FURTHER SPERMATOLOGICAL EVIDENCE FOR INCLUDING THE PENTASTOMIDA (TONGUE WORMS) IN THE CRUSTACEA

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Abstract—STORCH V. and JAMIESON B. G. M. 1992. Further spermatological evidence for including the Pentastomida (tongue worms) in the Crustacea. *International Journal for Parasitology* 22: 95–108. The ultrastructure of the spermatozoa of three species of the Pentastomida is investigated: the cephalobaenids *Raillietiella* sp. and *Cephalobaena tetrapoda*, and the porocephalid *Porocephalus crotali*. Phylogenetic relationships of pentastomids with their putative maxillopodan and remipedian relatives have been deduced from sperm ultrastructure intuitively and by parsimony analysis using the PAUP program of Swofford (1990, Version 3.01, Illinois Natural History Survey, Champaign, IL). A remarkable degree of homogeneity in the ultrastructure of the spermatozoa of cephalobaenids and porocephalids is demonstrated and a sister-group relationship of pentastomids and Branchiura, first proposed by Wingstrand (1972, *Kongelige Danske Videnskab Selskab Biologiske Skrifter* 19: 1–72), is confirmed. Spermatozoal synapomorphies of the Pentastomida and the branchiuran *Argulus* include: absence of an acrosome vesicle and filament in the mature spermatozoon; presence of a pseudoacrosome, about 30 µm long, divisible into a dorsal and a ventral component (the dorsal and ventral rod), and derived from pericentriolar structures; continuation of the dorsal rod with the dorsal ribbon of an axonemal sheath and with material embedding the centriolar doublets 1, 2 and 9; continuation of the ventral rod with material embedding the more ventrally situated doublets; presence of the axonemal sheath; presence of a connective from the dorsal ribbon to axonemal doublet 1; presence in the spermatid of nine dense fibres peripheral to the doublets; great elongation of the three mitochondria parallel to the nucleus; and total incorporation of the axoneme in the spermatozoon so that a free flagellum is absent. The sperm of the pentastome–branchiuran assemblage appear to be the most highly evolved of the flagellate crustacean sperm.

INDEX KEY WORDS: Phylogeny; Pentastomida; Branchiura: spermatozoa; ultrastructure; *Raillietiella*; *Cephalobaena*; *Porocephalus*; *Argulus*; Maxillopoda; Crustacea; *Philodryas*; *Vipera*; *Crotalus*.

INTRODUCTION

THE Pentastomida, tongue worms, are parasites of the respiratory tract of all classes of tetrapods, as definitive hosts, and utilize these or invertebrate intermediate hosts, though direct development is suspected in some (Riley, 1986). They are commonly regarded as a distinct and isolated phylum with obscure relationships. Among various proposed relationships, they have been considered to be related to the Tardigrada and Onychophora (von Graff, 1877; Cuénot, 1952; Vandel, 1949; Weber, 1949), or to be derived from annelids or pro-articulates independently of or with some affinity with tardigrades (Kaestner, 1955; Beklemishev, 1958; von Haffner, 1971).

The status of pentastomes as a distinct phylum was questioned by Osche (1963) who concluded, from their embryology, that pentastomids are tracheate arthropods with probable myriapod affinities. Nørrevang (1972) recognized chelicerate or chilopod relation-

ships from the structure of the ovary and Storch and Böckeler (1979) saw arthropod affinities in the ultrastructure of the head sensilla. Wingstrand (1972) had proposed, on the basis of spermatozoal ultrastructure, that pentastomes are arthropods but went so far as to state that they should be placed within the Branchiura, in the Crustacea. A crustacean relationship of pentastomids was endorsed, from somatic and other evidence by Riley, Banaja & James (1978). From a parsimony analysis of 18S ribosomal RNA sequences in the pentastome *Porocephalus crotali* and the branchiuran *Argulus nobilis*, Abele, Kim & Felgenhauer (1989) recognize a sister-group relationship between the two entities, thus supporting Wingstrand's proposed relationship.

Riley (1986) considered that the many refinements (for which we may substitute apomorphies) of sperm structure reported by Wingstrand (1972) as shared between the pentastomid *Raillietiella hemidactyli* and the branchiuran *Argulus foliaceus* seemed to preclude convergence as a complete explanation of the similarities of the sperm in the two taxa. He nevertheless

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stated that it would be imprudent to attach too much significance to sperm morphology but that if it were subsequently found that all pentastomids and all branchiurans produced essentially the same kind of sperm this would constitute powerful evidence of common ancestry. We would stress that particular attention needs to be focussed on the most plesiomorphic sperm type (the ground-plan) for pentastomids and branchiurans in evaluating a relationship between the two groups. Wingstrand (1972) examined, in *Raillietiella*, a genus which is considered to be relatively derived (von Haffner & Rack, 1971) and we therefore here examine the sperm of a species of *Cephalobaena*, a genus considered to be phylogenetically near the ancestry of the Pentastomida (von Haffner & Rack, 1971; von Haffner, 1973). To determine the extent of spermatozoal variation in pentastomes, sperm ultrastructure of a further cephalobaenid, *Raillietiella* sp., and a member of the remaining order, the advanced Porocephalida, *Porocephalus crotali*, is examined.

