

Phylogenetic Significance of Axonemal Ultrastructure: Examples from Diptera and Trichoptera

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ABSTRACT

The organization of the axoneme in various insects has been examined by using a tannic acid-containing fixative and been found to differ in a systematic fashion between different insect orders. Within each order, both protofilament number and other axonemal characteristics are relatively constant, however. There are two exceptions to this statement: Diptera and Trichoptera. These two orders were therefore examined in order to see whether the axonemal organization of the various species reflects their systematic position. About 24 dipteran species and 35 trichopteran species were examined in this respect. Axonemal structure in Brachycera is similar to that of Tipulomorpha, which indicates a derivation of Brachycera from that group. Mycetophilidae, rather than Tipulomorpha, seems to be the most primitive dipteran taxon, as judged from the axonemal organization. Axonemes of an examined bibionid species (but not of other members of Bibionomorpha) resemble those of culicomorph species. The axoneme in Trichoptera is characterized by a loss of the outer dynein arms and by the presence of accessory tubules with more than 16 protofilaments. The least modified sperm axoneme is found in the primitive families Rhyacophilidae and Glossosomatidae, which have motile spermatozoa and have 17 and 18 protofilaments, respectively. These families, as other members of Integripalpia (but not Hydroptilidae), have inner dynein arms only and sperm motility, whereas members of Annulipalpia have neither inner nor outer dynein arms and no progressive motility. The variability in axonemal organization is greater in Annulipalpia than in Integripalpia.

RÉSUMÉ

La signification phylogénétique de l'ultrastructure des axonèmes: exemples chez les Diptères et les Trichoptères

L'organisation de l'axonème a été étudiée, grâce à une fixation à l'acide tannique, chez de nombreux insectes et s'est montrée différente parmi les différents ordres d'Insectes, en accord avec la systématique. À l'intérieur de chaque ordre, toutefois, le nombre de protofilaments et les autres caractéristiques de l'axonème sont relativement constants, avec deux exceptions à cette règle, les Diptères et les Trichoptères. Ces deux ordres ont donc été examinés pour déterminer si l'organisation des axonèmes reflète leur position systématique. Environ 24 espèces de Diptères et 35 espèces de Trichoptères ont été étudiées. La structure de l'axonème chez les Brachycères est similaire à celle des Tipulomorphes, ce qui indique une origine des Brachycères dans ce groupe. D'après l'organisation de l'axonème, les Mycetophilidae, plutôt que les Tipulomorphes, semblent être le taxon de Diptères le plus primitif. Les axonèmes d'une espèce de Bibionidae étudiée (mais pas des autres membres des Bibionomorphes) ressemblent à ceux des Culicomorphes. L'axonème des Trichoptères est caractérisé par la perte des bras de dynéine externes et par la présence de tubules accessoires ayant plus de 16

protofilaments. L'axonème de spermatozoïde le moins modifié est trouvé dans les familles primitives Rhyacophilidae et Glossosomatidae, qui ont des spermatozoïdes motiles, et respectivement 17 et 18 protofilaments. Ces familles, comme les autres Integripalpia (mais pas les Hydroptilidae), ont seulement des bras de dynéine internes et des spermatozoïdes motiles, alors que les Annulipalpia n'ont ni bras internes ni bras externes de dynéine et n'ont pas de motilité permettant la progression. La variabilité de l'organisation des axonèmes est plus grande chez les Annulipalpia que chez les Integripalpia.

Most insect species have a sperm flagellum that contains a set of microtubules, which surround the nine microtubular doublets and which are named accessory tubules [21]. The axoneme is then said to have a 9+9+2 structure, which is a shorthand notation of 9 accessory tubules, 9 doublets and 2 central microtubules. It was long believed that 9+9+2 axonemes are of uniform appearance within the insect class, but this is far from true.

