The Royal Society

A robust molecular phylogeny of the Tricladida (Platyhelminthes: Seriata) with a discussion on morphological synapomorphies

Salvador Carranza[1], D. Timothy J. Littlewood[2,3], Karen A. Clough[2], Iñaki Ruiz-Trillo[1], Jaume Baguñà[1*] and Marta Riutort[1]

[1]*Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, Barcelona 08071, Spain*
[2]*Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
[3]*Division of Life Sciences, King's College London, Campden Hill Road, London W8 7AH, UK*

The suborder Tricladida (Platyhelminthes: Turbellaria, Seriata) comprises most well-known species of free-living flatworms. Four infraorders are recognized: (i) the Maricola (marine planarians); (ii) the Cavernicola (a group of primarily cavernicolan planarians); (iii) the Paludicola (freshwater planarians); and (iv) the Terricola (land planarians). The phylogenetic relationships among these infraorders have been analysed using morphological characters, but they remain uncertain. Here we analyse the phylogeny and classification of the Tricladida, with additional, independent, molecular data from complete sequences of 18S rDNA and 18S rRNA. We use maximum parsimony and neighbour-joining methods and the characterization of a unique gene duplication event involving the Terricola and the dugesiids to reconstruct the phylogeny. The results show that the Maricola is monophyletic and is the primitive sister group to the rest of the Tricladida (the Paludicola plus the Terricola). The Paludicola are paraphyletic since the Terricola and one paludicolan family, the Dugesiidae, share a more recent common ancestor than the dugesiids with other paludicolans (dendrocoelids and planariids). A reassessment of morphological evidence may confirm the apparent redundancy of the existing infraorders Paludicola and Terricola. In the meantime, we suggest replacing the Paludicola and Terricola with a new clade, the Continenticola, which comprises the families Dugesiidae, Planariidae, Dendrocoelidae and the Terricola.

Keywords: 18S rDNA; Tricladida; planarians; Platyhelminthes; phylogeny

1. INTRODUCTION

Within the free-living platyhelminthes the triclads or planarians are perhaps the best known group, largely as a result of intensive research concerning cellular regeneration, pattern formation and, most recently, *Hox* gene expression (Bayascas *et al.* 1997; for general reviews see Gremigni 1988; Baguñà *et al.* 1990, 1994). Planarians are numerous, diverse, and globally distributed, occurring in marine, limnetic and terrestrial environments (Ball & Reynoldson 1981). Recently, interest in the terrestrial planarians has been aroused as a result of the introduction of non-native predatory species in regions where they have achieved pest status, e.g. in the case of the New Zealand flatworm, *Artioposthia triangulata*, and its invasion of the British Isles and continental Europe (Boag *et al.* 1995; Jones & Boag 1996). In view of their biological importance in so many fields, a robust phylogenetic scheme for the planarians is required for both taxonomic and comparative purposes.

The Tricladida, which is best considered a suborder (Ehlers 1985), forms part of the order Seriata; other less well known seriates are grouped under the suborder Proseriata (Meixner 1938; Ax 1961; Karling 1974). Although most morphological data pointed to the Seriata as a monophyletic taxon, new data on the ultrastructure of the excretory system suggests instead a rather basal location for Proseriata and, therefore, the paraphyly of Seriata (Rohde 1990). Moreover, molecular data (18S rDNA sequences; Carranza *et al.* 1997) also indicate that the Seriata are possibly paraphyletic with the Tricladida grouping with the Rhabdocoela and the Proseriata occupying a rather basal position close to the parasitic Neodermata (*sensu* Ax 1996).

Systematists have recognized for a long time three major groups within the Tricladida, for which they used Hallez's (1890) ecological names: Paludicola (freshwater planarians), Terricola (land planarians), and Maricola (marine planarians). In a further entry on triclad taxonomy and phylogeny, Sluys (1990) proposed the new infraorder Cavernicola (represented by three species), which grouped taxa formerly assigned to the Maricola but with apparent closer affinities to the Paludicola. The taxonomic rank of these groups has shifted between that of suborder and infraorder, the latter being the accepted grouping today. The systematic and phylogenetic relationships of these infraorders have been discussed on the basis of morphological and ultrastructural characters by Ball (1977, 1981), Sopott-Ehlers (1985), and Sluys (1989*a*). Within triclads, Ball (1977, 1981; see figure 1*a*) considered

*Author for correspondence (bagunya@porthos.bio.ub.es).

