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The Contribution of the Sperm Structure to the Reconstruction of the Hexapod Phylogeny*

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Abstract

The sperm structure is a valuable character to infer insect phylogeny. In the present paper, after a short discussion on the main sperm components to be considered in the evaluation of the different taxa, some ideas relative to the insect order relationships are discussed. The importance of the structure of the accessory tubules of the flagellar axoneme, together with the expansion of the intertubular material between these tubules, is emphasized. The sperm structure often confirms the results obtained from morphological analysis; there are, however, a few insect groups in which the sperm structure is unable to give support to morphological evidences. A final schematic phylogenetic tree, based on the structure of the accessory tubules, is proposed.

The sperm structure often indicates the animal relationships and phylogeny better than does the external morphology, which is influenced by selective pressures and thus liable to variations. The best example in support of this claim comes from the analysis of sperm structure in animal parasites. Pentastomids were retained as a distinct taxon of Panarthropoda closely related to Tardigrada and Onychophora (Walossek *et al*., 1994). According to sperm morphology they are, instead, to be placed in a group related to branchiurans, within Crustacea. This conclusion, first proposed by Wingstrand (1972) is now supported by molecular studies (Abele *et al*., 1989; Giribet and Ribera, 2000). Similarly, Myzostomida (an ectoparasitic taxon living on Crinoidea and Ophiuroidea) were previously regarded to be related to Annelida, but are now considered to belong to a clade of organisms (Syndermata) including also Rotifera and Acantocephala. The name Prosomastigozoa was supposed for this new superphylum characterized by sperm that have an anteriorly directed flagellum (Zrzavý *et al*., 2001). Similar considerations can also be drawn for insects; *Braula caeca*, the dipteran parasite of honey bee, has lost many of the characters typical of the order. However, its sperm is remarkably similar to that of the suborder brachycerans such as *Drosophila melanogaster* or *Ceratitis capitata*. Similarly, *Coptosoma scutellatum* is a peculiar Heteroptera that looks like a beetle, but its sperm structure is that typical of the order, quite similar to that of *Pyrrhocoris apterus* or *Eurydema oleraceum* (Fig. 1). In all heteropteran species, irrespective of their morphological habitus, the sperm tail axoneme shows bridges between doublets 1 and 5 and the mitochondrial derivatives (Dallai and Afzelius, 1980; Afzelius and Dallai, 1989).

These considerations do not necessarily imply that sperm structure can resolve all phylogenetic problems; the sperm diversification obviously indicates that sperm structure is also a target of speciation, although its modifications are less conspicuous than those dealing with the external morphology. Thus, in certain groups sperm are of very diverse. This occurs, for instance, among basal dipterans or among the caddis-flies, where a great number of sperm models have been found and sperm cells give reliable cues for establishing different taxa.

Sperm diversification may be due to an adaptation to different modes of sperm transfer; to overcome the loss of female seminal receptacle or even to match the sequence of a post-copulatory sexual selection (Pitnick *et al*., 2003). Just for an example, the basal hexapod group Collembola, one of the main members of the soil fauna, have discoidal spermatozoa at maturity with the sperm components rolled around a central mass of extracellular material. This particular appearance of the sperm cell is clearly an adaptation to increase the efficiency of the indirect sperm transfer by spermatophores, which are laid on the soil by the male and then picked up by the female (Dallai *et al*., 2003a).

The insect sperm is an elongated cell that consists of a few structures, all specialized to transport the

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haploid set of chromosomes to the egg. It is provided with a long nucleus that often shows a hydrodynamic shape, well adapted for swimming in a viscous medium. The acrosome usually occupies an apical position anterior to the nucleus, which facilitates the penetration of the egg. The spermatid mitochondria fuse in the mature sperm to form two rigid rods parallel to the sperm axoneme. A careful examination of insect spermatozoon, however, reveals a great number of other features which make the sperm cell a complex cell-organism: the glycocalyx, the centriole adjunct, the accessory bodies, the accessory tubules, the intertubular material and other minor details around the flagellar axoneme.

An important consideration derived from comparative studies of the several sperm components in the different insect orders is that all the above structures do not always evolve in synchrony, thus the sperm cells have not been modified as a whole; rather each feature has been transformed independently from the others. For example, the axoneme of zygentoman sperm shows the same patterns in all members of the group and resembles that of the pterygotes; however, in the relic species *Tricholepidion gertschi* (Lepidotrichidae), as well as in Nicoletidae and Lepismatidae, the acrosome is bilayered as it occurs in the plesiomorphic status of the character, while Ateluridae have a derived pattern, provided with a monolayered acrosome (Dallai *et al*., 2004a). Similarly, in the dipteran cecidomyiids the flagellar axoneme is highly modified and the 9+2 pattern is no more recognizable, but mitochondria retain a conventional pattern (Baccetti and Dallai, 1976; Dallai *et al*., 2006). The result of this independent evolution of the several sperm components is that the sperm cell is unmodified, and retains a basic configuration for certain characters, while, for other characters, it is changed. This situation is of very common occurrence and needs to be carefully considered when phylogenetic relationships are discussed, to avoid wrong conclusions.

Moreover, a fact that has been previously underlined (Dallai, 1979) is that several insect orders display a tendency towards a progressive axoneme

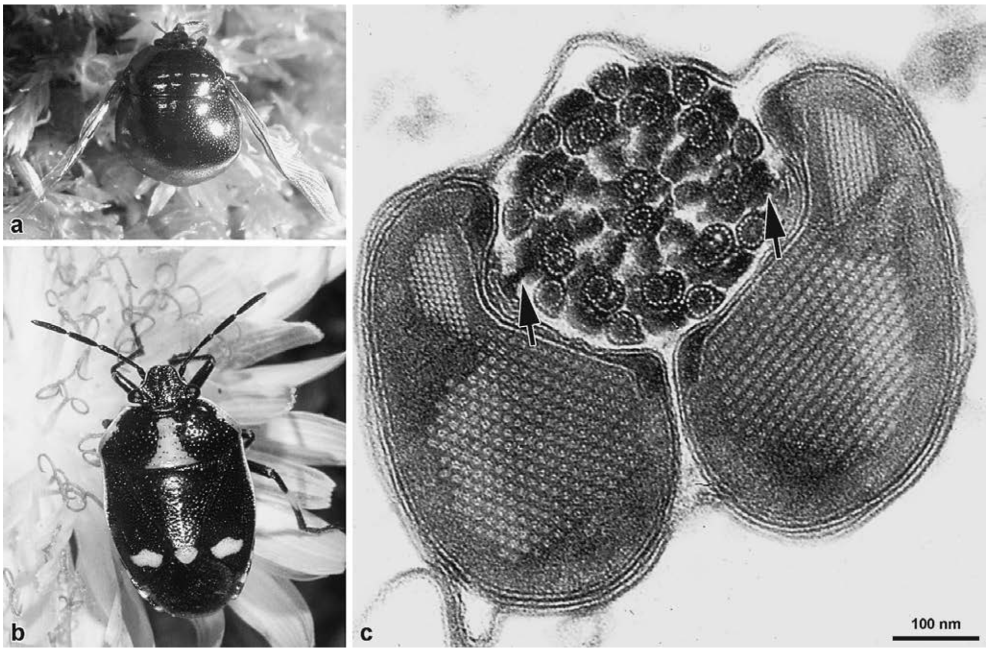

Fig. 1 a. Habitus of *Coptosoma scutellatum* (Heteroptera, Platispidae). b. Habitus of *Eurydema oleraceum* (Heteroptera, Pentatomidae). c. Cross section through the heteropteran *Pyrrhocoris apterus* (Pyrrhocoridae) sperm flagellum. Note the two bridges (arrows) connecting the axonemal doublets 1 and 5 to vesicles adherent to the mitochondrial derivatives.

