



Review Article

Approaches on Arthropod's Molecular Systematics Studies

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A B S T R A C T

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Phylogenies moleculars provide means for analyzing many old problems of behavioral and eco-epidemiology of arthropods, many of which are importance for public health. A number of molecular markers is used in studies of phylogeny, ecology and population dynamics. Mitochondrial DNA is the most widely used marker of DNA regions for insects as well as for animals in general. Nuclear ribosomal RNA genes have been widely used, especially due to their abundance in the genome and its ease of amplification and sequencing of highly conserved and variable regions (containing the 18S, 5.8S and 28S genes separated by non transcribed and transcribed spacers). Questions about the intraspecific population structure, species limits, and the diagnosis of species, can be often elucidated by techniques such as allozyme electrophoresis, restriction fragment length polymorphism (RFLP), or any variety of new techniques for DNA-based, as single strand conformation polymorphism (SSCP), amplified fragment length polymorphism DNA (AFLP) or random amplified polymorphic DNA (RAPD). Systematic studies on arthropods have analyzed about forty protein-coding genes, all major ribosomal RNA genes (mitochondrial and nuclear) as well as numerous non-coding regions. The regions most commonly sequenced on systematic in the arthropods are mitochondrial DNA and nuclear ribosomal DNA and have supported largely hypotheses based on morphology.

Introduction

Arthropods in general, and the fruit fly, *Drosophila melanogaster* (Figure. 1A), in particular, have traditionally served as models in developmental biology for understanding morphology or for biomedical reasons providing a key to the development

of studies on vertebrates, as an important model of genetic organism. All the fundamentals of classical genetics were built largely through studies on these insects, where molecular genetic testing with this Diptera became the basis for understanding

how all animals develop into adults from a fertilized egg (Alberts, 2008).

Specific changes in the hereditary information expressed in families of mutant flies, were correlated exactly in how the loss or modification of specific bands of their giant chromosomes (Figure. 1B) were processed. Due to DNA replication without intervention of a cell division, each chromosome in these cells contained more than 1,000 aligned identical DNA molecules. The genes were transcribed at a high rate corresponding to bands with a bloated appearance. The bands stained dark brown in micrograph appeared as sites where a regulatory protein was bound to DNA. Once all *Drosophila* genes were sequenced (genome with 13,000 genes presenting 180 megabases), more than in any other organism was possible to trace the chain of cause and effect of genetic instructions encoded in chromosomal DNA. Historically, studies by Thomas Hunt Morgan and colleagues in experiments with *Drosophila melanogaster* have proven the chromosomal theory of inheritance with the hypothesis that the genes were located on chromosomes and were arranged linearly along them (Gilbert, 2003).

The position of arthropods among animals has changed radically in the past two decades as a result of refinements in cladistic analysis and especially by the introduction of molecular systematic studies on insects, through the advent of polymerase chain reaction (PCR), the development of automated sequencing and the increasing computing power into a new science, bioinformatics (Giribet and Edgecombe, 2004).

Basically, any selection of gene region studied in molecular analysis becomes essential, since genes of rapid evolution (as

some genes of mitochondrial DNA) are useful for comparison of the relationship between taxa and genes that evolve more slowly are useful for analysis of distant taxa (Martins, 2007).

Phylogenies moleculars provide means for analyzing many old problems of behavioral and eco-epidemiology of insects, many of which are importance for public health. The principle of co-evolution was also examined from many perspectives. The phylogenetic research has showed that the association between organisms in many cases persisted for long periods of diversification. By examining the relationships between social insects and their relatives, the molecular trees casts a light on the origins of sociality in different groups, as well as the evolution of diversity of castes within certain social groups (Caterino *et al.*, 2000).

Review of Studies

Molecular data have revolutionized our understanding of arthropod relationships since when systematists constructed arthropod phylogenies using nuclear ribosomal genes, nuclear protein-encoding genes, or a combination of these with mitochondrial genes. Developments in sequencing technology and shotgun approaches following the sequencing of the first complete eukaryotic genomes changed our views on how to produce DNA sequence data (Giribet and Edgecombe, 2012).