Received 18 November 1997 *Accepted* 1 December 1997

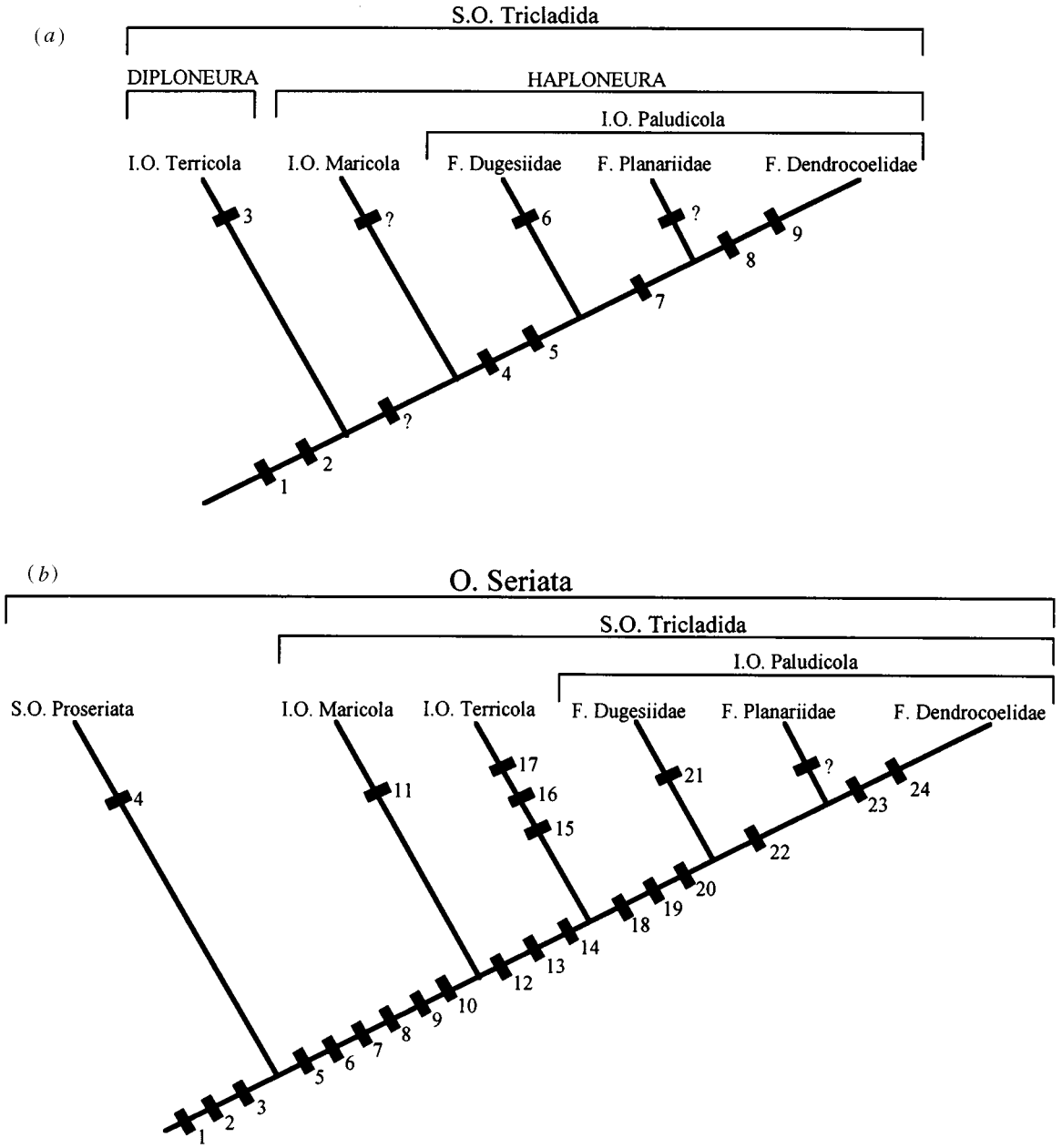

Figure 1. (*a*) Phylogenetic relationships of the Tricladida according to Ball (1977, 1981) with the hypothesized autapomorphies for each phyletic line (black rectangles). 1, germaria anterior in female gonads; 2, tricladoid intestine; 3, diploneural nervous system; 4, reduced cephalic duct; 5, probursal condition; 6, multicellular eye-cup; 7, common oviduct opening into atrium; 8, presence of anterior adhesive organ; 9, inner pharyngeal muscles intermingled; ?, unknown character. (*b*) Phylogenetic relationships of the Tricladida according to Sluys (1989) with the hypothesized autapomorphies for each phyletic line (black rectangles). 1, serial arrangement of testes and vitellaria; 2, backwards-directed tubiform and plicate pharynx; 3, elongated body shape; 4, loss of lamellated rhabdites; 5, tricladoid intestine; 6, crossing-over of pharynx muscles; 7, embryology; 8, cerebral position of female gonads; 9, serial arrangement of many nephridiopores; 10, marginal adhesive zone; 11, Haftpapillen in annular zone; 12, loss of Haftpapillen; 13, resorptive vesicles; 14, reduction number of longitudinal nerve cords; 15, creeping sole; 16, pharynx musculature; 17, diploneuran nervous system; 18, four subepidermal muscle layers; 19, spermatophore; 20, probursal condition; 21, dugesiid eye; 22, common oviduct opening into atrium; 23, dendrocoelid pharyngeal musculature; 24, anterior adhesive organ; ?, unknown character.

Terricola (defined by its complex diploneural nervous system; character 3, figure 1*a*) as the sister group of an undefined (no synapomorphies disclosed) clade made by Maricola and Paludicola (Haploneura). Whereas no synapomorphies were found for the Maricola, two presumed synapomorphies defined the Paludicola: their reduced precerebral diverticula and the probursal condition of the intestine (characters 4 and 5, respectively, in figure 1*a*). Biogeographical considerations, the apparent weaknesses of synapomorphies defining Paludicola, and the need to preserve a unique origin for the complex dugesiid eye led Ball (1981) to suggest a diphyletic origin of Paludicola and separate origins for Maricola and Terricola from marine proseriate-like ancestors.

Reassessment of previously used characters and the introduction of new ones were the basis of a phylogenetic scheme by Sluys (1989*a*) (figure 1*b*). The new characters were found to support the monophyly of the Tricladida,

the Terricola, the Maricola and the Paludicola, as well as to suggest the existence of a new clade, the Terricola–Paludicola clade (see figure 1*b*). All infraorders were considered monophyletic. The Cavernicola were provisionally placed between the Terricola and the Paludicola.