In a study from 1990 [4] we describe the axoneme of 49 insect species, representing 20 insect orders and show that there is a considerable diversity between different insects. Since that date we have extended the examination to many more species. In fact, axonemes from many species in most orders have been examined by now and this has been done with a new fixation technique that makes it meaningful to study the axoneme at high magnifications. In the resulting micrographs it is possible to count the number of protofilaments in the accessory tubules or in the microtubular doublets and singlets, and it is possible to see a substructure in the lumen of the microtubules or in the so called intertubular material (Intertubular material appears as one or two lumps of electron dense material external to the doublets and between the accessory tubules).

The main findings are summarized in Table 1. It can be concluded that diversity in axonemal structure is related to the systematic position of the animal. Many apomorphic traits can thus be found, such as accessory tubules with 17 protofilaments in the order Phasmida, accessory tubules with 13 protofilaments and no intertubular material in the order Ephemeroptera, and aberrant flagella in the order Thysanoptera. There are also some synapomorphic traits, such as accessory tubules with an elliptic cross-section recorded from the three insect orders Psocoptera, Mallophaga and Anoplura.

Although there is a marked diversity in axonemal structure within the class Insecta, there is a relative invariability within each insect order. There are two exceptions to this statement: Orders Diptera and Trichoptera have families and suborders with markedly different axonemal patterns. The purpose of the present study has been to examine the sperm tail from insects in these two orders in an attempt to see whether any phylogenetic conclusions can be drawn from the ultrastructural data.

MATERIAL AND METHODS

Spermatozoa from the insect species listed in Table 2 have been fixed for one or several days in a fixative that consists of 2 % glutaraldehyde, 1 % tannic acid, and 1.8 % sucrose in a 0.1 M phosphate buffer. This fixative is a modification of the fixative introduced by MIZUHIRA & FUTAESAKU [23]. Post-fixation and block-staining was performed with 1 % uranyl acetate in distilled water. No osmium tetroxide fixation was used. After dehydration in ethanols and embedding in epoxy resins, sections have been made and examined at a direct magnification of 50 000 or higher.

Tannic acid is thought to act as a mordant that encapsulates proteins; uranyl acetate is thought to contrast the tannic acid. Globular protein such as a tubulin in the microtubular wall will become visible as a lucid spot surrounded by an electron dense shell. Fibrous proteins, such as those in the spokes, may however appear as electron dense structures. Interpretation of the electron micrographs is not trivial.

OBSERVATIONS

Diptera

The axoneme of *Orfelia* sp. (Family Mycetophilidae) is of the 9+9+2 type. Its doublets have both inner and outer dynein arms. The accessory tubules have 16 protofilaments and the

intertubular material between two accessory tubules appears as two electron dense masses, i.e. a smaller unit attached to the accessory tubule and a larger one attached to the doublet; occasionally the two masses are confluent and form a single mass. In these respects the axoneme resembles that of examined members of several insect orders, such as Zygentoma, Plecoptera, Embioptera, Orthoptera, Dermaptera, Megaloptera, Raphidioptera, Planipennia, Coleoptera, Lepidoptera, and Hymenoptera. The cross-sectioned flagellum of an *Orfelia* spermatozoon differs from that of the other insects in having a single small mitochondrial derivative and differs also in other respects from those within other insect orders, but the axoneme has a morphology that can be regarded as plesiomorphic for insects.

The axoneme from some other members of the Mycetophilidae family has other patterns: *Tarnania* 2+9+2 and *Exechia seriata* 7+9+2 (both with 16 protofilaments in their accessory tubules) (Fig. 1), *Keroplatus reaumuri* 9+2, and *Boletina* sp. 9+9+2 with accessory tubules consisting of 15 protofilaments (Fig. 2). Such a variety of different axonemal patterns has not been described from any other insect family apart from Cecidomyiidae, in which there are highly aberrant spermatozoa.

The three examined members of family Chironomidae all have an axoneme of the 9+9+2 type and the accessory tubules have 15 protofilaments. The accessory tubules of examined members of the three families Dixidae, Culicidae (Fig. 3), and Bibionidae (Fig. 4) also have 15 protofilaments. However, their axoneme is of the 9+9+1 type as the two central microtubules are replaced by a central rod or cylinder in the three families.

