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REVIEW

Molecular studies in Brazilian malacology: Tools, trends and perspectives

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Abstract

The use of molecular tools in Brazilian malacological studies was analyzed. We searched for these molecular tools by screening the annals of malacological events in Brazil and Latin America within the last 15 years. Information was obtained from a Brazilian curricula data bank (*curriculum Lattes*), which enabled the identification of the main national research groups, their main researchers and research focuses. An estimate of the scientific production on the basis of the analysis of these curricula is also provided. The main techniques and molecular markers employed were indicated. The data showed that these studies were concentrated in institutions in the South and Southeast regions and emphasized gastropods of medical importance. However, molecular studies that examined other gastropods, cephalopods and bivalves were also performed. No research study was found in the Central-West region of the country. The growth of this field of study in the country is expected, considering the molecular studies that have been performed and the great diversity of the molluscan fauna in Brazil. Future molecular studies on some taxa in the country, which have not yet been studied, should be contemplated. The increase in the use of sequencing techniques will help to bridge the gaps in our taxonomic and phylogenetic knowledge, contributing to the studies on biodiversity and conservation.

Keywords: Molecular biology, malacology, snail, Brazil, DNA.

Introduction

Mollusca is the second largest phylum in species number, second only to Arthropoda. It includes animals with a wide diversity of body plans (e.g., octopi, mussels, snails and slugs), which inhabit all types of environments. The phylum also includes organisms of medical, veterinarian and economic importance. Despite this and despite the great biodiversity, extensive coastline and geographic area of Brazil, the country still lacks information on its invertebrate fauna, including mollusks (Simone, 1999 a,b,c). The refinement of taxonomic and phylogenetic proposals as well as studies on population genetics are important for the conservation and monitoring of molluscan diversity, and in this context, classic taxonomic studies associated with the use of molecular tools are very important (Santos et al., 2009).

In a review of Brazilian zoology, Santos et al. (2009) made important considerations on Brazilian terrestrial, freshwater and marine mollusks, highlighting their diversity and taxonomic, ecological, economic and public-health aspects. Among the most important problems in Brazilian malacology, they emphasized the following: 1) small attention has been given to terrestrial and freshwater gastropods, except for those of medical and veterinary importance; 2) studies on freshwater bivalves are concentrated on invasive species; 3) despite the abundance of marine mollusks in Brazil, knowledge on them is still limited; 4) invasive species must be further studied due to their potentially high environmental impact; and 5) malacofaunistic inventories are necessary, particularly in areas under strong anthropic pressure, for the detection of endangered species, among other reasons.

Molecular biology has been a recurrent theme in the last events of Brazilian malacology (e.g., EBRAM 2005-2011 and CBZ, 2010). This review intends to offer an overview on this subject and to complement that of Santos et al. (2009), addressing more broadly and in more detail the molecular studies in Brazilian malacology within the last 15 years. These studies were first evaluated through the abstracts published in the following meetings: "Encontro Brasileiro de Malacologia" (EBRAM), "Congresso Latino Americano de Malacologia" (CLAMA) and "Congresso Brasileiro de Zoologia" (CBZ), in which most of the studies by Brazilian malacologists were presented (Santos et al., 2009). The evaluation of these abstracts enabled the identification of the main Brazilian research groups. Consultation of the curricula bank, which is maintained by the Brazilian national council for scientific development (CNPq -

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http://lattes.cnpq.br), and the internet search engines of Google Scholar and PubMed enabled the identification of the researchers and research focuses of these groups. Research groups were identified on the basis of the target organism, on their research focus and authorship of scientific papers. Determination of each group's geographic region was on the basis of the locality of the home institution of most researchers.

The abbreviations used in the text, tables and figures in this review include the following: CPqRR – Centro de Pesquisas René Rachou, Fiocruz; ESALQ-USP – Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo; IBAG – Instituto de Biologia Roberto Alcântara Gomes; IECOS – Instituto de Estudos Costeiros; IOC – Fiocruz: Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro; PUCRS – Pontifícia Universidade Católica do Rio Grande do Sul; SUCEN – Superintendência de Controle de Endemias São Paulo; UFAC – Universidade Federal do Acre; UFF – Universidade Federal Fluminense; UFMG – Universidade Federal de Minas Gerais; UFRJ – Universidade Federal do Rio de Janeiro; UFRN – Universidade Federal do Rio Grande do Norte; UFSC – Universidade Federal de Santa Catarina; UMC – Universidade de Mogi das Cruzes, São Paulo; UNESP – Universidade Estadual Paulista Júlio de Mesquita Filho; UNICAMP – Universidade Estadual de Campinas.

Molecular tools employed in Brazilian malacology

Several molecular markers and techniques have been applied in the genetic studies of different organisms. Protein electrophoresis was largely employed in population studies in the 1970's (Murphy et al., 1996). Due to its low cost and high applicability in population genetics (Thorpe & Sole-Cava, 1994; Murphy et al., 1996; Weber & Silva, 2008), this technique is still in use. In Brazil, isozymes were employed in the population studies of marine gastropods in the families Litorrinidae and Acmaeidae (Andrade et al., 2003, 2005; José & Solferini, 2007); of bivalves in the genera *Crassostrea* Sacco, 1897 (Bivalvia, Ostreidae), *Mytella* Soot-Ryen, 1955 (Bivalvia, Mytilidae) and *Perna* Philipsson, 1788 (Bivalvia, Mytilidae); and of the freshwater mussel *Limnoperna fortunei* (Dunker, 1857) (Bivalvia, Mytilidae) (Ignácio et al., 2000; Oliveira et al., 2005; Moura-Neto et al., 2005; Duarte et al., 2005; Weber & Silva, 2008). Allozyme markers have also been used to identify the native or exotic origin of the most common mussel in southern and southeastern Brazil, *Perna perna* (Linnaeus, 1758) (Bivalvia, Mytilidae). Using these markers, Souza et al. (2004; 2009) suggested that Brazilian populations of this species most likely originated from an African stock. Brazilian specimens of *P. perna* were employed for the identification and sequencing of the main component protein of the byssal threads, which are valued in biotechnology due to its specific features such as resistance, elasticity and adhesion underwater (Clezar et al., 2008).

