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Sperm ultrastructure of an oviparous and an ovoviviparous onychophoran species (Peripatopsidae) with some phylogenetic considerations

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Abstract

The spermatozoa of the Australian oviparous *Ooperipatellus insignis* and the South African ovoviviparous *Opisthopatus cinctipes* (both: Onychophora, Peripatopsidae) were studied and compared with the spermatozoal patterns already described in the taxon. The spermatozoa of both species conform with the general plan described for the Onychophora: they are filiform cells formed, in sequence, by an elongated, fully condensed nucleus capped by an acrosome and surrounded by several spiral ridges; by a mitochondrial midpiece characteristically interpolated between the nucleus and a characteristic flagellum. Major differences between the spermatozoa of both species concern their acrosome organization. The correlation between the acrosomal pattern and the size and structure of the ovarian eggs (oocytes) in onychophorans has been investigated. A parsimony analysis was performed on 21 spermatozoal characters of the species considered. Its results are congruent with those of the traditional systematics. A new set of autapomorphies characterising onychophoran sperm is suggested and some of the spermatological homologies proposed between Onychophora and Euclitellata spermatozoa are critically discussed. Our analysis suggests that spermatozoal characters are good phylogenetic markers among onychophorans, also at low taxonomic level.

Key words: Onychophora – Peripatopsidae – *Ooperipatellus insignis* – *Opisthopatus cinctipes* – spermatozoa – ultrastructure – phylogeny

Introduction

Onychophorans (velvet worms) are terrestrial, soft-bodied, multi-legged invertebrates. They are nocturnal predators, confined to dark, humid microhabitats. They live within and under rotten logs, under stones and in leaf litter (Manton 1946). They are hard to find, because they are experts at hiding. Moreover they are extremely local, mostly occurring in low densities.

Taxonomic literature on onychophorans is still defective and incomplete. The most serious problem with this group is that at least 40% of the more recent publications on various aspects of onychophoran research are based on incorrect determinations. Unfortunately, based on this erroneous taxonomy, far-reaching conclusions have been drawn (e.g. Jamieson 1986, see Ruhberg 1992).

Initially onychophorans were described as aberrant (poly-pod) slugs (Guilding 1826), but their obvious combination of annelid and arthropod characters was soon recognized (Gervais 1837). Moseley (1874) discovered their tracheal system and hence established their arthropod affinities. Later investigations by Balfour (1883) and Sedgwick (1922) corroborated that *Peripatus*, albeit its affinities with the annelids, evidently was an arthropod (Walker 1986). Its major arthropodan features include a heart with ostia, a hemocoel, trachea and an ecdysone mediated moulting (ecdysis) of an α -chitin protein cuticle (Ax 2000).

Onychophora have often been considered a transitional form (connecting link) between Annelida and Arthropoda within the stem-group Articulata (Wheeler et al. 1993; Nielsen 1997). Whether the Onychophora and the Euarthropoda should be placed, together with the Cycloneuralia, in a clade called 'Ecdysozoa' (Giribet et al. 1996; Aguinaldo et al. 1997; Schmidt-Rhaesa et al. 1998; Eriksson et al. 2003) has recently been discussed.

The current consensus on the phylogenetic position supports the view of a monophyletic origin of the arthropods with the Onychophora being a sister group of the Euarthropoda

(Wheeler et al. 1993). This assumption is based on various strong criteria, such as morphological data (e.g. Weygoldt 1986; Zrzavý et al. 1998), fossil records (Hou et al. 1991) and molecular characters (Ballard et al. 1992; Giribet et al. 1996).

At present there are two families described: the Peripatidae Evans, 1901 and the Peripatopsidae Bouvier, 1904 with, respectively, a generally circumtropical and a south-temperate distribution (Peck 1975). It appears that the two taxa diverged prior to the breakup of Gondwana (Ghiselin 1984). A single phylogenetic analysis of taxa from Australasia was based on molecular data (Gleeson et al. 1998). It splits the Peripatopsidae in two major groupings: a monophyletic clade containing all species found in New Zealand and Tasmania, and a still unresolved group of Australian mainland taxa. On the other hand an extensive PhD-study on the Australian Peripatopsidae (Reid 1996) was based on a large set of morphological characters, including a single spermatological feature. It also suggests that the Australian Peripatopsidae may not be monophyletic: when the trees were not rooted at the Peripatidae as outgroup, they nested among the Peripatopsidae (Reid 1996, pp. 709–710).

The relationship between Peripatopsidae and Peripatidae is discussed controversial: Purcell (1900, pp. 94–95) was the first to reconstruct an ancestral form of the Onychophora. His phylogenetic assessment was based on the successive reduction of the posterior legs, glands and segments. In this respect he considered the 'American *Peripatus*' to be the ancestral form. Later Ruhberg (1985, p. 160) tentatively considered the Peripatopsidae as the derived family while other authors suggested the opposite.

Although morphologically very conservative, the Onychophora are astonishingly diverse both in their mating systems and in their reproductive functions. Sexes are separate and they reproduce for amphimixis, with a single exception: the peripatid *Epiperipatus imthurni* (Sclater, 1888) from Trinidad, where parthenogenesis has been reported (Read 1988). The sexes seem to have different demographics as shown in the

peripatopsid *Euperipatoides rowelli* Reid, 1996 from NSW, Australia (Sunnucks et al. 2000). Adult males are generally smaller than females of the same age. Sperm production occurs soon after birth, males mature in their first year of life (Lavallard and Campiglia 1975; Read 1988) while females mature much later, namely in their second or third year of life (Sunnucks et al. 2000). Onychophoran females show a remarkable spectrum of reproductive strategies. These span oviparity, ovoviviparity and non-placental viviparity in the Peripatopsidae and placental viviparity in the Neotropical Peripatidae (for definitions see Campiglia and Walker 1995; Brosius-Roggenbuck and Ruhberg 2000).

Insemination behaviour appears to be similarly diverse (Tait and Norman 2001). Most males produce spermatophores which can reach the female's reproductive tract/oocytes in different ways (Storch and Ruhberg 1977).

Onychophoran sperm ultrastructure has been thoroughly analysed in many species (an overview is given in Storch et al. 2000), but only few phylogenetic considerations based on these results have been published (e.g. Lavallard 1976; Jamieson 1986). To our knowledge sperm-based phylogenetic studies especially at lower taxonomic levels do not include onychophorans.