We do not in the present study attempt to corroborate all details of spermiogenesis and sperm ultrastructure given by Wingstrand (1972) in his long and comprehensive account but emphasize major ultrastructural features and such similarities and divergences from *R. hemidactyli* as occur in the taxa studied. Some comparison of the sperm of the three species examined here with those of *R. hemidactyli* will be made in the Results section in order to avoid repetition in the Discussion. Some inadequacies of fixation of the rare *Cephalobaena tetrapoda* necessitate emphasis on the better-fixed material of *Raillietiella* sp. despite the key position of *Cephalobaena* but data obtained from the latter are sufficient for determination of the chief features of its sperm.

In addition to Wingstrand's account of *R. hemidactyli*, brief accounts of spermiogenesis and the mature sperm have been given by Böckeler & Storch

FIG. 1. *Raillietiella* sp. Transmission electron micrographs of successive transverse sections, in anterior-posterior sequence, of the spermatozoon. A, The pseudoacrosome; B, the anterior region of the mitochondria at the extreme anterior end of the nucleus; C, the widest part of the nucleus; D-G, showing successive reduction in the width of the nucleus; H, immediately behind the posterior end of the nucleus; I, the arrangement of axonemal microtubules has become disrupted, the extreme posterior ends of the mitochondria are seen, and the ventral ribbon has ended. a, Axoneme; dr, dorsal ribbon of dense sheath of axoneme; dro, dorsal rod of pseudoacrosome; er, endoplasmic reticulum; m, mitochondrion; ms, dense sheath of mitochondrion; n, nucleus; om, oblique membrane (connective) connecting axonemal doublet I to the dorsal ribbon; vr, ventral ribbon of dense sheath of axoneme; vro, ventral rod of pseudoacrosome.

FIG. 1.

FIG. 2.

(1990) for *C. tetrapoda* and by Storch, Böckeler & Riley (1990) for *P. crotali* in descriptions of the male genital system from the specimens described here. Earlier Doucet (1965) published two electron micrographs of transverse sections of the spermatozoa of *R. boulengeri*. In addition to the account of the spermatozoa of *Argulus nobilis* by Wingstrand (1972), Brown (unpublished Doctoral thesis, University of Miami, University Microfilms Inc., Ann Arbor, Michigan, No. 66-12, 996, 1966) gave a brief description for an unnamed but different *Argulus* sp.

After description of the spermatozoa, an attempt will be made, using parsimony analysis, to place the Pentastomida in the wider perspective of maxillopod phylogeny in so far as this is suggested from sperm ultrastructure.

MATERIALS AND METHODS

Mature males of the following pentastomid species were obtained from the hosts and localities indicated: *Cephalobaena tetrapoda* Heymons, 1922, from the lungs of the Paraguayan tree snake, *Philodryas baroni*; *Raillietiella* sp., from *Vipera palaestinae*; and *Porocephalus crotali* (von Humboldt, 1808), from the lungs of the American rattlesnake, *Crotalus atrox*. The *Raillietiella* sp. awaits final identification but is either *R. mediterranea* or *R. spiralis* if it is not a new species (Riley, personal communication). Small pieces of testes and seminal vesicles were fixed in 3.5% glutaraldehyde in Sørensen's phosphate buffer (pH 7.4) at 4°C for 2 h; after the first fixation, the specimens were washed in the buffer. Then 1% phosphate-buffered osmium tetroxide (pH 7.2) was used for the second fixation, which lasted 2 h. Finally tissues were dehydrated with ethanol and embedded in Araldite. Silver sections were cut on a Reichert Ultracut ultramicrotome using glass and diamond knives. The sections, mounted on carbon coated copper grids, were stained for 5 min with uranyl acetate (saturated solution in 70% methanol) and for an additional 5 min with lead citrate. Transmission electron microscopy was carried out on Zeiss EM 9S2 and Hitachi 300 electron microscopes.

RESULTS

General

To facilitate understanding of pentastomid sperm structure, transverse sections are illustrated in anteroposterior sequence, throughout the length of the spermatozoon of *Raillietiella* sp., in Fig. 1A–I (see also spermatid in Fig. 3A–E).

The mature spermatozoon in the four investigated species is filiform, being 100–130 µm long in *R. hemidactyli*. Unlike 'primitive' sperm (aquasperm *sensu* Jamieson, 1986), the spermatozoon (Fig. 1) is not differentiated into head, midpiece and flagellum. The basal body of the axoneme is located anterior to the elongate nucleus and the mitochondria and, above these, the axoneme runs parallel to and 'dorsal' to the nucleus. The axoneme is wholly incorporated in the body of the spermatozoon, there being no free flagellum, and is enveloped by a bipartite dense sheath. A pseudoacrosome, in the terminology of Wingstrand (1972), is located at the anterior end of the spermatozoon, surmounting the basal body. There is no true acrosome. Further components are described in the following account. The relative positions of organelles as seen in transverse section are shown in Figs. 1A–I, 2A (*Raillietiella* sp.), 2B (*Cephalobaena tetrapoda*) and 2C (*Porocephalus crotali*).