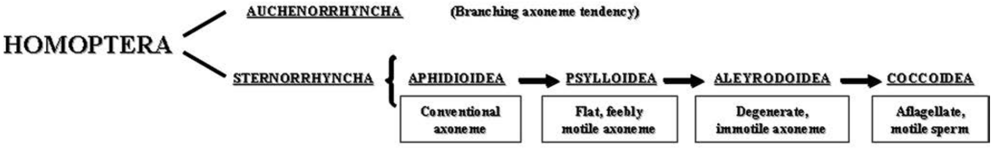

Fig. 2 Schematic drawing of the sperm evolution in Homoptera (from Dallai, 1979).

degeneration. Within each of these orders abnormalities in the flagellar axoneme are found, *e. g*., lack of the outer or the inner arms, lack of both these structures, or of the central tubules. The occurrence of an aflagellate and immotile sperm is the final step of such a degenerative process. The sperm evolution among Homoptera is a typical example in this respect (Dallai, 1979) (Fig. 2). Similar results can be found also among Protura, Isoptera, Ephemeroptera, Trichoptera and Diptera (Dallai *et al*., 2006).

The accessory microtubules of insects: a synapomorphic feature; their structure and diversity

While a 9+2 axonemal pattern is a widespread and invariant feature occurring in diverse organisms, many insect orders have an extra set of 9 singlet microtubules, outside the central 9+2 complex, hence known as a 9+9+2 axoneme (Fig. 3a). These nine accessory microtubules are formed during spermiogenesis as outgrowth on the B-subtubule of each microtubular doublet (Cameron, 1965; Dallai and Afzelius, 1993) (Fig. 3b, c). During the process of the accessory microtubule formation, an electrondense material is also stored in the intertubular space (Dallai and Afzelius, 1993). The extension of this material varies according to the insect orders and in few cases may be missing, as it occurs in Ephemeroptera (Fig. 7a).

Accessory tubules usually have 16 protofilaments (Figs. 1c, 3a), but microtubules with 13 or other different protofilament numbers have been described (Lanzavecchia *et al*., 1994; Dallai and Afzelius, 1999). Those with 13 protofilaments, thus similar to cytoplasmic microtubules occur in a few insect orders, such as Diplura, Ephemeroptera, Psocodea (Fig. 7a) and brachyceran Diptera (Fig. 11). Quite recently, accessory microtubules with 40 protofilaments were found in the atypical sperm of the neuropteran *Perlamantispa perla* (Dallai *et al*., 2005a).

The importance of accessory tubules derives from the consideration that, while the central 9+2 of the axoneme has a fairly conserved ultrastructure, there is a remarkable diversity in the structure of the accessory tubules and in the dense material between them. This diversity, if well considered, seems to be a useful marker of insect relationship, as the character is under strict genetic and molecular control. Raff *et al*. (1997) have studied the spermiogenesis of a sterile transgenic *Drosophila*, in which as little as 10% of the β-tubulin was replaced by its homologue isoform Hvβt from the moth *Heliothis*. These authors observed that the organization of some accessory tubules in the fly axoneme was of moth type, accessory tubules showing 16 rather than 13 protofilaments. The moth protein thus imposes a moth specific accessory microtubule architecture on the equivalent structure of fruit fly cell, even though only a small amount of the total β-tubulin pool was present (Fig. 4). Moreover, recent data have shown that a highly specialized β-tubulin is needed to construct *Drosophila* sperm tail axoneme (Dutcher, 2001; Nielsen *et al*., 2001; Nielsen and Raft, 2002).

Insects in which accessory microtubules are missing

Several insect species and whole taxa have axonemes devoid of accessory microtubules. For some groups, the lack of accessory microtubules is likely to be

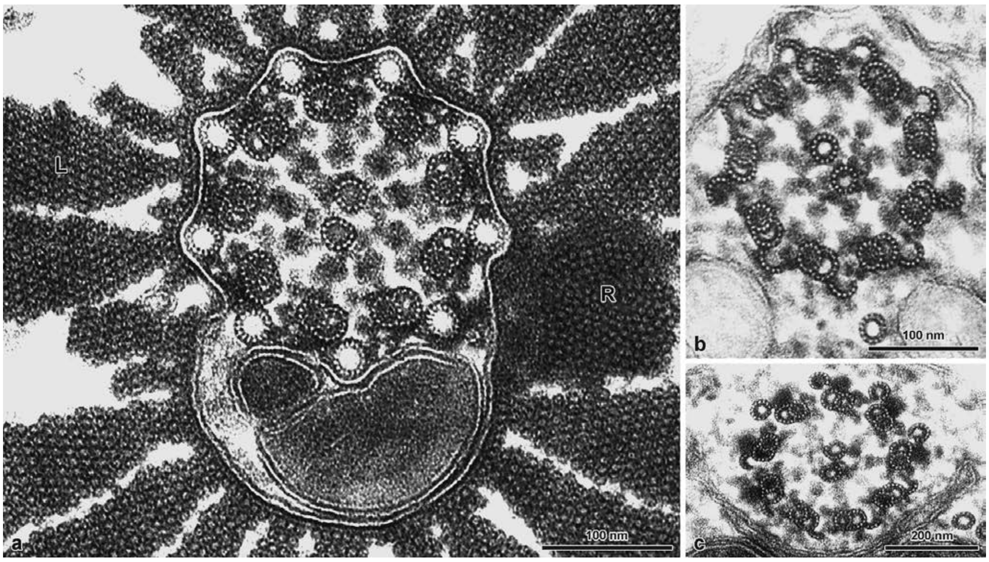

Fig. 3 a. Cross section through the lepidopteran *Apopestes spectrum* sperm flagellum. Note the reticulate (R) and the leciniate (L) appendages forming the glycocalyx in the eupyrene (functional) spermatozoa. b, c. Cross sections through the wingless dipluran *Campodea* sp. (b) and the medfly (*Ceratitis capitata*) (c) early spermatids showing the formation of the accessory tubules from the axonemal doublets.

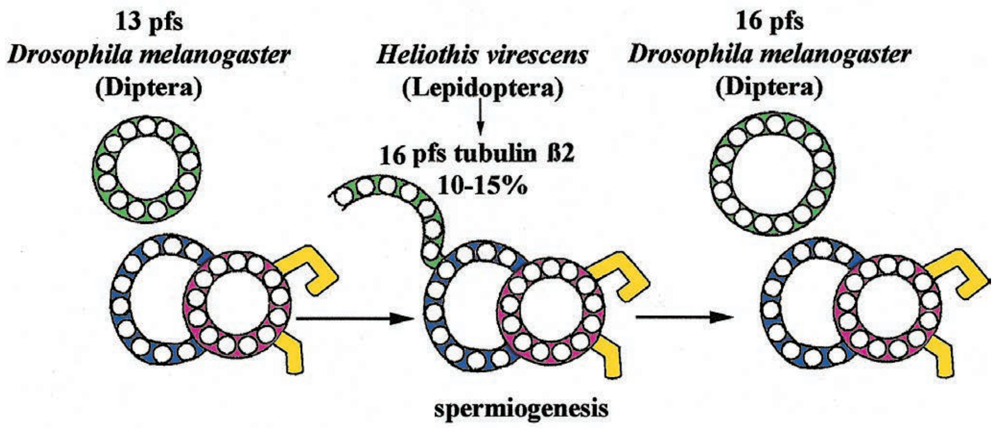

Fig. 4 Schematic drawing to show the experiment by Raff *et al*. (1997). The presence of a low concentration of β_2 tubulin homologue isoform from the moth *Heliothis virescens* during the spermiogenesis of *Drosophila* imposes the formation of an accessory tubule with 16 rather than 13 protofilaments in the fly sperm axoneme.