In the examined species of families Trichoceridae and Tipulidae (Fig. 5) the axoneme is of the 9+9+2 type but the accessory tubules have 13 protofilaments. In this respect they resemble all Brachycera species examined so far [15, 24] (Fig. 6). Another characteristic shared by the axonemes in Tipulidae, Trichoceridae and Brachycera is the prominence of the spoke head which usually seems to be double. Within Brachycera the intertubular material contains a straight row of inclusion bodies [15].

Trichoptera

Most specialists on Trichoptera agree that the order can be divided into two subgroups, Integripalpia and Annulipalpia, although some uncertainty remains as to whether families Rhyacophilidae, Glossosomatidae and Hydroptilidae belong to one suborder or the other. These three families are usually regarded as the most primitive ones within the Trichoptera. In the family Rhyacophilidae the axoneme has a 9+9+2 structure, the doublets have an inner dynein arm but no outer one, and the accessory tubules have 17 protofilaments along most of their length (Fig. 7), although 16 close to the posterior end. In the examined member of the family Glossosomatidae the axoneme has the same characteristics, except that there are 18 rather than 17 protofilaments along most of the length of the accessory tubules. Spermatozoa from Rhyacophilidae and Glossosomatidae show progressive motility, whereas spermatozoa from the examined members of the family Hydroptilidae lack a flagellum and are immotile.

The integripalpian family Leptoceridae also has a 9+9+2 axoneme, inner dynein arms only, and accessory tubules with 18 protofilaments (Fig. 8). The axoneme differs from that in Glossosomatidae, however, in that the two central microtubules always occupy an eccentric position. Axonemes in the three Integripalpia families Limnephilidae (Fig. 9), Goeridae and Odontoceridae (Fig. 10), also resemble those in the family Glossosomatidae, although the number of protofilaments is 19 along most of the length, decreasing to 16 distally. The two members of the family Sericostomatidae, finally, have 20 protofilaments proximally, the highest number recorded from any insect or other animal. Posteriorly the number decreases to 16 near the distal end. An accessory body lies lateral to the axoneme.

TABLE 1. — Some characteristics of the sperm tail axoneme in examined members of insects from the various insect orders

Abbreviations in the Table are: AT = accessory tubule, IM = intertubular material, OA = outer dynein arms, pfs = protofilaments, the formula 9+9+2 signifies the number of accessory tubules, microtubular doublets, and central microtubules, respectively.

Taxon	Axonemal features	Reference
Protura	no AT, 12+0, 16+0 or otherwise	[19]
Diplura	AT with 13 pfs, ATs arranged in straight line	[6]
Archeognatha	AT with 16 pfs, ATs arranged in two groups	[6]
Zygentoma	AT with 16 pfs	[6]
Odonata	AT with 16 pfs, one IM	[6]
Ephemeroptera	AT with 13 pfs, no IM, no OA, 9+9+0 or 9+9+1	[2, 6]
Plecoptera	AT with 16 pfs, two IMs	[2]
Embioptera	AT with 16 pfs	unpublished
Isoptera	no ATs, usually aflagellate	[6]
Blattodea	AT with 16 pfs, dense lumen in all microtubules	[6]
Mantodea	AT with 16 pfs, IM with 9 inclusion bodies	[6]
Phasmida	AT with 17 pfs, two IMs	[1, 6]
Orthoptera	AT with 16 pfs, prominent IM	[6, 9]
Dermaptera	AT with 16 pfs, prominent IM	[6]
Psocoptera	AT with 13 pfs, compressed ATs, AT lumen dense	[8]
Mallophaga	AT with 13 pfs, compressed ATs, AT lumen dense	[6]
Anoplura	AT with 13 pfs, compressed ATs, AT lumen dense	[6]
Thysanoptera	no AT, 27 microtubules of three kinds	[12]
Hemiptera	AT with 16 pfs, AT and A-tubule lumen dense	[6]
Homoptera	AT with 16 pfs, AT and A-tubule lumen dense	[6]
Megaloptera	AT with 16 pfs, two IMs	[3]
Raphidioptera	AT with 16 pfs, two IMs	[3, 6]
Planipennia	AT with 16 pfs, two IMs	[3]
Coleoptera	AT with 16 pfs, two IMs	[3, 6]
Strepsiptera	AT incomplete and containing an inclusion, no IM	[3, 9]
Mecoptera	no AT	[6]
Hymenoptera	AT with 16 pfs	[6]
Trichoptera	AT with more than 16 pfs, no OA	this paper
Lepidoptera	AT with 16 pfs	[6]
Diptera	AT with 13, 15, or 16 pfs	this paper
Siphonaptera	no AT	[21]

