

Molecular taxonomy and phylogeny of the Tricladida

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Within the free-living Platyhelminthes, the triclads or planarians are the best known group, partly as a result of being suitable for classroom studies but, largely, because they have been the subject of intensive research concerning the cellular bases of regeneration and pattern formation (for general reviews see Baguñà *et al.* 1994, and Baguñà 1998) and, most recently gene expression (Bayascas *et al.* 1997; Orii *et al.* 1999). The Tricladida Lang, 1884, which is best considered a suborder (Ehlers 1985a), forms, together with the suborder Proseriata Meixner, 1938, the Order Seriata. Autapomorphies for the Seriata are their backwards-directed tubiform and plicate pharynx and the division of testes and vitellaria into serially arranged follicles. Proseriata do not have obvious autapomorphies apart from their lack of lamellate rhabdites (Sopott-Ehlers 1985), whereas Tricladida are characterized by its three-branched intestine and its highly modified embryonic development with the presence of a transitory embryonic pharynx. A family of proseriates, the Bothrioplanidae Hofsten, 1907, was proposed by Sopott-Ehlers (1985) as the actual sister group of the Tricladida forming a taxon N.N. (Bothrioplanida + Tricladida) characterized by the presumed lack of epidermal collar-receptors, a triclaidoid intestine, and a crossing-over of muscle layers at the root and the tip of the pharynx. A general phylogenetic scheme of Seriata is summarized in Figure 6.1.

The monophyletic status of the Seriata has been questioned both on morphological and molecular grounds. The main morphological argument against the presumed autapomorphies of Seriata is that, without comparative ultrastructural studies, pharynx types are not useful for phylogenetic studies, whereas the serial arrangement of vitellaria is so general that homology is impossible to test (Sluys 1989a). Moreover, data on the ultrastructure of the excretory system indicates a basal location for Proseriata and, therefore, the paraphyly of Seriata (Rohde 1990). This paraphyly was reinforced from molecular data (18S rDNA sequences; Carranza *et al.* 1997). Neighbour-joining (NJ) distance, maximum-likelihood (ML) and maximum parsimony (MP) trees showed Tricladida clustering either with proleciophorans or rhabdoceles, whereas proseriates appeared as basal neophorans close to lecithoepitheliates or Neodermata. A more recent study, combining morphological and molecular characters (Littlewood *et al.* 1999a), confirmed the results of Carranza *et al.* (1997) and suggested a clade made by *Urastoma* Dörler, 1900, *Fecampiida* (Kronborgia, Christensen and Kannerworff, 1964) and *Ichthyophaga* Syriamiatnikova, 1949, as the actual sister group of the Tricladida. The morphological synapomorphies shared by the Bothrioplanida and the Tricladida proposed by Sopott-Ehlers (1985a), have been critically examined by Sluys (1989a). Lack of collar-receptor is a secondary absence and, in the lack of further information, has to be considered a weak character. Instead, the tripartite intestinal system and the muscle crossing-over at the pharynx, the latter not uncommon among other platyhelminths (see references in Sluys 1989a), were considered good synapomorphies.

Within the Tricladida, three infraorders usually have been recognized: Maricola (marine planarians), Paludicola (freshwater planarians) and Terricola (land planarians) (reviewed in Sluys 1989a), to which a new one, the Cavernicola was

further added (Sluys 1990). Relationships of these infraorders have been the subject of several morphological-based analyses. Sluys' (1989a) analysis is by far the most detailed and valuable. It supported the monophyly of the Tricladida, the Terricola, the Maricola and the Paludicola, and the existence of a new clade, the Terricola-Paludicola clade. Relationships within the infraorders have only been considered in detail within the Paludicola, in which three families are currently recognized: the Dugesiidae Ball, 1974, the Planariidae Stimpson, 1857, and the Dendrocoelidae Hallez, 1892. The Planariidae and Dendrocoelidae are considered derived groups and form the sister-group of the more primitive Dugesiidae. A radically different view of infraorder relationships of Tricladida emerged recently from molecular studies based on sequence data of the 18S ribosomal genes and the presence of an 18S gene duplication shared by the Terricola and the family Dugesiidae of the Paludicola (Carranza *et al.* 1998a,b). The resulting phylogenetic trees strongly indicated that the Paludicola is paraphyletic since the Terricola and one paludicolan family, the Dugesiidae, share a more recent common ancestor than the dugesiids with the other paludicolans (dendrocoelids and planariids). Therefore, it was suggested that the infraorders Terricola and Paludicola are redundant and should be replaced by a new taxon, the Continenticola (Carranza *et al.* 1998a). A comparison between Sluys' (1989a) and Carranza *et al.*'s (1998a) phylogenetic proposals is depicted in Figure 6.2.

We report here partial sequences of the Cytochrome Oxidase I (COI) gene from 21 taxa of the Terricola and the Paludicola, and new complete 18S rDNA sequences from five species of turbellarians. Published 18S rDNA sequences from the large data set of 18S rDNA sequences available from other Platyhelminthes, namely from the Proseriata, the Proleciophora and the Rhabdoceles, are also included in the phylogenetic analysis. The aims of this paper are to: 1) Reassess the taxonomic status and the phylogenetic position within the Platyhelminthes of the Proseriata, the Bothrioplanida and the Tricladida; 2) Further test the new triclaid phylogeny drawn from molecular data (Carranza *et al.* 1998a,b), in particular the paraphyletic status of the Paludicola and the monophyly of Terricola + Dugesiidae; and 3) Test the monophyletic or paraphyletic status of the Terricola and Dugesiidae and its internal phylogeny. Congruence and conflicts between the morphological and molecular phylogenies of the Tricladida are highlighted.

Materials and methods

The current taxonomic classification of the species used in this study is shown in Table 6.1.