Relationships within the infraorders have been considered in some detail within the Paludicola. Ball (1974*a*) recognized three families: Dugesiidae Ball 1974, Planariidae Stimpson 1857 and Dendrocoelidae Hallez 1894. The Planariidae and Dendrocoelidae are considered derived groups defined by one autapomorphy (character 22; figure 1*b*) (Ball 1974*a*). Together, the Planariidae and the Dendrocoelidae are the sister-group of the F. Dugesiidae (Ball 1981), defined by their peculiar eye structure (character 21, figure 1*b*). There are, however, some uncertainties. First, no autapomorphies defining the family Planariidae were presented. Second, the eye structure of the Dugesiidae resembles that of the land planarians (Terricola) more closely than the non-dugesiid members of the Paludicola which, in turn, are more similar to the Maricola and lower 'Turbellaria' (Ball 1981; Sluys 1989*a*,*b*). The internal phylogeny of the Maricola has been dealt with in detail by Sluys (1989*b*). No similar attempt has been undertaken for the Terricola.

An independent method to test the current phylogeny of the Tricladida, and to overcome some of the present difficulties associated with assessing morphological homology, is to employ molecular systematics. We have chosen 18S ribosomal genes. Molecular data obtained from ribosomal RNA or DNA offer an important new source of informative characters for inferring phylogenetic relationships at several levels, and provide an independent test of hypotheses based on morphological characters (Woese 1987; Field *et al.* 1988; Adoutte & Philippe 1993; Riutort *et al.* 1993; Carranza *et al.* 1997). The main reasons for using 18S rDNA or rRNA sequences are reviewed by Woese (1987) and Sogin (1991). The 18S rRNA gene belongs to a multigene family, copies of which remain homogeneous as a result of concerted evolution (Dover 1982; Hillis & Dixon 1991). Nevertheless, there are some exceptions to this rule (Gunderson *et al.* 1987; Carranza *et al.* 1996; Telford & Holland 1997). We showed previously (Carranza *et al.* 1996) that two types (types I and II) of 18S rDNA are present in the genome of *Schmidtea mediterranea*. A phylogenetic tree was drawn showing the position of the *S. mediterranea* type I and type II sequences clustering within the Tricladida. Moreover, the tree indicates that the duplication event should be ancient, affecting at least the genera *Schmidtea*, *Dugesia* and *Girardia*, but not affecting the representatives of the families Dendrocoelidae and Planariidae.