The development of the polymerase chain reaction (PCR) allowed considerable progress in molecular studies due to its simplicity and sensitivity. The amplification of nonspecific fragments to generate species-specific or population-specific band patterns has been widely used (Carvalho et al., 2001). Techniques based on the amplification of specific fragments of both mitochondrial and nuclear DNA have been widely employed and help to obtain more conclusive and consistent data in population genetics, taxonomy, and phylogeny of mollusks. The phylogenetic relationships among the classes of Mollusca and among their component groups are considered one of the most defying problems for molecular phylogeneticists (Giribet et al., 2007).

Amplification of nonspecific DNA regions:

The random amplified polymorphic DNA (RAPD) method was employed in the study of *Biomphalaria. tenagophila* (Orbigny, 1835) (Gastropoda, Planorbidae) lineages, which are susceptible and resistant to *Schistosoma mansoni* Sambon, 1907 (Trematoda, Digenea) and showed genetic variation between them (Abdel-Hamid et al., 1999). RAPDs were also employed as genetic markers of the populations of *Biomphalaria glabrata* (Say, 1818) and *B. tenagophila*, which are susceptible and resistant to *S. mansoni*, respectively (Spada et al., 2002; Silva et al., 2004b; Rosa et al., 2005; Oliveira et al., 2010), as well as in the study of genetic variability in populations of *Lymnaea* Lamarck, 1799 (Gastropoda, Lymnaeidae) (Cardoso et al., 2006). In bivalves, RAPD analyses were employed in the genetic characterization of the scallop *Nodipecten* Dall, 1898 (Pteriomorphia, Pectinidae) and of natural stocks of *P. perna* from the coast of the Brazilian state of Santa Catarina (Souza et al., 2006; Malaquias et al., 2008). In the mid 1990's, low-stringency polymerase chain reaction (LS-PCR) was used for the identification of species of *Biomphalaria* Preston, 1910 (Vidigal et al., 1996; Pires et al., 1997) and for the detection of *S. mansoni* infection in *B. glabrata* (Jannotti-Passos et al., 1997), *B. straminea* (Dunker, 1848) and *B. tenagophila* (Jannotti-Passos & Souza, 2000). LS-PCR was also employed to demonstrate the resistance of *B. occidentalis* Paraense, 1981 against infection by *S. mansoni* (Souza & Jannotti-Passos, 2001).

Specific DNA regions:

DNA regions mostly employed in systematics include mitochondrial (mtDNA) and ribosomal nuclear (rDNA) genes. Mitochondrial DNA regions appropriate for intra- and interspecific studies include the subunit 1 of the enzyme cytochrome c oxidase (COI) and the 16S region (Palumbi et al., 1996). Restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) using these DNA regions is one of the most employed auxiliary techniques in *Biomphalaria* systematics. Thus, PCR-RFLP of the Internal Transcribed Spacer of the rDNA (ITS - which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) was employed in the identification of 10 Brazilian species of *Biomphalaria* (Vidigal et al., 1998, 2000a), of other South American and Cuban species of the same genus (Caldeira et al., 2000; Vidigal et al., 2001; Velásquez et al., 2002) and of morphologically similar species in the *B. straminea* and *B. tenagophila* complexes (Caldeira et al., 1998; Spatz et al., 1999), among others (Spatz et al., 2000). *B. tenagophila guaibensis* Paraense, 1984, *B. oligoza* Paraense, 1974 and *B. peregrina* (Orbigny, 1835) of several localities in the state of Rio Grande do Sul were also identified using this technique (Pepe et al., 2009). Later, the molecular characterization of *B. amazonica* Paraense 1966 and *B. cousini* Paraense, 1966 was performed, and *B. cousini* was recorded in Brazil for the first time on the basis of PCR-RFLP of the ITS region, sequences of the 16S and ITS2 regions and morphology (Teodoro et al., 2010). PCR-RFLP was also employed in the

verification of the hybridization between *B. cousini* and *B. amazonica*, and the susceptibility of their hybrids to *S. mansoni* was evaluated (Teodoro et al., 2011). PCR-RFLP of the ITS region was used in the identification of species of the genus *Lymnaea* from different localities in Brazil and Argentina (Carvalho et al., 2004). The COI region was used in the differentiation of three species of *Biomphalaria,* which are the natural hosts of *S. mansoni* in Brazil (Vidigal et al., 2002b). The 16S region was employed in the identification of the native species of bivalves *Crassostrea brasiliana* (Lamarck, 1819) and *C. rhizophorae* (Guilding, 1828) and the introduced species *C. gigas* (Thunberg, 1793) (Pie et al., 2006b). Melo et al. (2010b) called attention to the use of this technique in the molecular identification of the oysters of the genus *Crassostrea*, which are economically important and are commonly cultivated in Brazil. Differences in the size of the fragments of the 5S region enabled the distinction of six species in five different genera (Sales et al., 2011) belonging to two families of Brazilian cephalopods.

Microsatellites (Weber & Silva, 2008) have been used in the analysis of genetic variability of different organisms due to their high rates of polymorphism. Thus, among mollusks, the genetic variability of species in the *B. straminea* species-complex was studied using "simple sequence repeat anchored polymerase chain reaction amplification" or SSR-PCR (Zietkiewicz et al., 1994; Caldeira et al., 2001). This technique was used to study the genetic variability of *B. glabrata* populations (Campos et al., 2002). Microsatellite analyses were also used to study the population genetics of the octopus *Octopus vulgaris* Curvier, 1797 (Cephalopoda, Octopoda) in the Brazilian coast (Moreira et al., 2011), in the study of the genetic variability of the bivalve *P. perna* (Appio & Weber, 2007) and in an evaluation of the population structure of the invasive gastropod *Achatina* (*Lissachatina*) *fulica* Bowdich, 1822 (Stylommatophora, Achatinidae) (Zanol et al., 2009). Microsatellite markers were employed for the characterization of Brazilian native oysters in the genus *Crassostrea* (Melo et al., 2002).