Pseudoacrosome

The centriolar rather than Golgi origin of the acrosome-like structure at the anterior end of the spermatozoon justifies terming it the pseudoacrosome (Wingstrand, 1972). It is seen in cross section in Figs. 1A, 2H (*R. sp.*), 2D (*C. tetrapoda*) and 2E and, spermatid, 5H (*P. crotali*) in which division into two components, described by Wingstrand (1972) for *Raillietiella hemidactyli* as a dorsal rod and a ventral rod, is seen. A longitudinal section of the pseudoacrosome of *Raillietiella* sp. is shown in Fig. 2F.

Nucleus

Extension of the nucleus through the length of the sperm, excepting the region of the pseudoacrosome and extreme anterior end of the mitochondria and of the extreme posterior end, is deduced from the presence of a section of the nucleus in all cross sections of the spermatozoon excepting this anterior region (Fig. 1A, B), see also the spermatid (Figs. 3AB, 4E) and posteriorly (Fig. 1H, I) in *Raillietiella* sp. In *R. sp.* the axoneme, though with disruption of the flagellar microtubules, continues posterior to the nucleus (Fig. 1I), as in *R. hemidactyli* but unlike *Argulus* (see discussion of characters in parsimony analysis).

FIG. 2. A–C, Transverse section (TS) through the nuclear region of the spermatozoon. A, *Raillietiella* sp.; B, *Cephalobaena tetrapoda*; C, *Porocephalus crotali*; note the block-like chromatin in *Raillietiella* and *Cephalobaena* and the homogeneous dense chromatin of *Porocephalus*; D, TS pseudoacrosomes (centre of field) of *Cephalobaena tetrapoda*; E, TS pseudoacrosomes, among nuclear sections, of *Porocephalus crotali*; F, *Raillietiella* sp. longitudinal section (LS) of pseudoacrosome; G, *Raillietiella* sp. LS of spermatozoa, showing cross striation of the ventral ribbon; H, *Raillietiella* sp. TS pseudoacrosomes of late spermatids. a, Axoneme; dr, dorsal ribbon of dense sheath of axoneme; er, endoplasmic reticulum; m, mitochondrion; ms, dense sheath of mitochondrion; mt, microtubule; n, nucleus; om, oblique membrane (connective) connecting axonemal doublet 1 to the dorsal ribbon; ps, pseudoacrosome; vr, ventral ribbon of dense sheath of axoneme.

In *R. hemidactyli* the chromatin is highly condensed and appears black or indistinctly granular (Wingstrand, 1972). In *R. sp.*, however, spermatids and mature sperm have chromatin consisting of large rounded dense blocks, narrowly separated from each other by clear spaces (Figs. 1C–E, 2A, transverse section, 2G, longitudinal section, *R. sp.*) as in *C. tetrapoda* (Fig. 2B). In *P. crotali*, the chromatin is more strongly condensed (Fig. 2C) and its spermiogenic stages do not include chromatin blocks, a few tortuous thick electron-dense chromatin ribbons being visible in cross section of the spermatid nucleus (Fig. 5F).

In the four investigated pentastomid species a nuclear envelope, consisting of inner and outer membranes with intervening cisterna, is retained in the mature spermatozoon.

Mitochondria

As noted by Wingstrand for *Raillietiella hemidactyli*, the mitochondria in *R. sp.*, *Cephalobaena tetrapoda* and *Porocephalus crotali*, are three filiform continuous rods, extending throughout the entire body with the exception of the pseudoacrosome region. The median rod lies between the axoneme and the nucleus while the other two form a pair of symmetrical wings, as seen in cross section, on each side of the median one (*R. sp.*, Figs. 1B–H, 2A; *C. tetrapoda*, Fig. 2B; *P. crotali*, Fig. 2C). Normal mitochondrial structure is seen in the presence of two bounding unit membranes but cristae are poorly developed or absent at maturity, being best developed in *R. sp.* and unrecognizable in *P. crotali* in which the mitochondrial matrix is strongly electron dense (Fig. 2C). *P. crotali* further differs from the three cephalobaenids in the large size of the mitochondria during spermiogenesis and the complex convolution and large number of pale membrane-like lacunae (presumably modified cristae) which outline dense rounded blocks of matrix (Fig. 5A–D). In tangential

FIG. 3. *Raillietiella* sp. Successive transverse sections, in anterior–posterior sequence, of the spermatid. The numerous microtubules, other than those of the centriole and axoneme, disappear by maturity. A and B, Pseudoacrosome; C, anterior region of centriole, showing doublet (not triplet) construction; D, more posterior region of centriole, with the three mitochondria and the nucleus now apparent; E, similar to D but the centriole is succeeded by the 9 + 2 arrangement of the axonemal microtubules. a, Axoneme; c, centriole; cd, centriolar doublet; dm, dense ring embedding centriole; dr, dorsal ribbon of dense sheath of axoneme; dro, dorsal rod of pseudoacrosome; ds, bipartite dense sheath of axoneme; m, mitochondrion; ms, dense sheath of mitochondrion; mt, microtubule; n, nucleus; om, oblique membrane (connective) connecting axonemal doublet 1 to the dorsal ribbon; vr, ventral ribbon of dense sheath of axoneme; vro, ventral rod of pseudoacrosome.