Caterino *et al.*, (2000) analyzed 567 studies of molecular systematic in insects checking large divergence in genes and regions already sequenced, indicating a need of an approach more coordinated and standardized for development of phylogenetic hypotheses in insects. They suggested the use of cytochrome oxidase I gene, 16S, 18S and the elongation factor 1-alpha, as patterns of

molecular systematics complementary to the morphological and ecological studies, on substantial contributions to evolutionary biological processes.

A number of molecular markers is used in studies of phylogeny, ecology and population dynamics, such as internal transcribed spacer (ITS). The ribosomal DNA (rDNA) is composed of long arrays of tandem repeat units separated by a non-transcribed intergenic spacer (IGS or NTS) which has a variable number of small internal sub-repetitions, and that are variables on length between related species closely and individuals of the same species, and external transcribed spacer (ETS). Each transcribed unit contains the sequences that encoding to three ribosomal genes 18S, 5.8S and 28S and which are highly conserved and essentially invariables. The first internal transcribed spacer (ITS1) is located between genes 18S e 5.8 S and the second internal transcribed spacer (ITS2) between genes 5.8S e 28S. Both can be used to differentiate strains and subspecies of arthropods, including mosquitoes and ticks.

Mitochondrial DNA (mtDNA) is the most widely used marker of DNA regions for insects as well as for animals in general. This popularity stems largely from their apparent ease of isolation and amplification, even from little preserved specimens. Revised data showed all mitochondrial regions used in Lepidoptera (COI, COII, ND5, Cyt b, ND1, 16S, 12S and A + T), Siphonaptera (COII) and Neuroptera (ND2 and COII), Entognatha (COI, COII, Cyt b, 16S and 12S), Paleoptera (COI, COII and 16S), Orthopteroidea (COI, COII, Cyt b, 16S and 12S), Hemipteroidea (COI, COII, Cyt b, ND1, 16S and 12S), Hymenoptera (ND2, COI, COII, ATPase6, COIII, ND6, Cyt b, ND1 and 16S), Diptera (ND2, COI, COII, COIII, ND5, ND4, Cyt b, ND1, 16S,

12S and A + T) and Coleoptera (COI, COII, COIII, ND5, ND4, Cyt b, ND1, 16S, 12S and A + T).

Due to the recognition that trees based on mitochondrial gene may represent only a partial and biased view on the phylogeny of organisms, many nuclear genes have been analyzed for the phylogeny of arthropods. Nuclear ribosomal RNA genes have been widely used, especially due to their abundance in the genome and its ease of amplification and sequencing of highly conserved and variable regions (containing the 18S, 5.8S and 28S genes separated by non transcribed and transcribed spacers).

Revised data also showed all regions of ribosomal DNA in Entognatha, Apterygota and Paleoptera (18S and 28S), Orthopteroidea (18S, ITS2 and 28S), Hemipteroidea (18S, ITS1, 5.8S and ITS2), Coleoptera (18S, ITS1, 5.8S, ITS2 and 28S), Lepidoptera (18S, ITS1 and 28S), Diptera (18S, ITS1, 5.8S, ITS2 and 28S), Hymenoptera (18S, ITS1, 5.8S, ITS2 and 28S), Neuropteroidea, Strepsiptera, Mecoptera, Trichoptera and Siphonaptera (18S, 5.8S and 28S).

Nuclear protein-encoding genes which exhibit a wide variety of evolutionary rates have been evaluated as phylogenetic tool. These loci showed a high possibility for resolving relationships in very conserved levels, in comparison to mitochondrial proteins. The rate variation among codon positions in genes encoding proteins suggests that many of these loci can be effective in elucidating the most recent divergences.

Caterino *et al.*, (2000) also reviewed studies that used as molecular markers, several nuclear gene regions that encoding proteins (Table 1).

