

**MOLECULAR PHYLOGENETIC METHODS IN ENTOMOLOGY
- NEW INSIGHTS ON THE EVOLUTIONARY RELATIONSHIPS
OF HEXAPODS AND ARTHROPODS**

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Traditional phylogenetic and taxonomic methods based on examining and comparing morphological characters have in the last few decades been supplemented or even replaced by molecular phylogenetic methods that utilize a great number of molecular markers - mitochondrial and nuclear genes and genomic regions. Molecular phylogeny has profoundly changed our perception of the taxonomic position of great number of hexapod taxa and posed some intriguing questions on the evolution of basal hexapod lineages, as well as hexapods in relation to other arthropod higher-level taxa (crustaceans, myriapods and chelicerates). Regarding the question of relationships between entognathous hexapod taxa, some of the analyses yielded monophyletic Ellipura, while the others suggest the non-monophyly of Entognatha. Recent investigations of both molecular and morphological data support a closer relationship between hexapods and crustaceans, a so called "Pancrustacea" clade, opposing the classical "Tracheata" (myriapods + hexapods) hypothesis. Some of the results even suggest the reciprocal paraphyly of Crustacea and Hexapoda. Regarding the other two major arthropod clades, myriapods and chelicerates, some investigations support the sister group of Pancrustacea + Myriapoda (so called "Mandibulata"), while in some analyses support was found for the pairing of myriapods with chelicerates (so called "Paradoxopoda" or "Myriochelata"). These hypotheses would imply that water-to-land transition occurred at least three times during the evolution of the Arthropoda. All these new phylogenetic topologies still need to be properly evaluated and compared with classical hypotheses about the evolutionary relationships of hexapods and arthropods.

Hexapoda, Arthropoda, insects, Ellipura, Entognatha, Pancrustacea, molecular phylogeny, molecular markers

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Tradicionalne filogenetske i taksonomske metode temeljene na istraživanju i usporedbi morfoloških obilježja u novije se vrijeme dopunjavaju ili čak i potpuno zamjenjuju molekularno-filogenetskim metodama koje upotrebljavaju veliki broj molekularnih markera - mitohondrijalnih i nuklearnih gena i genomskih regija. Molekularna filogenija znatno je promijenila znanja o taksonomskom položaju velikoga broja taksona kukaca (Hexapoda) te otvorila brojna pitanja o evoluciji bazalnih linija Hexapoda i o njihovu odnosu prema ostalim glavnim linijama člankonožaca (Arthropoda) - rakova (Crustacea), stonoga (Myriapoda) i klještaru (Chelicerata). Neke od analiza podržavaju monofiliju Ellipura, a neke upućuju na polifiliju ili parafiliju Entognatha. Brojna novija istraživanja molekularnih i morfoloških podataka podržavaju blisku vezu između Hexapoda i Crustacea, tzv. "Pancrustacea", koja je u suprotnosti s tradicionalnom hipotezom o bliskoj vezi između Hexapoda i Myriapoda (tzv. "Tracheata"). Neki od rezultata čak upućuju na uzajamnu parafiliju Crustacea i Hexapoda. Neke analize podržavaju vezu između Pancrustacea i Myriapoda (tzv. "Mandibulata"), a ostale daju podršku bliskoj vezi između Myriapoda i Chelicerata (tzv. "Paradoxopoda" ili "Myriochelata"). Te hipoteze upućuju na to da su tijekom evolucije člankonožaca barem tri puta neovisno pojedine linije prelazile iz vode na kopno. Sve ove nove pretpostavke moraju biti provjerene i uspoređene s klasičnim hipotezama o evolucijskim odnosima kukaca i člankonožaca.