FIG. 3.

FIG. 4. *Raillietiella* sp. Transverse sections of developing spermatids, A–C, showing that the number of mitochondria in the spermatid initially exceeds the three found in the spermatozoon; supernumerary mitochondria are progressively eliminated in the myelin-like bodies seen in B and C; D, transverse section of the centriole showing presence of dense peripheral fibres; E, showing that the mitochondria commence anteriorly to the nucleus in this species; F, as in E, showing outer and inner sacs between which the dorsal ribbon of the dense sheath of the axoneme begins to form before appearance of the ventral ribbon. df, Dense fibre peripheral to axoneme; is, inner sac; m, mitochondrion; my, sheath of myelin-like body; n, nucleus; os, outer sac.

longitudinal sections the mitochondria in the young elongating spermatid have a paracrystalline appearance in *P. crotali* (Fig. 5G). The internal membranes progressively disappear as the late spermatid matures (Fig. 5E, F).

The mitochondria extend anterior of the nucleus in *R. sp.* (Fig. 1B), as confirmed from the spermatid (Fig. 4E), whereas in *R. hemidactyli* the mitochondria and nucleus commence at the same level.

Each lateral mitochondrion is bordered anteriorly and laterally by a dense sheath which continues as one border of a narrow bridge which connects the mitochondrion with the lower half of a dense sheath which surrounds the axoneme (Figs. 1C, 2A, 3D, E, *R. sp.*,

2B, *C. tetrapoda*, 2C, *P. crotali*). This portion of the axonemal sheath is termed the ventral ribbon (see below).

There are several separate spherical mitochondria near the nucleus in the young spermatid. Figure 5A shows four mitochondria in the plane of section in the young spermatid of *P. crotali*. At this stage the nucleolus is still large and a remnant of the spindle of the second meiotic division is evident. Towards the end of spermiogenesis, the number of mitochondria is reduced to three. Wingstrand (1972) considered the reduction to occur wholly by fusion but in the present material supernumerary mitochondria appear to be eliminated in myelin-like bodies (*R. sp.*, Fig. 4B, C). In

FIG. 5.

the older spermatid the remaining mitochondria aggregate as a so-called nebenkern (*P. crotali*, Fig. 5D).

Stages in development of the nebenkern and neighbouring structures, as seen in cross section, are shown in Figs. 4A–C for *R. sp.* and 5B–F for *P. crotali*.

The nebenkern is initially situated posterior to the nucleus and near the point of outgrowth of the posterior, initially free part of the axoneme but each mitochondrion sends an extension posteriorly and anteriorly along the incorporated part of the axoneme between axoneme and nucleus (*P. crotali*, Fig. 5I).

Centriole

As noted by Wingstrand (1972), the triplets of the single surviving centriole which forms the basal body are replaced in early spermatogenesis with doublets. In transverse section of the mature spermatozoon these are seen to be embedded in a ring of dense material and are slightly displaced in a regular manner. This is shown for *Raillietiella sp.* in Fig. 3D for a late spermatid, identifiable by the fact that microtubules have not yet disappeared from the cytoplasm. Doublets 9, 1 and 2 are displaced into the dorsal half of the dense ring while the six other doublets (3–8) are cemented together in the lower half of the ring. The dorsal half of the ring is deduced to be continuous with dense material of the pseudoacrosome at the axial surface of the dorsal rod, while the ventral half is continuous with that of the ventral rod, of the pseudoacrosome (Fig. 3A). Connection with these rods was also shown in *R. hemidactyli* by Wingstrand (1972). In the early spermatid, when the dorsal ribbon is only incipient, as shown for *P. crotali* in Fig. 5B, the centriole is not embedded in dense material, though it may be surrounded by dense fibres, as seen in *Raillietiella* in Fig. 4D.

Axoneme

The axoneme has nine peripheral doublets and two central singlets. In favourable sections, two dynein arms are visible on the A tubules in all species. Doublet number 1 is always situated 'mid-dorsally', that is at the opposite side of the cross section relative to the

nucleus (e.g. Fig. 2A, *R. sp.*). The two central singlets may appear exactly transverse relative to this dorso-ventral plane or may be slightly tilted relative to it, suggesting a dynamic situation.

An oblique 'membrane' (connective) connecting doublet 1 with the dorsal ribbon is present in *Raillietiella hemidactyli*, Wingstrand (1972), as first demonstrated in *R. boulengeri* by Doucet (1965), and in *R. sp.* (Figs. 1B–H; 2A), *Cephalobaena tetrapoda* (Fig. 2B) and *Porocephalus crotali* (Fig. 2C).