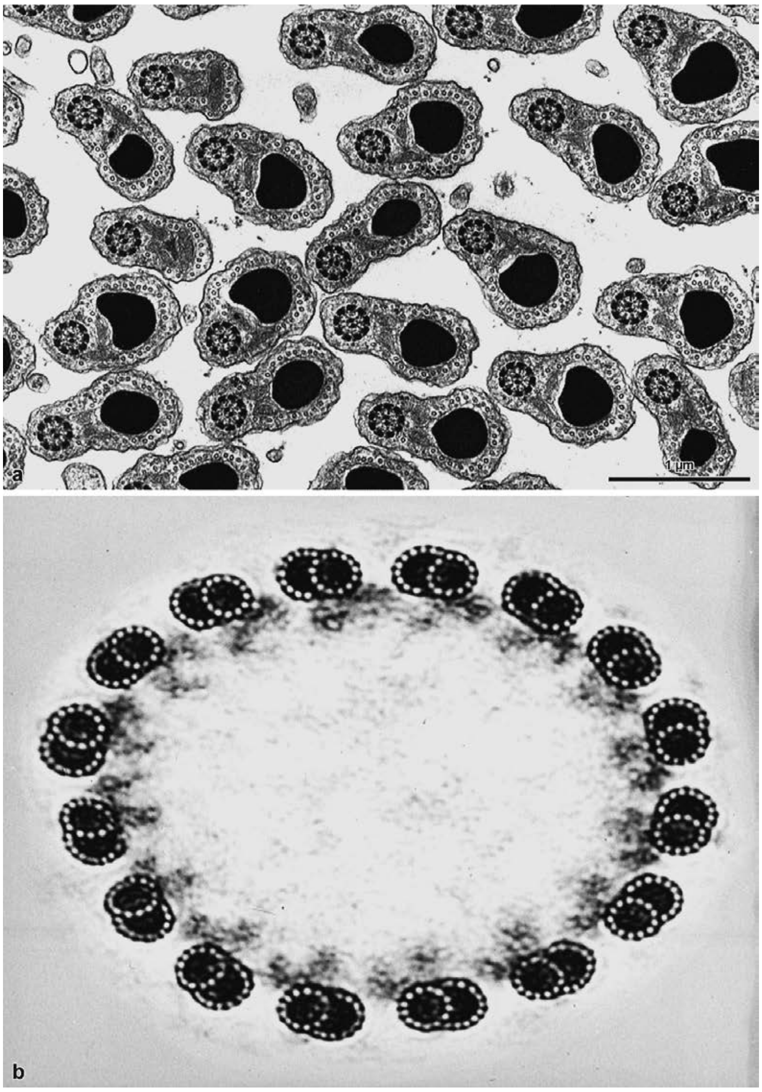

Fig. 5 a. Cross section through the collembolan *Allacma fusca* aged spermatids. Note the single 9+2 axoneme and the three mitochondria. b. Computer aided reconstruction of the sperm axoneme of the proturan *Acerentomon majus*. The two central microtubules are lacking. The number of axonemal doublet exceeds the conventional 9 doublets. Only inner dynein arms are present on each doublet.

considered a plesiomorphic tract; this is the case of the wingless Collembola and Protura, forming the taxon Ellipura. Collembola have a conventional 9+2 axoneme (Fig. 5a) (Dallai, 1970); Protura, however, exhibit an unusual flagellar axoneme consisting of several microtubule doublets (Fig. 5b). The proturan *Acerentomon majus* has a motile, flagellated sperm provided with 16 microtubular doublets but lacking central tubules; the doublets are devoid of outer dynein arms; similar sperm axonemal patterns are shown in related families, even though the number of doublets can be variable from 12 to 16. In the derived families Sinentomidae and Eosentomidae, however, the axoneme is missing, the sperm have become spheroidal or discoidal and are immotile (Dallai and Yin, 1983; Dallai *et al*., 1992).

In few other insect groups, the 9+2 pattern seems to be due to a secondary loss of accessory tubules. Among Isoptera, only *Mastotermes darwiniensis* shows a flagellate spermatozoon consisting of about 100 flagella, each one with a simple 9+2 axoneme (Baccetti and Dallai, 1978). The rhynchotoid order Thysanoptera is regarded as the sister group of Hemiptera. However, they have an odd axonemal pattern consisting of an amalgamation of 27 microtubular structures, none of which corresponds to an accessory tubule. This aberrant axoneme derives from the fusion, during late spermiogenesis, of three flagellar axonemes of the 9+0 type present in each single spermatid (Fig. 6a–d). This bizarre axoneme is, however, motile (Bode, 1988; Dallai *et al*., 1991; Paccagnini *et al*., 2007). The relationships of thrips with Phthiraptera (Mallophaga + Anoplura) is supported by the presence of two centrioles in the spermatids at the end of the spermiogenesis (Paccagnini et al., 2006). A third centriole is “*de novo*” formed only in thrips just before spermiogenesis and thus the process starts with a tri-flagellated cell, each flagellum provided with 9+0 axoneme (Paccagnini *et al*., 2006).

Among Holometabola, in addition to Mecoptera and Siphonaptera, accessory microtubules are also missing in a few basal dipterans, the large family Cecidomyiidae included, some Trichoptera Annulipalpia, and the Micropterygidae among Lepidoptera.

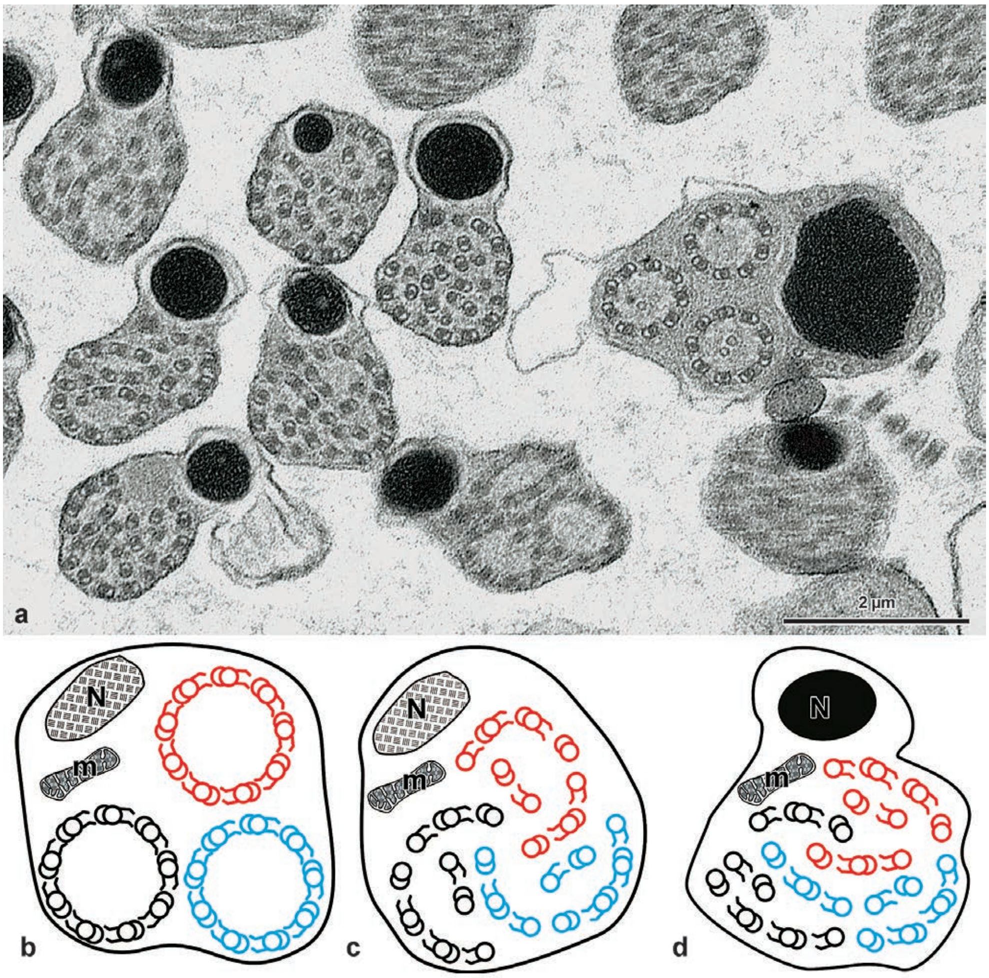

Fig. 6 a. Cross section through *Agrostothrips meridionalis* spermatids showing the still evident three axonemes in one cell and the progressive fusion of them. b–d. Schematic representation of the progressive amalgamation of the three spermatid axonemes into an aberrant complex of microtubular elements. N: nucleus, m: mitochondrion (from Paccagnini *et al*., 2007).

Comparative spermatology among pterygotan insects

Paleoptera (Ephemeroptera + Odonata) is an artificial group (Kristensen, 1998). Most phylogenetic analyses of hexapod relationships have suggested paraphyly of the paleopterans, with Odonata as the sister group to Neoptera (Hennig, 1953; Kristensen, 1981, 1998; Whiting *et al*., 1997; Carpenter and Wheeler, 1999; Wheeler *et al*., 2001). Members do not exhibit any shared spermatological features (synapomorphies). Furthermore, Ephemeroptera have realized several sperm autapomorphies: a monolayered acrosome; a 9+9+0 axoneme with microtubular doublets provided with only inner arms; the peculiar accessory microtubules with 13 protofilaments, intertubular material missing (Fig. 7a). Moreover, all Leptophloebidae have ovoidal immotile sperm devoid of sperm flagellum (Gaino and Mazzini, 1991; Dallai and Afzelius, 1999), a fact that could be expected from the modification occurred in the flagellar axoneme. On the contrary, Odonata have a conventional sperm model with a flagellum typical of pterygotan insects (Fig. 7b).