Sequencing of the 18S molecule

High molecular weight DNA was purified according to a modification (García-Fernández *et al.* 1993) of the guanidine isothiocyanate method initially described for RNA (Chirgwin *et al.* 1979) from live or ethanol-fixed specimens. The entire length of the 18S rDNA molecule was PCR-amplified applying specific primers and conditions described earlier (Carranza *et al.* 1996, 1998a; Littlewood and Smith 1995). Amplification

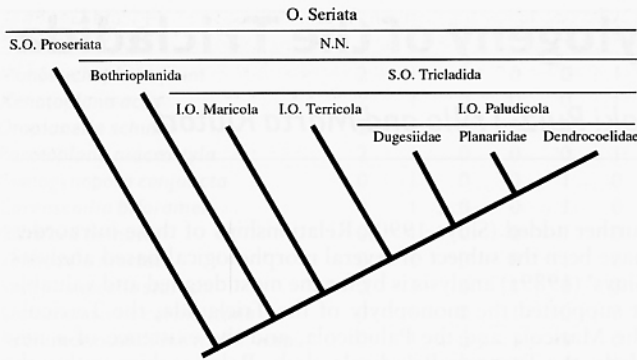


Figure 6.1 Internal phylogenetic relationships of the Seriata after Ehlers (1985), Sopott-Ehlers (1985) and Sluys (1989a), slightly modified. N.N.: unnamed taxon, according to Sopott-Ehlers (1985), formed by the Bothrioplanida and the Tricladida. For the sake of clarity, the Infraorder Cavernicola (Sluys 1990) of the Tricladida is not included.

products were sequenced directly. Sequencing of the clones and the PCR products was performed using an automated sequencer ABI Prism 377, following manufacturer's protocols.

Sequencing of the Cytochrome Oxidase I (COI) molecule

High molecular weight DNA was purified as described for the 18S rDNA gene (see above) from 21 species of Terricola and Paludicola (see Table 6.1). A fragment of approximately 450 nucleotides close to the centre of the cytochrome *c* oxidase subunit I mitochondrial gene was amplified. The primers used (pr-a2 and pr-b2) and the conditions of the PCR reaction were as described in Bessho *et al.* (1992). The PCR products were purified with GeneClean II kit (BIO 101 Inc.) and directly sequenced using the same primers as for amplification. Cycle sequencing using Dye-labelled terminators (Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit) was performed in a DNA Thermal Cycler Perkin-Elmer 480 according to the manufacturer's instructions and run on an automated sequencer ABI 377.

Sequence alignment

18S rDNA sequence data were aligned by hand with the help of a computer editor (GDE 2.2; Smith S.W. *et al.* 1994). Alignment gaps were inserted to account for putative length differences between sequences. A secondary structure model (Gutell *et al.* 1985) was used to optimize alignment of homologous nucleotide positions. Those positions that could not be unambiguously aligned were subsequently excluded resulting in a total of 1483 positions that could be used in the phylogenetic analyses.

CO I sequences were aligned by eye based on the protein sequences. There is a variable region in the middle of the fragment in which some species have one or two extra amino acids; this region has been excluded from the alignment. Given the variability of the third position of the codons it also was excluded from the phylogenetic analyses, resulting in a total of 232 positions that could be used in the subsequent studies.

The full sequence alignments used in these analyses have been deposited with EMBL under accessions ds41997 (18S) and ds42057 (CO I) and are available via anonymous FTP from FTP.EBL.AC.UK under directory pub/databases/embl/align

Phylogenetic analysis

Data sets were analysed using the programs in PHYLIP package v. 3.52 (Felsenstein 1993). A distance matrix was generated from the aligned sequences using the program DNADIST and corrected with the two-parameter method of Kimura (1980). The distances were then converted to phylogenetic trees using the NJ method of Saitou and Nei (1987) provided by the NEIGHBOR program. Bootstrap resampling (Felsenstein 1985) was accomplished with the use of the programs SEQBOOT (1000 replicates)

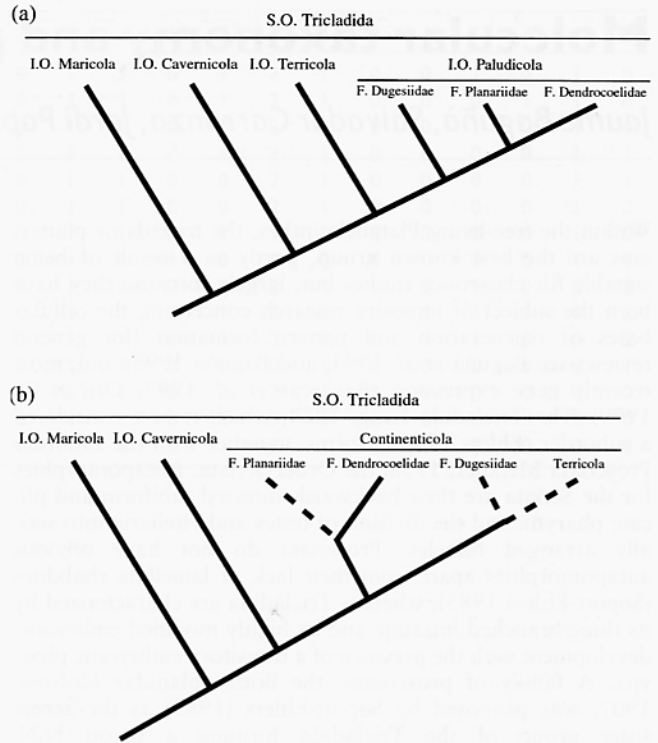


Figure 6.2 a) Phylogenetic relationships of the Tricladida based on morphological characters according to Sluys (1989a); b) Phylogenetic hypothesis for the Tricladida based on molecular characters (18S rDNA sequences and an 18S rDNA duplication event) (Carranza *et al.* 1998a). Dashed lines indicate groups that are not well-supported in the molecular phylogenetic analysis.

and CONSENSE. For the estimation of the maximum-likelihood (ML) trees we used the FastDNAmI program v. 1.1.1a (with global rearrangements and reordering of species) (Felsenstein 1981; Olsen *et al.* 1994).

To root the Platyhelminthes internal tree, representatives of lophotrochozoans and ecdysozoans (*sensu* Aguinaldo *et al.* 1997) and from deuterostomates were chosen. For the Terricola + Dugesiidae trees, representatives of families Dendrocoelidae and Planariidae were chosen because they are known to be its sister-group (Carranza *et al.* 1998a).