Questions about the intraspecific population structure, species limits, and the diagnosis of species, can be often elucidated by techniques such as allozyme electrophoresis, restriction fragment length polymorphism (RFLP), or any variety of new techniques for DNA-based, as single strand conformation polymorphism (SSCP), amplified fragment length polymorphism DNA (AFLP) or random amplified polymorphic DNA (RAPD). The best strategy will often be to use one or more of these techniques in combination with DNA sequencing, which has become a preference technique for generating comparative molecular data, consolidating the unique perspective to the conduction of evolutionary processes diversification of DNA.

RFLP – Restriction Fragment Length Polymorphism

With the advent of techniques for sequencing, the data generated with restriction enzymes disappeared on phylogenetic studies insects, though the technique is even commonly applied in parallel studies. With the mapping of restriction sites, however, they still provide a quick alternative for studying sequence variation in large cross-sections of the genome, and have proved very useful and reasonably accurate for phylogeny reconstruction. The most common use of restriction enzymes in systematic is for the diagnostic profiles that are sought within the DNA fragments (more often in mtDNA).

RAPD - Random Amplified Polymorphic DNA

The polymorphism of RAPD markers is revealed by amplification of chromosomal loci using primers composed of short sequences of oligonucleotides and are

generated by rearrangements or mutations (insertions or deletions) between the two locations or at the site of hybridization of the two primers. Differences in only one base pair (point mutations) may be sufficient to inhibit amplification. The low reproducibility of RAPD is a much debated question. Many insects of different orders were analyzed by RAPD with different approaches, among which include phylogenetic studies, taxonomic studies and epidemiological studies, to obtain genomic fingerprints of individuals, for analysis of structure and genetic diversity of populations, to construct genetic maps of high resolution and on studies related to the acquisition of insecticide resistance. Among the studied species can highlight the mosquito vector of malaria, *Anopheles*, the mosquito vector of dengue and yellow fever, *Aedes* and the transmitter triatomine of Chagas disease, *Triatoma*. Insects of economic interest, such as *Haematobia irritans* were also studied. In this technique a single oligonucleotide (containing 10 to 20 bases) is randomly constructed, resulting in the amplification of a target sequence unknown. Each primer used directs the amplification of several DNA segments simultaneously in different parts of the genome, resulting in several bands in the gel.

The polymorphism produced by RAPD technique appears in the form of presence or absence of fragments or bands in the gel. Unlike other methods, RAPD does not require preliminary work, such as construction of genomic banks, since it does not require the previous development of a library of probes specific for the organism of interest, eliminating the need to transfer DNA to membranes (Southern Blot) and the use of radioactive isotopes or non-radioactive marking. Another feature of this molecular tool is that these markers are

inherited as dominant markers. This inheritance model does not allow differentiating heterozygous and homozygous dominant individuals without subsequent crossings. By observing a band on the gel, it is not possible to distinguish whether that segment was originated of one or two copies of the amplified sequence.

SSCP – Single Strand Conformation Polymorphism

These techniques rely on differences in the DNA secondary structures due to the primary sequence variation and the consequent variation in the rate of electrophoretic mobility. SSCP may be sensitive to the differences of a single base pair. In entomological applications SSCP is typically used in conjunction with amplification of a specific fragment for the purpose of diagnosing species. SSCP also provides a quick method of differentiation of polymorphisms between populations and species that can be sequenced for phylogenetic effects. A particularly interesting application employs SSCP as an alternative to cloning to isolation nuclear alleles.

AFLP - Amplified fragment length polymorphism

AFLP is a technique developed that as RAPD, quickly locates genetic polymorphisms dispersed among organisms. Despite the apparent promise for applications entomological, AFLP has not been widely applied on studies of systematic of insects. The technique is based on the property of certain restriction enzymes to leave, after cleavage of the DNA, cohesive ends of the known sequence. Thus, it is possible to construct nucleotide sequences of double-stranded that bind at the ends of the restriction fragments.

