



## Molecular markers in studies on fish parasites (Platyhelminthes): Review

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**ABSTRACT.** Studies with molecular markers are currently more common for all groups of living organisms. Molecular techniques used in Platyhelminthes parasites of fishes do not merely reveal complex life cycles, but are important for species distinction and the elucidation of the phylogenetic hypothesis. Current research verified which molecular markers were mainly used phylogenetic studies on Platyhelminthes parasites of fish so that subsidies for further phylogenetic studies in Ichthyoparasitology could be provided. Data base of CAPES Journals platform was employed for bibliometric analysis comprising the keywords “fish” and “phylogeny” associated with “Cestoda”, “Digenea” or “Monogenea”. Information retrieved was quantified and tabulated. Most studies were on Monogenea (43%), followed by Digenea (37%) and Cestoda (18%). Ribosomal molecular markers were the most used in the phylogenetic studies for fish parasites. Due to the advance of molecular biology techniques and of bioinformatics, with more robust phylogenetic analysis, the use of these techniques in other areas such as Ichthyoparasitology is on the increase. In fact, molecular phylogenetics and morphological structures analysis have efficiently contributed towards the understanding of phylogenetic relationships among the groups.

**Keywords:** Digenea, Cestoda, Monogenea, ribosomal DNA, COI.

## Marcadores moleculares aplicados a estudos de Platyhelminthes parasitos de peixes: revisão

**RESUMO.** Estudos com marcadores moleculares são cada vez mais comuns em todos os grupos de seres vivos. Para os platelmintos parasitos de peixes, as técnicas moleculares possibilitam desvendar ciclos de vida complexos, sendo importantes também na distinção de espécies e na elucidação de hipóteses filogenéticas. Neste sentido, este trabalho teve como objetivo verificar quais são os principais marcadores moleculares utilizados nos estudos de platelmintos parasitos de peixes, visando fornecer subsídios para futuros estudos filogenéticos na Ictioparasitologia. Para a análise bibliométrica foi utilizado o banco de dados dos Periódicos da CAPES, tendo como palavras-chave “fish” e “phylogeny” associadas a “Cestoda”, “Digenea” e “Monogenea”. As informações obtidas nos trabalhos foram tabuladas e quantificadas. Dos 143 trabalhos obtidos 43% foram com monogenéticos, 37% com digenéticos e 18% com cestóides. Os marcadores moleculares ribossômicos foram os mais utilizados nos estudos filogenéticos com estes parasitos de peixes. Com o avanço das técnicas de biologia molecular e da bioinformática, com análises filogenéticas mais robustas, é crescente a utilização destas técnicas na Ictioparasitologia. A filogenética molecular, juntamente com a análise de estruturas morfológicas tem contribuído de maneira mais eficiente para o entendimento das relações de parentesco, entre estes grupos de parasitos de peixes.

**Palavras-chave:** Digenea, Cestoda, Monogenea, DNA ribossômico, COI.

### Introduction

In the 1960s, Willi Hennig introduced the phylogenetic system or the reconstruction of a common evolution offspring of a group of living organisms (Bueno-Silva, 2012). The variations of characteristics with a genetic origin are investigated within a certain species groups. Hypotheses of

homology are thus formulated, compared and contrasted with congruency tests to verify kinship (Nelson & Platnick, 1981).

Homologous structures comparing different species should be understood to formulate the phylogenetic hypotheses. According to Amorim (2002), structure is any section of a living organism or a ny genotypic expression. Phylogenetic studies

also comprise genetic sequences, chromosomes and proteins. Molecular phylogenetics is the use of information from DNA, RNA sequences or protein sequences for the study of kin relationship between organisms (Patwardhan, Say, & Roy, 2014). Phylogenetic reconstructions based on molecular data may be inferred from the comparative analysis of DNA or from protein homologous sequences (Lemey, Salemi, & Vandamme, 2009).

Great progress occurred during the last decades on the employment of genetic information in phylogenetic studies due to new technologies, development of genetic data bases, computer programs for phylogenetic inferences, computer infrastructure and statistical methods for phylogenetic inferences (Bueno-Silva, 2012). Current advances in bioinformatics and molecular biology have triggered studies on species' derivation relations in evolution, population, epidemiological and genealogical investigations (Yang & Rannala, 2012).

Although current phylogenetic studies that use molecular markers are highly common in all groups of living organisms, the phylogenetic relationship of the species has been mainly based on the species's morphological features (Bueno-Silva, 2012). Sequencing of specific regions is one of the most efficacious manners to obtain data that may elucidate phylogenetic hypotheses (Gasques, Souza, & Graça, 2013). As a rule, the most employed regions for such ends have been the ribosome DNA region for the organism's genome, especially ITS regions, 18S and 28S (Bueno-Silva, 2012; Gasques et al., 2013).