Multiplex-PCR has been used in the molecular epidemiology of schistosomiasis (Caldeira et al., 2009). First, primers for the identification of the three natural hosts of *S. mansoni* in Brazil were designed from ITS2 sequences (Vidigal et al., 2002a) and used in the same reaction to produce a characteristic fragment for each target species. Posteriorly, a pair of specific primers for the mtDNA of *S. mansoni* was added to the reaction, enabling the simultaneous identification of the *Biomphalaria* species and detection of intra-mollusk infection (Janotti-Passos et al., 2006).

Specific primers from sequences of the regions 18S and COI were used in the early detection of *L. fortunei* larvae (Pie et al., 2006a; Boeger et al., 2007). The early detection of the invader and the knowledge of its population structure are considered important factors in the management of invasive species (Junqueira et al., 2009). The golden mussel, *L. fortunei,* caused a series of environmental damages and threatened the electric sector, irrigated agriculture, fishery and water supply in different South American countries, including Brazil (Darrigran et al., 2007; Boltovskoy et al., 2009)

DNA sequence data of mollusks:

Sequence analysis is a very informative tool used in the molecular systematics of different mollusk groups in Brazil, with an emphasis on regions such as ITS, COI and 16S. Universal primers, which amplify part of the COI of 11 invertebrate phyla, including Mollusca, have been used in species identification and phylogenetic studies (Folmer et al., 1994). This molecular approach has helped classic taxonomy and phylogenetic analysis, and its standardization has been facilitated when morphological knowledge is well established, for example, in the genera *Biomphalaria* and *Phyllocaulis* Colosi, 1922 (Gastropoda, Veronicellidae) (Vidigal et al., 2001; Gomes et al., 2010c). When specific identification is made difficult by variations in morphological characteristics and ecological similarity, COI analysis may direct the studies and contribute to the elucidation of taxonomic and phylogenetic studies (*e.g.,* bivalves in the genus *Crassostrea*) (Melo et al., 2010c; Lazoski et al., 2011). Herbet et al. (2003), after analyzing the COI sequence polymorphism among and inside species belonging to diverse animal groups, found this marker suitable for species diagnosis, giving birth to DNA barcoding techniques. This technique aims at the characterization of species using sequences of a short standard DNA region and is a promising strategy for the evaluation of the biodiversity on Earth (Hajibabaei et al., 2007; Miller, 2007). However, some researchers argue that it would be better to employ molecular information as an additional tool in taxonomic studies, instead of, as originally conceived, DNA barcoding, and confine them to the molecular information of a single DNA region (Sole-Cava, 2008). Moreover, the difficulty in considering intraspecific variability is another negative aspect of DNA barcoding (Sole-Cava, 2008). Despite all of the current controversy, this tool is being widely employed, including in the study of *B. tenagophila* complex species (Tuan et al., 2012).

In cephalopods, two studies can be highlighted: the use of COI sequences for the molecular distinction of two species of *Octopus* (Cephalopoda, Octopodidae) (Moreira et al., 2007) and the characterization of the 16S region with the morphological description of *Octopus insularis* (Leite & Haimovici, 2008). This latter paper, combined with those of Moreira et al. (2011), employing microsatellites, and Sales et al. (2011), using the rDNA 5S region, are some of the most relevant contributions to the knowledge of Brazilian cephalopods.

Sequence analyses have also been employed for species identification and phylogenetic studies of bivalves. COI sequences were employed in phylogeographic studies of bivalves in the genus *Donax* Linnaeus, 1758 (Bivalvia, Veneroidea) (Silva et al., 2005) and in the phylogenetic study of four Amazonian species of Hyriidae (Santos-Neto et al., 2007). The 16S region was used in the study of the Teredinidae (Santos et al., 2005) and has helped in the identification of *Crassostrea* of the Brazilian coast, as well in the determination of their distribution. It was also employed in phylogenetic studies of the genus (Varella et al., 2007). In addition, the ITS1 region was employed in the identification of Brazilian native species of *Crassostrea* (Melo & Tagliaro, 2010).

Arruda et al. (2009) showed the viability of using the COI region in the study of the genetic diversity of *Anomalocardia brasiliana* (Gmelin, 1791) (Bivalvia, Veneroidea), which also has potential for commercial production. COI sequencing of four Brazilian populations of this species has revealed great haplotype diversity. Conservation of such genetic diversity is important in the management and conservation of this species, as well as in the development of new lineages suitable for cultivation. Furthermore, genetic studies employing COI have been applied

to ostreiculture. In fact, the propagation and sharing of genetic information may help in population management because interchange among genetically homogeneous populations minimizes the impact of genetic depression (Paula et al., 2010). In addition, this region has been employed in studies of populations of *Mytella guyanensis* (Lamarck, 1819) (Gomes et al., 2010b) and in the molecular identification, phylogeny and geographic distribution of Brazilian species of *Crassostrea* (Melo et al., 2010b).

Sequences of several DNA regions (ITS1, ITS2 and 16S) were used in the study of the phylogenetic relationships of *Biomphalaria* in different regions of South America, including Brazil, Colombia and Argentina (Vidigal et al., 2000b; Vidigal et al., 2004b; Estrada et al., 2006). In a phylogenetic study involving Neotropical and African species, the African lineages were derived in relation to the Neotropical lineages, suggesting an American origin of the genus (DeJong et al., 2001). The ITS2 and 16S regions showed considerable length variation (larger in the 16S) and were informative for the study of phylogenetic relationships of *Biomphalaria* (Teodoro et al., 2010). Tuan & Santos (2007) compared the ITS2 of *B. glabrata, B. tenagophila, B. occidentalis* and *B. peregrina* obtained in the Paranapanema valley (São Paulo state) with sequences of *Biomphalaria* available in GenBank. They addressed topics on the population structure and phylogenetic relationships in the genus and highlighted the relationships among species in the *B. tenagophila* complex. Although still considering gastropods of medical interest, the analysis of 16S, COI and ITS2 sequences have been important in phylogenetic inferences of *Phyllocaulis*, of which some species are considered as agricultural pests and as intermediate hosts of the nematodes *Angiostrongylus costaricensis* Moreira & Cespedes, 1971 and *A. cantonensis* (Chen, 1935) (Nematoda, Protostrongylidae) (Gomes et al., 2010c). COI sequences were also employed in the systematic studies of *Omalonyx* d'Orbigny, 1837 (Gastropoda, Succineidae) (Coscarelli & Vidigal, 2009). These mollusks are widely distributed in South America and are intermediate hosts of trematodes in the genus *Leucochloridium* Carus, 1835 (Digenea: Leucochloriidae) (Lutz, 1921) and potential hosts of *Angiostrongylus* species (Montresor et al., 2008; Mozzer et al., 2011).