Hexapoda, Arthropoda, kukci, Ellipura, Entognatha, Pancrustacea, molekularna filogenija, molekularni markeri

Introduction

Hexapods are the largest and most abundant group of organisms – 1.5 million species have been described, and according to some recent assumptions, up to 30 million species might still be hidden and waiting to be discovered and identified (Saier Jr., 2006). With their enormous diversity of forms and the ability to evolutionarily adapt to almost every possible terrestrial (and not only terrestrial) environment and climate conditions, from extreme biotopes such as polar and desert biomes to rain forests and high mountains, insects are by far the most successful and progressive form of eukaryotes. Moreover, insects have historically been tightly connected to human society because of their great economic and me-

dical significance. Due to the great interest in insects, most of the major lineages of the group have long been described or identified, and currently 33 orders of hexapods, most of them insects, are commonly recognized (Naumann, 1994).

Traditionally, the taxonomy and systematics of hexapods and their relationships to other arthropods have been studied mainly based on the examining, scoring and comparison of anatomical and morphological features. Phylogenetic trees of a great number of hexapod taxa constructed several decades and centuries ago, based on those characters, resulted in robust and reliable phylogenetic assumptions, but still many taxa were not so easy to accommodate because of the lack and/or inadequacy of the characters used. The reason for this mainly lies in the extensive occurrence of convergence of morphological characters, quite often found in insects and arthropods in general.

With the advent of molecular techniques, traditional approaches to taxonomy and systematics of the whole living world have been challenged, and new horizons have also suddenly opened in the field of entomology. Thanks to rapid and efficient polymerase-chain-reaction based amplification and sequencing of DNA, numerous molecular markers were developed that proved useful in various taxonomic ranges in hexapods, from species to orders, and new genes and genomic regions with promising abilities are still constantly emerging. Highly informative molecular datasets can easily be built for taxa under question by combining genes and genomic regions with different evolutionary rates, such as mitochondrial and nuclear genes, protein coding genes, noncoding regions (introns, regulatory regions, microsatellites etc.).

These datasets can be aligned and analysed with different computer-based algorithms and methods of phylogenetic analyses, by using various applications usually available free of charge on internet. The most commonly used methods are multiple alignment of nucleotide or protein sequences and their analyses by parsimony, maximum likelihood, Bayesian and distance algorithms, implemented in programs such as ClustalW, Paup, Phylip, Mega, MrBayes and others (Thompson et al., 1994, Swofford, 2002, Tamura et al. 2007, Huelsenbeck and Ronquist, 2001, Kumar et al., 2008). The large quantity of molecular data, combined with powerful analytical methods, most usually result in high resolution and confidence of phylogenetic trees.

Molecular markers in phylogenetic studies of hexapods and arthropods

Probably the most commonly used molecule in phylogenetic analyses is mitochondrial DNA (mtDNA). The mitochondrial chromosome is a circular, supercoiled, double stranded DNA molecule, present in multiple copies in each cell, and is inherited almost exclusively in maternal fashion. It contains genes with different functions and thus different evolutionary rates - some are quite conserved while the others are more variable. Generally it evolves faster and contains a much larger number of length mutations and transitions than single-copy nuclear DNA, because mtDNA does not code for proteins directly involved with its own replication, transcription or translation, and because mitochondria lack proofreading (i.e. error-repair) machinery during DNA replication or after DNA damage. Mitochondrial genes lack introns, intergenic regions are usually small or absent, and heteroplasmy (i.e. the coexistence of more than one type of mtDNA within a cell or individual) is considered to be very rare in natural populations. All these features make mtDNA an ideal candidate for phylogenetic investigations on different taxonomic levels (Alberts et al, 2002).

The mitochondrial chromosome of *Drosophila yakuba*, 18,5 kb in length, contains 37 genes: 2 are rRNA, 22 are tRNA, and 13 are protein genes that code for subunits of enzymes functioning in electron transport or ATP synthesis (Clary & Wolstenholm, 1985). Complete mitochondrial genomes of many other hexapods have been investigated and published so far, including several species of *Drosophila*, mosquitoes, bees, grasshopper, silkworm, collembolans, diplurans and others, while partial mitochondrial sequences of a very large number of hexapods, coding for different mtDNA genes, are deposited in and can be easily retrieved from the GenBank. MtDNAs of hexapods are generally similar in length, base composition, gene order and other features to *D. yakuba* mtDNA, although there are examples of larger and/or more A-T rich mitochondrial genomes (e.g. Boyce et al., 1989).