Results

The phylogenetic position of the Tricladida within the Platyhelminthes

Both NJ and ML methods of phylogenetic reconstruction gave similar results as regards the position of the Tricladida and the Proseriata and the paraphyly of Seriata, only the NJ tree being shown (Figure 6.3). First of all, the Seriata appears as a polyphyletic assemblage, because Proseriata, Bothrioplanida and Tricladida never cluster together. Second, Bothrioplanida never clusters with proseriates and triclads, appearing with poor bootstrap support (NJ tree) as the sister-group of a vast assemblage of neophorans and in the ML tree as the sister-group of the Neodermata. Third, Proseriata and Tricladida, represented by four or more sequences fall into clear recognizable monophyletic groups, with very high bootstrap support, regardless of the phylogenetic method used. Fourth, although their positions are variable, proseriates fall in both types of trees as a basal clade of neophorans. And

Table 6.1 Species used in the analysis with classification. Classification of turbellarians following Cannon (1986). 18S sequence (+) and GenBank accession numbers, and COI sequences (*) and GenBank accession numbers (in brackets), are indicated. New 18S rDNA sequences reported in this paper are marked #. Note: The terricolan genus *Artioposthia* is now known as *Arthurdendyus* (Jones and Gerard 1999).

Classification	18S rDNA	COI	Accession number	Classification	18S rDNA	COI	Accession number
Phylum Chordata				Of uncertain status (see text)			
<i>Xenopus laevis</i>	+		X04025	Family Urastomidae			
Phylum Echinodermata				<i>Urastoma cyprinae</i>	+		U70085
<i>Asterias amurensis</i>	+		D14358	<i>Ichthyophaga</i> sp.	+		AJ012512
Phylum Arthropoda				Order Rhabdocoela			
<i>Odiellus troguloides</i>	+		X81441	Dalyelliida			
<i>Scolopendra cingulata</i>	+		U29493	Family Dalyelliidae			
Phylum Annelida				<i>Microdalyellia rossi</i>	+		AJ012515
<i>Eisenia foetida</i>	+		X79872	Family Graffillidae			
<i>Lanice conchilega</i>	+		X79873	<i>Graffilla buccinicola</i>	+		AJ012521
Phylum Mollusca				Family Pterascalidae			
<i>Acanthopleura japonica</i>	+		X70210	<i>Pterastericola australis</i>	+		AJ012518
<i>Nerita albicilla</i>	+		X91971	Family Fecampiidae			
Phylum Platyhelminthes				<i>Kronborgia isopodicola</i>	+		AJ012513
Order Catenulida				Temnocephalida			
Family Stenostomidae				Family Temnocephalidae			
<i>Stenostomum leucops</i>	+		U70085	<i>Temnocephala</i> sp. #	+		AF051332
Family Catenulidae				<i>Temnocephala</i> sp.	+		AJ012520
<i>Suomina</i> sp.	+		L41129	Typhloplanida			
Order Macrostomida				Family Trigonostomidae			
Family Dolichomacrostomidae				<i>Mariplanella frisia</i>	+		AJ012514
<i>Paramalostomum fuscum</i>	+		AJ012531	Family Typhloplanidae			
Family Macrostomidae				<i>Bothromesostoma personatum</i>	+		M58347
<i>Macrostomum tuba</i>	+		U70080	<i>Mesocastrada</i> sp.	+		U70081
<i>Macrostomum hystricinum</i> #	+		AF051329	Kalyptorhynchia			
Family Microstomidae				Family Polycystidae			
<i>Microstomum lineare</i>	+		U70082	<i>Gyatrix hermaphroditus</i>	+		AJ012510
Order Polycladida				Schizorhynchia			
Acotylea				Family Schizorhynchidae			
Family Leptoplanidae				<i>Diascorhynchus rubrus</i>	+		AJ012508
<i>Notoplana australis</i>	+		AJ228786	Family Karkinorhynchidae			
<i>Notoplana koreana</i>	+		D85097	<i>Cheliplana</i> cf. <i>orthocirra</i>	+		AJ012507
Family Planoceridae				Order Seriata			
<i>Planocera multitentaculata</i>	+		D17562	Suborder Proseriata			
Family Discocelidae				Family Bothrioplanidae			
<i>Discocelis tigrina</i>	+		U70078	<i>Bothrioplana semperi</i> #	+		AF051333
Cotylea				Family Monocelidae			
Family Pseudocerotidae				<i>Monocelis lineata</i>	+		U45961
<i>Thysanozoon brochii</i>	+		D85096	<i>Archiloa rivularis</i>	+		U70077
<i>Pseudoceros tritriatus</i>	+		AJ228794	Family Otoplanidae			
Order Haplopharyngida				<i>Otoplana</i> sp.	+		D85090
Family Haplopharyngidae				<i>Parotoplana renatae</i>	+		AJ012517
<i>Haplopharynx rostratus</i>	+		AJ012511	Suborder Tricladida			
Order Lecithoepitheliata				Infraorder Maricola			
Family Prorhynchidae				Family Bdeleouridae			
<i>Geocentrophora</i> sp.	+		U70079	<i>Bdeleoura candida</i>	+		Z99947
<i>Geocentrophora baltica</i>	+		AF065417	Family Procerodidae			
<i>Geocentrophora spyrocephala</i>	+		D85089	<i>Ectoplana limuli</i>	+		D85088
<i>Geocentrophora wagini</i>	+		AJ012509	<i>Procerodes littoralis</i>	+		Z99950
Order Proleclithophora				Family Uteriporidae			
Combinata				<i>Uteriporus</i> sp.	+		AF013148
Proporata				Infraorder Terricola			
Family Pseudostomidae				Family Geoplanidae			
<i>Pseudostomum gracilis</i>	+		AF065423	Subfamily Caenoplaninae			
<i>Reisingeria hexaoculata</i>	+		AF065426	<i>Artioposthia</i> sp.		*	(AF178325)
Opisthoporata				<i>Artioposthia testacea</i>		*	(AF178305)
Family Cylindrostomidae				<i>Artioposthia triangulata</i>	+		AF033038
<i>Cylindrostoma fingsalianum</i> #	+		AF051330	<i>Caenoplana caerulea</i>	+		AF033040
<i>Cylindrostoma gracilis</i>	+		AF065416	<i>Australoplana sanguinea</i>	+		AF033041
Separata				Subfamily Geoplaninae			
Family Plagiostomidae				<i>Geoplana ladislavi</i>		*	(AF178313)
<i>Plagiostomum cinctum</i>	+		AF065418	Family Bipaliidae			
<i>Plagiostomum striatum</i>	+		AF065420	<i>Bipalium adventitium</i>		*	(AF178306)
<i>Plagiostomum vittatum</i> #	+		AF051331	<i>Bipalium kewense</i>	+		AF033039
<i>Plicastoma cuticulata</i>	+		AF065422	<i>Bipalium</i> sp.		*	(AF178307)
<i>Vorticeros ijimai</i>	+		D85094	Family Rhynchodemidae			
Family Ulianiniidae				Subfamily Microplaninae			
<i>Ulianinia mollissima</i>	+		AF065427	<i>Microplana nana</i>	+	*	AF033042 (AF178317)