Quite recently, a new insect order has been described from South Africa: the Mantophasmatodea (Klass *et al*., 2002). As suggested by the name the external morphology of the new taxon is reminiscent of

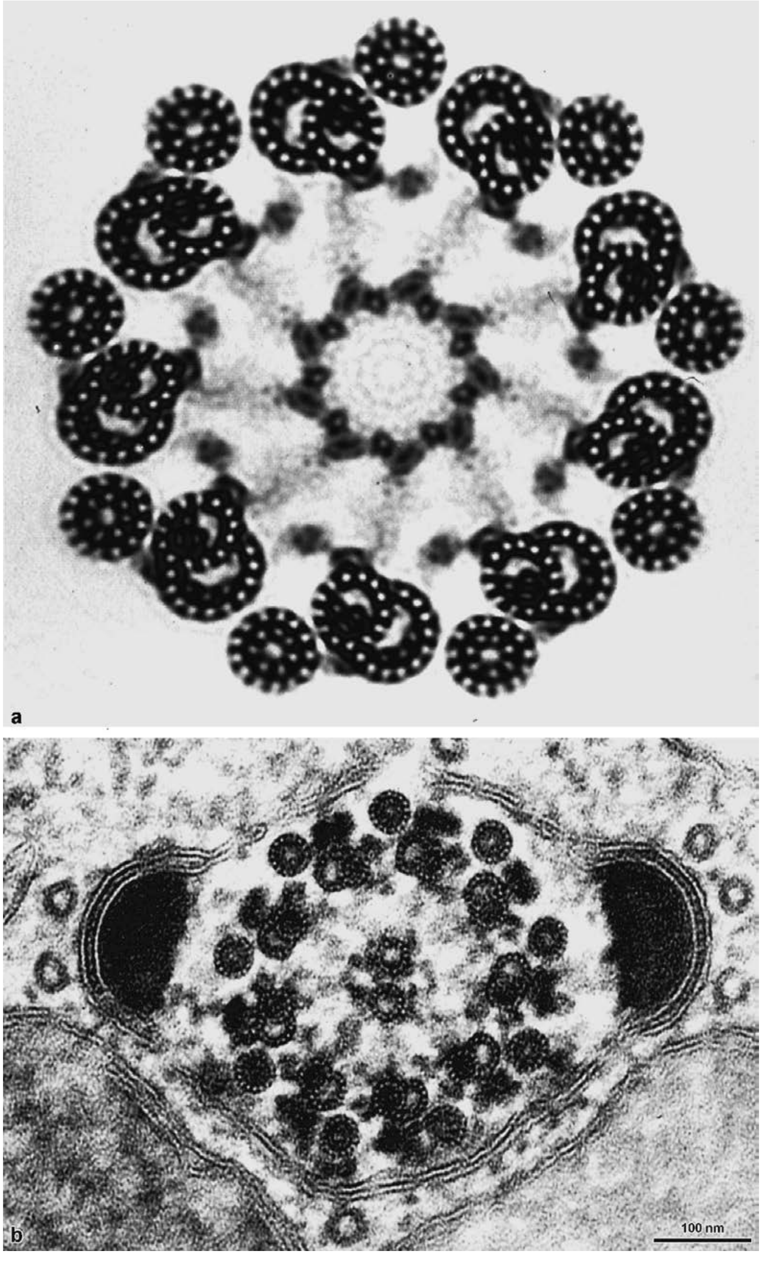

Fig. 7 a. Computer aided reconstruction of the sperm axoneme of the ephemeropteran *Cloëon dipterum*. Note the lack of central tubules and the presence of only inner dynein arms on microtubule doublets (from Dallai and Afzelius, 1999). b. Cross section through a spermatid of the dragonfly *Calopteryx* sp. Note the 9+2 axoneme pattern flanked by two dense accessory bodies.

Mantodea and Phasmatodea. A careful comparative spermatological analysis (Dallai *et al*., 2003b) has allowed the conclusion that the new order could be related to Mantodea, as both groups share the peculiar feature of 3 connecting bands between the flagellar axoneme and the mitochondrial derivatives. On the contrary, Phasmatodea are not related to Mantophasmatodea, as the group exhibits several derived characters: the lack of mitochondria, the presence of 17 protofilaments in the tubular wall of their accessory tubules; expanded intertubular material and accessory bodies of unusual shape and dimension not observed in any other insect order. Mantodea, however, have an apomorphic character in their axoneme that makes them easily distinguishable: the presence of a row of nine filaments that are connected to the B-subtubule, somewhat similar to an extra accessory tubule during its formation (Dallai and Afzelius, 1999). The recent finding of the sperm structure of the order Grylloblattodea has allowed us to suggest that this taxon, together with Mantophasmatodea, Mantodea and Orthoptera, could form a cluster of related orders (Dallai *et al*., 2005b) (Fig. 8).

The molecular data support the monophyly of Psocodea. They do not group with Hemiptera and could be the sister group of Thysanoptera (Whiting *et al*., 1997). Psocodea (Psocoptera + Phthiraptera) sperm share the peculiar feature of elliptic accessory tubules provided with 13 protofilaments in their tubular wall and a reduced intertubular material. These are important characters that together with the tendency in Psocoptera towards the presence of a biaxonemal flagellum support well the monophyly of the group (King and Ahmed, 1989). In fact, Phthiraptera also share a biaxonemal flagellum.

Among Endopterygota (Holometabola) the sperm structure supports the relationships between Neuropterida (Megaloptera, Raphidioptera, Planipennia) and Coleoptera. They share a sperm axoneme of almost the