Dewilde et al. (1998) reported on the use of globins as molecular markers and characterized sequences of myoglobins (Mbs) and of the hemoglobin (Hb) of *B. glabrata.* On the basis of these data, Coscarelli et al. (2009) began the analysis of the Mb intron and exon regions of *B. glabrata* and other Brazilian species of *Biomphalaria.* Despite the large size variation observed in the intron sequences of some species, this was considered a promising approach to analyze genetic variability and to identify species in this genus. Importantly, Mb sequences were employed in studies of systematics and population genetics of mollusks of different classes, in which the structural role of different polymorphisms in these proteins were investigated (Dewilde et al., 1998; Medeiros et al., 1998; Suzuki et al., 2003a, b; Lieb et al., 2006). Recently, the molecular and biochemical characterization of the myoglobin of the Brazilian species *Biomphalaria* was performed (Teixeira et al., 2011).

Analysis of the COI region of ampullariid populations, including Brazilian ones, was employed to determine the origin of *Pomacea* Perry, 1811 (Caenogastropoda, Ampullariidae), which was introduced into Asia (Hayes et al., 2008). The use of COI sequences in the phylogeographic and population structure studies of the invasive gastropod *A. fulica* was highlighted by Zanol et al. (2009). The introduction of a land snail, *Meghimatium pictum* Stoliczka, 1873 (Gastropoda, Phylomycidae), from China was confirmed from the study of morphological characteristics and analyses of COI sequences (Gomes et al., 2011).

The simultaneous use of several techniques and DNA regions is prominent in the search for consistent, reliable results. This was the case in the study of natural populations of *C. gigas* in Brazil by Melo et al. (2010a), who employed morphology, PCR-RFLP of nuclear and mitochondrial genes and the sequencing of ITS2 and 16S regions. In a study of the phylogenetic relationships among *Crassostrea* species of the Atlantic sea, COI, 16S and ITS2 sequences were analyzed (Lazoski et al., 2011). In studying the mytilid *P. perna,* Weber et al. (2009) constructed a restriction map of the 16S region, sequenced this same region, and analyzed the mtDNA inheritance and phylogeny of the genus. In a phylogenetic study of ampullariid snails, among which Brazilian species were included, Hayes et al. (2009) sequenced the COI, 16S and 18S regions. Recently, Tuan et al. (2012) studied the pattern of genetic divergence of COI and 16S sequences and analyzed the morphology of *B. tenagophila* complex species.

Current status of the studies on Brazilian malacofauna

The molecular techniques used in the study of Brazilian malacofauna, their basic characteristics, applications (e.g., taxonomic, phylogenetic or population research), target genera and the most relevant Brazilian studies related to each technique are listed in Tab. 1. The research groups, research lines, studied organisms, main researchers and their institutions, and each group's scientific publications are listed in Tab. 2. The analysis of the scientific publications on Brazilian malacofauna and the mollusk genera studied revealed that the most employed techniques were the PCR-RFLP of the ITS region and sequence analysis of the ITS, 16S and COI regions (Tab. I). Most publications were produced in institutions in the South and Southeastern regions (Fig. 2). The Midwest region was the only one where molecular methods were not employed. Brazilian research groups working on molecular malacology have restricted their studies on the classes Cephalopoda, particularly in Gastropoda and Bivalvia (Fig. 1), despite the occurrence of the classes of Aplacophora, Polyplacophora and Scaphopoda in the country. The analysis of target organisms (genera) listed in Tab. 2 also shows that more emphasis is given to gastropods of medical importance. In fact, among the 11 research groups working on Gastropoda, nearly 60% (numbers 1 to 4, 6, 7 and 9 in Tab. 2) of the groups work with species of medical and veterinary importance. Data show the important contribution of the 20 Brazilian research groups in the molecular study of molluscan fauna. The different molecular approaches used to solve several problems (Tab. 1) demonstrate the great potential of this investigative area in Brazil.

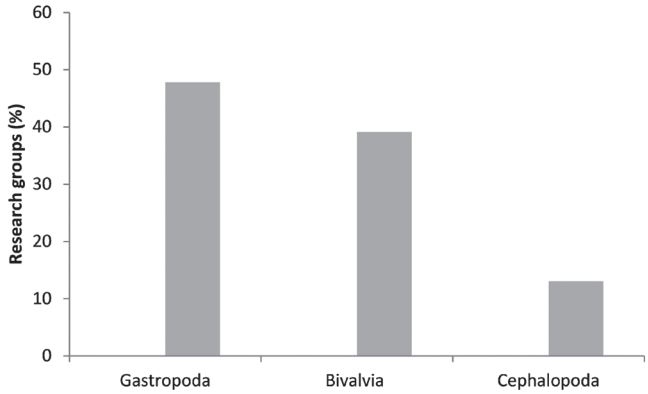

Figure 1 - Percentage of research groups involved in the molecular studies of each mollusk class.