In this paper, we describe, for the first time, the sperm ultrastructure of an oviparous onychophoran, the Australian peripatopsid *Ooperipatellus insignis* (Dendy, 1890) and compare this with *Opisthopatus cinctipes* Purcell, 1899, a South African ovoviviparous peripatopsid. Although spermiogenesis has been investigated before in this species (Storch and Ruhberg 1977), new material and techniques have resulted in additional data, especially concerning its acrosomal organization. In a comprehensive approach including species from both families we compare our new findings to the ultrastructural data obtained from the literature. Using sperm characters, we here attempt to construct a phylogenetic analysis at low taxonomic level inside onychophorans with particular attention to the relationship between Peripatopsidae and Peripatidae. Among the Metazoa, the ultrastructure of spermatozoa has proved useful for phylogenetic assessment; among clitellates, for example, both at higher (Jamieson et al. 1987; Ferraguti and Erséus 1999) and at lower taxonomic level (Cardini et al. 2000; Marotta et al. 2003).

The aim of this work is to contribute to a better understanding of the general ground plan of onychophoran sperm and to propose a new set of characters, the spermatozoal ones, that could be useful to a better resolution of the onychophoran systematics and taxonomy. Some of the spermatological homologies proposed between onychophoran and euclitellate spermatozoa (Jamieson 1986) and the proposed correlation between the acrosomal pattern and the size and structure of the eggs in onychophorans (Baccetti et al. 1976) will be critically discussed.

Materials and methods

Specimen collection and microscopical techniques

Specimens of *O. insignis* (Dendy, 1890) were hand collected by A. L. Reid and C. Brockmann in February, 1997 from rotten logs at its type locality Mt. Macedon, Victoria, Australia, 37°23'S, 144°35'E, 1.001 m. Specimens of *O. cinctipes* Purcell, 1899 were obtained by M. Hamer and H. Ruhberg in August, 2002 from rotten logs at Karkloof, Kwa-Zulu-Natal, South Africa, 29°36'S, 30°75'E, 1.226 m. Specimens of both species were maintained in the laboratory of the junior author in Hamburg.

Efferent and deferent vasa and the ejaculatory duct of *O. insignis* containing mature sperm were fixed in 0.1 M phosphate buffered 2% glutaraldehyde with 1% tannic acid (Afzelius 1988). After a 5 days fixation, the specimens were first washed for 12 h in phosphate buffer, and later in distilled water; *en bloc* stained for 2 h in the dark in 2% aqueous uranyl acetate, dehydrated in a graded ethanol series and embedded in Spurr's resin. The corresponding regions of male *O. cinctipes* containing mature sperm were fixed in a saturated solution of picric acid SPAFG (Ermak and Eakin 1976). The specimens were washed overnight in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, washed in distilled water, pre-stained in 2% uranyl acetate in water, dehydrated in a graded ethanol series and embedded in Spurr's resin.

Thin sections were cut with a Reichert Ultracut E; stained with uranyl acetate and lead citrate and observed with a JEOL 100SX transmission electron microscope.

Selected taxa for phylogenetic analysis

Ingroup and outgroup taxa used for the phylogenetic analysis are listed in Table 1.

The ingroup comprises 10 onychophoran species representing two genera of Neotropical Peripatidae and five genera of Peripatopsidae. In order to root the tree, the ground plan of the insect spermatozoon (*sensu* Jamieson et al. 1999) has been considered. For comparative purposes – i.e. to critically discuss some of the proposed synapomorphies between onychophorans and clitellates (Jamieson 1986) – the 'plesiomorphic' spermatozoon for the oligochaetes (*sensu* Jamieson et al. 1987) has been added to the outgroup.

Taxonomic problems

As already mentioned (see Introduction), there is a serious problem with the current taxonomy of the Onychophora. At least 40% of the more recent publications are based on incorrect determinations (Ruhberg 1992). As far as our publication is concerned, Baccetti and Dallai (1977) succeeded in finding an acrosome in '*Peripatoides leuckartii*' (syn.: *Euperipatoides leuckartii*), a composite species, which needs to be revised (Reid 1996). A detailed report on an acrosome of

Table 1. Ultrastructural descriptions of onychophoran spermatozoa and list of taxa used in the present study

Taxa	References
Ingroup taxa	
Family Peripatidae Evans, 1901	
Genus <i>Peripatus</i> Guiling, 1826	
<i>P. acacioi</i> (Marcus & Marcus, 1950)	Lavallard 1976
<i>P. sedgwicki</i> Bouvier, 1899	Storch and Ruhberg 1990
Genus <i>Epiperipatus</i> Clark, 1913	
<i>E. biolleyi</i> Bouvier, 1902	Storch and Ruhberg 1993
Family Peripatopsidae Bouvier, 1904	
Genus <i>Peripatoides</i> Pocock, 1894	
<i>P. novaezealandiae</i> (Hutton, 1879)	Jamieson 1986
Genus <i>Euperipatoides</i> Ruhberg, 1985	Baccetti and Dallai 1977
<i>E. leuckartii</i> (Saenger, 1869)	Dallai and Afzelius 1993
Genus <i>Peripatopsis</i> Pocock, 1894	
<i>P. capensis</i> (Grube, 1860)	Camatini et al. 1979
<i>P. moseleyi</i> (Wood-Mason, 1879)	Baccetti et al. 1976
Genus <i>Opisthopatus</i> Purcell, 1899	
<i>O. cinctipes</i> Purcell, 1899	Storch and Ruhberg 1977, 1983; and this study
Genus <i>Ruhbergia</i> Reid, 1966	
<i>R. bifalcata</i> Reid 1996	Storch et al. 2000
Genus <i>Ooperipatellus</i> , Ruhberg, 1985	
<i>O. insignis</i> (Dendy, 1890)	This study
Outgroup taxa	
Hexapoda 'ancestor'	Jamieson et al. 1999
Euclitellata 'ancestor'	Jamieson et al. 1987

an onychophoran, a composite species from New Zealand, was given by Jamieson (1986) under the name '*Peripatoides novaezealandiae*'. As there are no voucher specimens available, the taxonomic status of Jamieson's research material remains unsolved.

Spermatozoal characters and character states

Twenty-one spermatozoal characters, all treated as unordered, are considered (see Appendix 1). For a detailed and critical revision of the character and character states considered, see Appendix 1 and Discussion.

Parsimony analysis

Phylogenetic analyses were performed using Phylogenetic Analysis Using Parsimony (PAUP), version 4.0b10 for 32 bit Microsoft Windows (Swofford 2002). The exact branch-and-bound search algorithm was selected, with the following options: furthest addition sequence (ADDSEQ = FURTHEST), save all minimal trees found during the branch and bound search (MULTREES = YES) and collapse a branch if its minimum possible length is zero (COLLAPSE = MINBRLEN). To evaluate the support for tree topologies, the data set was analysed by jackknifing (Farris et al. 1996). One thousand replicates were subjected to a separate branch and bound search, using PAUP. Jackknife frequencies = 50% were calculated.