FIGS 1-6. Axonemes from various dipterans: 1: *Exechia seriata*; 2: *Boletina* sp.; 3: *Culex pipiens*; 4: *Bibio* sp.; 5: *Tipula* sp.; 6: *Scatophaga* sp. Scale bar 0.1 μ m.

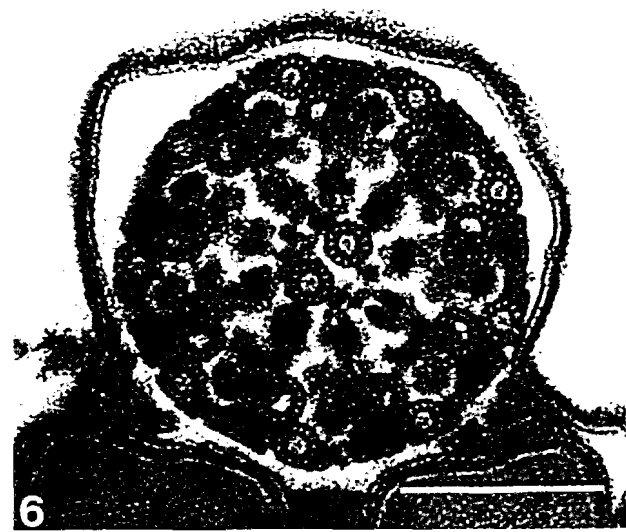
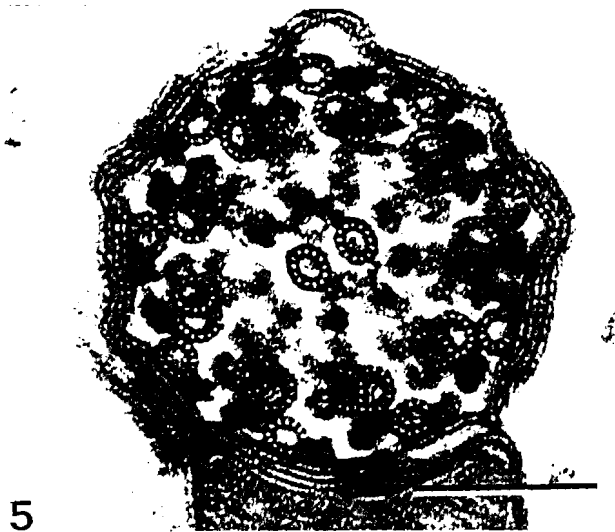