Dense sheath of axoneme

The axoneme in all four species is surrounded by a dense sheath which consists of two components, each a half tube, the dorsal and ventral ribbons in the terminology of Wingstrand (1972). The dorsal ribbon, embracing the dorsal half of the axoneme, meets the ventral ribbon lateral to the axoneme, the junctions being in line with doublets 3 and 8 (Figs. 2A, *R. sp.*, 2B, *C. tetrapoda*, 2C, *P. crotali*) where the rounded lower limit of the dorsal ribbon fits into the concave upper limit of the ventral ribbon. The ventral ribbon, embracing the ventral half of the axoneme, is continuous laterally, via the mitochondrial bridge referred to above, with the incomplete dense sheath of the adjacent lateral mitochondrion. The ventral ribbon is also, but less distinctly, continuous with dense material between the lateral and median mitochondria. In longitudinal section the ventral ribbon presents a regular cross striated structure connecting mitochondrion and axoneme (Fig. 2G), as also seen in micrographs of sperm of *R. hemidactyli*.

In all species, in a cross section of the sheath, the dense material of the dorsal ribbon is interrupted in the midline by a pale crescentic area, representing a longitudinal pale core, and the ventral ribbon has two pale elliptical areas, one on each side of the midline near the apposition of the lateral mitochondria with the ventral mitochondrion (Figs. 1B–H, 2A, *R. sp.*, 2B, *C. tetrapoda*, 2C, *P. crotali*). In the spermatid, the dorsal ribbon of the dense sheath of the axoneme begins to form between outer and inner sacs before appearance of the ventral ribbon (Figs. 4F, *R. sp.*, 5C, D, F, *P. crotali*).

FIG. 5. *Porocephalus crotali*. Sections of developing spermatids. A, At least 4 separate, spherical mitochondria with tortuous cristae are present near the nucleus; the nucleolus is still large and remnants of the nuclear spindle are visible; B, the centriolar doublets are each accompanied by an indistinct peripheral dense fibre but at this stage the doublets are not yet embedded in dense material; C and D, progressive fusion of four mitochondria in C to give the three definitive mitochondria in D; E and F, showing loss of cristae in maturing mitochondria; G, paracrystalline appearance of mitochondria of nebenkern in tangential section; H, transverse section of developing pseudoacrosome; I, showing the mitochondria of the nebenkern beginning to send an extension posteriorly and anteriorly between axoneme and nucleus. a, Axoneme; c, centriole; df, dense fibre peripheral to axoneme; dr, dorsal ribbon of dense sheath of axoneme; er, endoplasmic reticulum; is, inner sac; m, mitochondrion; n, nucleus; no, nucleolus; os, outer sac; pa, paracrystalline array; ps, pseudoacrosome; sr, residue of spindle of meiosis II; vr, ventral ribbon of dense sheath of axoneme.

FIG. 6. Phylogeny of those maxillopodans with flagellate sperm. Derived 'intuitively' and by PAUP analysis. Branch-and-bound search for 14 characters; consistency index (CI) = 0.947; CI excluding uninformative character 7 = 0.944; rescaled consistency index (RC) = 0.904.

Dense periflagellar fibres

In the late spermatid of *Raillietiella* sp., nine dense fibres are present peripherally to the centriole (Fig. 4D) and anterior axoneme, one at each doublet. We confirm Wingstrand's observation that dense fibres 9, 1 and 2 disappear on the formation of the dorsal ribbon which presumably develops by their fusion, that fibres 3 and 8 seem to disappear, and that the ventral ribbon develops at the site of dense fibres 4–7, between the axoneme and the nucleus (Fig. 4C). In the spermatid of *Porocephalus crotali*, development of the dense fibres is similar to that in *R. sp.*, but two fibres (apparently 3 and 8) can sometimes be seen to persist and to contribute to the bridge from the axoneme to

the dense sheath of each lateral mitochondrion (Fig. 5D, F).

Endoplasmic reticulum

Endoplasmic reticulum cisternae are present in all four species in the approximately delta-shaped area of cytoplasm, as seen in cross section, between the lateral mitochondria and the nucleus (Figs. 2A, *R. sp.*, 2C, *P. crotali*).

PHYLOGENETIC ANALYSIS AND DISCUSSION

In the present study, phylogenetic relationships of pentastomids with their putative maxillopodan and remipedian relatives have been deduced intuitively

from sperm ultrastructure but this has also been subjected to parsimony analysis using the PAUP program, version 3.01 of Swofford (1990) (Fig. 6). The analysis must be regarded only as preliminary, pending more detailed investigation of the spermatozoa of maxillopodan taxa.