Results

Sperm morphology of *Ooperipatellus insignis* and *Opisthopatus cincitipes*

The following description refers to the mature spermatozoa as observed inside the vasa efferentia and the vas deferens. In these regions, a helical, granular band of secretion envelops the head region of the spermatozoa (Fig. 1A).

The spermatozoon of both *O. insignis* and *O. cincitipes* are filiform cells formed, in sequence, by an acrosome, an elongated cylindrical nucleus, and a mitochondrial midpiece characteristically interpolated between the nucleus and the flagellum (Fig. 2a and b).

Acrosome

Ooperipatellus insignis has a small acrosome (Fig. 1A and 2a; Table 2) formed by a thick, conical electron dense structure, the conical subacrosomal structure *sensu* Jamieson (1986), of uniform thickness, ending at its posterior extremity in a prominent ring (Fig. 1D). The conical subacrosomal structure surrounds the flattened apical portion of the nucleus (Fig. 1E). A membrane-bound acrosomal vesicle lies lateral to the conical subacrosomal structure. Some scattered electron dense material surrounds the outer side of the acrosome vesicle (Fig. 1D).

On the contrary, *O. cincitipes* has an acrosome formed by a long, filiform vesicle, tapering from the base to the apex (Fig. 1R; Table 2). The acrosome vesicle is basally coiled around the nuclear apex (Fig. 1S), and it extends to form the apical portion of the spermatozoon (Fig. 1L and m). A tube-like, electron dense structure, starting around the apical portion of the nucleus (Fig. 1N and O), surrounds the acrosomal vesicle along all its length (Fig. 1R).

Nucleus

In both species, the nucleus is an elongated cone, with a diameter decreasing considerably from the nuclear base to the apex (Table 2) and with a deep conical indentation in the middle region of the base of the nucleus (Fig. 1F and P). In *O. insignis* the nucleus ends in a flat apical portion, and in

O. cincitipes the nucleus has a convex apex. Three parallel ridges of chromatin run spirally around the nucleus of *O. insignis* along its entire length; their pitch, in accordance with the reduction of the nuclear diameter, is greater in the basal than in the apical nuclear region (Fig. 1A). Each chromatin ridge is triangular in cross-section, with the wide basis close to the nucleus, and tapering at its extremity. There is an electron transparent area at the centre of the ridges (Fig. 1B). *Opisthopatus cincitipes* also has four parallel ridges of chromatin, but only in late spermatids. In both species the nucleus is straight (Fig. 1C and T), although in *O. cincitipes* it is characteristically twisted at the apex (Fig. 1S).

Midpiece

Ooperipatellus insignis and *O. cincitipes* both have a round to oval shaped midpiece containing respectively six and four irregularly coiled mitochondria with conspicuous cristae (Fig. 1G and Q; Table 2). A central indentation, present in the basal portion of the midpiece, the centriolar fossa *sensu* Jamieson (1986), houses the basal body of the flagellum (Fig. 1C and T).

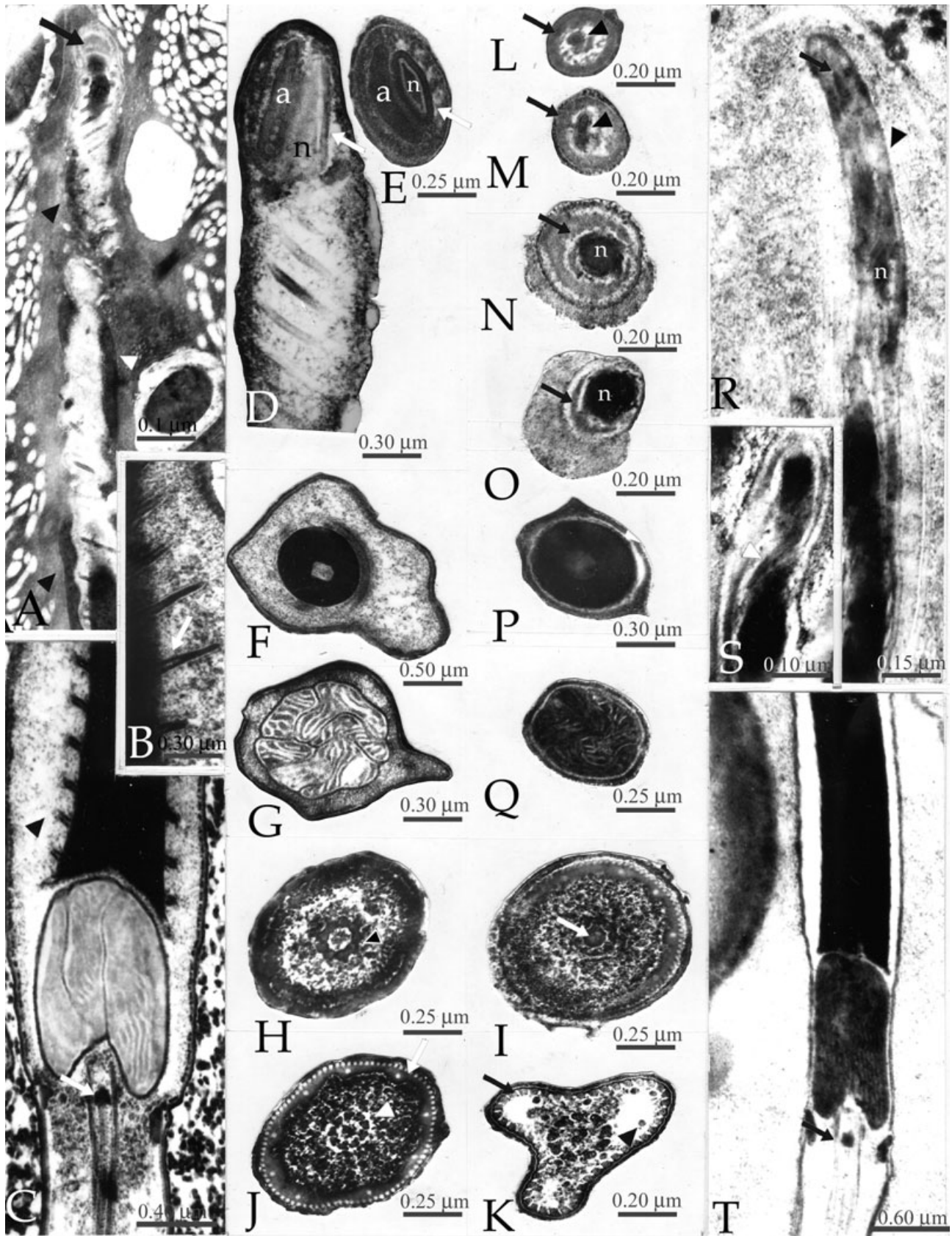
Flagellum

The flagellum, with a simple $9 \times 2 + 2$ axoneme, is surrounded by nine, irregularly distributed, peripheral singlets and, external to these, beneath the cell membrane a manchette of microtubules (Fig. 1K).