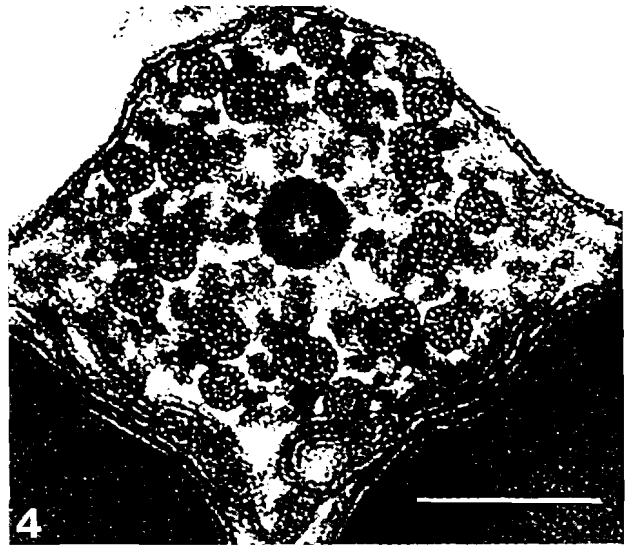
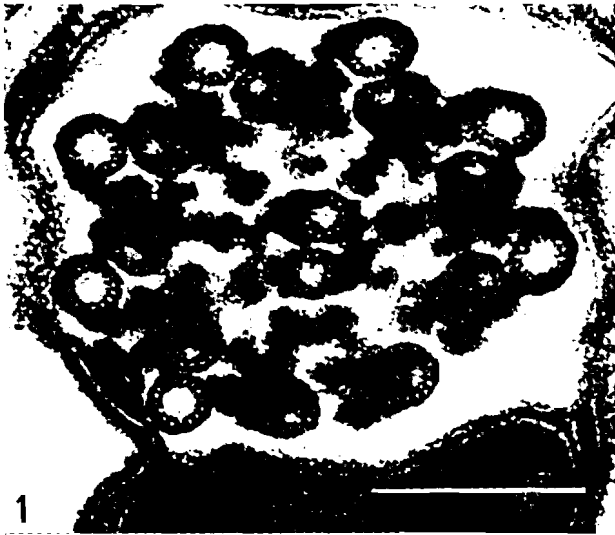
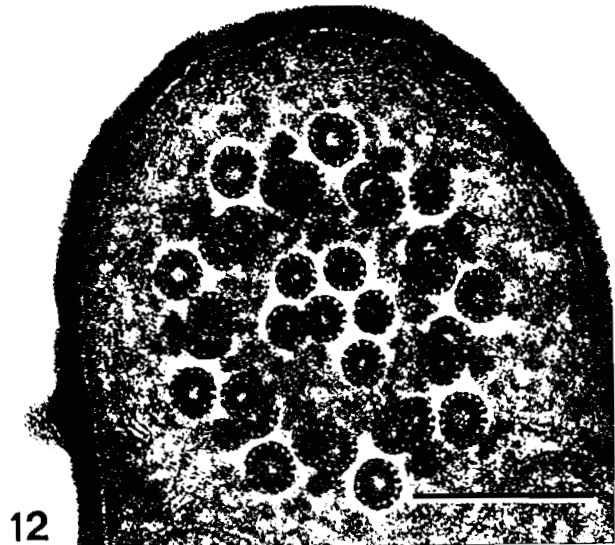
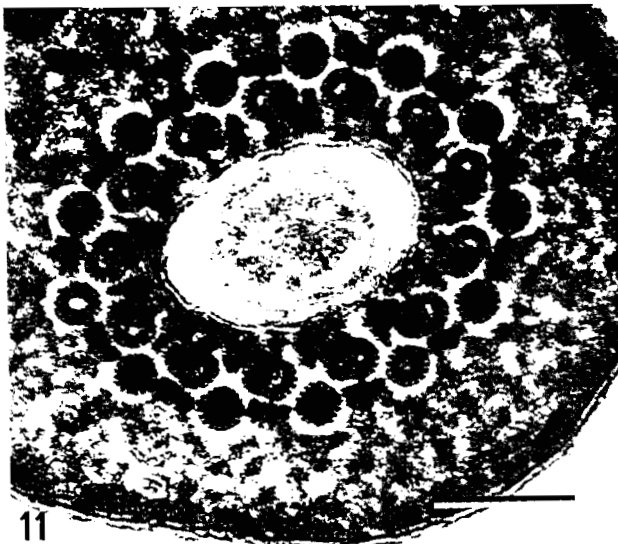
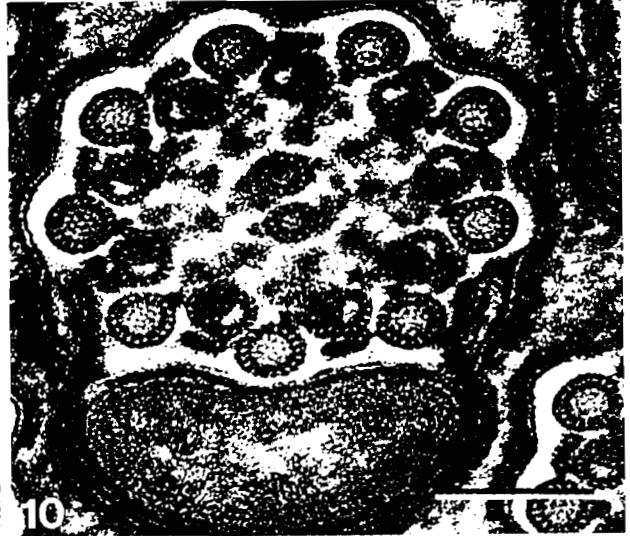
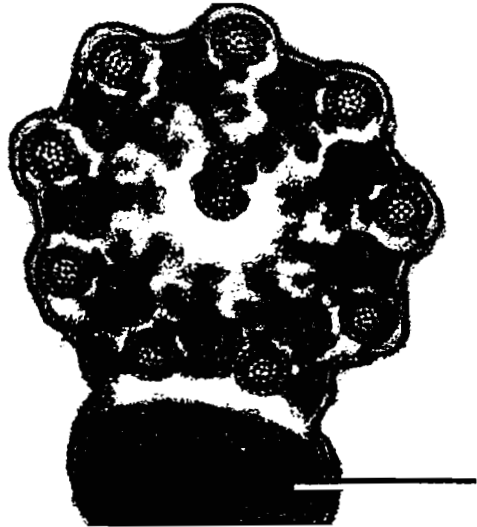