We report here the complete 18S rRNA and/or rDNA sequences of three species of Maricola, five species of Terricola, and nine species of Paludicola. Published 18S rDNA sequences from two Proseriate and from three Paludicola were also included in the phylogenetic analysis. The aims of this paper are to (1) analyse the phylogenetic relationships between the three infraorders (Maricola, Terricola and Paludicola); (2) test the monophyletic or paraphyletic status of the three infraorders; and (3) analyse the internal relationships within the Paludicola. New data are interpreted in the light of existing morphological evidence.

2. MATERIAL AND METHODS

Table 1 shows the current taxonomic classification of the species used in this study.

(a) *Sequencing of the 18S molecule from RNA*

Total cellular RNA was isolated by the guanidine isothiocyanate method (Chirgwin *et al.* 1979) from live individuals from all the representatives of the infraorder Terricola sampled for this study, four representatives of the family Dugesiidae, and five representatives of the F. Planariidae and F. Dendrocoelidae (table 1). All the RNA extracted from three individuals of each species was resuspended in 20 µl of H_2O DEPC to which 15 units of DNAse (RNAse-free) were added. The mixture was kept at 37 °C for 20 mins. After the incubation period, the RNA was precipitated. Around 1 µg of RNA from each species was retrotranscibed to single-stranded cDNA with the AMV reverse transcriptase enzyme. The reaction conditions were as follows: 5 µl of annealing mix (1 µg of RNA, 10 pmol of the 9R primer and H_2O DEPC to 5 µl) were heated to 65 °C for 10 min, transferred to 37 °C for 10 min and subsequently chilled on ice. 20 µl of reaction mix (1 µl of dNTP's 10 mM, 40 units of RNAse inhibitor, 25 units of AMV reverse transcriptase, 5 µl of AMV buffer (× 5) and H_2O DEPC to 20 µl) were added to the tube, and the mixture was kept at 42 °C for 45 min. The tube was then transferred to a water bath at 100 °C for 5 min to inactivate the AMV enzyme, before adding 75 µl of distilled water to the mix. PCR amplifications covering the entire length of the 18S molecule were performed with 1 µl of this 18S cDNA single-strand solution using specific primers and following PCR conditions described earlier (Carranza *et al.* 1996; Littlewood & Smith 1995). The PCR fragments were directly sequenced using an automated sequencer ABI 377 following the manufacturer's protocols.

(b) *Sequencing of the 18S molecule from DNA*

High molecular weight genomic DNA (gDNA) was purified using a standard phenol–SDS extraction procedure (Sambrook *et al.* 1989) from live or ethanol-fixed specimens from the same species for which RNA extraction was performed, and also from *Polycelis tenuis*, *Phagocata sibirica*, *Baikalobia guttata*, *Bdelloura candida*, *Uterioporus* sp., and *Procerodes littoralis*. The entire length of the 18S rDNA molecule was PCR-amplified, applying specific primers and conditions described earlier (Carranza *et al.* 1996; Littlewood & Smith 1995). The PCR fragments amplified from taxa which were used for RNA extraction were cloned into the pUC 18 vector as described in Carranza *et al.* (1996): other amplification products were sequenced directly. Sequencing of the clones and the PCR products was performed using an automated sequencer ABI 377, following the manufacturer's protocols.

(c) *Sequence alignment and phylogenetic analysis*

Sequence data were aligned with ClustalW (Thompson *et al.* 1994), and subsequently by hand, with reference to published secondary structure using an alignment editor (GDE; Smith *et al.* 1994). Alignment gaps were inserted to account for putative length differences between sequences. A total of 1562 unambiguously aligned positions were used in the phylogenetic analyses, 696 being variable and 390 being parsimony informative when all the taxa are compared. The full sequence alignment used in these analyses is available on request from the authors. Full data sets were analysed using both maximum parsimony (MP) and neighbour-joining (NJ) under a maximum likelihood model using the algorithms in PAUP* (Swofford 1998). For MP we

Table 1. *Type of 18S sequence, accession numbers of the sequences and taxonomic classification for the 22 species sampled for this study*
(Note: new sequences reported in this paper are marked #. O, order; S.O., suborder; F, family; I.O., infraorder.)