A series of cross-sections of the neck region has allowed us to investigate, in more detail, the fine structure of this portion of the sperm. The axoneme originates from an unconventional basal body, formed by nine triplets of microtubules immersed in a dense material (Fig. 1H). Remnants of an anchoring apparatus, connecting the basal body to the plasma membrane, and many electron dense spots, considered to be glycogen granules (Jamieson 1986), are observed inside the cytoplasm surrounding the basal body (Fig. 1H). A single, thick-walled cylinder (Table 2), is present inside the distal portion of the basal body (Fig. 1I). From it the central apparatus of the flagellum arises, connected to the peripheral microtubules by prominent radial links. The centriolar region is surrounded by an electron dense, subplasmalemmal structure, the annulus *sensu* Jamieson (1986). Around the basal body the annulus forms an irregular electron dense band, from which the microtubules of the subplasmalemmal manchette emerge (Fig. 1H). At a more distal level, the annulus shows nine regularly spaced thickenings, roughly in correspondence with the nine axonemal doublets, housing the nine accessory microtubules (Fig. 1I). The same structure illustrated for *O. insignis*, is also present in *O. cincitipes*.

Parsimony analysis

The parsimony analysis of the 21 selected spermatozoal characters resulted in four most parsimonious trees, with 36 steps, a consistency index (CI) of 0.889, and a retention index (RI) of 0.879. The strict consensus tree is shown in Fig. 3. The two outgroups, the 'Hexapoda ancestor' and the 'Euclitellata ancestor', group by their own at the base of the tree. The examined ingroup species group together to form a strongly supported monophyletic Onychophora. The Peripatidae clade (comprising *Peripatus acacioi* (Marcus & Marcus, 1955), *P. sedgwicki* Bouvier, 1899 and *Epiperipatus biolleyi* (Bouvier, 1902) is strongly supported and it turns out to be the sister



group of a less supported monophyletic Peripatopsidae. Within the Peripatopsidae, *Peripatopsis capensis* (Grube, 1866) and *P. moseleyi* (Wood-Mason, 1879) from South Africa group together to form the sister group of the other

peripatopsid species. Among them, the ovoviparous *Peripatopsis novaezealandiae* (Hutton, 1876) (*sensu* Jamieson 1986) from New Zealand and the oviparous *O. insignis* from its type locality in Victoria (Australia) turn out to be sister groups.

Fig. 1. Spermatozoon of *Ooperipatellus insignis* (a–k) and *Opisthopatus cinctipes* (l–t). (a–d), (r–t): longitudinal sections. (e–q): cross-sections. (A) Anterior portion of head. Note acrosome (arrow) and helical band of secretion around the nucleus (arrowhead). (B) Helical ridges around nucleus: note electron transparent area at centre of ridge (arrow). (C) Nucleus, midpiece and tail. Note ridges of chromatin (arrowhead) and basal cylinder (arrow). (D and E) Acrosome showing vesicle (a), conical subacrosomal structure (arrow), and flattened extremity of nucleus (N). (F) Basal portion of nucleus showing the nuclear indentation. (G) Midpiece. (H–J) Neck region of axoneme. (H) Basal body of flagellum (arrowhead). (I) Distal portion of basal body and cylinder within (arrow). (J) $9 \times 2 + 2$ axoneme (arrowhead), nine thickenings housing nine accessory microtubules (arrow) and subplasmalemmal manchette. (K) Tail showing a simple axoneme, nine accessory microtubules (arrowhead) and subplasmalemmal manchette (arrow). (L and M) Acrosome, showing tube-like structure (arrow) and acrosomal vesicle (arrowhead). (N and O) Apical and middle nuclear portion showing tube-like structure (arrow) around nuclear apex (N). (P) Basal portion of nucleus showing nuclear indentation. (Q) Midpiece. (R) Acrosome. Note tube-like structure (arrowhead), nucleus (N) and acrosomal vesicle (arrow). (S) Spirally coiled apical portion of nucleus. Note basal portion of acrosome vesicle (arrowhead). (T) Basal portion of nucleus, midpiece and proximal portion of tail. Note basal cylinder (arrow)

Discussion

The spermatozoon

Onychophoran spermatozoa are filiform cells, formed by an elongated, fully condensed nucleus surrounded by several spiral ridges, and in some species capped by an acrosome vesicle. A mitochondrial midpiece is characteristically interpolated between the nucleus and the flagellum. The tail extends from a highly modified basal body, containing a characteristic basal cylinder *sensu* Ferraguti (1984). Its tail is a simple $9 \times 2 + 2$ axoneme, surrounded by nine fibres and a peripheral subplasmalemmal manchette, consisting of a variable number of close microtubules.

The general plan of the spermatozoon of *O. insignis* and *O. cinctipes* conforms with that of other onychophorans, confirming the already suggested great constancy of sperm ultrastructure inside the group (Storch et al. 2000). However, major differences concern the organization of their acrosome (Table 3).

Data on onychophoran acrosomes present an intriguing pattern for phylogenetic considerations. An acrosome, present in the spermatid only, is a common feature supposed to be shared by all Neotropical viviparous peripatid species investigated to date (Storch et al. 2000, and see Appendix 1). Within the Peripatopsidae, a complete acrosome in the mature sperm is present only in those species characterized by ovoviviparity: e.g. in *Euperipatoides leuckartii* (Saenger, 1869) (Baccetti and Dallai 1977), *Peripatoides novaezealandiae sensu* Jamieson (1986), and *Ruhbergia bifalcata* Reid, 1986 (Storch et al. 2000). Among the South African peripatopsid species characterized by non-placental viviparity and hypodermal insemination, e.g. *Peripatopsis capensis* (Grube, 1866) (Camatini et al. 1979) and *P. moseleyi* (Wood-Mason, 1879) (Baccetti et al. 1976) the acrosome is completely absent; unless the formation – during spermiogenesis – of vesicles that do ‘not produce an acrosome-like structure, but rather fuse into an amorphous mass that is eliminated from the mature sperm’ (Baccetti et al. 1976).