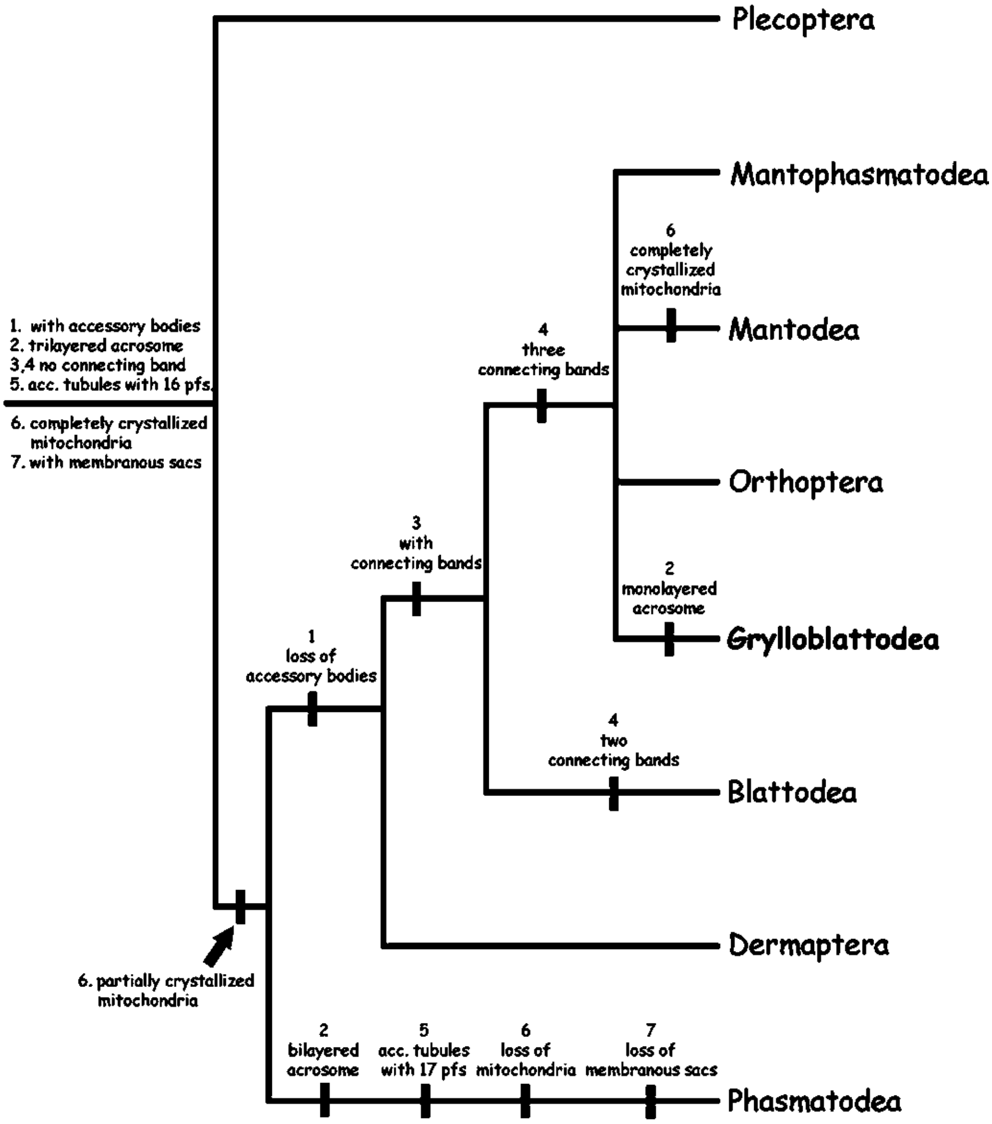

Fig. 8 Sperm cladogram of the relationships between orthopteroid orders as resumed from their sperm structure (from Dallai *et al*., 2003b).

same kind: their accessory microtubules have 16 protofilaments, and the intertubular material is divided in two portions: one part is in contact with the external side of the doublets and another one projects as a beak protruding from the accessory tubules (Afzelius and Dallai, 1994; Dallai and Afzelius, 1999). Furthermore, both groups have well developed accessory bodies (Fig. 9).

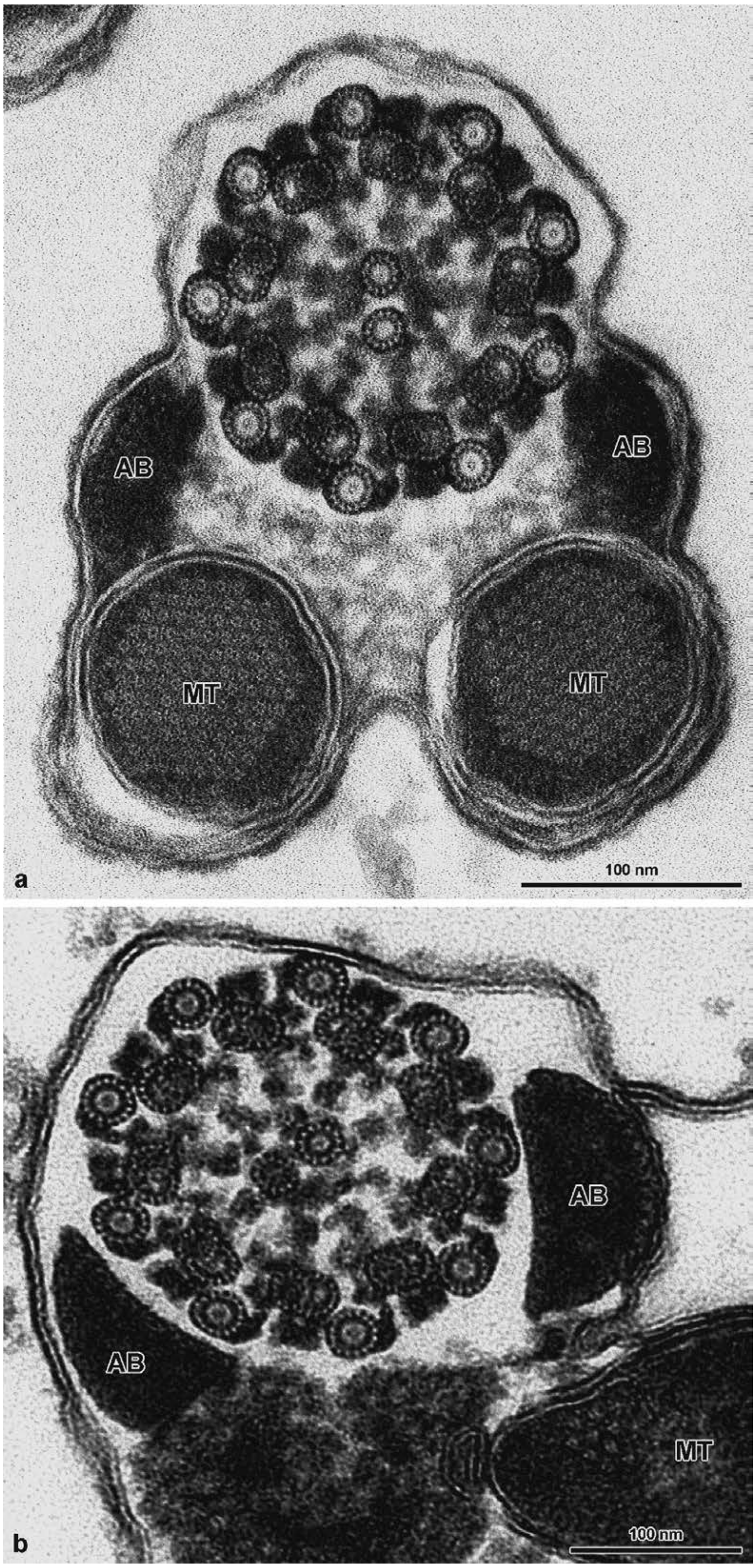

Fig. 9 Cross sections through the neuropteran *Ascalaphus* sp. (a) and the coleopteran *Nemonyx lepturoides* (b) sperm tails. Note the similar organization of the intertubular material in the two taxa, the two mitochondrial derivatives (MT) and the two large accessory bodies (AB) (from Jamieson *et al*., 1999).

The presumed sister groups Mecoptera and Siphonaptera share some somatic synapomorphies (Kristensen, 1991), even though some doubts were raised on the monophyly of Mecoptera. Such doubts were reinforced by molecular analysis (Whiting *et al*., 1997; Whiting, 2002) which supported the paraphyly of Mecoptera with Boreidae as the sister taxon of Siphonaptera. A recent study on the sperm structure of both mecopteran and siphonapteran species (Dallai *et al*., 2003c) have evidenced three apomorphic character states supporting the monophyly of Mecoptera. These consist in: 1) a similar organization of the external glycocalyx, 2) the presence of two extra-axonemal rods and 3) the presence of a short acrosome. These findings do not support the placement of *Boreus hyemalis* within the Siphonaptera, as suggested by Whiting (2002).

Diptera have a variable sperm structure. In the basal nematocerans, the accessory tubules have 16 protofilaments, the most common number among insects; it has been found in Mycetophilidae (Dallai *et al*., 1995), whereas Chironomidae, Dixidae, Culicidae, Bibionidae and Simulidae (Dallai *et al*., 2007) all have accessory tubules with 15 protofilaments, irrespective of the number of central tubules in their axonemes (Fig. 10a). This finding clearly indicates a close relationship between these nematoceran taxa. Tipulidae and Trichoceridae, which also belong to the nematoceran suborder, by contrast, have 13 protofilaments in the tubular wall (Fig. 10b). This number is shared by all higher brachyceran dipterans (Dallai *et al*., 1993; Dallai and Afzelius, 1999). This feature is of great interest as it supports the hypothesis that the brachycerans have evolved from a tipulimorph group (White, 1973) with some further modifications of the axoneme, such as the extension of the intertubular material, which is reduced in Tipulidae, but is large and contains peripheral features in the different brachyceran groups (Fig. 11). Interestingly, in the brachyceran suborder, what seems to have greatly changed is the sperm length rather than the cross sectioned axonemal profile. For example, within the genus *Drosophila*, sperm length varies by more than two order of magnitude and gigantic sperm (*i. e*., up to 5.8 cm) have independently evolved (Pitnick *et al*., 2003).