Phylum Platyhelminthes	type of 18S sequences	accession number
O. Seriata		
S. O. Proseriata		
F. Monocelididae		
Monocelis lineata	only one type	U45961
Archiloa rivularis	only one type	U70077
S.O. Tricladida		
I.O. Maricola		
F. Uteriporidae		
Uteriporus sp. #	only one type	AF013148
F. Procerodidae		
Procerodes littoralis #	only one type	Z99950
F. Bdellouridae		
Bdelloura candida #	only one type	Z99947
I.O. Terricola		
F. Geoplanidae		
Artioposthia triangulata #	type I and type II	AF033038, Z99945
Caenoplana caerulea #	type I and type II	AF033040, AF033046
Australoplana sanguinea #	type I and type II	AF033041, AF033047
F. Bipaliidae		
Bipalium kewense #	type I and type II	AF033039, AF033045
F. Rhynchodemidae		
Microplana nana #	type I and type II	AF033042, AF033048
I.O. Paludicola		
F. Planariidae		
Polycelis nigra #	only one type	AF013151
Polycelis tenuis #	only one type	Z99949
Crenobia alpina	only one type	M58345
Phagocata ullala #	only one type	AF013149
Phagocata sibirica #	only one type	Z99948
F. Dendrocoelidae		
Dendrocoelum lacteum	only one type	M58346
Baikalobia guttata #	only one type	Z99946
F. Dugesiidae		
Schmidtea mediterranea	type I and type II	U31084, U31085
Schmidtea polychroa #	type I and type II	AF013152, AF013154
Cura pinguis #	type I and type II	AF033043, AF033049
Dugesia subtentaculata #	type I and type II	M58343, AF013155
Girardia tigrina #	type I and type II	AF013157, AF013156

determined the most parsimonious solutions with the branch and bound option; gaps were considered as fifth state. In the distance analysis we first reconstructed trees under the LogDet/paralinear option (Lake 1991; Lockhart *et al.* 1994), and then optimized a maximum likelihood model onto this tree by multiple iterative remodelling, estimating in turn the following parameters: the transition–transversion ratio, the proportion of invariable sites, and gamma distribution, until we reached the lowest log-likelihood value for the tree. We then reconstructed a NJ (minimum evolution) tree using these model parameters under a maximum likelihood model. Both MP and NJ (minimum evolution) trees were bootstrap resampled ($n=1000$) to indicate branch support. To root the trees, two representatives of the suborder Proseriata (*Monocelis lineata* and *Archiloa rivularis*) were chosen. Although proseriates are not the sister-group of the triclads, they are nevertheless a closely-related taxon (Sluys 1989*a*; Ax 1996; Carranza *et al.* 1997).

To determine the statistical difference between the most parsimonious solutions suggested by the molecular data and the morphologically based tree suggested by Sluys (1989*a*), we used a backbone constraint tree (outgroup (Maricola (Terricola (Dugesiidae (Planariidae (Dendrocoelidae)))))) to determine the tree length of the most parsimonious solution of the molecular data. The unconstrained and constrained trees were compared using the test of Kishino & Hasegawa (1989).

3. RESULTS

Two types of 18S ribosomal genes homologous to the type I and type II genes, already described (Carranza *et al.* 1996), have been found in all the dugesiid and all the Terricola sampled for this study. As stated in Carranza *et al.* (1996) the 18S type I sequences were obtained when PCR amplification and sequencing were carried out from