For most insect mitochondrial genes PCR-primers have been designed, whether universal or taxonomic group-specific, proving useful in a great number of phylogenetic analyses of hexapods and arthropods (e.g. Simon et al., 1994, 2006). Commonly used mitochondrial genes are protein-coding cytb, COI, COII, NADH, ATP, as well as genes coding for mitochondrial ribosomal subunits, 16S

rDNA, 12S rDNA, and so called D-loop (mitochondrial regulatory region) - depending on the evolutionary divergence of taxa and subsequent nucleotide differences, either whole or only parts of these genes can be used in low-level to moderately deep phylogenies (e.g. Shao et al., 1999, 2000, Frati et al., 1997, Garcia-Machado et al., 1999, Carapelli et al., 2000). On the other hand, more conserved parts of mitochondrial genes as well as complete mitochondrial genomes proved useful even in elucidating deep-level phylogenies among distantly related hexapod and arthropod taxa (e.g. Boore et al., 1995, Boore & Brown, 2000, Black & Roehrdanz, 1998, Lavrov et al., 2000, 2002, Wilson et al., 2000, Hwang et al., 2001).

Other types of frequently used molecular markers are nuclear genes: protein-coding (most of them being single-copy genes), ribosomal genes, introns and intergenic regions. Due to the lower level of nucleotide divergence, many nuclear protein-coding genes are particularly useful in moderate to deep-level phylogenies, from family to order level. Such genes are, for example, those coding for EF1- α , EF2, RNA-polymerases, histone proteins, Wingless, Hox, Ubiquitin genes and others, which have been used in a great number of deep-phylogeny investigations in insects, hexapods and arthropods (e.g. Regier & Schultz, 1997, 2001, Schultz & Regier, 2000, Regier et al., 2008, Edgecombe et al., 2000, Cook et al., 2001). Nuclear ribosomal genes, those coding for 18S and 28S ribosomal subunits, according to need for maintenance of their functional tertiary structures (i.e. stems and loops in folded rRNA), contain highly conserved regions as well as regions of greater diversity between related taxa, and are thus useful in a range of phylogenetic investigations, from genus to order level (e.g. Turbeville et al., 1991, Friedrich & Tautz 1995, 2001, Spears & Abele, 1997, Giribet & Ribera 2000, Wheeler et al., 1993, 2001, D'Haese 2002).

On the other hand, some more divergent parts of the nuclear genome, mostly those non-coding, can be used in lower-level phylogenies - such as introns, internal transcribed spacers (ITS1, ITS2) and different classes of repetitive DNA (e.g. Fairley et al., 2005, Mahendran et al. 2006, Carapelli et al., 2000, Flook & Post, 1997). Most commonly used markers for investigating intraspecific relationships or relationships between very closely related species (so called tokogenetic relationships), are microsatellites. For a great number of insect species, mostly those that are of economic or medical importance, specific microsatellite loci have been isolated and developed for intraspecific or population studies (InSatDb: Insect

microsatellite database: <http://sunserver.cdfd.org.in:9999/PHP/INSATDB/home.php>).

New insights on the evolution of hexapods and arthropods

Molecular phylogeny brought new insights and posed some intriguing questions on the evolution and taxonomy of basal hexapod lineages, as well as hexapods in relation to other arthropod higher-level taxa (crustaceans, myriapods and chelicerates).