Continued overleaf

Table 6.1 Continued.

Classification	18S rDNA	COI	Accession number	Classification	18S rDNA	COI	Accession number
<i>Microplana terrestris</i>		*	(AF178318)	<i>Dugesia ryukyuensis</i>		*	(AF178311)
Subfamily Rhynchodeminae				<i>Neppia montana</i>		*	(AF178319)
<i>Platydemus manokwari</i>		*	(AF178320)	<i>Spathula</i> sp.		*	(AF178324)
Infraorder Paludicola				<i>Girardia tigrina</i>	+	*	AF013157
Family Planariidae				<i>Girardia dorotocephala</i>		*	(AF178316)
<i>Polycelis nigra</i>	+		AF013151	<i>Girardia anderlani</i>		*	(AF178314)
<i>Polycelis tenuis</i>	+	*	Z99949				(AF178315)
			(AF178321)	Neodermata			
<i>Crenobia alpina</i>	+		M58345	Class 'Monogenea' — incerta sedis			
<i>Phagocata ullala</i>	+		AF013149	<i>Udonella caligorum</i>	+		AJ228796
<i>Phagocata</i> sp.	+		AF013150	Class Trematoda			
Family Dendrocoelidae				Order Strigeida			
<i>Dendrocoelum lacteum</i>	+	*	M58346	Family Schistosomatidae			
			(AF178312)	<i>Schistosoma mansoni</i>	+		M62652
<i>Baikalobia guttata</i>	+		Z99946	Order Echinostomida			
Family Dugesiidae				Family Fasciolidae			
<i>Schmidtea mediterranea</i>	+	*	U31084	<i>Fasciolopsis buski</i>	+		L06668
			(AF178322)	Order Plagiorchiida			
<i>Schmidtea polychroa</i>	+	*	AF013152	Family Gyliauchenidae			
			(AF178323)	<i>Gyliauchen</i> sp.	+		L06669
<i>Cura pinguis</i>	+	*	AF033043	Class Eucestoda			
			(AF178309)	Order Cyclophyllidea			
<i>Dugesia subtentaculata</i>	+		M58343	Family Taeniidae			
<i>Dugesia etrusca</i>	+	*	(AF178310)	<i>Echinococcus granulosus</i>	+		U27015
<i>Dugesia japonica</i>	+	*	AF013153				
			(D499166)				

finally, Tricladida forms a monophyletic group with very high bootstrap support (100%) and appears as the sister-group, with a rather weak support (42%) in NJ trees, to the Prolecithophora. Using both phylogenetic methods, the new clade formed by Tricladida + Prolecithophora, shifts the clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* (Littlewood *et al.* 1999a) to a more external position forming the sister-group of the Tricladida + Prolecithophora and altogether a new clade with a 79% bootstrap support.

Molecular phylogeny of the Tricladida: the monophyly of the Maricola, the paraphyly of the Paludicola, and evidence for a clade Dugesiidae + Terricola

All dugesiids and all the Terricola sampled so far show two types of 18S ribosomal genes homologous to the type I and type II genes described in the dugesiid *Schmidtea mediterranea* Benazzi *et al.* 1975, by Carranza *et al.* (1996). Using this duplication event and the sequences of either type I or type II, it was found that: 1) the Maricola form a monophyletic primitive group; 2) the Terricola and the Dugesiidae cluster together with high support irrespective of the phylogenetic method used (NJ, MP and ML; Carranza *et al.* 1998a,b); and 3) the other paludicolan families, the Planariidae and Dendrocoelidae form the sister-group of the Terricola + Dugesiidae clade. A NJ tree using only type I 18S rDNA sequences is shown in Figure 6.4. The clade Terricola + Dugesiidae has a 100% bootstrap support, and its sister group a support of 99%. ML trees (not shown) had also both sister-groups very well supported.

Although the topology of the tree drawn from COI sequences is different from that derived from 18S rDNA sequences, it also supports with very high bootstrap support (100%) the clustering of Terricola and Dugesiidae and the sister-group character of the Planariidae and Dendrocoelidae (Figure 6.5).

Are the Terricola and the Dugesiidae monophyletic clades?

In both NJ trees drawn from 18S rDNA sequences (Figures 6.3 and 6.4) the monophyly of the Terricola is not supported or very weakly supported. Likewise, trees derived from COI sequences show monophyly of the Terricola, but with very weak support. A similar situation holds for the Dugesiidae, the anomaly in 18S trees being the clustering of the dugesiid *Girardia tigrina* Girard, 1850 (Figure 6.3) within the Terricola, and in COI trees the early branching of *Spathula* sp. Nurse, 1950 (Figure 6.5). Nevertheless, both sets of trees reproduce with high bootstrap support monophyletic assemblages such as the dugesiid genera *Schmidtea* Ball, 1974, and *Dugesia* Girard, 1850. In addition, the COI tree supports the family Bipaliidae, the subfamily Microplaninae and the genus *Girardia* Ball, 1974. It is worth noting the 'anomalous' position in COI trees of the rhynchodemid *Platydemus manokwari* de Beauchamp, 1962, within the Geoplanidae and of the dugesiid species *Girardia anderlani* Kawakatsu and Hauser, 1983, within the genus *Dugesia* and not with the other *Girardia* species.