The following characters were employed:

1. Basal body relative to nucleus: (0) posterior; (1) anterior
2. Nucleus length: (0) short; (1) moderate; (2) long
3. Acrosome vesicle: (0) present in sperm; (1) in spermatid only; (2) absent from sperm
4. Acrosome filament: (0) present in sperm; (1) in spermatid only; (2) absent from sperm
5. Axonemal sheath: (0) absent; (1) present
6. Connective at doublet 1: (0) absent; (1) present
7. Connective at 3 & 8: (0) absent; (1) present
8. Dense fibres: (0) absent; (1) present
9. Pseudoacrosome: (0) absent; (1) represented as centriolar crown; (2) highly developed
10. Mitochondrial position: (0) periflagellar; (1) 'ventral'
11. Mitochondrial number: (0) several; (1) three; (2) one
12. Mitochondria form: (0) rounded; (1) moderately long; (2) very long
13. Mitochondria position relative to nucleus: (0) behind; (1) parallel
14. Free flagellum: (0) present; (2) absent.

Examination of the characters used provides the opportunity to discuss the similarities carefully enumerated by Wingstrand and regarded by him as true synapomorphies between pentastomes and branchiurans and to include additional deductions.

We will also discuss characters of pentastomid sperm, unique to the group and shared with branchiurans, in the wider context of those Maxillopoda with flagellate sperm; the Ascothoracida, Cirripedia, Mystacocarida, Branchiura, and the Pentastomida, here included. The Remipedia will be provisionally included in this assemblage in view of the claim, on the basis of molecular biology, that they are the nearest relatives of the group (Abele *et al.*, 1989) despite their placement by Schram (1986), from a careful parsimony analysis of general anatomy, as the plesiomorphic group of all other Crustacea. The remipede may thus be used as an outgroup in determining apomorphies for the other maxillopods or, in the parsimony analysis, in contributing to formulation of the characteristics of a hypothetical ancestor. Lest the hypothetical ancestor be considered too subjective a concept, it should be noted, in anticipation, that an identical tree topology is obtained if the remipede is used as the outgroup in the absence of a hypothetical ancestor.

Although the flagellate condition is limited in crustacean sperm to the maxillopods mentioned above, including the Remipedia, it would appear to be a symplesiomorphy and is not in itself significant in phylogenetically unifying the taxa possessing it. In view of the fact that some maxillopodans (ostracods and copepods) have aflagellate sperm, there is therefore some possibility that the maxillopods with flagellated sperm, though undoubtedly interrelated, are a paraphyletic assemblage. If this were so the phylogram (Fig. 6) would still record the trends in evolution of flagellate sperm in the group. Nevertheless, maxillopods with aflagellate sperm do not appear to be as closely related to those with flagellate sperm as these are to each other and will not be considered in the following discussion.

Acrosome

The presence in *Argulus* spermatids of a small acrosome vesicle and acrosome filament, both lost at maturity, was noted by Wingstrand as a peculiarity of *Argulus* relative to *Raillietiella*. As an acrosome and filament are present in the remipede, where the filament penetrates the nucleus as in *Argulus*, and in cirripedes, their absence from pentastome sperm and mature *Argulus* sperm must be regarded as synapomorphic and their presence in *Argulus* spermatids as a plesiomorphic condition which does not define the Branchiura. Total suppression of the acrosomal apparatus in the spermatid may be considered a pentastome autapomorphy. In the parsimony analysis, presence in the mature sperm of the acrosome vesicle (character 3) and of the acrosome filament (character 4) are scored as plesiomorphies, with restriction to the spermatid as an initial apomorphy (as in *Argulus*); and total absence from spermatid and mature spermatozoon as a second apomorphy. Absence of the filament in the presence of the vesicle is seen in Ascothoracida (though there is possibly some weak development of a filament in the subacrosomal material in these) and in mystacocarids. The filament takes the form of a stout rod posterior to the acrosome vesicle in the remipede.

Pseudoacrosome

A pseudoacrosome, about 30 μm long, divisible into ventral and dorsal components and derived (Wingstrand, 1972) from pericentriolar structures (granulosome, apical membrane, granular material, and the associated dorsal ribbon), is present in pentastomids and *Argulus*. A phylogenetic precursor appears to be the osmiophile crown observed on the basal body of the sperm of the cirripede *Trypetesa nassarioides* by Turquier & Pochon-Masson (1969) and alluded to by Wingstrand (1972). We may thus

recognize a transformation series from this crown to the more apomorphic elaboration in the pentastomid-branchiuran assemblage. Absence, as in other Crustacea, of the pseudoacrosome or crown is regarded as plesiomorphic (character 9).

The detailed agreement in structures transitional between the pseudoacrosome and the remainder of the sperm demonstrated by Wingstrand, and confirmed here, constitutes several highly distinctive synapomorphies between the two groups and autapomorphies for the combined assemblage. These similarities include continuation of the dorsal rod of the pseudoacrosome with the dorsal ribbon and with the centriolar doublets 1, 2 and 9 and of the ventral rod with the more ventrally situated doublets. They further cement the relationship between pentastomes and branchiurans but have not been used in the parsimony analysis.

Nucleus

A short, subspheroidal nucleus is typical of ect-aquasperm and is a plesiomorphic condition. The ellipsoidal condition in the remipede, though somewhat derived, is plesiomorphic for the assemblage investigated while the slightly elongate condition in the Ascothoracida is apomorphic. Mystacocarids, cirripedes and Branchiura-*Argulus* show a progressive elongation which in the latter two taxa is extreme; this has, however, been grouped as a single further apomorphy (character 2).