Perspectives

Currently, molecular biology techniques have been widely used for the purpose of assisting in the identification of insects of forensic importance. The mitochondrial DNA (mtDNA) has proved an excellent molecular marker, particularly due to the simple and uniform organization of the mitochondrial genome, the low number of recombinations and the high rate of nucleotide substitutions. In addition, a wide range of universal primers is available for mtDNA of insects, thereby permitting trusted amplifications of genes or homologous regions in several groups of insects. The ability to recover, efficiently, genetic information from damaged or poorly preserved samples also facilitates the use of mtDNA markers in forensic investigations. The combination of molecular methods such as polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) has been very useful for identifying of cryptic species or at different stages of life (Thyssen *et al.*, 2005; Thyssen, 2008).

In studies in Diptera (Calliphoridae), two specific regions of the mitochondrial DNA, the cytochrome oxidase subunit I (COI) and the control region (CR) were amplified by PCR and digested using restriction endonucleases. The cleavage patterns generated by endonucleases *DraI* and *SspI* were suitable to distinguish the two species *Hemilucilia* (Thyssen *et al.*, 2005).

Low *et al.*, (2014) presented in their studies a molecular evidence of that the closely related species *Haematobia irritans* Linnaeus and *Haematobia exigua* de Meijere are genetically distinct and could be differentiated based on multilocus sequence analysis of five mitochondrial genes, mitochondria-encoded cytochrome c oxidase subunit I (COI), cytochrome B (CytB),

NADH dehydrogenase subunit 5 (ND5); nuclear encoded 18S ribosomal RNA (18S) and 28S ribosomal RNA (28S) genes. Results of these analyses demonstrated that ND5 and CytB genes were more variables and informatives in resolving the interspecific relationships of both species, the COI gene was more valuable in inferring the intraspecific relationships and the ribosomal 18S sequences and 28S of the two species were highly conserved with intra- and inter-specific variation limited.

Mitochondrial DNA fragment of about 700 bp, comprising part of the cytochrome oxidase I, tRNA of leucine-full and part of the cytochrome oxidase II, was amplified by PCR, demonstrating that the application of molecular tools has been extremely useful for separating species and groups of species of ants of the genus *Linepithema* (Martins, 2007).

Rampelotti *et al.*, (2008) studied the genetic diversity of *Tibraca limbativentris* (Hemiptera: Pentatomidae) of Santa Catarina and Rio Grande do Sul, using RAPD markers, aiming to test DNA extraction protocols and characterize populations of this important insect pest of rice. It wasn't observed relationship between geographical distance and the genetic similarity of populations, reflecting a dispersion model of *T. limbativentris* still unknown where studies exploring strategies of dispersal of the species could help in understanding the insect distribution, evidencing which one the main source of genetic variability.

Borba *et al.*, (2005) analyzed the genetic dissimilarity of lineages by *Trichogramma* (Hymenoptera: Trichogrammatidae) by molecular marker ISSR, measuring the level of genetic differentiation of these lineages by employing the technique of ISSR. Five

lineages were studied with extraction protocols and standardization of DNA and PCR using 11 markers selected by greater polymorphism and consistency of 26 ISSR markers, which produced 172 polymorphic bands with a variable genetic similarity between 19% to 96%, allowing us to conclude that the ISSR technique can efficiently identify DNA polymorphism in *Trichogramma*, with demonstration of results that suggest an accentuated variation inter and intraspecific.

According to Wei *et al.*, (2014), in animal mitochondrial genomes, gene arrangements are usually conserved across major lineages but might be rearranged within derived groups and provide valuable phylogenetic characters. The mitochondrial genomes of *Cephalonomia gallicola* (Chrysoidea: Bethyloidea) and *Wallacidia oculata* (Vespoidea: Mutillidae) were sequenced, where in *Cephalonomia* at least eleven tRNA and two protein-coding genes were rearranged and in the Hymenoptera three events of rearrangement occurred in the protein-coding genes (reversal, transposition and reverse transposition).