TABLE 2. — Species investigated and sources of data

Group, Species	Family	Reference
DIPTERA NEMATOCERA		
<i>Orfelia</i> sp.	Mycetophilidae	[15]
<i>Exechia seriata</i> (Meigen)	Mycetophilidae	[15]
<i>Boletina</i> sp.	Mycetophilidae	[15]
<i>Keroplatus reaumurii</i> Dufour	Mycetophilidae	[15]
<i>Asphondylia ruebsaameni</i> Kertész	Cecidomyiidae	[18, 22]
<i>Sciara</i> sp.	Sciariidae	[15]
<i>Chironomus</i> (three unidentified species)	Chironomidae	[15]
<i>Culex pipiens</i> L.	Culicidae	[15]
<i>Anopheles maculipennis</i> Meigen	Culicidae	[15]
<i>Dixa</i> sp.	Dixidae	[15]
<i>Biblio</i> sp.	Bibionidae	[2, 15]
<i>Trichocera hiemalis</i> De Geer	Trichoceridae	[15]
<i>Tipula</i> sp.	Tipulidae	[15]
DIPTERA BRACHYCERA		
<i>Bombylius</i> sp.	Bombyliidae	[15]
<i>Ramphomyia</i> sp.	Empididae	[15]
<i>Drosophila melanogaster</i> Meigen	Drosophilidae	[7]
<i>Bactrocera</i> (= <i>Dacus</i>) <i>oleae</i> (Gmelin)	Tephritidae	[7]
<i>Ceratitis capitata</i> (Wied.)	Tephritidae	[9, 15]
<i>Scatophaga</i> sp.	Scatophagidae	[15]
<i>Calliphora vomitoria</i> L.	Calliphoridae	[15]
<i>Gasterophilus intestinalis</i> De Geer	Gasterophilidae	[15]
TRICHOPTERA INTEGRIPALPIA		
<i>Rhyacophila</i> (<i>Rhyacophila</i>) <i>foliacea</i> Moretti	Rhyacophilidae	[17]
<i>Rhyacophila</i> (<i>Rhyacophila</i>) <i>dorsalis</i> (Curtis)	Rhyacophilidae	[17]
<i>Rhyacophila</i> (<i>Pararhyacophila</i>) <i>italica</i> Moretti	Rhyacophilidae	[17]
<i>Rhyacophila</i> (<i>Hyporhyacophila</i>) <i>tristis</i> Pictet	Rhyacophilidae	[17]
<i>Catagapetus nigrans</i> McLachlan	Glossosomatidae	[17]
<i>Stactobia caspersi</i> Ulmer	Hydroptilidae	[11]
<i>Orthotrichia costalis</i> (Curtis)	Hydroptilidae	[11]
<i>Hydroptila aegyptia</i> Ulmer	Hydroptilidae	[11]
<i>Hydroptila angulata</i> Mosely	Hydroptilidae	[11]
<i>Hydroptila forcipata</i> Eaton	Hydroptilidae	[11]
<i>Hydroptila tineoides</i> Dalman	Hydroptilidae	[11]
<i>Oecetis furva</i> Rambur	Leptoceridae	[10]
<i>Leptocerus tineiformis</i> Curtis	Leptoceridae	[10]
<i>Mytacidis azurea</i> L.	Leptoceridae	[10]
<i>Odontocerum albicorne</i> Scopoli	Odontoceridae	[10, 13]
<i>Sericostoma italicum</i> Moretti	Sericostomatidae	[10]
<i>Sericostoma pedemontanum</i> McLachlan	Sericostomatidae	[10]
<i>Silo mediterraneum saturniae</i> Moretti	Goeridae	[10]
<i>Leptodrusus budtzi</i> Ulmer	Limnephilidae	[10]
<i>Limnephilus bipunctatus</i> Curtis	Limnephilidae	[10]
<i>Limnephilus rhombicus</i> (L.)	Limnephilidae	[10]