Discussion

In his detailed analysis on the phylogenetic relationships of the triclads, Sluys (1989a) stated that research in this group 'is in a state of flux'. The advent of molecular studies, largely based on 18S rDNA sequences, has increased the rate of flux. This reflects the apparent instability of the morphological framework due to increasing difficulties of distinguishing homologies from recurrent convergences or homoplasies but, at the same time, also reflects the inconsistencies of most molecular data often based on incomplete sampling and, so far, on a single (namely 18S rDNA) or a few genes.

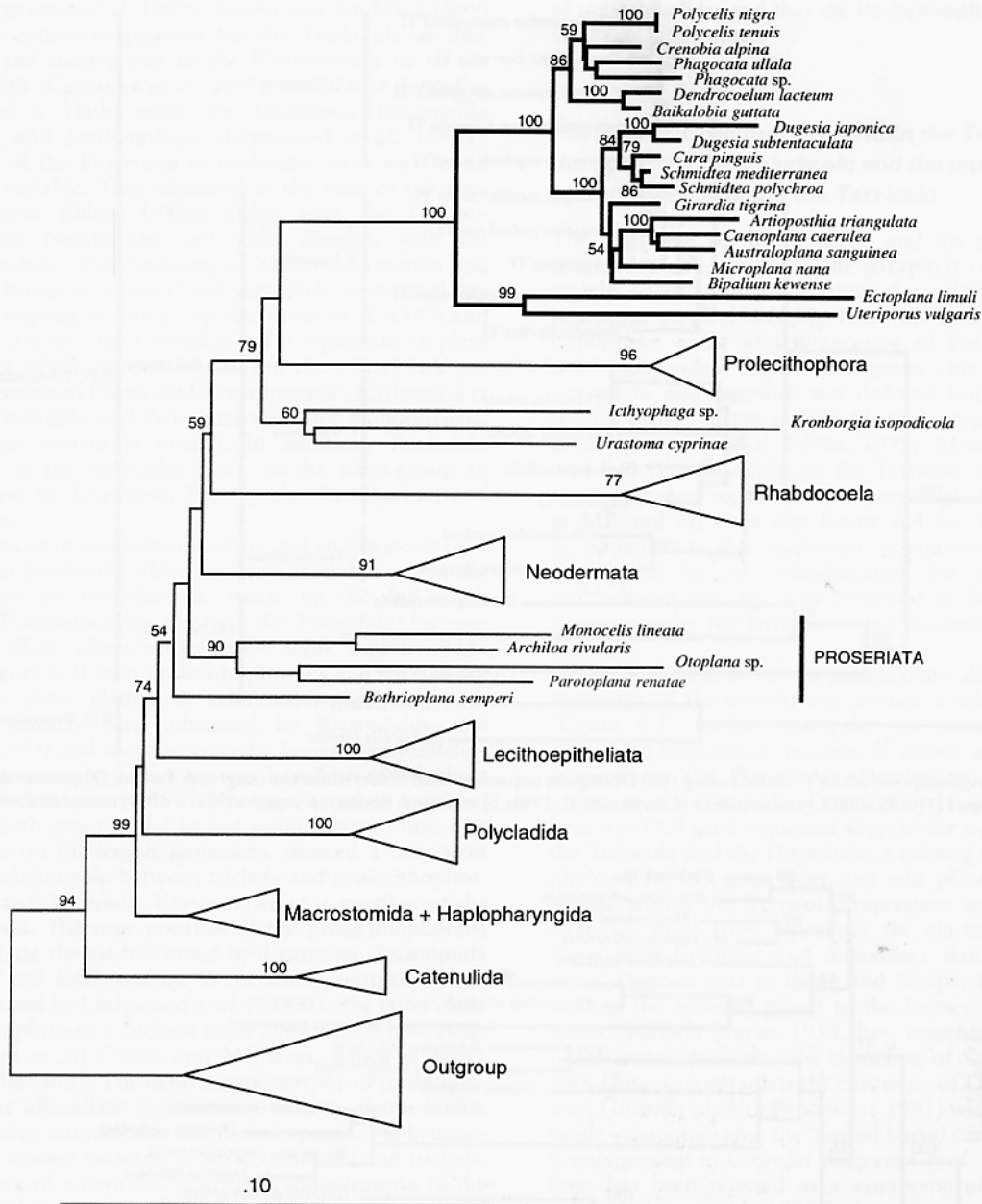


Figure 6.3 Neighbour-joining tree of the Platyhelminthes and other triploblasts (outgroup) based on 18S rDNA data set. Numbers on branches represent bootstrap percentages ($n = 1000$), only those over 50% being indicated. The tree illustrates the position of the Tricladida (top of the tree, bold lines) and its sister relationship to the Prolecithophora and the paraphyly of the Seriata. For taxa and species names, see Table 6.1. The complete tree with all species names is available on request from the authors. Scale indicates substitutions per position.

The validity of the taxon Seriata and the position of the Tricladida within the Platyhelminthes

The phylogenetic placement of the Tricladida within the Platyhelminthes is an example of these uncertainties. Most taxonomists consider the Tricladida a highly derived group of turbellarians, largely due to the rather complex morphological structure and to the advanced features of embryonic development. The precise phylogenetic position of the Tricladida and its sister-group relationships, however, have not been fully resolved. On the basis of a large set of morphological and embryological characters, Karling (1974) placed the Tricladida close to the Proseriata and not far from Prolecithophora and the

Rhabdocoela, a position also supported by Ehlers (1985a) and by Smith *et al.* (1986). Based on protonephridial structure, Rohde (1990) placed them close to the Polycladida whereas the Proseriata fell close to Neodermata. Finally, using a matrix of 65 equally weighted and unordered morphological characters, the 50% majority-rule consensus solution found also suggested that Tricladida and Proseriata do not constitute a monophylum (Littlewood *et al.* 1999a). The Tricladida was found, however, albeit with very poor support, within a clade together with Polycladida, Macrostomida and Haplopharyngida, whereas Proseriata appeared as the sister group of the Fecampiida. Recoding of some characters to take into account proposed synapomorphies for spermiogenesis between the