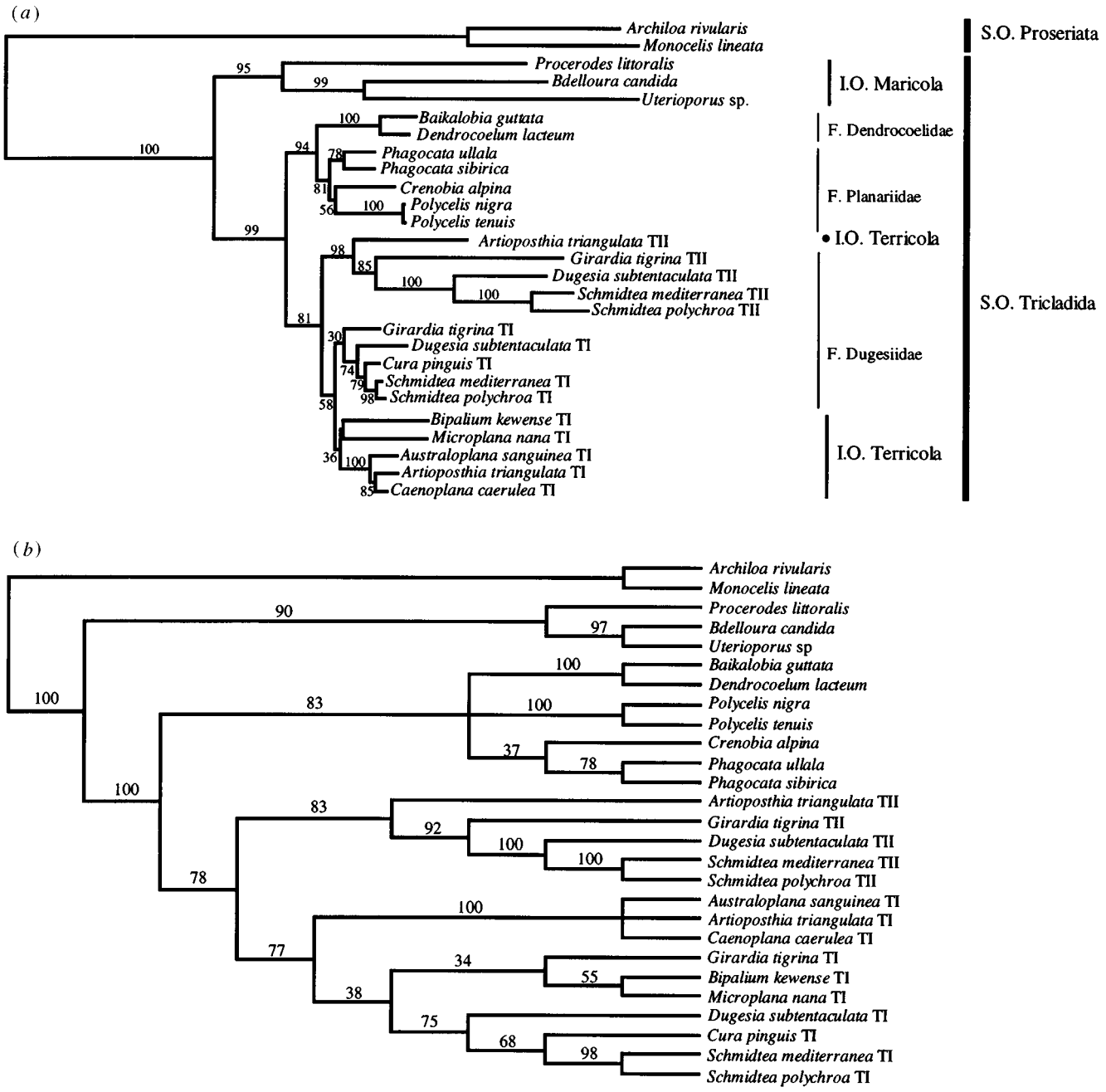

Figure 2. (*a*) Neighbour-joining tree using the minimum evolution HKY85 model in PAUP* (see text for details; Swofford 1998); transition/transversion ratio = 1.31201; ln likelihood = 8547.51398; gamma rate distribution with shape parameter = 0.91403. Bootstrap support (%; $n = 1000$) indicated at nodes. (*b*) Strict consensus tree of the four most parsimonious trees found using the branch-and-bound algorithm in PAUP*. Tree length = 1317; consistency index excluding uninformative characters = 0.5312; retention index = 0.6318. Bootstrap support (%; $n = 1000$) indicated above the nodes.

the RNA source, and the 18S type II sequences were obtained when PCR amplification and sequencing were carried out from the DNA source. In all the rest of the Tricladida only one type of 18S molecule was found, although in some of them (see § 2) the 18S molecule was also independently PCR-amplified and sequenced from both DNA and RNA sources.

While full length sequences of the type I 18S molecule were obtained for all the Dugesiidae and Terricola included in this study, full length sequences of the 18S rDNA type II genes were only obtained for three representatives of the Dugesiidae (*S. polychroa*, *D. subtentaculata*, and *G. tigrina*) and one representative of the Terricola (*Artioposthia triangulata*). For the other four Terricola (see table 1) and for *C. pinguis* we sequenced a small fragment (200 bp) of the most variable zone of the type II 18S gene that clearly shows that they are polymorphic with respect to the type I gene (see table 1 for accession numbers).