<i>Glyphotaenius pellucidus</i> Retzius	Limnephilidae	[10]
<i>Potamophylax cingulatus</i> (Stephens)	Limnephilidae	[10]
<i>Melampophylax melampus</i> McLachlan	Limnephilidae	[10]
<i>Stenophylax permistus</i> McLachlan	Limnephilidae	[2, 10]
<i>Micropterna sequax</i> McLachlan	Limnephilidae	[10]
<i>Chaetopteryx gessneri</i> McLachlan	Limnephilidae	[10]
TRICHOPTERA ANNULIPALPIA		
<i>Philopotamus ludificatus</i> McLachlan	Philopotamidae	[16]
<i>Philopotamus montanus</i> Donovan	Philopotamidae	[2, 16]
<i>Wormaldia occipitalis</i> Pictet	Philopotamidae	[16]
<i>Wormaldia copiosa</i> McLachlan	Philopotamidae	[16]
<i>Plectrocnemia geniculata</i> McLachlan	Polycentropodidae	[16]
<i>Polycentropus mortoni</i> Moseley	Polycentropodidae	[16]
<i>Polycentropus irroratus</i> Curtis	Polycentropodidae	[16]
<i>Cyrnus trimaculatus</i> Curtis	Polycentropodidae	[16]
<i>Hydropsyche pellucidula</i> Curtis	Hydropsychidae	[16]

FIGS 7-12. — Axonemes of some trichopterans. 7: *Rhyacophila foliacea*; 8: *Leptocerus tineiformis*; 9: *Potamophylax cingulatus*; 10: *Odontocerum albicorne*; 11: *Wormaldia copiosa*; 12: *Philopotamus montanus*. Scale bar 0.1 μ m.



Members of the suborder Annulipalpia lack inner and outer dynein arms. Hence they have no regular, flagellar movements. Weak vibrations have been observed in *Philopotamus* spermatozoa, when remaining in the testicular fluid, however. The philopotamid species *Wormaldia copiosa* has a unique axonemal pattern, 13+13+v, where v stands for a cylindrical vesicle along the central axis of the axoneme (Fig. 11), whereas another member of the same genus, *W. occipitalis*, has a similar axoneme although with the formula 9+9+v. In examined members of *Philopotamus* the axonemal pattern is 9+9+7 and the seven central microtubules consist of 13 or 14 protofilaments (Fig. 12).

The family Polycentropodidae is related to the Philopotamidae. Its members have lost the accessory tubules and have a 9+7 axoneme, in which the central microtubules have 13 or 14 protofilaments. Also related to these families are the Hydropsychidae, whose members have a highly aberrant sperm morphology. Whereas a section just distal to the centriole shows nine axonemal doublets, sections posterior to that level show a great number of microtubular singlets and doublets, some enclosed in the cell body, others contained in finger-like rodlets projecting from the cell body.