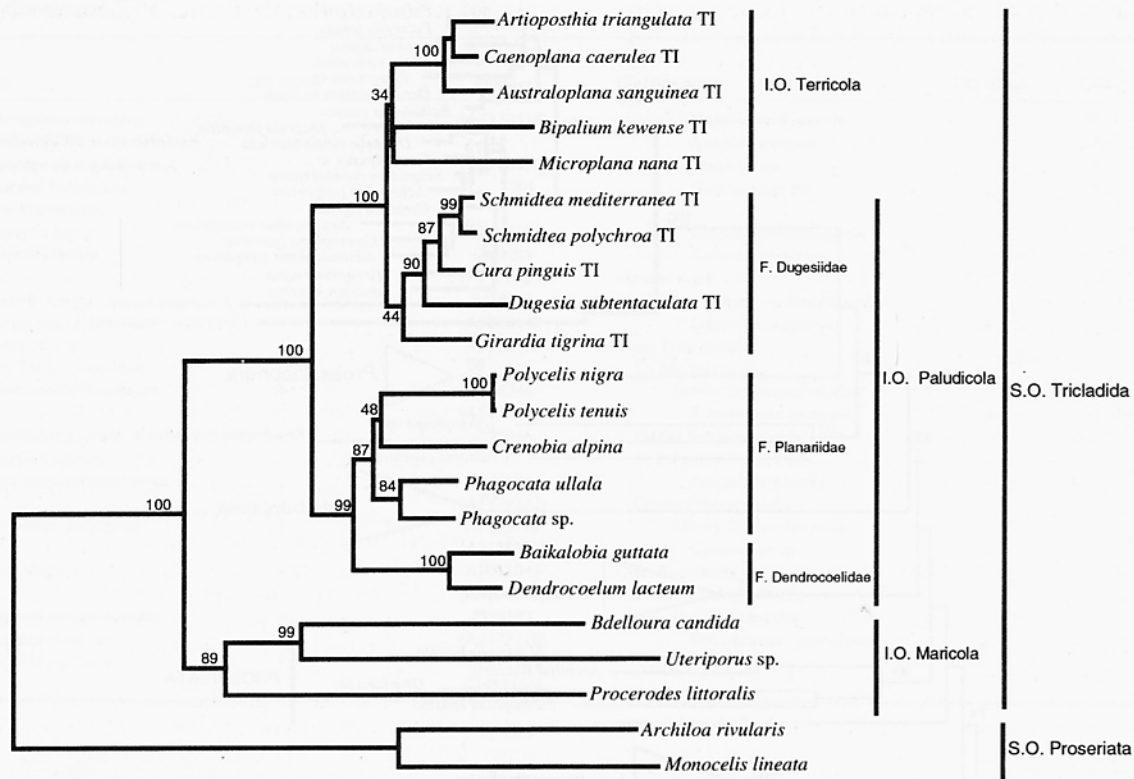


Figure 6.4 Neighbour-joining tree of the Tricladida using 18S rDNA gene sequences with Proseriata as the outgroup. For the Dugesiidae and the Terricola only the type I (TI) 18S rDNA gene sequence (Carranza *et al.* 1998a,b) was used. Bootstrap support (%; n = 1000) indicated above the nodes.

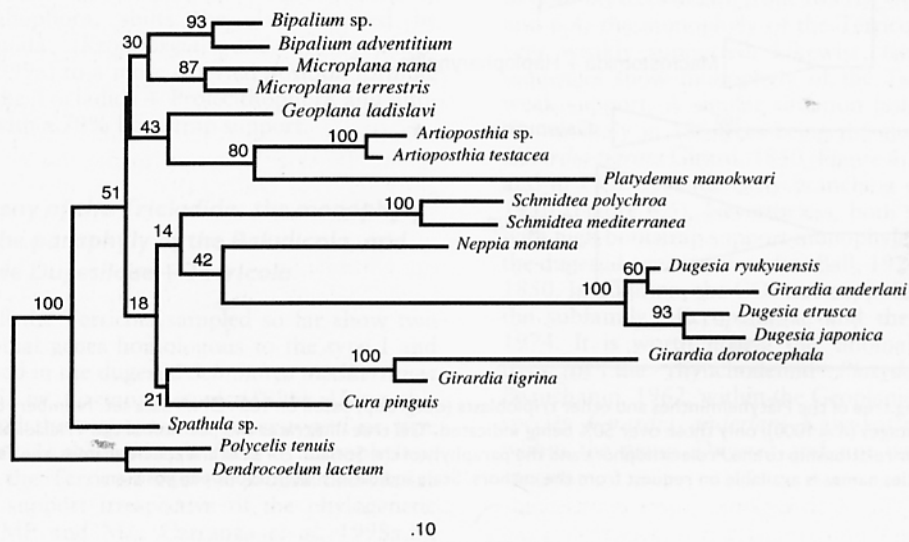


Figure 6.5 Neighbour-joining tree based on partial sequences of the cytochrome oxidase I gene of 21 species of Terricola and Paludicola. Only first and second positions were used for analysis. Numbers on branches represent bootstrap percentages (n = 1000). For taxa and species names, see Table 6.1. Scale indicates substitutions per position.

Fecampiida, *Urastoma* and the Neodermata (Joffe and Kornakova 1998), supported a new tree that, as regards the position of the Tricladida, did not differ substantially to those from previous analyses (Littlewood *et al.* 1999a).