Molecular techniques have been employed for phylogeny of fish parasites in the early 1990s (Rohde, et al., 1993; Bray, Soto, & Rollinson, 1994). They have also an important role in taxonomic relationships with species characterized by difficult morphological separation and complex life cycles (Clark, 2006).

Analyses of DNA sequences in the protein's codifying and non-codifying regions, have been largely used in phylogenetic studies on fish helminths parasites (Clark, 2006), with special reference to investigations on the three main Platyhelminthes groups: Cestoda, Digenea and Monogenea. Baverstock, Fielke, Johnson, Bray, and Beveridge (1991) performed pioneer studies on platyhelminthes with molecular phylogeny, in which the efficiency of the 18S marker was used to test phylogenetic hypotheses. In fact, phylogenetic studies employing molecular biology techniques for fish parasites have been recently very common, featuring studies on Monogenea, by King, Marcogliese, Forest, McLaughlin, and Bentzen

(2013); Yoon et al. (2013), Sarabeev and Desdevises (2014), Sepúlveda and González (2014); on Digenea by Locke, Daniel McLaughlin, and Marcogliese (2010); Cai et al. (2012); Miller and Cribb (2013) and Georgieva et al. (2013); on Cestoda by Bazsalovicsová, Králová-Hromadová, Štefka, and Scholz (2012); Waeschenbach, Webster, and Littlewood (2012); Caira, Marques, Jensen, and Ivanov (2013); Scholz et al. (2014).

Current study investigates the main molecular markers in phylogenetic studies on Monogenea, Digenea and Cestoda fish parasites to support future phylogenetic studies with molecular markers in Ichthyoparasitology and related areas.

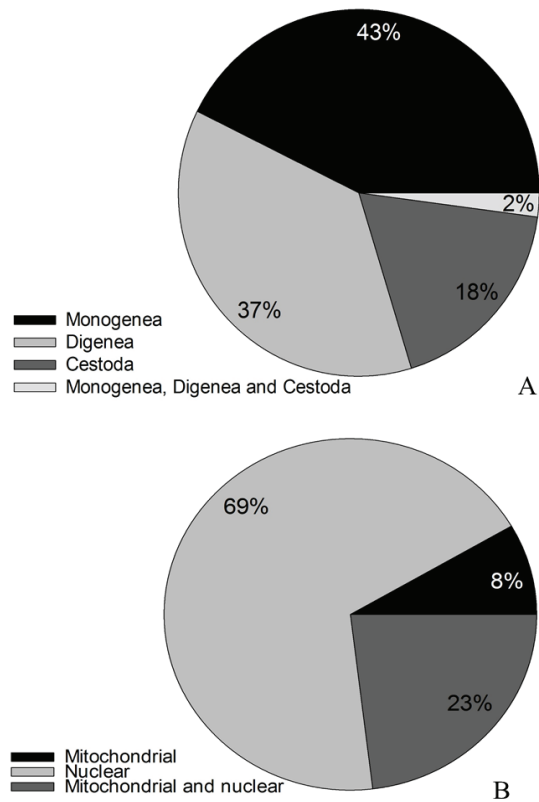
## Material and methods

A bibliometric survey (Machado, 2007; Macias-Chapula, 1998) of the main markers used in phylogenetic analyses on monogeneans, digeneans and cestodes parasites was undertaken to establish research methodologies with fish parasites. Publications available at the data base of the CAPES Journals platform were searched through keywords 'fish' and 'phylogeny' associated to one of the three classes of fish parasites under analysis: 'Monogenea', 'Digenea' and 'Cestoda'. CAPES Journals' data base was employed due to its more than 21,500 international and Brazilian journals available, coupled to 126 data bases currently available (retrieved from <http://www.periodicos.capes.gov.br>, 2016).

The specific articles were read and those featuring molecular markers were selected. These molecular markers were used in phylogenetics studies, cladograms reconstruction for distinction and characterization of species. Titles, name of authors, year of publication, groups of parasites studied and molecular markers for the preparation of phylogeny and cladograms were retrieved from the articles in the sample (supplementary appendix material). Survey occurred in 2016 and the characterized articles available till June 20, 2016 were retrieved.