(a) *Phylogeny of the Tricladida*

Figure 2*a*,*b* shows the NJ (minimum evolution) and MP trees, respectively. Both trees have the same general topology and show that:

(1) the Maricola (Procerodidae, Bdellouridae, and Uteriporidae) are monophyletic, with high bootstrap support, and appear to be the primitive sister-group to the rest of the Tricladida (the Terricola plus the Paludicola).

(2) the Paludicola are paraphyletic, since the Terricola and the Dugesiidae share a more recent common ancestor than the Dugesiidae with the other representatives of the Paludicola (dendrocoelids and planariids).
(3) both trees support the hypothesis that the 18S duplication event, giving the type I and type II 18S sequences, took place in the common ancestor of the Dugesiidae–Terricola clade.
(4) the monophyly of the Terricola (inferred from the type I sequences) is supported by a very low bootstrap value in the NJ tree (36%), and is not supported in the MP analysis where *G. tigrina* is the sister taxon of the clade formed by *Bipalium kewense* and *Microplana nana*.
(5) the monophyly of the family Dugesiidae (inferred from the type I sequences) is supported by a very low bootstrap value in the NJ tree (30%), and is not supported in the MP analysis for the same reasons as discussed in point (4).
(6) within the family Dugesiidae, the genera *Dugesia*, *Cura*, and *Schmidtea* cluster together with a rather high bootstrap support (74 and 75% for the NJ and MP analyses, respectively).
(7) the Planariidae–Dendrocoelidae clade is monophyletic (94 and 83% bootstrap support for the NJ and MP analyses, respectively)
(8) the Planariidae and Dendrocoelidae appear as monophyletic groups in the NJ tree (81 and 100% bootstrap support respectively), while in the MP analysis only the Dendrocoelidae is strongly supported (100% bootstrap value).

A backbone constraint analysis forcing the topology of Sluys (1989*a*) was performed using type I sequences only and resulted in 11 equally parsimonious solutions (length=1046; CI=0.7563; RI=0.659). These trees were 15 steps longer than the unconstrained solution and were statistically different from the most parsimonious solution under the Kishino–Hasegawa test ($p<0.03$), suggesting the molecular data were inconsistent with the tree topology proposed by Sluys (1989*a*), thus preventing a total evidence approach (Huelsenbeck *et al*. 1996)

4. DISCUSSION

In his morphologically based phylogenetic analysis of the triclads, Sluys (1989a) accumulated 'the strongest possible evidence available' and put forward a hypothesis which we have been able to test with additional, independent molecular data based on complete sequences of 18S rDNA and 18S rRNA. Our new robust hypothesis suggests a taxonomic revision of the group that includes a remarkable molecular synapomorphy involving a gene duplication event.

(a) Evidence for a Terricola–Dugesiidae clade

There are two important features that support the Terricola–Dugesiidae clade. First, in both distance and MP analyses (inferred either from the type I or the type II 18S sequences), the Terricola and the dugesiids cluster together as a monophyletic group with very high bootstrap support (see figure 2*a*,*b*). Second, they share an 18S gene duplication that can be explained only by accepting a common origin for the Terricola and the Dugesiidae (see figure 2*a*,*b*). This is because divergence between the type I and the type II genes within any species is greater than the divergence among the type I or the type II genes between species. Had independent duplications occurred, i.e. duplications after the split between dugesiids and terricolans, type I and II genes within any species would be more similar than type I or type II genes between species. This duplication event, as well as data from the distance and the MP trees, provides very strong evidence for the paraphyly of the Paludicola and support for the Dugesiidae and the Terricola as a monophyletic clade.

The conclusions from our molecular data are inconsistent with previous phylogenetic morphology-based analyses of the suborder Tricladida (Ball 1981; Sluys 1989*a*) (see figure 1*a*,*b*). Sluys (1989*a*) described three characters that supported the monophyly of the Paludicola: (i) a probursal condition; (ii) sperm transfer through spermatophores; and (iii) subepidermal musculature consisting of four layers.