Among nematoceran dipterans, the gall-midge family Cecidomyiidae is the largest one. According to the conventional systematics, its subfamily Cecidomyiinae consists of four supertribes: Stomatosematidi, Asphondyliidi, Cecidomyiidi and Lasiopteridi (Gagné, 1981; 1994; Skuravá, 1986) (Fig. 12). The supertribes Asphondyliidi and Cecidomyiidi are characterized by giant sperm axonemes provided with numerous microtubular doublets (Fig. 13); among cecidomyiidi doublets are orderly arrayed to form single rows, while in Asphondyliidi they give rise to a double parallel spiral. In both groups, however, each doublet is provided with only the outer arm, a feature that is unique in the animal kingdom (Dallai *et al*., 1996). Spermatological studies using the quick freeze deep-etching technique allow the characterization of the outer dynein arm (Lupetti *et al*.,

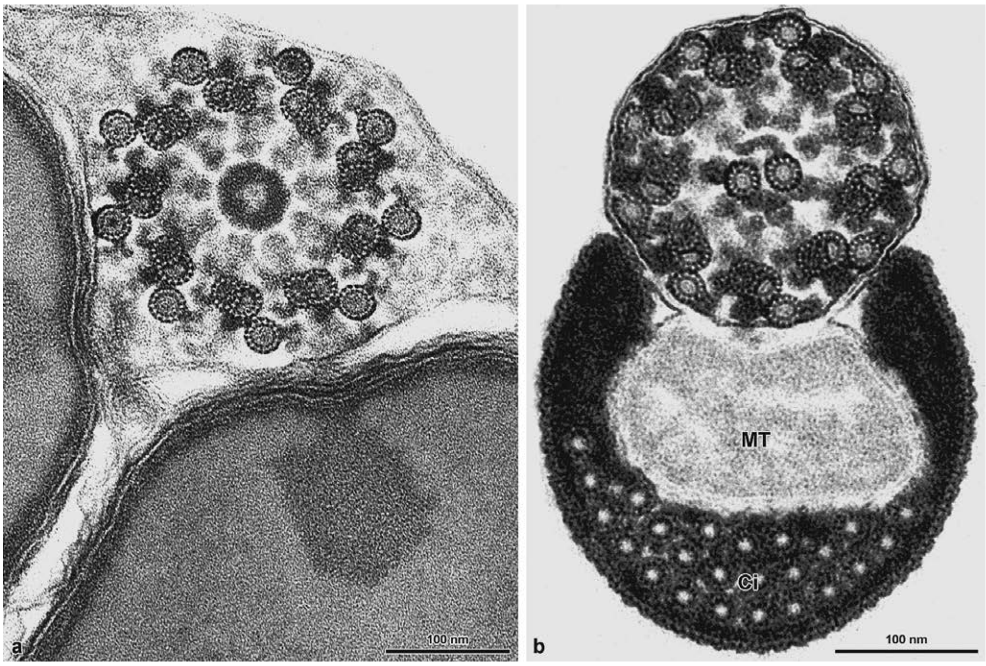

Fig. 10 a. Cross section through the bibionid *Bibio* sp. sperm tail to show the axoneme pattern characterized by the presence in the axoneme of a central cylinder rather than two microtubules and by the presence of accessory tubules provided with 15 protofilaments in their tubular wall (from Jamieson *et al*., 1999). b. Cross section through the nematoceran *Tipula* sp. with accessory tubules provided with 13 protofilaments in the tubular wall. Ci: centriole adjunct material, MT: mitochondrion (from Dallai *et al*., 2008).

1998; Mencarelli *et al*., 2001).

Final considerations

The origin and phylogenetic relationships of the hexapods is one of the most controversial issues in the animal kingdom. This is due to the ancient origin of the group that occurred already long ago in the Devonian (Whalley and Jarzembowsky, 1981) or even in the Silurian (Grimaldi and Engel, 2005).

According to the recent molecular data, it is estimated that the radiation of Arthropoda began before Ordovician (Pisani *et al*., 2004). Moreover, the time of divergence between Myriapoda and Chelicerata was estimated to be about 642±63 My ago and that between Insecta and Crustacea about 666±58 My ago. Thus, in a relatively short period the main arthropodan taxa differentiated. The consistent support of molecular data for a close relationship between Crustacea and Insecta on one side (Boore *et al*., 1995, 1998) and between Myriapoda and Chelicerata (Myriochelata) on the other (Cook *et al*., 2001; Hwang *et al*., 2001; Negrisolo *et al*., 2004), has provided a new scenario of hexapodan evolution.

Moreover, it is now clear that hexapody has been realized more than once; it is certainly not the consequence of water-to-land transition which has occurred at least three times, if not more, during Arthropoda evolution (Haas *et al*., 2003; Nardi *et al*., 2003; Negrisolo *et al*., 2004).

Finding of ancestor of hexapods is thus a quite difficult matter. It is debated whether Hexapoda evolved from a myriapodan or a crustacean organism. Sperm structure does not give any clue in this respect: among myriapods, the flagellate sperm of Symphyla, Pauropoda and Chilopoda have different structures than the insect sperm has and none of them is provided with accessory tubules. Only the symphylan eusperm has a superficial similarity with that of some basal zygentoman insects (Dallai and Afzelius, 2000; Dallai *et al*., 2004a). The presence of two centrioles in *Scutigerella* sperm, however, indicates that spermiogenesis proceeds in a different way from that of insects. Furthermore, all examined millipeds have aflagellate, immotile spermatozoa (Baccetti *et al*., 1979; Jamieson, 1987). Within Crustacea, Ascothoracica have flagellated sperm with a 9+2 axoneme. This flagellate condition is elsewhere restricted to the related maxillopod group. Thus, the Crustacea sperm structure does not help to find a clear relationship with any other arthropodan groups. Even more important, Malacostraca, which are considered to be the sister group of Hexapoda (Wilson *et al*., 2000; Hwang *et al*., 2001; Nardi *et al*., 2003) have a very

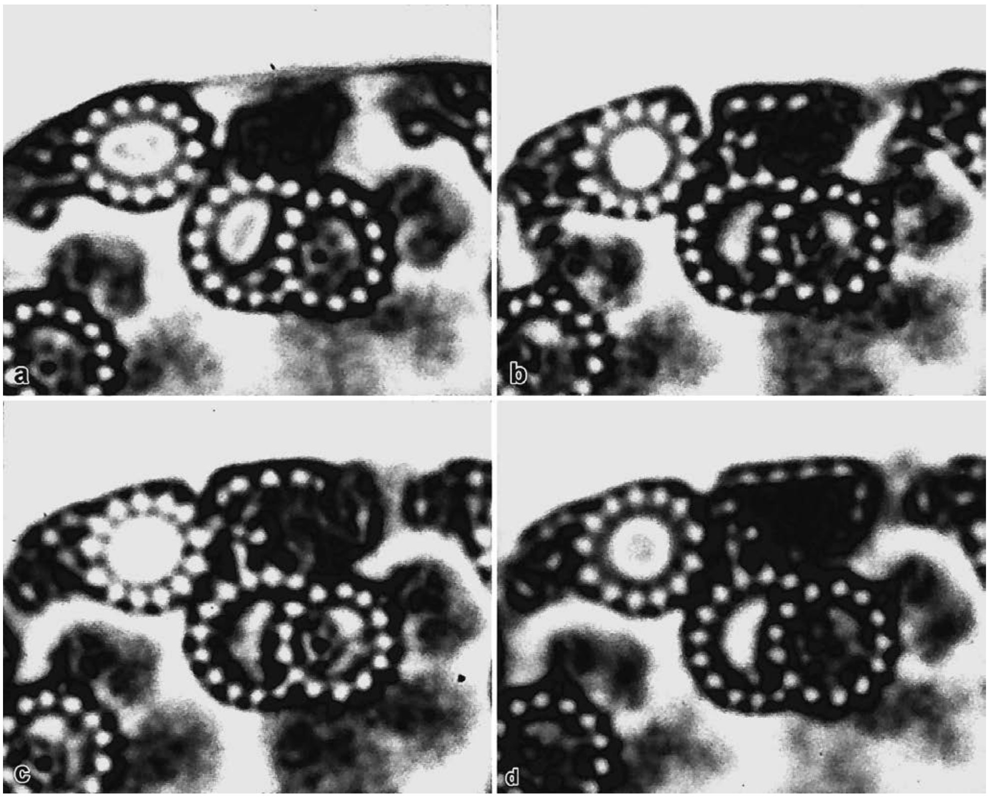

Fig. 11 Computer aided elaborations of axoneme detail of dipterans. a. The nematoceran Tipulidae. b–d. The brachycerans Empididae, Drosophilidae and Scatophagidae, respectively. All members are provided with accessory tubules with 13 protofilaments, but the intertubular material is more elaborated in brachycerans than in the nematocerans.

modified aflagellate sperm.