Molecular data based on partial 18S and 28S rDNA sequences have added very little but confusion. Tricladids were found either: 1) close to Rhabdocoela (Rohde *et al.* 1995); 2) as a group close to the prolecithophorans (Kuznedelov and

Timoshkin 1995); 3) as a basal platyhelminth group branching early, second only to the acoels (Katayama *et al.* 1996); 4) as forming the sister-group to a clade made up of the Acoela and the rhabdocoel fecampiid *Kronborgia* (Jondelius 1998); 5) as the sister-group to the Acoela (Campos *et al.* 1998); and 6) as the sister-group of the Fecampiidae, both forming a sister-group to some rhabdocoels (Litvaitis and Rohde 1999). Studies based on complete 18S rDNA sequences (Carranza *et*

al. 1997; Littlewood *et al.* 1999a; Norén and Jondelius 1999) gave a more consistent position for the Tricladida as they appeared as the sister-group to the Rhabdocoela or to the Prolecithophora (Carranza *et al.* 1997; Norén and Jondelius 1999), or to a clade made by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* (Littlewood *et al.* 1999a). The position of the Proseriata in molecular trees was found to be rather variable. They clustered at the base of the neophorans (*sensu* Ehlers 1985a) either with the Lecithoepitheliata, the Neodermata or, oddly enough, with the Nemertodermatida. The clustering of Nemertodermatida and Proseriata is, however, unusual and very likely artefactual due to the poor sampling of this group (Carranza *et al.* 1997) and because there are no sound morphological arguments to place them together. When morphological and the 18S rDNA evidence were combined ('total evidence approach'; Littlewood *et al.* 1999a), Tricladida and Proseriata were not monophyletic, i.e., the taxon Seriata is invalid. In addition, Tricladida appeared, as in the molecular trees, as the sister-group to a clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga*.

New sequences of prolecithophorans and rhabdocoels now available from GenBank, added to those available within the large data set of turbellarians, made up 72 full-length sequences of Platyhelminthes (20 from the Tricladida) for analysis. Results show, albeit with a rather weak support (42% bootstrap; Figure 6.3), that prolecithophorans and triclads are monophyletic sister clades. A relationship between both groups had already been advanced by Kuznedelov and Timoshkin (1995) and more recently by Norén and Jondelius (1999). The first study, however, was based on rather short stretches (340–368 bp) of 18S rDNA from a rather restricted sampling of both groups, and lacked suitable outgroups. The second, based on full-length sequences, showed a consistent sister-group relationship between triclads and prolecithophorans and rejected the family Urastomidae as a member of the Prolecithophora. The new position of the prolecithophorans seems to exclude the clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* as the sister-group of the triclads as suggested by Littlewood *et al.* (1999a). The latter clade and prolecithophorans + triclads form altogether a new clade well supported in NJ (79%) and ML trees, which is worth analysing in the future. The main autapomorphy of prolecithophorans is the aflagellate spermatozoa with no dense bodies and an extensive intracellular membrane system. No synapomorphies are known between prolecithophorans and triclads, though the lack of sclerotized structures, some features of the protonephridial system and the pharynx, and the structure of the female copulatory apparatus are worth exploration. NJ and ML trees also supported the monophyly and the basal position of the Proseriata within the neophorans, confirming molecular analyses by Carranza *et al.* (1997) and Littlewood *et al.* (1999a). In addition, the Bothrioplanida, here represented by *Bothrioplana semperi*, and considered a proseriate (see Cannon 1986) or the sister-group of the Tricladida (Sopott-Ehlers 1985), was never seen clustering with the Tricladida. Instead it appears as a sister-group, albeit with weak support, to the Neodermata (ML trees), or to a large group of neophorans (NJ tree; Figure 6.3). Hence, the presumed synapomorphies linking the Bothrioplanida and the Tricladida (a tricladioid intestine and a crossing over of muscle layers at the pharynx; Sopott-Ehlers 1985) may be convergences and should be reassessed. To summarize, available evidence gathered from this as well as from previous molecular analyses, strongly suggests that the Order Seriata is not a valid taxon because the Proseriata and Tricladida are monophyletic unrelated taxa, that the Bothrioplanida is not the sister taxon

of the Tricladida, and that the Prolecithophora and Tricladida are likely sister-taxa.

Phylogenetic relationships within the Tricladida: the paraphyly of the Paludicola and the internal phylogeny of the Dugesiidae and the Terricola

The clustering of the Terricola and the paludicolan family Dugesiidae and, therefore, the paraphyly of the Paludicola is mainly based on their sharing of an 18S gene duplication (Carranza *et al.* 1998a,b). That this resulted from a single duplication event in the ancestor of both clades and not from independent duplication events after the split between terricolans and dugesiids was deduced from intra- and inter-specific genetic distances found between type I and type II 18S genes (Carranza *et al.* 1998a, 1999). Moreover, using either type I or type II sequences, the Terricola and the Dugesiidae always cluster together with very high bootstrap support in MP and NJ trees (see Figure 6.4 for Type I sequences). In addition to this molecular synapomorphy, a character thought to be an autapomorphy for the dugesiids, the multicellular eye cup, was proposed to be a morphological synapomorphy for terricolans and dugesiids (Carranza *et al.* 1998a).

The phylogenetic tree of paludicolans derived from partial sequences of the cytochrome oxidase c subunit I (COI) gene (Figure 6.5) further reinforces the evidence for a clade Terricola+Dugesiidae, because it shows a 100% bootstrap support for the Planariidae+Dendrocoelidae as the sister-group to the Terricola+Dugesiidae. However, neither 18S gene nor COI gene sequences support the monophyly of either the Terricola and the Dugesiidae rendering them, so far, paraphyletic. In 18S gene trees, the odd placement of *Girardia tigrina* within the Terricola, represents its main drawback. Even so, these trees reproduce the clustering of the genus *Cura* Strand, 1942, and *Schmidtea* Ball, 1974, with the genus *Dugesia* next to them, and *Neppia* Ball, 1974, usually making the external group to the former three. Instead, the genus *Spathula* Nurse, 1950, has, together with *Girardia*, a rather erratic position. The clustering of *Schmidtea* and *Cura* with *Dugesia* contradicts the clustering of *Cura* and *Schmidtea* with *Girardia* (de Vries and Sluys 1991) which was based on a single synapomorphy: the 'angled bursal canal'. This character is only present in *Girardia* (Carranza *et al.* 1998a) and, therefore, has been rejected as a synapomorphy for these three genera (Sluys *et al.* 1998a). In contrast to *Schmidtea* and *Dugesia*, for which more than one species have been sequenced for the 18S gene, only a single species each is so far available for the genera *Girardia* and *Neppia*. This makes the sampling too poor to draw any firm conclusion. Besides, *Neppia* and *Spathula* are poorly defined genera (Sluys 2001, this volume). A similar situation holds for the Terricola, only the clustering of the Geoplanidae (type I gene), represented by three species, being highly supported.