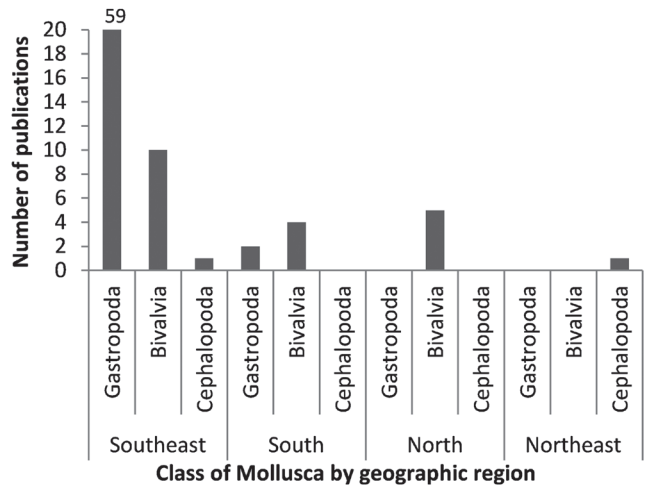

Figure 2 - Number of papers presenting molecular studies of each mollusk class, grouped by Brazilian geographical regions.

Expansion of molecular studies: opportunities and challenges

The expansion of molecular studies has dramatically changed our understanding of the phylogenetic relationships inside and among different animal groups. Advances in the knowledge of metazoan phylogeny have been produced by molecular systematics, particularly by phylogenomic methods. A few examples of such methods are *a)* the complete sequencing of the mitochondrial genome; *b)* the study of exclusive properties of genomes, such as the order and number of genes; and *c)* the study of partial transcriptomes, for example, DNA expressed sequence tags (ESTs) (Edgecombe et al., 2011). The analysis of ESTs has been widely used to accelerate the phylogenetic studies of different animals, including mollusks (Edgecombe et al., 2011; Smith et al., 2011). Examples of the use of ESTs in malacology are found in the international literature and include mollusks of medical and veterinary importance (*Biomphalaria* and *Lymnaea*) as well as mollusks of economic importance such as oysters (*Crassostrea*) (Lockyer et al., 2007).

Important steps are being taken on phylogenomic studies in Brazil, including the complete sequencing of mtDNA of *B. tenagophila* (Janotti-Passos et al., 2010), the ESTs databank for *B. glabrata* (http://www.snaildb.org/) (Tab. 1) and the participation of Brazilian researchers in the *B. glabrata* genome project (Raghavan & Knight, 2006; http://biology.unm.edu/biomphalaria-genome/consortium.html). The phylogenomic tools have generated conclusive data on the evolutionary relationships among Mollusca (Smith et al., 2011), and the availability in data banks of mitochondrial genomes of several genera of different classes allow for the study of phylogenetic relationships in the phylum (Serb & Lydeard, 2003). Twenty complete sequences of mtDNA were available at the National Center for Biotechnology (www.ncbi.nlm.nih.gov/genome) in 2006, belonging to the classes Bivalvia (6), Gastropoda (7), Cephalopoda (4), Polyplacophora (1) and Scaphopoda (1) (Vallés & Boore, 2006). Simison & Boore (2008) in a revision of the evolutionary genomics of Mollusca found 37 complete sequences of mitochondrial genomes: Bivalvia (13), Gastropoda (12), Cephalopoda (9), Polyplacophora (1), Scaphopoda (2). However, the mitochondrial genomes of Monoplacophora and Aplacophora have not been reported. Currently, 130 complete sequences of molluscan mtDNA are available at NCBI (data obtained in June 2012), representing the following classes: Bivalvia (52), Gastropoda (55), Cephalopoda (19), Polyplacophora (1), Scaphopoda (2) and Aplacophora (1). Monoplacophora was the only class that did not present any sequences until now. These results highlight advances in the research of the mitochondrial genome of Mollusca, as suggested by Vallés & Boore (2006) and Simison & Boore (2008). Although maternal inheritance of mtDNA is observed in almost all of the animal groups, there were exceptions (Breton et al., 2007). Among the mollusks, the doubly uniparental inheritance (DUI) of mtDNA was observed in some bivalves (the marine bivalves Mytiloida and Veneroida; the freshwater bivalve Unionoidea), and this fact stimulated the study of mtDNA genome in mollusks (Vallés & Boores, 2006; Breton et al., 2007). In Brazil, maternal and paternal mitochondrial lineages were found in one species of *Mytella* (Alves et al., 2007); however, no evidence of DUI could be found in *P. perna* (Weber et al., 2009).

Conclusions and perspectives

The analyses conducted here showed that several methods and molecular markers have been used in the study of mollusks in Brazil. They showed that the frequency of studies involving nucleotide sequences (e.g., of a gene or specific genomic regions) is increasing, while indirect techniques such as protein electrophoresis and others, such as RAPD and PCR-RFLP, are being abandoned.

The method selection should be carefully conducted, taking in consideration its adequacy to resolve target problems because no technique is optimal for all purposes (Palumbi, 1996; Moritz & Hillis, 1996; Silva & Russo, 2000). Several molecular methods can be applied to the study of mollusk systematics and phylogeography and each one has its peculiarities, which should be considered given the biological differences among different

Table 1 - Main molecular techniques employed in Brazilian malacology, their characteristics, applications, mollusk genera studied and bibliographical information sources. Based on the summaries published in the annals of the Brazilian Meeting of Malacology (EBRAN – 1999, 2001, 2003, 2005 and 2007, 2009 and 2011), the Brazilian Congress of Zoology (CBZ – 2000, 2002, 2004, 2006, 2008 and 2010), the Latin-American Congress of Malacology (CLAMA – 2002, 2005 and 2008) and the analysis of the publications in the curricula in the *Lattes* data bank (see text) for the period between January 1997 and March 2012.