Thus, no convincing spermatological evidence occurs to establish from which group, either Crustacea or Myriapoda, the ancestor of Hexapoda arisen.

Nevertheless, considering the basal Hexapoda, the sperm structure highlights the following questions:

1) Are Protura really hexapods? Can this taxon without antennae belong to the Antennata? Are they really related to Collembola? Few external morphological characters indicate a relationship between these taxa (Ellipura), but both have differentiated apomorphic characteristics (Bitsch and Bitsch, 2000). They could be relics of the rich palaeozoic fauna somewhat related to the cheliceratans, and in particular to Pantopoda, together with which they share unusual sperm; an hypothesis that was primarily suggested by Yin (1984). Berlese (1910), long ago, suggested the new taxon Myrientomata to emphasize the relationship of Protura with Myriapoda. Recent molecular data have indicated a relationship between Myriapoda and Chelicerata (Friedric and Tautz, 1995; Cook *et al.*, 2001; Hwang *et al.*, 2001; Negrisolo *et al.*, 2004), a suggestion that would have been considered heretic a few years ago. If this is the case, it is reasonable to hypothesize a relationship between Protura and Pantopoda: the former being aberrant hexapods, and the second aberrant cheliceratans (Dunlop and Selden, 1998). Both exhibit a common and peculiar motile sperm flagellar axoneme consisting of a crown of doublets devoid of outer arms and also lacking central tubules. Furthermore, sperm of both Protura and Pantopoda have several conventional mitochondria, a feature that is unusual for true insect

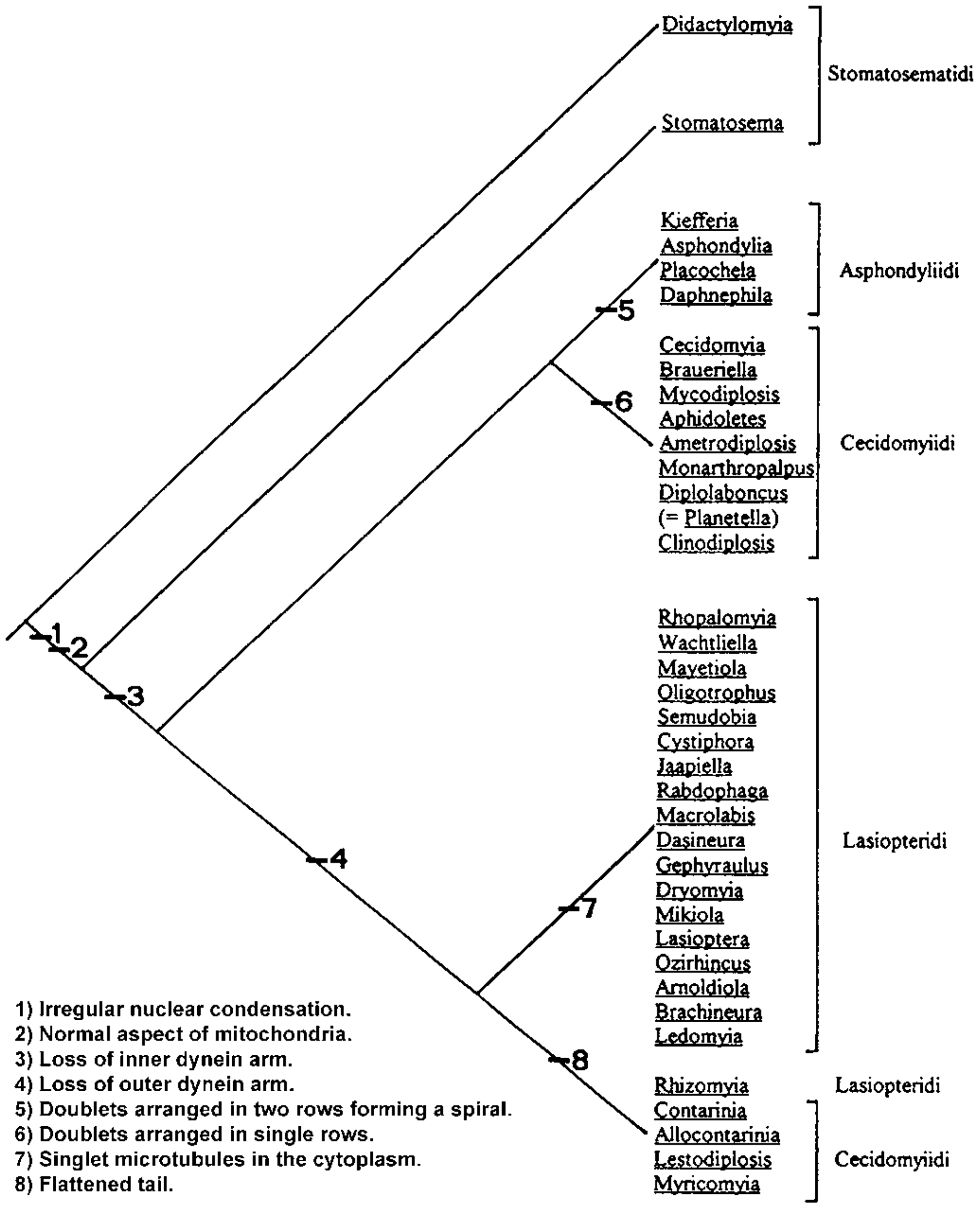

Fig. 12 Cladogram of the phylogenetic relationships among the gall-midge dipterans of the subfamily Cecidomyiidae (from Dallai *et al.*, 1996).

sperm (Van D'Eurs, 1974a, b; Dallai *et al*., 1992) and have a single centriole, which is unusual for a cheliceratan. Finally, in both taxa a tendency towards aflagellarity and sperm immotility is evident.

2) As to Collembola, they could be an independent evolutionary lineage that possibly colonized the numerous soil habitats during the early Devonian (Ghilarov, 1958; D'Haese, 2002). Their sperm structure shows the 9+2 pattern in their flagellar axoneme, but they have also some apomorphies, such as the long peduncle extending over the acrosome, the presence of three postnuclear mitochondria and a peculiar rolling process in late spermiogenesis (Dallai *et al*., 2003a, 2004b), leading to the formation of discoidal sperm. Recent molecular data obtained with mitochondrial DNA suggested their position outside insects (Nardi *et al*., 2003).

3) Diplura share with the whole Ectognatha the synapomorphic feature of accessory tubules outside the central 9+2 sperm axoneme. Both Campodeidae and Japygidae have accessory tubules with 13 protofilaments. In Japygidae, however, only five accessory tubules are visible all along the sperm tail; the remnant four tubules are confined only in a short post-nuclear region. The dislocation of the accessory microtubules, which migrate from their usual position around the 9+2 axoneme, is a character shared by the two groups, Campodeidae and Japygidae (Dallai and Afzelius, 1999). The findings suggest the monophyly of the taxon, in contrast with data proposing the paraphyly of Diplura (Štys and Biliński, 1990). The relationship of Diplura with Ectognatha was already suggested by Kukalová-Peck (1987). This is an important point that, if validated by further results, would confirm the hypothesis that Entognatha are not a monophyletic assemblage.