In COI trees, all terricolans and most dugesiids cluster together. However, the very low bootstrap support found does not permit any firm conclusion to be drawn regarding the monophyly of these two clades. Within the Terricola, and despite insufficient sampling, the family Bipaliidae and the subfamily Microplaninae of the Rhynchodemidae appear highly supported. However, the position of the rhynchodemid *Platydemus manokwari* (subfamily Rhynchodeminae) within the Geoplanidae (also found for type II 18S gene trees; Figure 6.4) suggests that the rhynchodemids are polyphyletic and the geoplaniids are paraphyletic. The family Rhynchodemidae has

always been considered an artificial assemblage, with subfamilies Rynchodeminae and Microplaninae being only loosely related (personal communication, Leigh Winsor 1998). The non-clustering of both subfamilies in both 18S and COI gene trees may reflect this situation, and deserves a better sampling and further studies. As regards the Dugesiidae, the clustering of *Dugesia* and *Schmidtea* and, more loosely, *Neppia* is reproduced as it was for the 18S gene tree, but the single *Cura* species, *Cura pinguis*, does not cluster with them. In addition, the single *Spathula* species falls outside the Dugesiidae.

A special mention must be made of the odd placement in COI trees of *Girardia anderlani* within the genus *Dugesia* (Figure 6.5). Taken at face value it may be either an artefact, a misclassified specimen, or that *Girardia anderlani* does not actually belong to *Girardia* but to *Dugesia*. *Girardia anderlani*, described so far from Brazil (Kawakatsu *et al.* 1983), is externally similar to other species belonging to the genus *Girardia* living in South America. However, it differs internally in the presence of dorsal testes (usually ventral in other *Girardia*, though a few also have dorsal testes) and in their large and very asymmetrical penial bulb. Moreover, its chromosome number of $2x = 18$; $n = 9$, differs to those of most *Girardia* species, usually bearing chromosome numbers of $2x = 8, 16$ or 24 ; $n = 4$ or 8 , the only exception being *Dugesia* (*Girardia*) *cubana* Codreanu and Balcesco, 1973, with $2x = 18$. Chromosome numbers of $2x = 18$ have been described for all species of the genus *Dugesia* belonging to the *Dugesia sicula* group (Baguñá *et al.* 1999). Therefore, given the clustering of *Girardia anderlani* within the *Dugesia* species in COI trees, and its specific karyotype, we suggest it may actually belong to the genus *Dugesia*. Otherwise, and considering that the genus *Dugesia* has not been reported from North or South America (Sluys *et al.* 1998a) some misclassification or cross-contamination may have occurred.

Main conclusions and prospects

To summarize, phylogenetic analyses of 18S ribosomal sequences of 72 Platyhelminthes species, including those of 20 species of triclads, together with the phylogenetic analyses of cytochrome oxidase subunit I partial gene sequences from 21 species of triclads show that: 1) the Tricladida is a monophyletic taxon not related to the Proseriata; therefore, validity of the taxon Seriata is rejected; 2) 18S sequence analyses show for both NJ and ML trees a sister-group relationship between the Tricladida and the Order Prolecithophora; 3) a new clade made by Tricladida + Prolecithophora and a clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* is well supported in both NJ and ML trees deserving further studies; 4) the Bothrioplanida does not appear in any of the analyses as the sister-group of the Tricladida; 5) 18S rDNA sequence analyses, the duplication event involving the 18S ribosomal gene, and COI sequence analyses, strongly support the paraphyly of the Paludicola and the validity of the Terricola + Dugesiidae clade; and 6) 18S rDNA and COI sequences do not validate so far, probably because of insufficient sampling, the monophyly of the Terricola and the Dugesiidae.

Altogether, the new sister-group relationship between the Tricladida and the Prolecithophora, together with 18S rDNA

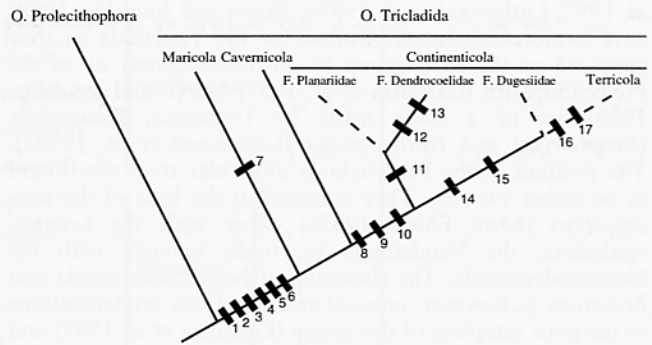


Figure 6.6 A new phylogenetic hypothesis for the Tricladida proposed in the present study. It illustrates the monophyly of the Tricladida and its sister-group relationships to the Order Prolecithophora, the monophyly of the Maricola, the clade formed by the Dugesiidae and the Terricola, and the recently proposed clade, the Continenticola, grouping the present families Dugesiidae, Planariidae, Dendrocoelidae and the Terricola. Selected morphological characters from Sluys (1989a) and the molecular apomorphy (character 15) from Carranza *et al.* (1998a) have been mapped onto the tree, with black prisms referring to derived characters. 1, tricladoid intestine; 2, crossing-over of pharynx muscles; 3, embryology; 4, cerebral position of female gonads; 5, serial arrangement of many nephridiopores; 6, marginal adhesive zone; 7, Haftpapillen in annular zone; 8, loss of Haftpapillen; 9, resorptive vesicles; 10, reduction in number of longitudinal nerve cords; 11, common oviduct opening into atrium; 12, dendrocoelid pharyngeal musculature; 13, anterior adhesive organ; 14, multicellular eye cup with numerous retinal cells; 15, two types of 18S rDNA genes (type I and type II); 16, creeping sole; 17, diploneuran nervous system.

and COI data reported in Carranza *et al.* (1998a,b) and in this work, support a new phylogenetic hypothesis for the Tricladida which is depicted in Figure 6.6.

To further support the conclusions here obtained, and to resolve the contradictions posed, further studies will be needed, including a denser sampling of Tricladida taxa for molecular data, complementary sequences from independent genes, and the broad and thorough morphological database already available for the Tricladida (Sluys 1989a, 1990; Sluys *et al.* 1998a), used together in a combined 'total evidence' approach.

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