Recent study in *Anopheles cruzii* (Diptera: Culicidae), the primary vector of malaria in humans and nonhuman primates in Brazil, through a multilocus analysis using six different fragments of nuclear genes was conducted to compare two populations (Itaparica and Florianópolis), representing respectively species of the northeast and south. Were used, three fragments of genes involved in the control of circadian rhythms, *timeless (tim)*, *Clock (Clk)* and *cycle (cyc)*, and three genes encoding ribosomal proteins, *Rp49* (Ribosomal protein 49, also known as *RpL32* - Ribosomal protein L32), *RpS2* (Ribosomal protein S2) and *RpS29* (Ribosomal protein S29). This study was crucial for analysis of differences between populations and helped to verify when the

differentiation in the circadian genes is greater than the divergence in the constitution of loci, such as the genes encoding ribosomal proteins *Rp49*, *RpS29* and *RpS2* (Rona *et al.*, 2010).

Carapelli *et al.*, (2006) demonstrated the existence of conflicts between morphological and molecular data, as well as between different data sets and methods of analysis, in respect to the basal phylogeny of hexapods. However, molecular data have led to reconsideration of the hypothesis of systematists of hexapods by longtime, based on morphological data. This leads directly to the question whether it would be Hexapoda a monophyletic taxon, or alternatively, one or more independent lineages within Pancrustacea (Figure. 2), suggesting the development of more molecular markers and the obtaining of a sampling of taxon denser for both genes mitochondrial and nuclear, well as a reassessment of new and established morphological characters.

On molecular studies in populations of "horn fly" was cloned and sequenced the gene that confers one of types of resistance to pyrethroids ("knock down resistance gene" – *kdr*). Since then it is possible to detect this type of resistance (*kdr*) to pyrethroids from a PCR, determining the frequency of *kdr* for susceptible and resistant populations (Sabatini, 2003).

Although much biological research to depend on the diagnosis of the species, the taxonomic expertise is collapsing. The only prospect for a sustainable identification capability will be to build systems that employ DNA sequences as taxon 'barcodes'. The mitochondrial gene cytochrome c oxidase I (COI) may serve as the nucleus of a global system for bio-identification animal. A COI profile model, based on the analysis of a single individual from each of the 200 species of Lepidoptera, was 100%

successful in correctly identifying specimens. This construction of a COI identification system will also enable important new insights into the diversification of life and in the rules of molecular evolution (Hebert *et al.*, 2003).

The use of DNA barcode (Hebert *et al.*, 2003) has been widely discussed by those who successfully apply this approach to identify and diagnose species, and those who believe that there are so many problems in using this molecular marker that is not justified its use. For insects of the order Lepidoptera neither side seems completely right or wrong, and although some groups have been solved by taxonomically unique or additional use of this marker, for others the "barcode" helped little to solve taxonomic problems. The "DNA barcode" is a small genic region with 648 bp of the mitochondrial DNA gene cytochrome c oxidase 1 (COI) (Silva-Brandão, 2009).

Hiragi *et al.*, (2009) analyzed the genetic variability of different populations of *Aedes aegypti* using RAPD markers (Random Amplified Polymorphic DNA), where DNA to ten larvae collected from three different populations were analyzed using ten RAPD primers, indicating the existence of genetic variability inter and intrapopulation.

Oliveira *et al.*, (2002) compared simple methods of preservation of specimens of *Dalbulus maidis* (Hemiptera: Cicadellidae) regarding to quantity and quality of DNA for use in RAPD-PCR after periods successive of storage.

Arias *et al.*, (2003) emphasized the importance of the mitochondrial genome and the different analysis applied to it, to solve issues of population and evolutionary character in stingless bees addressing a population analysis of *Plebeia remota*, a phylogenetic analysis among species of

genera *Plebeia*, *Melipona* and *Schwarziana* and the characterization of the mitochondrial genome of *Melipona bicolor* by sequencing, evidencing translocations of genes into tRNA. Restriction maps for all of these species were determined using 17 restriction enzymes and the Southern blot technique to identification of the fragments. For a better positioning of restriction sites, was used a RFLP-PCR, which also allowed to locate restrictions sites with relation to the genes present in the amplified fragment.

Ceretti-Junior *et al.*, (2008) performing an analysis of taxonomic and systematic relationships among species of triatomine bugs (Hemiptera, Reduviidae) from colonies of the Special Health Service Araraquara, demonstrated the utility of the 16S gene as a marker of species of triatomine and their importance in matters of systematic and taxonomy, emphasizing the need to be further studies involving other markers associated with classic systematic characters of morphology, ecology and behavior for appropriate systematic decisions, since it would impact not systematic only but also for control strategies.