Phylogenetic relationships among basal hexapods, as well as the question of a hexapod sister group, have become a matter of controversial debate in the last decade (e.g. Deuve, 2001, Giribet et al., 2001, Caterino et al., 2000). The difficulties encountered in the interpretation of the relationships among the basal lineages of hexapods and their relatives most probably result from the very ancient diversification of major clades. Hexapoda (or *Insecta sensu lato*) includes four main groups: Protura, Collembola, Diplura and *Insecta sensu stricto* (the latter including Archaeognatha, Zygentoma and pterygote insects). Protura, Collembola and Diplura are ancient groups, known as fossils from Devonian, Carboniferous and Cretaceous periods up to 400 Mya (e.g. Whalley, 1995). Based on their mouthparts, Hennig (1953) joined Protura, Collembola and Diplura into Entognatha (with enclosed mouthparts), in contrast to Ectognatha (*Insecta sensu stricto*, with exposed mouthparts).

Perhaps the biggest question is the one on phylogenetic relationships of the five high-ranking apterygotan taxa: the ectognathous Archaeognatha and Zygentoma, and entognathous Diplura, Protura and Collembola. Controversies regard the grouping of Protura and Collembola as a taxon Ellipura, the monophyly of Diplura, a single or multiple origins of entognathy, and the monophyly and relationships among the zygentoman families. It is generally accepted that Archaeognatha and Zygentoma are closely related to the pterygotan insect lineage. With respect to entognathous taxa, several competing hypotheses have been proposed regarding their monophyly-paraphyly, their mutual relationships and for their possible sister-group taxa (reviewed in Bitsch & Bitsch, 1998, 2000, Dunger, 2003). The most common hypothesis based on morphological characters assumed a sister-group relationship between Protura and Collembola, forming so called "Ellipura", with Diplura being their closest relative and thus making Entognatha monophyletic (Kristensen, 1981, 1991, Kukulova-Peck, 1987, Hennig, 1981, Figure 1A).

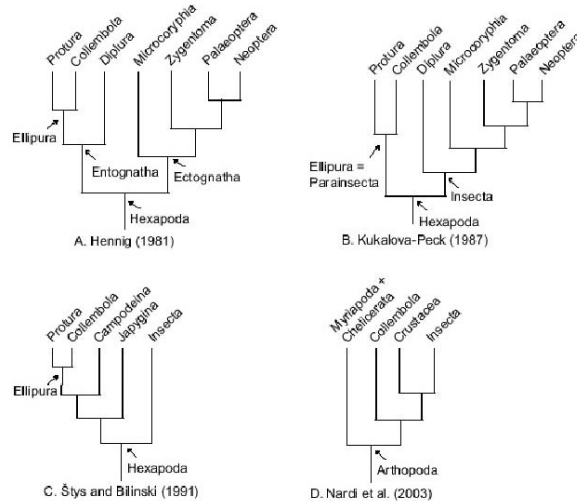


Figure 1. Four hypotheses of basal hexapod relationships, based on morphological (A, B and C) and molecular (D) characters. From: Luan et al., 2005.

However, some authors found Diplura to be more closely related to Insecta s.str. (Kukalova-Peck, 1987, Figure 1B), or paraphyletic with respect to Ellipura (Štys & Bilinski, 1991, Figure 1C). Moreover, recent molecular analyses afforded some new topologies, depending on the markers used, that put hexapod subgroups in completely new relationships among each other and with other higher arthropod taxa (e.g. Nardi et al., 2003, Figure 1D).

Regarding the question of relationships between entognathous hexapod taxa, molecular data gave no definite results. Some of the analyses yielded monophyletic Ellipura (e.g. Regier et al., 2004, Carapelli et al., 2000), while the others contradict this classical hypothesis, with either Protura or Collembola being closer to Diplura, or only a part of it (e.g. Nardi et al., 2003, (Figure 1D), Giribet et al., 2004, Luan et al., 2003, 2004, 2005, Cameron et al., 2005, Carapelli et al., 2007). In molecular analyses Diplura sometimes appears to be paraphyletic with respect to other entognathous taxa or more closely related to ectognathous taxa (Regier et al., 2004), while Collembola in many investigations also groups outside entognatha, closer to ectognathous insects or even to crustaceans, thus suggesting the non-monophyly of Entognatha (Giribet et al., 2004, Kjer, 2004). Many of these

new phylogenetic hypotheses received significant support in molecular analyses, but the stability of results is highly sensitive to choice of markers, optimality criteria and outgroup taxa (e.g. Cameron et al., 2005).