In conclusion, accessory tubules appeared early in hexapod evolution (Fig. 14). Their presence may be considered to be a synapomorphic feature uniting all ectognathan orders plus Diplura, thus excluding Collembola and Protura. After the 13-protofilament accessory tubules of Diplura, a perhaps more stable 16-protofilament model might have evolved, prior to the

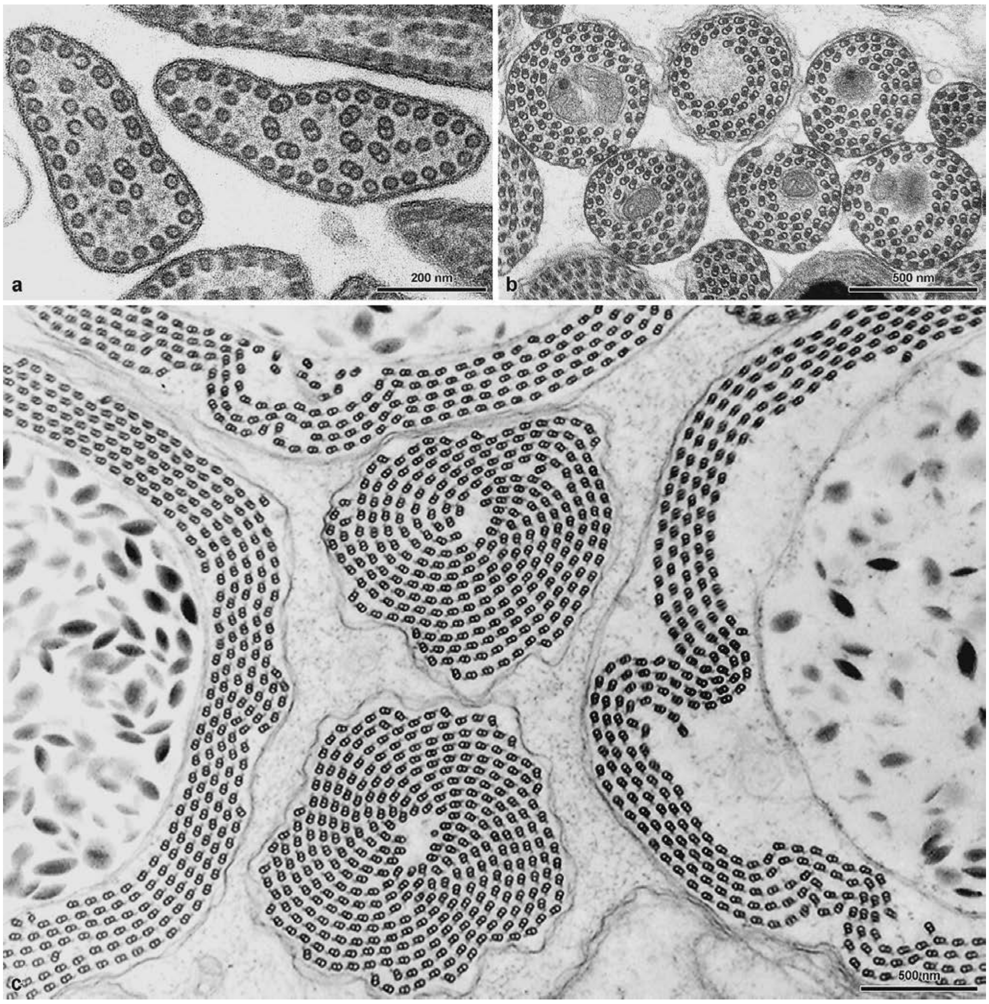

Fig. 13 Cross sections through the sperm tails of *Gephyraulus* sp. (Lasiopteridi) (a), *Placochela nigripes* (Asphondyliidi) and (b) *Massalongia bachmaieri* (Cecidomyiidi) (c) (from Baccetti and Dallai, 1976; Dallai, 1988; Dallai *et al*., 1997).

diversification of ectognathan orders. The fact that the 16-protofilament model is the most common one, may suggest that this is the plesiomorphic condition among pterygotan insects; it needs, however, to hypothesize that the protofilament number could secondarily be changed, as it occurred in Phasmatodea and Trichoptera. These changes also include either loss of accessory tubules or an independent return to a 13-protofilament condition, as in Ephemeroptera, Psocodea and higher Diptera.

As a final remark, it can be said that sperm structure, as it occurs for other characters, agrees in many cases with the results of systematics based on external morphology, whilst in a few other cases, it contradicts consolidated positions established by many convergent morphological or/and molecular observa-

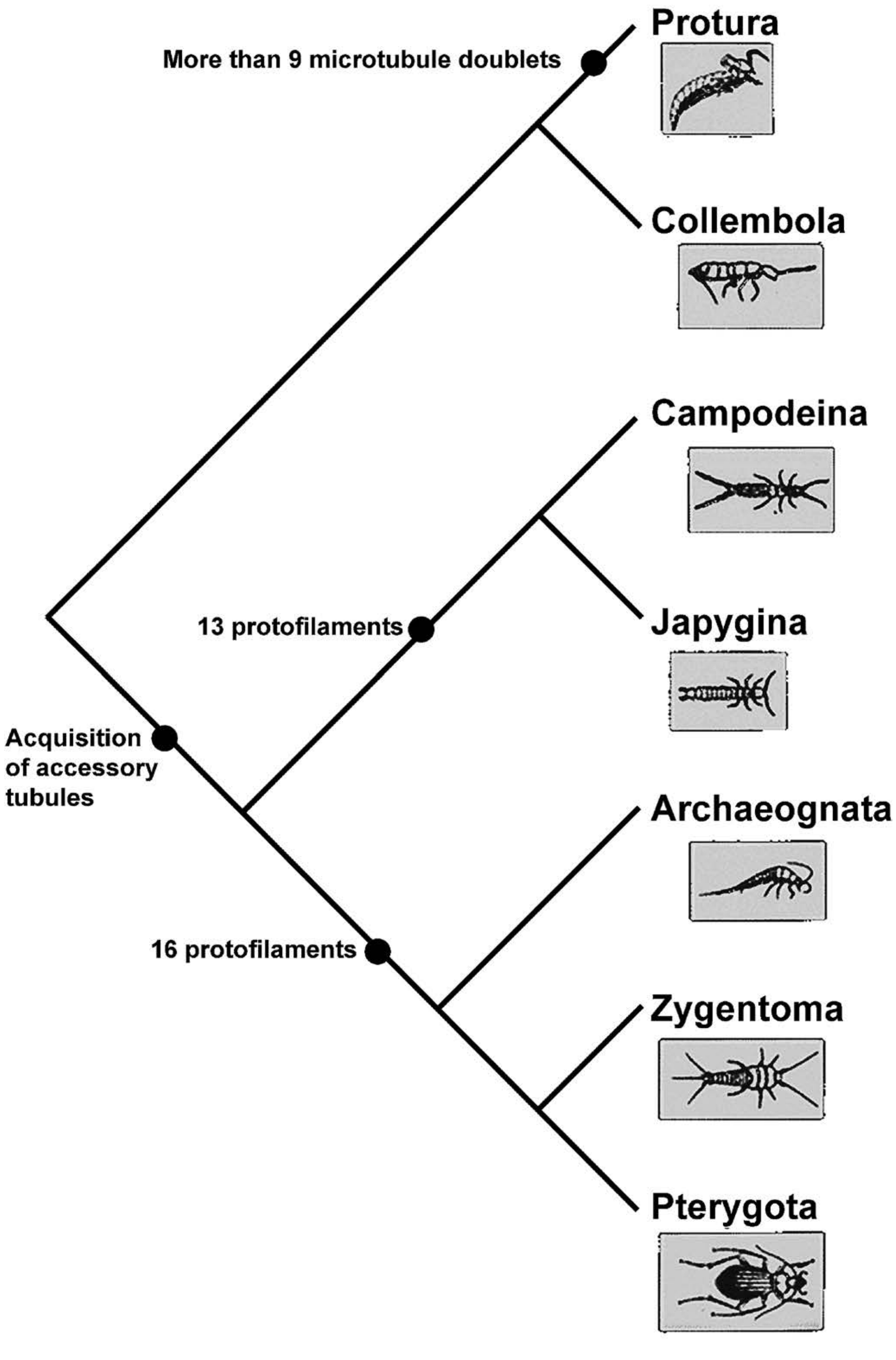

Fig. 14 Phylogenetic relationships among Hexapoda based on the accessory tubule structure of the sperm tail.

tions. Why, for instance, do Trichoptera, which undoubtedly are closely related to Lepidoptera, show so many sperm models, while Lepidoptera have a rather uniform type of spermatozoon? Analogously, why have Nematocera Diptera, but not Brachycera differentiated into so many different sperm axoneme?

New techniques and new improved methods for the preparation of the material will provide us, in future, with additional sperm characters to be used for a better understanding of Hexapoda evolution.

"Evolutionary biology is an endless frontier and there is still plenty to be discovered" (Mayr, 2004). We must be aware that the fascinating story of Hexapoda evolution is, in many aspects, still unknown but that new perspectives conflict with what we once thought we knew. In the next future we have to study this difficult matter with the aim of reconciling morphological data with the new findings from molecular, embryological and paleontological studies.

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