs, rodents and birds as definitive hosts (Beveridge, 1994; Chilton et al., 2007). While rarely associated with clinical disease, it has been implicated as a cause of unthriftiness and emaciation in ungulates (Kutz et al., 2012). A strong support for cryptic species within two groups of *Moniezia benedeni* and two groups of *Moniezia expansa* from sheep and cattle in Southern Australia was demonstrated in a study employing MEE (Chilton et al., 2007). In this study, MEE analysis showed a 92% and 33% genetic divergence within two *M. benedeni* and two *M. expansa* groups, respectively, while divergence between the two species reached 77%. Those results support the existence of true cryptic lineages in the groups; however, mitochondrial analyses are needed to further validate the cryptic species status (Chilton et al., 2007).

## 5. Concluding remarks

Cryptic diversity in parasitic helminths commonly results from several evolutionary events. Speciation occurs in large parasite and host

communities, driven by climate, space and time, and aided by the influence of human activities which facilitates parasite spillover. Currently, the wide availability and relative low cost of molecular techniques and bioinformatic tools has facilitated the sequencing of mitochondrial and whole parasite genomes leading to the identification of cryptic diversity within various helminth species of human and veterinary importance, as outlined throughout this review. In this regard, mitogenomes and mitochondrial markers have proven extremely valuable in cryptic species discrimination, as they evolve rapidly and do not recombine. Moreover, these sequences are useful in identifying the appearance of monophyly before being detected by ribosomal markers. In spite of this, when delimiting cryptic species, molecular divergence data should not be used as yardsticks, due to the absence of such universal reference range, considering the existing variation in evolutionary rates for markers in specific parasite lineages.

Identification of cryptic diversity and cryptic species should be assessed as a general medical priority, since these taxa may vary in clinical and epidemiological features such as distribution, pathogenicity, virulence, drug resistance or susceptibility, and may weigh in clinical decision making. The recognition of this diversity may influence patient management, course of treatment (drug and dose of choice), anthelmintic resistance emergence, disease control and prevention, and the development of educational programmes to mitigate parasite transmission and infection. Moreover, cryptic diversity monitoring is necessary to detect emergent zoonotic species and further study their implications in human medicine. Furthermore, the burden of cryptic diversity in the veterinary setting could reflect the expansion of infections in a wider host range by novel cryptic species, most likely due to host colonization. The repercussions of these phenomena could manifest as a heavy economic loss in animal productive systems, or a decline in wildlife populations.

Lastly, we highlight the need for the correct use of definitions and concepts when describing new cryptic species in parasitology, as molecular and morphological data should be integrated whenever possible. A standardized nomenclature will ease the description of the status of potential cryptic entities to the scientific community.

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## Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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