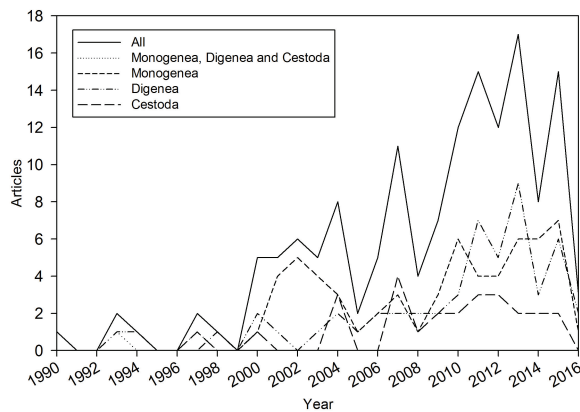
## Results

One hundred and forty-three articles were selected, featuring monogeneans as the most studied parasite group, followed by digeneans and cestodes. Only three studies investigated the three groups of parasites (Figure 1A). Nuclear markers were the most used in phylogenetic studies on these parasites, whereas mitochondrial markers were only scantily employed in phylogenetic studies with fish flatworm parasites (Figure 1B).



**Figure 1.** A) Proportion of papers, published between 1991 and 2016, on molecular markers in studies with Monogenea, Digenea and Cestoda fish parasites. B) Proportion of papers, published between 1991 and 2016, on Monogenea, Digenea and Cestoda fish parasites, according to the genome region of the molecular markers.

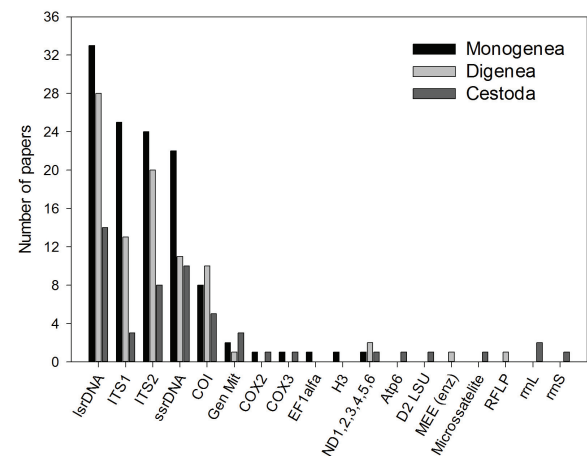
The first article to use molecular markers in phylogenetic studies on fish platyhelminthes parasites dated from 1991. However, published research works involving fish parasites and molecular markers increased only after 2000 (Figure 2).



**Figure 2.** Amount of papers between 1991 and 2016 using molecular markers in studies on Monogenea, Digenea and Cestoda fish parasites.

The most employed molecular markers for phylogenetic purposes were related to ribosomal nuclear genes (Figure 3). They were grouped under two headings: Large-Subunit Ribosomal DNA (lsrDNA), which comprised molecular markers 28S, 5.8S and complete fragment, and Small Subunit Ribosomal DNA (ssrDNA), which comprised molecular markers 18S and complete fragment. Non-transcribed internal spacers were grouped separately (ITS 1 and ITS 2) when used alone.

The most employed mitochondrial molecular markers was Cytochrome C Oxidase I (COI) gene followed by mitochondrial genome.



**Figure 3.** Amount of papers, published between 1991 and 2016, featuring molecular markers used in studies on Monogenea, Digenea and Cestoda fish parasites.

## Discussion

It should be underscored that nuclear genes are mostly employed when dealing with molecular markers-based phylogenetic studies. Various evolution rates among the different ribosomal DNA regions, the presence of several copies in most rDNA sequences per genome and the concerted evolution among the repeated copies are among the genes' characteristics which justify their utilization in studies on different groups of living organisms (Álvarez & Wendel, 2003; Hillis & Dixon, 1991).

Results show that lsrDNA in nuclear genes was the most employed region in phylogenetic studies, followed by ssrDNA, ITS 1 and ITS 2 regions. In fact, lsrDNA and ssrDNA genes were preserved throughout the evolution process. Since they contain information on the phylogenetic relations during the eukaryotes' evolution history, these characteristics make them relevant in phylogenetic studies on great groups (Perkins, Martinsen, & Falk, 2011).

As part of the *ssrDNA* region, 18S gene has been employed since 1991 by Baverstock et al. (1991) to test phylogenetic hypotheses within the Platyhelminthes group. It has also been employed to identify species and evidence cryptic species (Curran, Tkach, & Overstreet, 2013; Marigo, Thompson, Santos, & Iñiguez, 2011; Snyder, 2004). 28S gene involves the *lsrDNA* region and has been used to determine phylogenetic positions (Alama-Bermejo, Montero, Raga, & Holzer, 2011) and identify species. This is due to difficulties in identifying some parasite species during certain stages of their life cycle (Born-Torrijos, Kostadinova, Raga, & Holzer, 2012). Another characteristic of these genes is the fact that they contain a nucleotide replacement pattern that provides statistically reliable information for phylogenetic analyses and thus sufficient information for the study of phylogenetic relations during the early evolution of eukaryotes (Hasegawa, Kishino, & Yano, 1985; Hillis & Dixon, 1996).