DISCUSSION

Diptera

The suborder Nematocera is generally considered to be more primitive than the Brachycera and its subgroup Tipulomorpha (= Tipulidae + Trichoceridae) and to be the sister group to all other Diptera [5, 20]. Tipulomorpha is thus regarded as the lowest branch of the dipteran phylogenetic tree. Our data on the sperm axoneme is not compatible with this opinion. Rather, it seems to us that the family Mycetophilidae is the most primitive dipteran taxon. Only in this family has the plesiomorphic axonemal pattern - 9+9+2 and accessory tubules with 16 protofilaments - been found. Whether data from molecular biology will confirm that Mycetophilidae is the most primitive extant dipteran group, or whether data will show the Tipulomorpha to be so, is a matter that probably will be settled soon.

As mentioned above, some axonemal characteristics are shared between the examined members of Tipulomorpha and the Brachycera: accessory tubules with 13 protofilaments and spoke heads that appear double. We believe that these are synapomorphies shared by these groups and that Brachycera is derived from the tipulomorph group. According to HENNIG [20] Nematocera contains four large groups: Tipulomorpha, Psychodomorpha, Culicomorpha (containing, amongst other families, Dixidae, Culicidae, Simulidae and Chironomidae), and Bibionomorpha (with Bibionidae, Mycetophilidae, Cecidomyiidae, Sciaridae). Members of Psychodoidea have an axoneme of the 9+9+0 type [14], but nothing is known about the number of protofilaments in the accessory tubules.

The examined members of the culicomorph families Dixidae, Culicidae, and Chironomidae share an axonemal character: accessory tubules with 15 protofilaments. This number of protofilaments has been found also in the examined member of Bibionidae and in the mycetophilid *Boletina*, but otherwise is a rare feature. Whereas the chironomids have a 9+9+2 axoneme and the simuliid a 9+9+3 one [4], culicid and bibionid flies have a central rod rather than two central microtubules, a 9+9+'1' or sometimes a 9+9+0 pattern. This finding indicates that Bibionidae may be closer related to Culicomorpha than to other families of Bibionomorpha or that Culicomorpha is derived from Bibionomorpha. The sperm tails of members of Cecidomyiidae and Sciaridae, finally, have highly aberrant axonemes.

Trichoptera

Based, *inter alia*, on their free-living larvae, the families Rhyacophilidae, Glossosomatidae, and Hydroptilidae are considered to be the most primitive trichopterans. The loss of a sperm tail in Hydroptilidae prevents any comparative study based on those characters that are treated here. The

relatively few modifications from the plesiomorphic insect axoneme in the other two families is compatible with the opinion that they are primitive.

The division of Trichoptera into two suborders, Integripalpia and Annulipalpia, is based on the morphology of the palps. If only the sperm axoneme were considered, Trichoptera could also be divided into two groups, one possessing inner dynein arms and flagellated spermatozoa, the other without these characteristics. This division would be identical to the classical one and the two families Rhyacophilidae and Glossosomatidae would be classified as belonging to Integripalpia. The inclusion of these families (and of Hydroptilidae) into Integripalpia is also advocated by ROSS [25].

The various species of Integripalpia have axonemal patterns that are characteristic of the families to which they belong. No significant differences have been found between the three families Limnephilidae, Goeridae and Odontoceridae. Within Annulipalpia there may be a greater variability between members of the same family or even the same genus. Since the flagellum is unable to propagate the spermatozoon, any mutation that changes the pattern from a regular 9+9+2 pattern can be regarded as neutral, in that it will not affect sperm functions. A greater diversity of the axonemal pattern can hence be expected and has thus been recorded.

ACKNOWLEDGEMENTS

We are grateful to Professor MORETTI for collecting and determining most of the caddisflies and to Professor HIPPA for determining some of the dipteran flies. The study has been supported by a grant from MURST 60% to R.D.

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