From the studies of Garcia and Powell (1998) and Stothart *et al.*, (1998) was inaugurated the era cladistic molecular on the systematic studies of Triatominae, solving many impasses related to the status of some species or in respect to the cryptic species with intensification of the discussion about the evolutive origin of this group and support for their evidences of polyphyly or paraphyly. New analyzes with the use of a greater number of genetic markers associated with the classic systematic patterns of morphology, ecology and behavior, must be added for a suitable phylogenetic decision, since haven't only important systematic, but serious implications regarding to measures to control of vector species.

Phylogenetic relationships of 75 arthropod and five species "outgroup" were analyzed under likelihood criteria using four strategies, where the aligned sequences for 62 nuclear genes encoding proteins showed results that strongly support the monophyly of Hexapoda; in contrast to mitochondrial studies that place Collembola among crustaceans, instead of other hexapods (Regier *et al.*, 2010).

Fernandez *et al.*, (2005) analyzed the genomic polymorphism in *Bombyx mori* through RAPD profile generated from the genomic DNA of two lineages of Japanese caterpillars (which had a high content of the silk cocoon) and two lineages of Chinese caterpillars (who had greater resistance to field cultivation); using the CSGE technique (Conformation Sensitive Gel Electrophoresis) which allows the identification of polymorphisms in target genes correlated with phenotype. Through this methodology was established the detection of co-dominance, allowing the identification of two alleles of the same locus and allowing the detection of polymorphic sequences of DNA (heteroduplex or homoduplex), with potential applications in breeding programs of these lineages. Fet *et al.*, (2003) discussed the first phylogenetic hypotheses about scorpions of the family Buthidae (17 genera) presenting arguments based on molecular data (16S rRNA mitochondrial DNA).

Borges *et al.*, (2010) developed a phylogenetic hypothesis based on mitochondrial DNA from 21 species of *Tityus* collected in Venezuela, Trinidad, Brazil and Panama, including 12 toxics taxa to humans, through phylogenetic reconstruction based on 850 nucleotides of the cytochrome oxidase subunit I (COI) and 16S rRNA genes for the most of these species of scorpions.