Further, ribosomal 5.8S gene is generally employed with markers ITS1 and ITS2, which together form the complete region of the transcribed internal spacer (Jousson, Bartoli, Zaninetti, & Pawlowski, 1998). The fragments are separated by the fragment of 5.8S gene; 18S gene lies before ITS1 and 28S gene after ITS2. The regions ITS1 and ITS2 are also molecular markers highly employed in phylogenetic studies and share some characteristics with 18S and 28S genes. However, they have more accelerated evolution rates, with high variations in the spacers among the specimens, following the group of organisms under analysis (Perkins et al., 2011). Accessibility is an asset in the choice of ITS markers since the fragments are close to conserved genes. Consequently, the primers design for the amplification of the region becomes easy (Luton, Walker, & Blair, 1992).

When dealing with phylogenetic studies between parasite genera and species, the more adequate nuclear markers would be those with variability, although they conserve the phylogenetic signal, such as markers ITS1 and ITS2. However, great groups may not be adequate due to high variability which may limit the alignment process of the sequences obtained and directly affect the analysis. In studies on great groups such as order and family, the most indicated would be the conserved genes, such as 18S and 28S, since they maintain a conservation rate throughout the evolution of the eukaryotes, as previously demonstrated (Hillis & Dixon, 1991).

A similar situation to that of ITS regions may be perceived with mitochondrial markers. Highly dissimilar evolution rates, sometimes very fast and

sometimes very slow, may occur in these regions (Vawter & Brown, 1986). Several problems may arise, however, to elucidate deeper relationships between the groups or even between the species which have been formed in very fast speciation processes (Springer et al., 2001).

Some mitochondrial markers, such as COI and D-Loop, are indicated for phylogeographic, separation or not of con-genus species, population studies and adaptive diversification studies (Bueno-Silva, 2012). Mitochondrial genes are haploids since they are inherited from the mother. In fact, they do not have genic recombination and may contain important information on phylogenetic studies between strictly related species (Perkins et al., 2011).

Results revealed that COI was the most employed mitochondrial gene in the reconstruction of the phylogeny of the three fish parasite groups. The gene is characterized by high variability and genetic divergence among the species, besides occurring in the most different taxa (Bueno-Silva, 2012; Kress & Erickson, 2008).

Although it is the most employed mitochondrial marker, gene COI in parasites may have great variability and may not favor a reliable phylogenetic reconstruction (Huyse, Audenaert, & Volckaert, 2003). Due to the previously mentioned characteristics, the marker is not the most adequate to investigate kin relations at order and family levels. However, mitochondrial markers, especially COI, have been extensively used in phylogenetic studies of small groups in fish parasites, perhaps due to the fact that studies on molecular, population and biogeographic systems employ phylogeny but fail to solve kin relationships at higher taxonomic levels. It may be also due to the easiness in amplifying the region by polymerase chain reaction and to positive results obtained by Hebert, Cywinska, Ball, and DeWaard (2003) in the use of the region as a universal barcode in species distinction. Consequently, the gene is one of the most employed for the characterization of animal species for phylogenetic studies among close species.

When possible contributions in the use of molecular techniques are taken into consideration, it may be observed that the number of studies in the area and in phylogenetic studies has increased. In fact, since 2000, there has been an increase in the number of phylogenetic studies with phylogenetic markers, triggered by the comprehensiveness and advance in the molecular biological techniques and their availability (Gasques et al., 2013; Patwardhan et al., 2014), coupled to the difficulty in the morphological identification of fish parasites which are frequently microscopically sized and

characterized by a simple morphology, hindering the obtaining of synapomorphy for phylogenetic reconstructions (Luton et al., 1992; Perkins et al., 2011).

## Conclusion

Progress in molecular biology and bio-computer techniques, coupled to more robust phylogenetic programs and analyses, has triggered an increase in the use and acknowledgement of such techniques in phylogenetic and taxonomic investigations of fish parasites. Molecular phylogenetics has provided new information on kinship among parasite groups, in species identification and in population and biogeographic studies. Nevertheless, molecular phylogeny has not solved all taxonomic and phylogenetic issues in Ichthyoparasitology. The use of such tools is recommended when possible and when required in the hypothesis test, coupled to morphological information. The employment of more than one gene is also recommended for greater reliability in phylogenetic reconstructions, developing evolution rates of the organism and not merely that of one isolated gene.

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