Techniques		Applications			Studied Genera[2]	Main References[3]
Name	Characteristics[1]	Species identification	Genetic variability	Phylogeny		
ARAPD - PCR	Use of primers of randomly selected sequences.		+		*Biomphalaria, Lymnaea, Nodipecten, Perna.*	Abdel-Hamid 1999; Spada et al. 2002; Silva et al. 2004b; Cardoso et al. 2006; Souza et al. 2006; Malaquias et al. 2008; Oliveira et al. 2010.
Sequencing of nuclear DNA markers ITS/18S/-DNAr	Use of universal primers.	+	+	+	*Biomphalaria, Crassostrea, Litorrina, Phyllocaulis, Physa, Succinea.*	Paula et al. 2002; Silva et al. 2004a; Vidigal et al. 2000b, 2004, 2007b; Ramires et al. 2006; Estrada et al. 2006; Tuan & Santos 2007; Gomes et al. 2010c; Melo et al. 2010a, Melo & Tagliaro 2010; Teodoro et al. 2010; Dayrat et al. 2011, Lasoski et al. 2011.
Sequencing of mtDNA markers (COI)	Use of universal primers.	+	+	+	*Anomalocardia, Biomphalaria, Castalia, Corbula, Crassostrea, Diplodon, Donax, Limnoperna, Meghimmatium, Mesodesma, Mytella, Octopus*, *Omalonyx, Paxydom, Phyllocaulis, Sarasinula, Triplodon.*	Silva et al. 2004a; Pie et al. 2006a; Alves et al. 2007; Boeger et al. 2007; Hilsdorf 2007; Melo et al. 2007; Moreira et al. 2007; Paula et al. 2007; Santos-Neto et al. 2007; Tuan et al. 2008; Arruda et al. 2009; Coscarelli & Vidigal 2009; Darrigran et al. 2009; Santos-Neto et al. 2009; Silva et al. 2009, 2010; Quast et al. 2009; Gomes et al. 2010a,c; Gomes et al. 2011, Melo et al. 2010c; Tagliaro et al. 2010; Dayrat et al. 2011; Lasoski et al. 2011, Tuan et al. 2012
Sequencing of mtDNA markers (16S)	Use of universal primers.	+		+	*Biomphalaria, Bankia, Bostryx, Castalia, Crassostrea, Diplodon, Nausitora, Neoteredo, Octopus, Paxyodon, Phyllocaulis, Psiloteredo, Sarasinula, Succinea, Triplodon.*	Silva et al. 2001, 2004a; Ramires et al. 2003 a.b; Santos et al. 2005; Varela et al. 2007; Leite et al. 2008; Gomes et al. 2010c, b; Melo et al. 2010a, Dayrat et al. 2011, Lasoski et al. 2011, Tuan et al. 2012
Sequencing of complete mitochondrial genome			+	+	*Biomphalaria.*	Janotti-Passos et al. 2010.

Table 1 - Continued.

Techniques		Applications			Studied Genera[2]	Main References[3]
Name	Characteristics[1]	Species identification	Genetic variability	Phylogeny		
conventional PCR PCR/ RFLPs (rDNA, and mtDNA)	Use of universal primers. Use of restriction enzymes.	+			*Biomphalaria, Crassostrea, Illex, Loligo, Lollinguncula, Lymnaea, Ornithoteuthis, Perna, Sepioteuthis*.	Caldeira et al. 1998, 2000, 2001, 2009, 2010; Vidigal et al. 1998, 2000a, 2001, 2002b, 2004a; Spatz et al. 1999,2000; Velásquez et al., 2002; Carvalho et al. 2004; Rosa et al. 2004; Pie et al. 2006b,; Sousa et al. 2009; Weber et al. 2009; Melo et al. 2010a, Sales et al. 2011, Teodoro et al. 2010, 2011.
LS-PCR	One pair of specific primers (20 bp) low stringency PCR	+	+		*Biomphalaria.*	Jannotti-Passos & Souza, 2000; Souza & Jannotti-Passos, 2001.
Microsatellites	Specific primers for microsatellite regions.		+		*Crassostrea, Octopus, Perna.*	Appio & Weber 2007; Galvão & Hilsdorf 2009. •Weber & Silva 2008; Moreira et al. 2011
SSR-PCR	Primers directed for intra- and inter- satellite regions.		+		*Biomphalaria.*	Caldeira et al. 2001; Campos et al. 2002.
Multiplex-PCR	Use of several specific primers simultaneously.	+			*Biomphalaria, Lymnaea.*	Vidigal et al. 2002a; Janotti – Passos et al. 2006; Magalhães et al. 2008; Caldeira et al. 2009.
Sequencing of cDNA and exons and introns of the gene encoding myoglobin	Use of several specific primers for *Biomphalaria glabrata* (Dewilde et al. 1998)	+	+	+	*Biomphalaria.*	Coscarelli et al. 2009; Teixeira et al. 2011.
EST analysis	Short sequences obtained from clones selected from cDNA libraries.	Identification of mollusk genes homologous to the genes of other species	+	+	*Biomphalaria.*	*Biomphalaria glabrata* data base (www.snaildb.org/)
Isozyme/ allozyme electrophoresis	Protein physical chemistry. Use of different enzyme systems and evaluation of their variations.		+		*Anomalocardia, Collisella, Crassostrea, Limnoperna, Litorrina, Littoraria, Nodilittorina, Mytella, Perna.*	Ignácio et al. 2000; Andrade et al. 2003; Oliveira et al. 2005; Duarte et al. 2005, Moura-Neto al. 2005; José & Solferini 2007; Andrade & Solferini, 2007; Weber & Silva 2008.

[1] Compiled from Jones et al. (1999) with modifications.
[2] Includes the main summaries in the annals of scientific meetings (published articles underlined).
[3] A few exotic genera were included.

Table 2 - Main Brazilian research groups employing molecular techniques in malacological studies: Research lines, studied mollusks, researchers and institutions. Based on the summaries published in the annals of the Brazilian Meeting of Malacology (EBRAN – 1999, 2001, 2003, 2005 and 2007, 2009 and 2011), the Brazilian Congress of Zoology (CBZ – 2000, 2002, 2004, 2006, 2008 and 2010), the Latin-American Congress of Malacology (CLAMA – 2002, 2005 and 2008) and the analysis of the publications in the curricula in the *Lattes* data bank (see text) for the period between January 1997 and March 2012. A few exotic genera were included. Superscript numbers indicate the researcher's institutions.