Even a number of recent morphological investigations support some of these new hypotheses: e.g., Bitsch & Bitsch (2000, 2003) examined new morphological characters (mainly of external morphology) in large number of apterygote taxa, and found no support for monophyly of Ellipura, as well as very little support (or even none at all) for the monophyly of Entognatha, because Diplura grouped with monophyletic Ectognatha (Archaeognatha, Zygentoma and Pterygota).

Recent advances in molecular phylogenetics are also continuously changing our perception of the phylogenetic relationships among the main arthropod lineages: crustaceans, hexapods, chelicerates, and myriapods. The position of hexapods within arthropods appears most problematic (e.g. Klass & Kristensen, 2001). The classical view of the relationships of hexapods to other arthropods asserts that the myriapods are a sister group to the hexapods, or even that myriapods are paraphyletic with respect to hexapods - e.g., that Symphyla were closer to hexapods than to other labiate arthropods (Snodgrass, 1938). This view assumed the monophyly of the so called "Tracheata", i.e. "Myriapoda + Hexapoda", as a consequence of presumed single evolutionary event that led to the development of a tracheal system during terrestrialization of the last common ancestor of these taxa. Many recent morphological works support this hypothesis (e.g. Wagele & Stanjek, 1995, Kukulova-Peck, 1991, Kraus, 2001, Koch, 2001). However, by 1995, Averof & Akam had suggested, based on comparative developmental and molecular studies, that insects could emerge from a crustacean-like ancestor independently of the myriapods, and after the major crustacean radiations.

Recent investigations of both molecular and morphological data has supported a closer relationship between hexapods and crustaceans, a so called "Pan-crustacea" (or "Tetraconata") clade (e.g. Boore et al., 1998, Giribet et al., 2001, 2004, Dohle, 2001, Kjer, 2004, Lavrov et al., 2004, Hassanin, 2006, Carapelli et al., 2007, Nardi et al., 2003, Cook et al., 2005, Regier et al., 2008, and many others). The discrepancy between traditional taxonomy and new molecular evidence suggests that many of the morphological characters used to build arthropod phylogenies have been subject to convergence, particularly between hexapods and myriapods. In this context, even the monophyly of hexapods, which had remained largely unchallenged, was seriously called into question (e.g. Giribet et

al., 2004, Kjer, 2004, Hassanin, 2006, Carapelli et al., 2007, Nardi et al., 2003, Cook et al., 2005). Thanks to these molecular findings, the taxon joining hexapods and crustaceans (the Pancrustacea) is now widely accepted among zoologists. However, the relationships among its basal lineages, and particularly the supposed reciprocal paraphyly of Crustacea and Hexapoda, continues to represent a challenge (e.g. Garcia-Machado et al., 1999, Cook et al., 2006, Carapelli et al., 2007) (Figure 2).

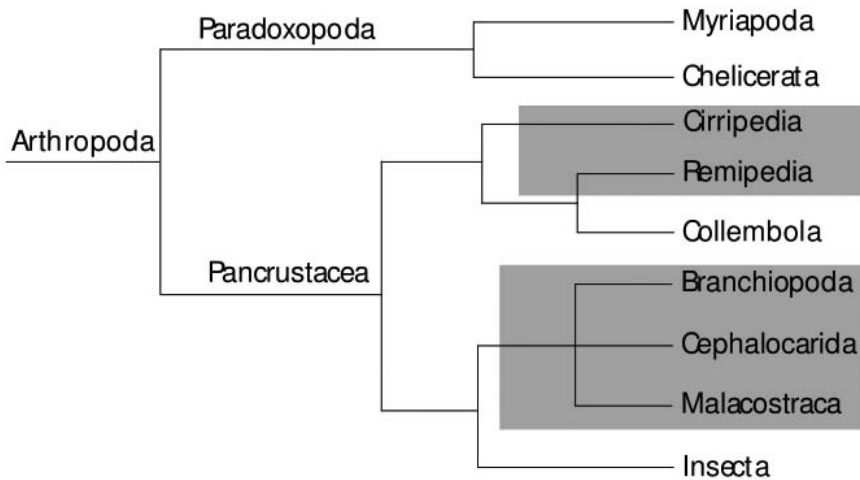


Figure 2. Phylogenetic relationships of major arthropod groups inferred from mitochondrial genes. From: Hassanin et al., 2006.