Baccetti et al. (1976) proposed a correlation between acrosomal organization, the size and structure of eggs, and the modes of reproduction among onychophorans. Since the acrosome is the organelle responsible for the sperm penetration into the egg, ‘the appearance of viviparity, which usually implies unprotected eggs, reduced the importance of the acrosome... as it is evident in

Onychophora, which either have or do not have an acrosome depending on the type of egg’ (Baccetti 1985). A re-examination of the spermatozoal data present in literature together with the observation of the spermatozoon of *O. insignis* and *O. cinctipes*, corroborates the suggested hypothesis. The placental (*Epiperipatus biolleyi*, *Peripatus acacioi* and *P. sedgwicki*) and non-placental (*Peripatopsis capensis*, *P. moseleyi*) viviparous onychophorans, with eggs almost devoid of yolk and envelopes, have spermatozoa without acrosome. On the other hand, the oviparous *O. insignis*, with large, thick-walled, yolk-filled eggs, has spermatozoa with a complete acrosome (acrosome vesicle and conical subacrosomal structure). Among ovoviviparous species, the spermatozoa of both *Ruhbergia bifalcata* and *O. cinctipes* have an acrosome showing a condition in some way intermediate between that observed in viviparous and oviparous ones: they have an acrosome vesicle, but lack any subacrosomal structures. The defectiveness and incompleteness of the onychophoran taxonomy, as already mentioned, makes the interpretation of the spermatozoa of *Peripatoides novaezealandiae* and *Euperipatoides leuckartii* problematic. Both are composite species, which need to be revisited (Storch et al. 2000).

Among the Metazoa a complete, secondary loss of the acrosome has occurred independently several times in phylogenetically unrelated groups, as for example in all the teleost fishes (Mattei 1991) and, among crustaceans, in Branchiopoda (Wingstrand 1978); sometimes this loss is associated with internal fertilization, as in some insects (Baccetti 1984). In all such cases, the fertilization of the eggs occurs via a special micropile (Baccetti and Afzelius 1976). Baccetti et al. (1976) proposed that the loss of the acrosome is a secondary event also in onychophorans, as proved by the formation of the acrosome during spermiogenesis in those species, in which it is absent in mature spermatozoa. In accordance with this hypothesis, this analysis suggests that the presence of the acrosome vesicle in mature spermatozoa is the ancestral state (outgroup condition) and that the loss of the acrosomal vesicle arose independently in Peripatidae and Peripatopsidae clades (Fig. 3). This result is congruent with the most widely accepted evolutionary view that the ancestral form of Onychophora is an oviparous or ovoviviparous representative with large, yolky eggs (Anderson 1973; Reid 1996). Moreover our analysis suggests that viviparity arose two times independently: placental viviparity, associated with minute eggs almost devoid of yolk and envelopes at the base of Neotropical Peripatidae; non-placental viviparity within the Peripatopsidae.

Character analysis

Rieppel and Kearney (2002) emphasized the importance of character analysis, the generation of character hypotheses – i.e. primary conjecture of homologies – stemming from comparative anatomical studies, in morphological systematics. They proposed to use the classical criteria of homology as a test of hypotheses on the morphological characters. Following this insight, the homology and analogy criteria of the homology theorem applied to the ultrastructural research (Rieger and Tyler 1979) have been used to test the proposed synapomorphies between the spermatozoa of oligochaetes and that of onychophorans (Jamieson 1986).

An elongated, cylindrical nucleus, starting from the mid-piece and ending apically in an acrosome (*equivalence in position*), strongly condensed and with helical ridges (*equivalence of component parts*) is present in both onychophorans and oligo-