Axonemal characters

The basal body, from which the axoneme arises, is plesiomorphically at the posterior end of the nucleus in ent-aquasperm, as in the introsperm of the remipede and the ect-aquasperm of the Ascothoracida. Apomorphically it lies at the anterior end of the nucleus immediately behind the acrosome vesicle in the mystacocarid, in cirripedes, pentastomes and *Argulus* (character 1). In cirripedes the plesiomorphic sequence acrosome-nucleus-axoneme is nevertheless retained by insertion of a narrow plate-like extension of the nucleus between the acrosome and the basal body. This has not been represented in the data matrix, chiefly because of uncertainty as to the condition in other taxa and because the anterior location of the basal body remains a valid apomorphy.

Orientation of the plane of the two central singlets at right angles to the 'dorso-ventral' axis of the sperm is a significant synapomorphy in view of the different orientation in other similarly bilaterally symmetrical sperm, those of insects (see Jamieson, 1987). In view of the uncertainty of this character in some other maxillopodans, it has not been used in the parsimony analysis.

A connection, observed in the four pentastomid species (Wingstrand, 1972; present study), of axonemal doublet 1 to the dorsal ribbon is a synapomorphy, as is its oblique tilt in a clockwise direction, linking the Pentastomida and *Argulus foliaceus* (not examined in *A. sp.*) and unknown in other maxillopods. In view of the overall similarity of pentastomid and branchiuran sperm so meticulously demonstrated by Wingstrand (1972), it is improbable that the connective has been acquired convergently in the two groups. It is therefore an autapomorphy of the pentastomid-branchiuran assemblage and correspondingly a symplesiomorphy for the Cephalobaenida and Porocephalida. It is nevertheless noteworthy that connectives occur independently, though at doublets 1 and 5 and connecting with mitochondrial derivatives, in Heteroptera, in the Insecta (references in Jamieson, 1987). In the parsimony analysis presence of the connective is regarded as apomorphic and its absence as plesiomorphic (character 6).

Argulus foliaceus differs from the pentastomids in possessing additional membranous connections of doublets 3 and 8 with the dorsal ribbon, connections unknown in other maxillopods. Whether this is a synapomorphy of an ancestor of the Branchiura and Pentastomida and has been lost in the Pentastomida or is a unique specialization (autapomorphy) of the Branchiura cannot readily be determined. However, disruption of the junction between the dorsal and ventral halves of the axonemal sheath was frequently observed in pentastomid sperm, so that the dorsal half (ribbon) was displaced and, although this was probably a fixation artifact, suggested a weakness at the junctions. It is therefore tempting to speculate that the membranous connectives at doublets 3 and 8 in *Argulus*, corresponding with the junctions in the sheath, may represent an apomorphic advance over the pentastomid and presumed ancestral condition, stabilizing the junction. In the parsimony analysis presence of the connective is regarded as apomorphic and its absence as plesiomorphic (character 7). This character is uninformative as it is unique to a single taxon computed.

An axonemal sheath has not been reported for the sperm of maxillopods other than the pentastome-branchiuran assemblage. Its detailed agreement in the two taxa, including division into interdigitating dorsal and ventral ribbon and continuation of the dorsal ribbon with the dorsal component (rod) of the pseudoacrosome, establishes the sheath as a complex of convincing autapomorphies for the assemblage. Nevertheless, Wingstrand suggests that there is a precursor in cirripede sperm of the endoplasmic reticulum cisterna which is associated with development of the ribbon in pentastomes and *Argulus*.

Presence of the sheath is regarded as apomorphic in the parsimony analysis (character 5); the putative cirripedian precursor has not been scored but if included would not alter the tree topology.

The dense peripheral axonemal fibres constitute a further synapomorphy of the pentastomes and *Argulus* as does association of those at doublets 1, 2 and 9 with the dorsal ribbon. These are again pentastome-branchiuran autapomorphies. In the parsimony analysis the dense fibres are scored as an apomorphy (character 8).

Termination of the nucleus caudally before termination of the axoneme, confirmed here for *Raillietiella*, contrasts with *Argulus* in which the nucleus is present, with the dorsal ribbon, in caudal sections which lack the axoneme (Wingstrand, 1972). The pentastomid condition is presumably plesiomorphic relative to that of *Argulus* in view of the presence of a free, postnuclear flagellum in remipedian through to cirripedian sperm, as in ect-aquasperm. Nevertheless, in both taxa the axoneme is incorporated in the sperm body throughout its length insofar as there is no terminal free flagellum. In the remipede, ascothoracids, the mystacocarid and cirripedes there is a long free flagellum despite incorporation of an extensive anterior region of the axoneme in all but the first two. The axoneme incorporated condition is scored as apomorphic and the free condition as plesiomorphic (character 14).