Groups[1]	Research Line	Class/Genera	Main Researchers	Institution/State	Articles
01	Population genetics. Molecular phylogeny and systematics. Phylogeography.	Gastropoda: *Achatina, Asolene, Marisa, Pomacea*	Cláudia A. M. Russo; [1]Joana Zanol P. da Silva, [2]Silvana A. R. C. Thiengo	Instituto de Biologia, Depto Genética, UFRJ. [2]Depto. Malacologia, IOC/Fiocruz - Rio de Janeiro - (RJ).	-
02	Molecular/morphological phylogeny and systematics.	Gastropoda: *Biomphalaria, Lymnaea*	[3]Omar dos S. Carvalho, [3]Roberta L. Caldeira, [3]Liana K. Jannotti-Passos	[3] Lab. Helmintologia e Malacologia Médica/ CpqRR - Minas Gerais - (MG).	41
03	Molecular/morphological phylogeny and systematics. Myoglobins.	Gastropoda: *Biomphalaria, Omalonyx, Physa*	[4]Teofânia H. D. A. Vidigal, [5]Cristiana A. Brito, [6]Marcelo M. M. Santoro	[4] Depto. Zoologia, Lab. Malacologia / UFMG; [5] Lab. Malaria/ CPqRR. [6] Depto Bioquímica e Imunologia/ UFMG - (MG).	25*
04	Molecular systematics, phylogeny and ecology.	Gastropoda: *Phyllocaulis, Sarasinula*	[7]Suzete Rodrigues Gomes, [8]Sandro Luís Bonatto, [8]Fernanda Britto da Silva, [9]José Willibaldo Thomé, [10]Eduardo Colley, [11]Eliana Nakano, [12]Juliane B. Picaço	[7]*United States Department of Agriculture USA*. [8]Instituto de Biociências, Biotecnologia Genômica e Molecular/PUCRS, Rio grande do Sul - (RS). [9] Escr. de Malacologia e Biofilosofia (RS). [10] Depto. de Zoologia/ UFPR - Paraná (PR). [11] Lab. Parasitologia/Malacologia/ Butantan (SP). [12] Lab. Genética humana, Faculdade de Biociências (RS).	2
05	Molecular systematics.	Gastropoda: *Bostryx, Succinea*	[9,13]Rina L. Ramírez Mésias, [8]Sandro L. Bonatto, [9]José Willibaldo Thomé	[13] Malacologia, PUCRS. [8] Instituto de Biociências, Biotecnologia Genômica e Molecular/ PUCRS; [9] Escr. Malacologia e Biofilosofia - (RS).	-
06	Molecular genetics.	Gastropoda: *Biomphalaria*	[14] Rodolfo G. M. Spada, [15]Eliana M. Zanotti-Magalhães	[14] ICB/UNESP. [15] Depto Parasitologia/ UNICAMP, São Paulo - (SP)	6
07	Molecular genetics and phylogeny.	Gastropoda: *Biomphalaria*	[16] Roseli Tuan	[16] Lab. de Bioquímica e Biologia Molecular/ SUSCEN- (SP).	3
08	Molecular genetics / Molecular/morphological systematics.	Gastropoda: *Litorrina*	[17] Gisele Lobo-Hajdu, [18]Ricardo S. Absalao	[17] IBAG, Depto. de Genética/ UERJ. [18]IBAG, Depto Zoologia/ UERJ - (RJ).	-

Table 2 - Continued.

Groups[4]	Research Line	Class/Genera	Main Researchers	Institution/State	Articles
09	Transcriptome/ molecular characterization	Gastropoda: *Biomphalaria*	[19] Guilherme Corrêa de Oliveira***	[19] Parasitologia Celular e Molecular/CpqRR - (MG).	
10	Population genetics. Genetic variation. Morphological systematics.	Gastropoda: *Collisella, Echinolittorina, Litorrina, Littoraria, Nodilittori*na	[20] Vera Nisaka. Solferini, [20]Juliana José, [21]Sônia C. Silva Andrade**** [22] Claudia Alves de Magalhâes	[20] Instituto de Biologia, Genética e Evolução/ UNICAMP, São Paulo - (SP). [21] Lab. de Biotecnologia Animal ESALQ, USP [20, 22] Ministério da Ciência e Tecnologia, Distrito Federal.	4
11	Applied molecular genetics. Molecular phylogeny and systematics.	Bivalvia: *Nodipecten, Perna.* Gastropoda*: Patel*la	[23] Laura I. Weber da Conceição	[23] Núcleo de Pesquisas Ecológicas e Desenvolvimento Sócio-Ambiental de Macaé /UFRJ - (RJ).	4
12	Phylogeography.	Bivalvia: *Donax, Mesodesma*	[8] Fernanda B. da Silva, [8]Sandro L. Bonatto	[8] Biotecnologia Genômica e Molecular /PUC-RS - (RS).	-
13	Molecular markers applied to environmental problems. Larval detection.	Bivalvia: *Crassostrea, Limnoperna*	[10] Márcio R. Pie, [10] Walter A. P. Boeger	[10] Depto. de Zoologia/ UFPR - Paraná (PR).	4
14	Molecular genetics/ bioinvasion and conservation.	Bivalvia: *Anomalocardia, Corbicula, Isognomon, Limnoperna, Mytilus, Per*na	[24] Edson P. da Silva	[24] Lab. Genética Marinha e Evolução Instituto de Biologia, Depto. Biologia Marinha/ UFF - (RJ).	2
15	Molecular genetics. Molecular phylogeny and systematics.	Bivalvia: *Paxyodon, Prisodon, Triplodon, Castalia, Diplodon, Crassostrea, Anomalocardia, Mytella, Bankia, Nausitora, Neoteredo, Psilotere*do	[25] Ana Claudia P. Muller, [26]Sonia Maria L. S. Vale, [27]Colin R. Beasley, [28]Claudia H. Tagliaro, [29]Maria Iracilda da C. Sampaio	[25] Instituto Paranaense de Desenvolvimento Econômico e Social, Paraná (PR). [26] União Educacional do Norte, UFAC. [27] Lab. de Moluscos, Campus de Bragança/ UFPA – Pará (PA). [28] Lab. Conservação e biologia evolutiva, Campus de Bragança/ UFPA - (PA). [29] Campus de Bragança, IECOS, UFPA - (PA).	5
16	Molecular genetics and systematics.	Bivalvia: *Corbula*	[30] Vera N. Solferini, [31]Mônica P. Quast	[30] Instituto de Biologia, Genética e Evolução/ UNICAMP. [31] Depto de Biologia Animal/ UNICAMP - (SP).	-
17	Genetic characterization of Oysters - Molecular systematics. Molecular identification of larvae. Conservation genetics.	Bivalvia: *Crassostrea, Mytella, Perna*	[32]Antônio M. Sole Cava, [32]Cristiano V. S. Lazoski, [33]Cláudio M. R. de Melo, [1]Cláudia A. M. Russo	[32] Biodiversidade Molecular, Instituto de Biologia, Genética/ UFRJ - (RJ). [33] Lab. Moluscos marinhos, UFSC. Santa Catarina - (SC). [1] Instituto de Biologia, Genética, UFRJ.	4