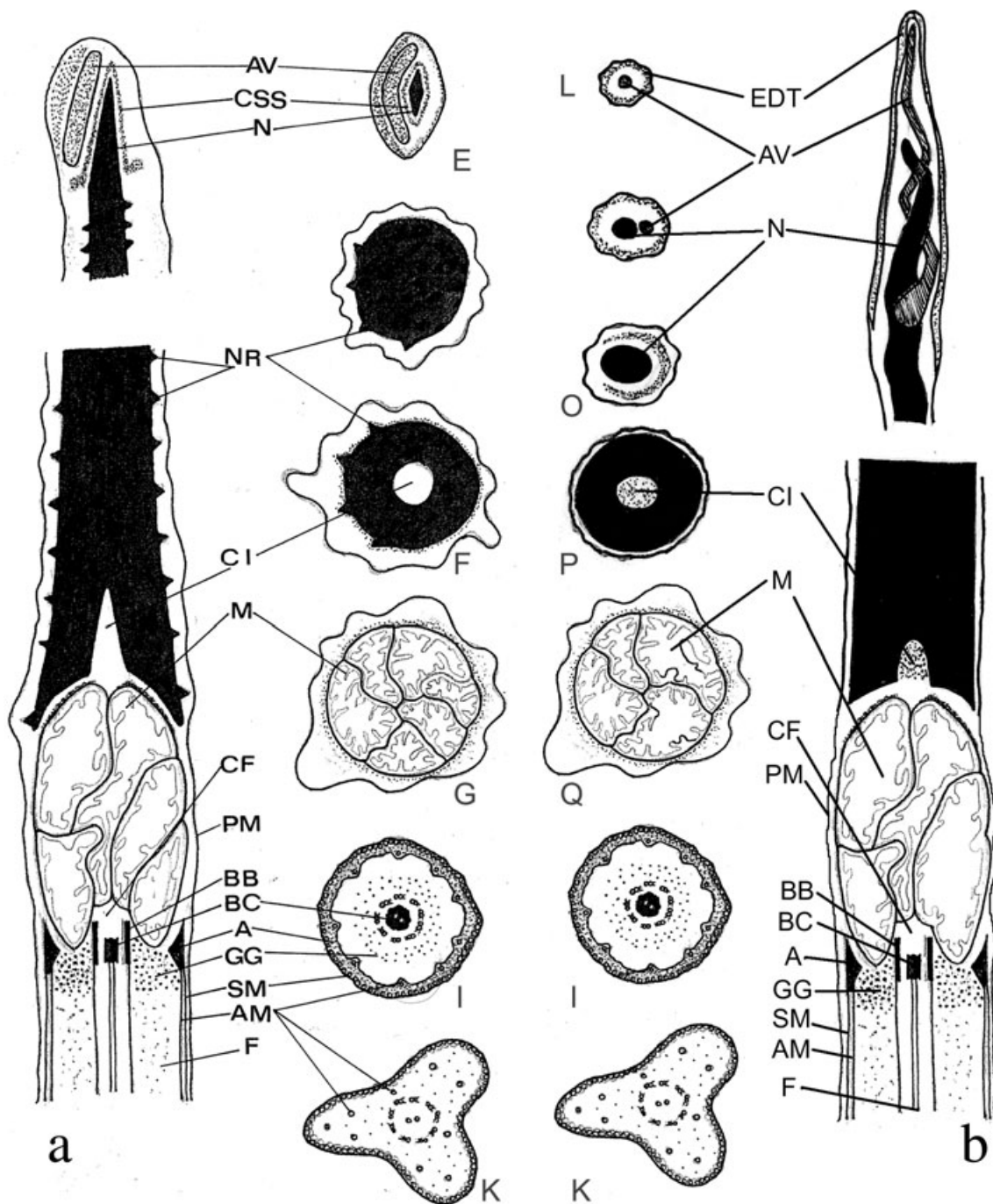


Fig. 2. Diagrams of mature spermatozoa of *Ooperipatellus insignis* (a) and *Opisthopatus cincitipes* (b). A, annulus; AM, accessory microtubules; AV, acrosome vesicle; BB, basal body; BC, basal cylinder; CI, conical indentation; CF, centriolar fossa; CSS, conical subacrosomal structure; EDT, electron-dense tube; F, flagellum; GG, glycogen granules; M, mitochondrion; N, nucleus; NR, nuclear ridges; PM, plasma membrane; SM, subplasmalemmal manchette. Cross-sections are lettered following Fig. 1

	<i>Ooperipatellus insignis</i>	<i>Opisthopatus cincitipes</i>
Acrosome vesicle length	$0.60 \pm 0.07 \mu\text{m}$, $n = 3$	$1.34 \pm 0.06 \mu\text{m}$, $n = 2$
Nucleus diameter at the base	$0.60 \pm 0.10 \mu\text{m}$, $n = 8$	$0.54 \pm 0.03 \mu\text{m}$, $n = 6$
Nucleus diameter at the apex	$0.16 \pm 0.02 \mu\text{m}$, $n = 6$	$0.16 \pm 0.04 \mu\text{m}$, $n = 4$
Mitochondrial length	$1.22 \pm 0.19 \mu\text{m}$, $n = 6$	$1.29 \pm 0.12 \mu\text{m}$, $n = 7$
Mitochondrial diameter	$0.67 \pm 0.16 \mu\text{m}$, $n = 6$	$0.52 \pm 0.07 \mu\text{m}$, $n = 7$
Basal cylinder diameter	$110 \pm 0.009 \text{ nm}$, $n = 9$	$100 \pm 0.02 \text{ nm}$, $n = 6$
Basal cylinder length	$100 \pm 0.016 \text{ nm}$, $n = 8$	$90 \pm 0.010 \text{ nm}$, $n = 6$

Table 2. Metric characters of the sperm of *Ooperipatellus insignis* and *Opisthopatus cincitipes* compared

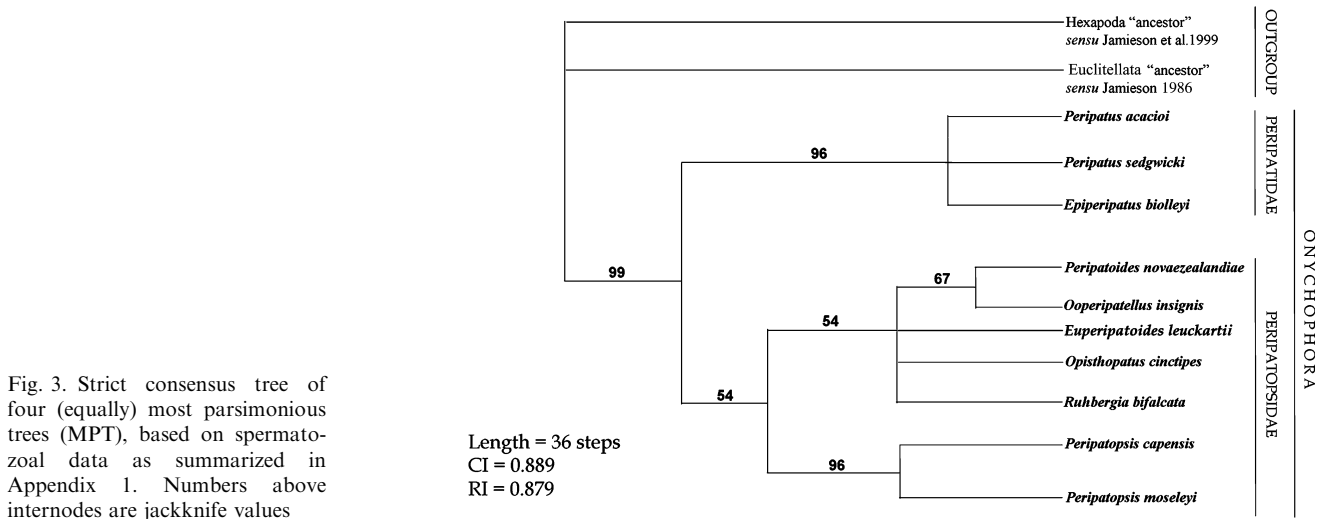


Table 3. Acrosomal patterns among onychophorans

Way of reproduction	Type of ovarian eggs	Species	Acrosome
Oviparity	Large, yolk-filled, thick-walled (Ruhberg 1985)	<i>Ooperipatellus insignis</i>	Present with subacrosomal structure
Ovoviviparity	Large, yolk-filled, thick-walled (Ruhberg 1985)	<i>Peripatooides novaezealandiae</i> <i>Ruhbergia bifalcata</i>	Present with subacrosomal structure Present without subacrosomal structure
		<i>Euperipatooides leuckartii</i> <i>Opisthopatus cinctipes</i>	Present without subacrosomal structure Present without subacrosomal structure
Viviparity: non-placental	Small to medium size, nearly devoid of yolk (Ruhberg 1985)	<i>Peripatopsis capensis</i> <i>Peripatopsis moseleyi</i>	Absent Absent
Viviparity: placental	Minute, nearly devoid of yolk (Ruhberg 1985)	<i>Peripatus sedgwicki</i> <i>Peripatus acacioi</i> <i>Epiperipatus biolleyi</i>	Absent Absent Absent

chaetes. Nevertheless this character is present in many unrelated metazoan sperm. In effect the elongation and reduction in diameter of the sperm head and its equipment with possible perforatorial structures (i.e. the spiral ridges), may well represent parallel adaptation in animals in which the sperm are transferred during copulation in seminal receptacles or through spermatophores, as in oligochaetes and in onychophorans respectively. The needle-shape of the head and the helical ridges may be an adaptation to a better sperm packaging or to increase the penetration capacity of sperm through the tegument and tissue or other viscous media (Jamieson and Rouse 1989). Indeed, the ultrastructural analysis of the spiral ridges surrounding the nuclei in both taxa, reveals that their similarity is only superficial. In onychophorans the spiral ridges are just peripheral formations already considered by Baccetti et al. (1976) as 'opposite punctuate relief of the cariotheca'. Differently, in oligochaetes the spiral ridges are chromatin formations.