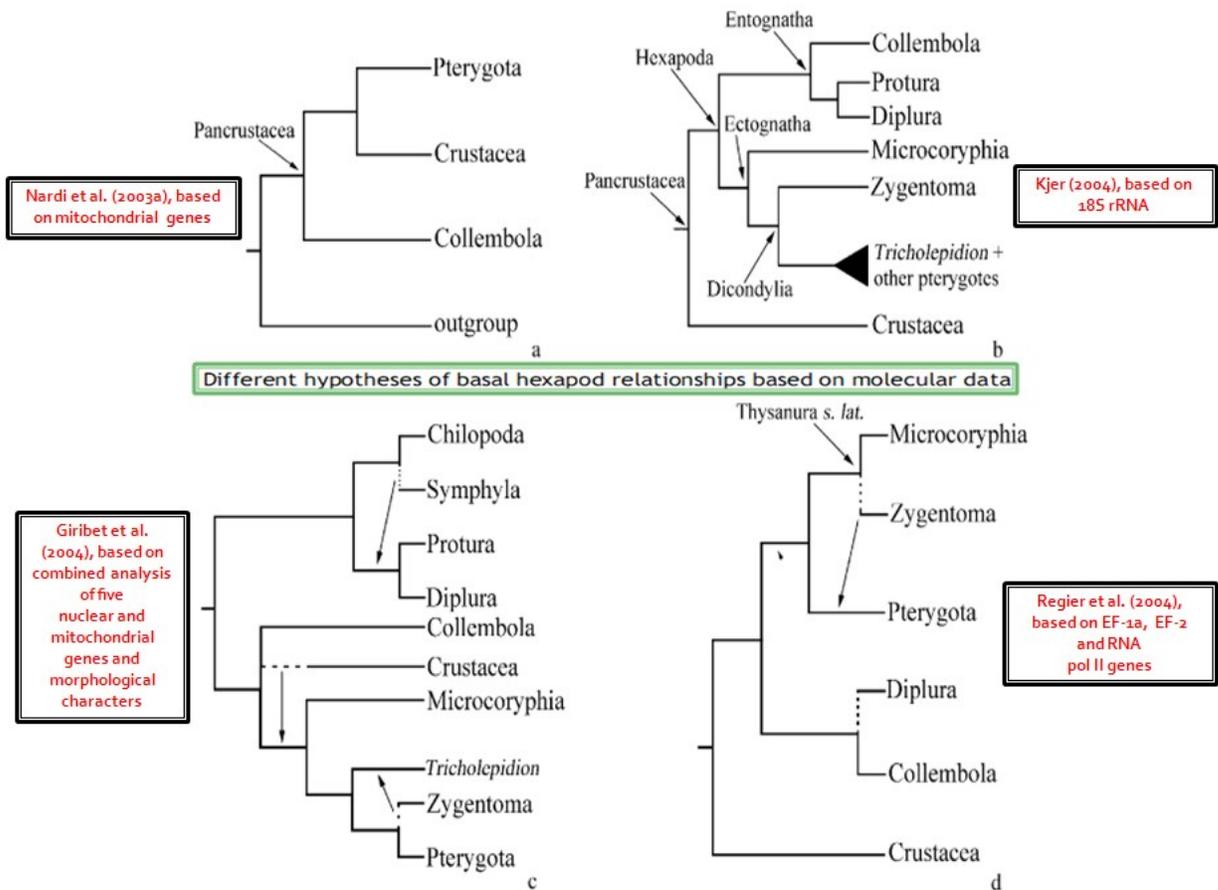
Table.1 Nuclear protein-encoding genes used in molecular systematics of insects
(Caterino *et al.*, 2000)

Locus	Sequences	Taxa
α -amylase (<i>amy</i>)	38	Diptera, Coleoptera
Acetylcholine esterase (<i>achE</i>)	21	Diptera
Actin	5	Hymenoptera, Lepidoptera
Alcohol dehydrogenase (<i>adh</i>)	157	Diptera
Arylphorin (hexamerin family)	12	Lepidoptera
Cecropin family	36	Diptera
Chorion genes: <i>s18</i> , <i>s15</i> , <i>s19</i>	5	Diptera
Cpnl-1 (anonymous nuclear DNA)	8	Orthoptera
Dopa decarboxylase (<i>ddc</i>)	57	Lepidoptera
Elongation factor-1-alpha (<i>EF-1α</i>)	196	Lepidoptera, Hymenoptera, Hemiptera, Collembola, Archaeognatha, Blattodea
<i>EF-1α</i> (copy 2)	1	Hymenoptera
Esterase (<i>est</i>)	31	Diptera
Glycerol-3-phosphate dehydrogenase (<i>gpdh</i> ou <i>g3pdh</i>)	39	Diptera
Glycerol-6-phosphate dehydrogenase (<i>g6pdh</i>)	13	Diptera, Lepidoptera, Thysanura
Guanylate cyclase	13	Diptera
Globin family	33	Diptera
Histone H1	8	Diptera
Histone H4	7	Hymenoptera
Hunchback (<i>hb</i>)	39	Diptera
Kruppel	3	Diptera
Luciferase	2	Coleoptera
Lysozyme intron	16	Lepidoptera
Myosin alkali light chain (<i>Mic 1</i>) intron	30	Diptera
Nullo	7	Diptera
Opsin	28	Hymenoptera, Diptera
Period (<i>per</i>)	111	Diptera, Lepidoptera
Phosphoglucose isomerase (<i>Pgi</i>)	2	Orthoptera
Phosphoenolpyruvate carboxykinase (<i>PEPCK</i>)	12	Lepidoptera, Trichoptera, Diptera, Mecoptera, Siphonaptera
Prune	10	Diptera
Resistance to dieldrin (<i>Rd1</i>)	17	Coleoptera
Retrotransposon reverse transcriptase	30	Hymenoptera
Ribosomal protein 49 (<i>rp49</i>)	9	Diptera
Serendiptiy- α	7	Diptera
Cu, Zn – superoxide dismutase (<i>sod</i>)	30	Diptera
Sodium channel para locus 1	27	Lepidoptera, Hemiptera, Blattodea, Coleoptera
Snail	3	Diptera
Timeless	1	Diptera
Triosephosphate isomerase (<i>Tpi</i>)	39	Diptera
Vestigial (<i>vg</i>)	5	Diptera
White	15	Diptera
Wingless (<i>wg</i>)	57	Lepidoptera, Diptera
Xanthine dehydrogenase (<i>xdh</i>)	37	Diptera
Yolk protein 1 (<i>yp1</i>)	55	Diptera
Yolk protein 2 (<i>yp2</i>)	40	Diptera
Zeste	24	Diptera