Regarding other two major arthropod clades, myriapods and chelicerates, some investigations support the sister group of Pancrustacea + Myriapoda (so called “Mandibulata”) (e.g. Giribet et al., 1996, Giribet & Ribera, 1998, Regier et al., 2008), while in some analyses a support was found for the unconventional pairing of myriapods (millipedes and centipedes) with chelicerates (spiders, scorpions, horseshoe crabs, etc.), and even the paraphyly of myriapods with respect to chelicerates cannot be completely ruled out (e.g. Friedrich & Tautz, 1995, Giribet et al., 1996, Hwang et al., 2001, Negrisolo, 2004, Pisani et al., 2004). The names Paradoxopoda and Myriochelelata were proposed for this group of animals, which

includes many that immobilize prey with venom (Figure 2). If this hypotheses turn out to be true, it would imply that water-to-land transition occurred at least three times (hexapods, myriapods, arachnids) during the evolution of the Arthropoda.

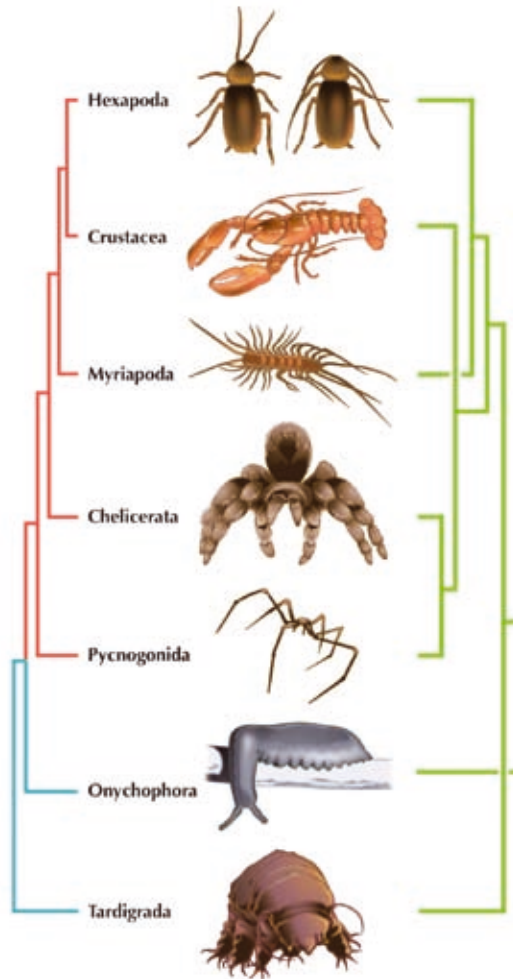


Figure 3. Two views of the interrelationships of arthropod groups based on molecular data (left; red indicates Arthropoda) and morphology (right). Adapted from: Blaxter, 2001.

Conclusion

Although some authors dispute the reliability of new phylogenetic topologies of hexapods and arthropods obtained by the sole use of molecular markers (e.g. Bitsch et al 2004), our understanding of the evolution of these fascinating organisms has been profoundly changed since molecular methods brought new insights in this field and caused radical reordering among extant arthropod classes (reviewed in Blaxter 2001, Budd & Telford 2009, Figure 3). New and unexpected phylogenetic topologies which emerge from molecular methods of analyses should be properly evaluated and compared with the classic hypothesis about the evolutionary relationships of the investigated taxa, as is the case with the molecular phylogenetic investigations of insects briefly presented here. It is evident, therefore, that all these intriguing questions will require intensive efforts in order to reach their definite answers.

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