Despite minor differences, both onychophoran and oligochaete spermatozoa have a mid-piece characteristically interposed between nucleus and axoneme. Although a similar condition, among modified metazoan sperm models, has been observed in the alvinellid polychaete *Paralvinella pandorae* Desbruyères & Laubier, 1986 (McHugh 1995) and among xenotrichulid gastrotrichs (Ferraguti et al. 1995), it is difficult to find an adaptive explanation for it. Perhaps an axoneme that is not enclosed by mitochondria or mitochondrial derivatives could be more free in locomotion (Rouse and Jamieson 1987). On the other hand, as pointed out by Jamieson (1986), the interpolation of the midpiece between nucleus and axoneme cannot be interpreted as the secondary end product of a

constrained developmental process, due to the loss of the proximal centriole during spermatogenesis.

An annulus-like structure, extending at the end of the midpiece and appearing as a ring of subplasmalemmal dense material, is present in both oligochaetes and onychophorans. Despite obvious similarities, these structures differ remarkably. In oligochaetes the annulus or annuloid *sensu* Jamieson (1982), when present, is a simple 'periodically interrupted ring of dense material' (see Fig. 22 in Jamieson 1982), not comparable with the highly complex structure of the onychophorans.

A basal cylinder *sensu* Ferraguti (1984) is present at the anterior end of the axoneme in both onychophorans and oligochaetes. In both taxa this structure, of comparable dimensions (diameter ranging from 60 to 100 nm and length from 0.1 to 0.3 μm in oligochaeta Ferraguti (2000); Table 2), lies deep inside the basal body of the flagellum (*equivalence in position*) and the central apparatus of the axoneme emerges from it (*equivalence of component parts*). Although this structure has a relatively low order of complexity, known the relationship between complexity of similar structures and improbability to arise independently (Grimstone 1959), it is difficult to find a functional role for it. The fact that, to our knowledge, the basal cylinder is not present in any other metazoan sperm, is a confirmation of its low adaptive value.

Phylogenetic analysis

Congruence test

Two of the four proposed synapomorphies between the spermatozoa of oligochaetes and that of onychophorans

(Jamieson 1986), an elongated cylindrical nucleus with helical ridges and an annulus-like structure, failed the criteria of the homology theorem applied to the ultrastructural research (see above); they cannot be considered putative homologies. On the contrary, the basal cylinder and a mid-piece characteristically interposed between nucleus and axoneme, passed the criteria of the homology theorem, and have been considered primary homologies. The assumption that both are synapomorphies (i.e. secondary homologies) between euclitellates and onychophorans spermatozoa is corroborate, among sperm characters, when tested by congruence (Patterson 1982).

Phylogenetic analysis

This is the first phylogenetic analysis among onychophorans based on sperm ultrastructure. It reveals that spermatozoal characters are good phylogenetic markers among onychophorans, also at low taxonomic level, as shown from the high consistency index value. The results of this analysis are largely congruent with those already known from the traditional systematics. The poor jackknife support and the large polytomy inside the Peripatopsidae clade as well as the incongruent close relationship between *P. cf. novaezealandiae* and *O. insignis* could be due to an incorrect determination for *P. novaezealandiae* by Jamieson (1986) or to the small sample of onychophoran species considered in this analysis (see Material and Methods). There are about 10 genera of the Peripatidae and 40 genera of the Peripatopsidae described at present, and our study has only covered but a fraction of the total character variation in the onychophoran taxa.

A new set of homologies characterizing onychophoran sperm is suggested. Jamieson (1986) proposed two autapomorphies characterising onychophoran sperm, both referring to the organization of the flagellum: a subplasmalemmal manchette and nine accessory microtubules surrounding the axoneme. Here we propose new potential autapomorphies for the Onychophora (Fig. 1): an annulus *sensu* Jamieson (1986) inside the neck region of the mature spermatozoon and an extracellular helical band of secretion surrounding the head of the spermatozoon. The new spermatozoal autapomorphies that we propose for the Neotropical Peripatidae, besides the presence of prominent helical ridges sculpturing the nucleus (Storch and Ruhberg 1993) are an acrosome vesicle present in late spermatids but absent in the mature spermatozoon (Storch et al. 2000), a chromatin ring at the base of the nucleus, and a mid-piece formed by a single mitochondrion.

The new single autapomorphy for the Peripatopsidae, besides the presence of helical ridges surrounding the nucleus (Storch and Ruhberg 1993), is the presence of a centriolar fossa *sensu* Jamieson (1986) at the base of the midpiece. New autapomorphies for the genus *Peripatopsis* are the complete absence of an acrosome vesicle also in spermiogenesis (Storch et al. 2000) and a long coiled midpiece formed by three mitochondria. A comma-shaped acrosome vesicle flanking a conical subacrosomal structure are the synapomorphies for *O. insignis* and *Peripatoides cf. novaezealandiae*.

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Zusammenfassung

Ultrastrukturelle Untersuchungen an Spermien eines oviparen und eines ovoviviparen Onychophoren (Peripatopsidae) und phylogenetische Einschätzungen

Die Ultrastruktur der Spermien zweier Onychophoren-Arten (Peripatopsidae) wird vergleichend untersucht. Erstmals wird ein oviparer Vertreter berücksichtigt; nämlich *Ooperipatellus insignis* vom *Locus typicus*; Mt. Macedon, Victoria, Australien. Seine Spermatozoen werden mit denen der ovoviviparen südafrikanischen Art *Opisthopatus cinctipes* verglichen. Die Ergebnisse dieser Untersuchung werden mit verfügbaren Literaturdaten abgeglichen und ein Grundmuster für Onychophoren-Spermien entwickelt. Die Spermien der beiden hier untersuchten Arten stimmen in ihrer Morphologie und Ultrastruktur im Wesentlichen mit dem bereits für Onychophoren bekannten Muster überein. Es sind fädige Zellen, die recht einheitlich aufgebaut sind. Sie weisen einen länglichen, kompakten Kern mit Akrosom-Kappe auf und sind spiralförmig von Furchen umgeben. Der mitochondriale Mittelbereich der Spermien liegt zwischen dem Nukleus und einem für Onychophoren typischen Flagellum. Unterschiede betreffen dagegen die Organisation des Onychophoren-Akrosoms. Die mögliche Korrelation zwischen Akrosom-Ausbildung und Größe und Struktur der Ovarialeier wird untersucht. Basierend auf 21 Spermatozoen-Charakteren wird eine Parsimonie-Analyse durchgeführt. Die so gewonnenen Daten decken sich mit denjenigen der traditionellen Systematik. Die Spermien der Onychophoren weisen Merkmale auf, welche Autapomorphien des Taxon Onychophora sein können. Darüber hinaus werden mutmaßliche Spermien-Homologien, die von verschiedenen Autoren für die Taxa Onychophora und Euclitellata vorgeschlagen wurden, kritisch bewertet. Unsere Analyse zeigt, dass Spermien-Merkmale für Onychophoren gute phylogenetische Marker darstellen, sogar auf Art-Niveau.