Table 2 - Continued.

Groups[4]	Research Line	Class/Genera	Main Researchers	Institution/State	Articles
18	Molecular identification and genetic diversity.	Cephalopoda: *Octopus vulgaris.* Bivalvia: *Crassostrea*	[34]Alexandre W. S. Hilsdorf	[35] UMC - (SP).	1**
19	Applied genetics/ Taxonomic revisions.	Cephalopoda complex: *Octopus vulgaris, Illex, Loligo, Lollinguncula, Ornithoteuthis, Sepioteuthis*	[36]Manuel Haimovici, [36] Tatiana S. Leite,[29] Maria Iracilda da C. Sampaio	[36] Lab. de Recursos Demersais de cefalópodes, UFRN - (RN). [29] Campus de Bragança, IECOS, UFPA - (PA).	1
20	Applied genetics/ Cephalopod population evaluation.	Cephalopoda: *Octopus vulgaris*	[30] Acácio Ribeiro G. Tomás	[30] Instituto de Pesca, Centro de pesquisa pesqueira marinha - (SP).	1**

[1] The order of the research groups is arbitrary.

Note: articles produced in collaboration among different groups: *20 papers in collaboration with Group 2; **The same paper is listed for Group 20. *** Researcher responsible for the *Biomphalaria glabrata* database (www.snaildb.org/) also works with Group 2. ****Member of Harvard University – Mollusca Phylogenomics and ESTs research Group.

groups (Lydeard & Lindberg, 2003; Silva & Russo, 2000; Silva, 2011). Another aspect that should be carefully considered to obtain consistent results is taxonomic sampling (Maronna & Marques, 2009). More reliable and informative data are achieved when different approaches are used in the study (i.e., morphological characteristics and molecular tools) (Ponder & Lindberg, 2008; Teodoro et al., 2010). Despite the increasing accessibility of both equipment and reagents, cost is a factor of consideration in tool choices. Thus, the analysis of ESTs should be considered an alternative to genome sequencing, which is much more informative, but very expensive, particularly when it is employed as a taxonomic sample that is sufficiently large for reliable results (Edgecombe et al., 2011).

The popularization of DNA sequencing technologies has generated large amounts of molecular data (Giribet et al., 2007). In discussing the phylogeny and evolution inside Mollusca, Ponder & Lindberg (2008) highlighted the advances made in computational-analysis methods and noticed that analyses combining molecular and morphological data in mollusks are still uncommon. To emphasize the advance and incorporation of molecular studies in malacology, Ponder & Lindberg (2008) compared the works published in their edited book with studies on the origin and radiation of Mollusca edited by Taylor (1996). In the latter, only 17% of the chapters discussed molecular data, while in the former, 76% of the chapters discussed molecular data. Giribet et al. (2007) discussed the abundance of molecular works in modern literature, recognizing that molecular data could be more easily obtained with lower costs compared to morphological data and by an individual with no previous knowledge of the target organism. However, they emphasized that these presumably advantageous characteristics should be observed with care because they do not contribute to the formation of professionals with extensive knowledge of the studied group.

Phylogenomic tools were used to investigate the evolutionary relationships among mollusks, in which a Brazilian researcher collaborated in this work (Tab. 2) (Smith et al., 2011). In another recent study, Dayrat et al., (2011) revealed the phylogenetic relationships among pulmonate gastropods using a nuclear marker (18S) and two mitochondrial markers (16S and COI) for 96 species. It is relevant to mention that a member of Group 4 collaborated in this work and that Brazilian specimens (Veronicellidae) were employed in this study. Brazilian scientists also collaborated in a study employing ETS to compare the expression patterns in *S. mansoni* infected and noninfected *B. glabrata*. The EST analysis was used to study the expression of genes involved in the host-parasite relationship (expression patterns in *B. glabrata* infected and noninfected by *S. mansoni*) (Lockyer et al., 2007). Such a collaboration between Brazilian researchers with foreign scientists shows that they are engaged in the internationalization of molecular studies.

The development of the necessary infrastructure for molecular studies, as indicated by the number of research groups and methods employed in Brazilian malacology, and the knowledge gaps in several areas of mollusk research suggest a rapid development of molecular research in Brazilian malacology in the near future. This development should include the emergence of new research groups focusing on other mollusks, particularly those belonging to classes that have not yet been studied with molecular tools. Moreover, it is with hope that

many of the papers that were recently presented as abstracts at scientific meetings will be published as full scientific papers, increasing the available information on the molecular biology of Brazilian mollusks. It is also expected that the molecular studies involving sequencing (including complete mitochondrial-genome sequencing), EST analysis and DNA barcoding will be expanded to different mollusk groups and applied to bridge the gaps in knowledge on the systematics and phylogeny, as well as on the elucidation of the population structure of native, invasive and threatened species, in addition to the studies of biodiversity and its conservation and management. Importantly, the expansion of molecular biology over morphological studies is a problem for Brazilian malacology. Considering its current status, the lack of knowledge on mollusk biodiversity is noticeable as is the lack of incentive to train new malacologists (Santos et al., 2009). Incentives for the incorporation of different types of data in the studies should be given, as this would generate more reliable and informative data, as previously discussed.

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