Figure.1 Giant chromosomes (B) in cells of the salivary gland of *Drosophila melanogaster* (A) (Alberts, 2008)



Figure.2 Different hypotheses of phylogenetic relationships in Hexapoda based on molecular data (Carapelli *et al.*, 2006)



According to Burger *et al.*, (2012), our understanding of the phylogenetic relationships among lineages of ticks has been limited by the lack of resolution provided by phylogenetic markers most commonly used. Mitochondrial genomes have been increasingly used to address the controversial phylogenetic relationships. In this sense, presented a study on the nucleotide sequences of the complete mitochondrial genomes of five species of ticks metastriata: *Amblyomma elaphense*, *Amblyomma fimbriatum*, *Amblyomma sphenodonti*, *Bothriocroton concolor* e *Bothriocroton undatum*, aiming at addressing the phylogenetic position of two morphologically "primitive" species - *A. elaphense* e *A. sphenodonti* - with regard to the genus *Amblyomma*. The analysis of these five mitochondrial genomes and more eleven other mitochondrial genomes of ticks, as well as the analysis of rRNA nuclear genes, generated strong evidence that genus *Amblyomma* would be polyphyletic with the inclusion of the species *A. sphenodonti* and *A. elaphense*.

Burger *et al.*, (2014a) using phylogenetic analyses of mitochondrial genome sequences, to approach the phylogenetic relationships among the species of the subgenus *Boophilus*, revealed a species complex called *Rhipicephalus microplus* complex (*R. annulatus*, *R. australis* and two clades of *R. microplus*), concluding that *cox1* and 16S rRNA were more successful in resolving the phylogenetic relationships than the 12S rRNA or the ITS2 nuclear marker.

Genetic analyses of soft ticks (Argasidae) have been limited to 16S rRNA which is not highly informative, phylogenetically. The sequence of entire mitochondrial genomes of seven species of soft ticks and

the partial mitochondrial genomes of a further five species of soft ticks was used to test the controversial classification for the genus-level in this group. The comparison of mitochondrial rRNA, nuclear rRNA and the analyses of mitochondrial genome showed that only mitochondrial genome sequences have the potential to resolve the controversial phylogenetic relationships within the major soft tick lineages (Burger *et al.*, 2014b).

According to Nava (2009) the molecular markers most used in studies at population level and to infer inter and intra-specific variation of ticks were the ITS1 and ITS2 and the mitochondrial genes 16S, 12S, cytochrome oxidase I (COI) and cytochrome oxidase III (COIII), and other genetic markers such as the microsatellites, allozyme polymorphism, random amplified polymorphic DNA (RAPD), polymorphism of the *bm86* gene and 28S rDNA gene.

The diversity of available markers, no doubt, has favored the cause of the systematic, however, this pleiad of markers also carries on the risk of plurality, to the extent that the use of different estimators between the studies difficults the comparison and makes it difficult their synthesis. The essence of science is repeatability, and into their interest we should reiterate some principles integrated to the molecular assays for phylogenetic studies. The objective should be always identify a relatively small set of markers to serve as a reference standard for possible comparisons.

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