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Appendix 1. Spermatozoal characters

1. *Acrosome vesicle*: absent (0); present in spermatid only (1); present in mature sperm (2). The character state 'complete absence of an acrosome vesicle' (character 1, state 0) has been coded for *Peripatopsis moseleyi* and *P. capensis*. Indeed, in both species, a complete absence of the acrosome in mature spermatozoa is reported in the literature (Baccetti et al. 1976; Camatini et al. 1979). The character state 'presence of an acrosome vesicle only in spermatids' (character 1, state 1) has been coded for *Peripatus acacioi* and *P. sedgwicki* spermatozoa, since in *P. acacioi* a complete acrosome vesicle lateral to the nucleus is reported in spermatids only (Lavallard 1976; Storch et al. 2000); a similar condition is also stated for *P. sedgwicki* (see Figs 16–17 in Storch and Ruhberg 1990).

This character state has been coded as missing for *Epiperipatus biolleyi*, since there are no data available concerning its acrosome. The character state 'presence of an acrosomal vesicle in mature sperm' (character 1, state 2) has been coded for *Peripatoides novaezealandiae sensu* Jamieson (1986), *E. leuckartii* (Baccetti and Dallai 1977), *Ruhbergia bifalcata* (Storch et al. 2000), and *Ooperipatellus insignis*, *Opisthopatus cinctipes* (this study), since a well-defined acrosome vesicle is present in mature spermatozoa of all these species. 2. *Periacrosomal structures*: absent (0); conical or tube-like (1); acrosomal tube (2). 3. *Acrosome vesicle shape*: straight, at the top of the nucleus (0); comma-shaped, lateral to the nucleus (1); filiform and spirally coiled around the nucleus (2). Since for *Peripatoides cf. novaezealandiae* the presence of both states 1 and 2 has been reported (Jamieson 1986), we codify here the presence of a filiform acrosome vesicle, spirally coiled around the nucleus (character 3, state 2), following Storch et al. (2000). 4. *Acrosome length*: less than 3 µm (0); between 3 and 6 µm (1); more than 6 µm (2). 5. *Sub-acrosomal structures*: absent (0); conical subacrosomal structures (1); perforatorium (2). 6. *Helical ridges of the nuclear envelope*: absent (0); peripatid-like (1); peripatopsid-like (2). Referring to Fig. 24 in Storch and Ruhberg (1993), the nuclear cristae in the Peripatidae are longer and less tilted around the nucleus than in Peripatopsidae species. 7. *Number of ridges of the nuclear envelope*: between 1 and 3 (0); between 4 and 5 (1); more than 5 (2). 8. *Basal nuclear portion*: flat (0); slightly concave (1); deeply concave (2). 9. *Nuclear indentation*: absent (0); present (1). 10. *Nuclear basal ring*: absent (0); present (1). 11. *Mitochondrial organization*: regularly arranged (0); irregularly arranged (1). In clitellates mitochondria are arranged symmetrically to form the midpiece, as cylindrical sectors (character 11, state 0). On the contrary, in onychophorans they are irregularly arranged (character 11, state 1). 12. *Number of mitochondria*: 1 (0); 2 (1); 3 (2); more than 3 (3). 13. *Shape of mitochondria*: nearly parallel (0); coiled (1). 14. *Position of mitochondria*: lateral to the tail (0); interpolated between nucleus and flagellum (1). 15. *Mitochondrial slenderness*: less than 1 (0); between 1 and 2 (1); between 2 and 3 (2); more than 3 (3). 16. *Fossa at the basis of the midpiece*: absent (0); superficial (1); deep (2). 17. *Basal cylinder*: absent (0); present (1). 18. *Annulus posterior to mitochondria*: absent (0); present (1). 19. *Accessory microtubules*: absent (0); present (1). 20. *Subplasmalemmal manchette*: absent (0); present (1). 21. *Extracellular helical band of secretion*: absent (0); present (1).

Appendix 2. Data matrix

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Hexapoda 'ancestor'	2	0	0	0	2	0	?	0	0	0	?	?	?	0	?	?	0	0	1	0	0
Euclitellata 'ancestor'	2	2	0	0	2	0	?	0	0	0	0	3	0	1	0	0	1	0	0	0	0
<i>Peripatus acacioi</i>	1	0	?	?	0	1	1	1	0	1	1	0	?	1	2	0	1	1	1	1	?
<i>Peripatus sedgwicki</i>	1	0	?	?	0	1	1	1	0	1	1	0	?	1	2	0	1	1	1	1	1
<i>Epiperipatus biolleyi</i>	?	0	?	?	0	1	?	1	0	1	1	0	?	1	2	0	1	1	1	1	?
<i>Peripatoides cf. novaezealandiae</i>	2	1	1	0	1	2	2	2	0	0	1	3	0	1	1	2	1	1	1	1	1
<i>Euperipatoides cf. leuckartii</i>	2	?	2	0	0	2	1	2	0	0	1	1	0	1	2	?	1	1	1	1	?
<i>Peripatopsis capensis</i>	0	0	?	?	0	2	?	0	0	0	1	2	1	1	3	1	1	1	1	1	?
<i>Peripatopsis moseleyi</i>	0	0	?	?	0	2	1	0	?	0	1	2	1	1	3	1	1	1	1	1	?
<i>Opisthopatus cinctipes</i>	2	1	2	0	0	2	1	0	1	0	1	3	0	1	2	2	1	1	1	1	1
<i>Ruhbergia bifalcata</i>	2	1	2	1	0	2	1	0	0	0	1	1	0	1	1	1	1	1	1	1	1
<i>Ooperipatellus insignis</i>	2	1	1	0	1	2	1	1	1	0	1	3	0	1	2	2	1	1	1	1	1