Mitochondrial characters

In the parsimony analysis the arrangement of mitochondria around the base of the flagellum, typical of 'primitive' sperm, is regarded as plesiomorphic and the 'ventral' position as apomorphic. The condition in Remipedia is uncertain. In the mystacocarid the mitochondria occur above and below the axoneme, a condition which might have been given a separate scoring (though uninformative as not convincingly to be considered precursory to the 'ventral' position seen in cirripedes, pentastomes and *Argulus*) but has been scored as periflagellar (character 10).

The presence of several, rounded mitochondria behind the nucleus is clearly a plesiomorphic condition in aquasperm and presumably in crustacean sperm. Restriction to three mitochondria in pentastomes and *Argulus* is apomorphic, while reduction to 1, in cirripedes, is seen as a further apomorphy (character 11). The occurrence of three mitochondria in the collembolan *Anurida maritima*, alluded to by Wingstrand, is clearly homoplastic. They differ there in being arranged at the points of a triangle and almost in line with, rather than at right angles to, the plane of the central singlets (see Jamieson, 1987).

The moderate elongation of the cirripede mitochondrion is regarded as apomorphic while the great

elongation in pentastomes and *Argulus* is scored as a further apomorphy (character 12). Cirripedes, Ascothoracida, and, less certainly, the remipede, retain the plesiomorphic location of the chondriome behind the nucleus whereas it parallels the nucleus, the apomorphic condition, in the mystacocarid, pentastomes and *Argulus* (character 13).

Hypothetical maxillopodan ancestor

As stated above, the remipede would serve as a suitable plesiomorphic outgroup in a parsimony analysis of those maxillopods with flagellate sperm but the above consideration of spermatozoal characters allows us to envisage the following characters for a hypothetical ancestor: 1. basal body behind nucleus; 2. nucleus short; 3. acrosome vesicle present; 4. acrosome filament present; 5. axonemal sheath absent; 6. connective at doublet 1 absent; 7. connectives at doublets 3 and 8 absent; 8. dense fibres absent; 9. pseudoacrosome absent; 10. mitochondria periflagellar; 11. mitochondria several; 12. mitochondria rounded; 13. mitochondria behind nucleus. Only character states 10 and 13 do not apply with certainty to the Remipedia, as the location (and persistence at maturity) of the mitochondria has not been definitely established.

These ultrastructural observations from the present study together with a survey of the literature on crustacean sperm (references in Jamieson, 1987) permit the construction of the following data matrix:

Hypothetical ancestor	00000000000000
Remipedia	000000000?0000
Ascothoracida	010?0000000000
Mystacocarida	12020000000010
Cirripedia	12000000112100
Branchiura	12111111211211
Pentastomida	12221101211211

The following options were employed in the analysis: the branch-and-bound option was used with the search settings: initial upper bound: unknown (computed via stepwise); addition sequence: furthest; initial MAXTREES setting = 100; branches having maximum length zero collapsed to yield polytomies; outgroup rooting; outgroup defined as the Remipedia (*Speleonectes benjamini*). Characters were all ordered; character-state optimization was accelerated transformation (ACCTRAN); character 7 proved uninformative but was included for its descriptive value. Character 4 (acrosome filament) was excluded and was added to the tree *a posteriori*.

The results of the analysis are shown in Fig. 6. The topology of this tree corresponds with that intuitively deduced from sperm ultrastructure. A single most parsimonious tree was obtained with a length of 19

steps and with the following characteristics: consistency index (CI) = 0.947; homoplasy index (HI) = 0.056; CI excluding uninformative character 7 = 0.944; HI excluding uninformative character 7 = 0.056; retention index (RI) = 0.955; rescaled consistency index (RC) = 0.904; f value = 6; f -ratio = 0.0330.

The present investigation confirms Wingstrand's description for *Raillietiella* in all significant respects and has shown that his observations can be extended to other pentastomids as it has demonstrated a remarkable degree of homogeneity in the ultrastructure of the spermatozoa of cephalobaenids and porocephalids. Leaving aside metric considerations, the only differences appear to be the larger size of the developing mitochondria and complexity of their internal structure (convoluted membranes), together with greater electron density of the mature mitochondrial matrices, in *Porocephalus crotali*, in comparison with the three investigated cephalobaenids, differences which require confirmation from further material. This spermatozoal homogeneity of both divisions of the Pentastomida, the putatively plesiomorphic cephalobaenids and apomorphic porocephalids, retrospectively vindicates Wingstrand's reliance on the ultrastructure of a single cephalobaenid species, *Raillietiella hemidactyli*, in drawing a comparison with the branchiuran *Argulus*.

No unique features of pentastome sperm which were not previously recognized by Wingstrand have emerged from the present study. The large number of similarities carefully enumerated by Wingstrand and regarded by him as true synapomorphies between pentastomes and branchiurans are corroborated here and leave no reasonable doubt of the sister-group relationship of the two groups and this is supported by the parsimony analysis.

A tentative representation of morphological trends in the flagellate sperm of maxillopods which is heuristic in terms of phylogenetics of the group, has been given in Fig. 6. It will be seen that the sperm of the pentastome-branchiuran assemblage appear to be the most highly evolved of the flagellate crustacean sperm. The position of maxillopod groups with aflagellate sperm has yet to be investigated.

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