(i) *Probursal condition*

As Ball (1981) noted, probursal condition is a positional character and, by the generally accepted criteria of homologous relationships (Riedl 1978), must be considered a weak defining character. Although it is clear that within the Tricladida the probursal condition is a derived feature, this character has been acquired independently in some marine triclads of the genera *Probursa* (Hyman 1944) and *Pacificides* (Holmquist & Karling 1972; Ball 1981) as well as in species of *Balliana*, *Opisthobursa*, *Eviella* and *Mitchellia* that belong to the Cavernicola. Conversely, there are several marine triclads in which the bursa lies above the male atrium or above the genital duct (e.g. *Cercyra hastata*, *Obrimoposthia wandeli*, *Pacifides psammophilus*; Sluys 1989*a*). The copulatory bursa in the terrestrial planarians (known as receptaculum seminis in most literature; Minelli 1977) is a highly variable character (Minelli 1974; Ball & Reynoldson 1981; Ball & Sluys 1990). Some species present a probursal condition, while in others the bursa canal curves antero-dorsally, so that the bursa lies above the atrium or elsewhere. In other species the copulatory bursa is absent. The variability in the morphology of the bursa copulatrix in the maricolan and terricolan planarians, in addition to its absence in several terricolans and maricolans, supports the idea that the probursal condition is a poor morphological character for phylogenetic inference in the Tricladida.

(ii) *Sperm transfer through spermatophores*

This character has been described for species of the genera *Dugesia*, *Planaria*, *Polycelis*, *Phagocata*, *Neppia* and *Spathula,* although in *Dugesia seclusa* only free sperm were observed (Sluys & De Vries 1988; for a review, see Sluys 1989*a*). In *Opisthobursa mexicana*, a cavernicolan, a spermatophore-like structure was described in the copulatory bursa (Benazzi & Giannini 1973). In both the Maricola and the Terricola it seems that free sperm is discharged into the partner during the copulation process (Sluys 1989*a*; Meixner 1928). Nevertheless, Heinzel (1929) observed a spermatophore-like structure in the atrium of the terricolan *Platydemus victoriae* (Sluys 1989*a*). Although this character seems a good synapomorphy for the Paludicola (Ball 1981; Sluys 1989*a*), our analysis suggests that it could be a convergence for the freshwater planarians.

(iii) *Subepidermal musculature consisting of four layers*

In the Paludicola, the subepidermal musculature consists of a layer of circular muscles directly beneath the basement membrane, a thin layer of (outer) longitudinal muscles, a diagonal layer, and an inner longitudinal layer. The Terricola and the Maricola lack the outer longitudinal muscle layer and only three subepidermal muscles layers are present (Sluys 1989*a*). The presence of an extra (outer) longitudinal muscle layer in the subepidermal musculature was proposed as a synapomorphy for the Paludicola (Sluys 1989*a*). As noted by Sluys (1989*a*), the extra layer of outer longitudinal muscles is very thin, consisting of only one row of fibres, and it is usually difficult to discern. Moreover, this layer may not be present on every part of the body, and Sluys (1989*a*) pointed out that some individuals of a given species had it whereas others did not. It is evident that within the Paludicola this character should be checked carefully using new and more powerful techniques, such as confocal microscopy.

Apart from the morphological characters mentioned above that have been hypothesized as synapomorphies that apparently support the monophyly of the Paludicola (Sluys 1989*a*), there is one other character that warrants consideration under our new phylogenetic scheme, namely (iv) the dugesiid eye structure.

(iv) *Dugesiid eye structure*

The eye structure of planarians consists of a retinal cup that may be a single cell or may be multicellular, and a part consisting of bipolar neurons, from which the photosensitive dendritic ends project into the pigment cup (Ball 1981; Sluys 1989*a*,*b*). Eye structure in the Maricola and in the non-dugesiid members of the Paludicola is characterized by a unicellular eye cup with, usually, only a few retinal cells. In the Dugesiidae, however, the pigment cup is multicellular and contains numerous retinal cells (Ball 1981; Sluys 1989*a*,*b*). This peculiar eye structure has been considered a derived feature, constituting a synapomorphy for the Dugesiidae (Ball 1977, 1981; Sluys 1989*a*,*b*). Nevertheless, the Terricola, with the exception of bipaliids, have always complicated the picture since they also possess multicellular eye cups and numerous retinal cells. Although the character of a multicellular eye cup and numerous retinal cells was considered as evidence for convergence in the Terricola and the Dugesiidae in morphological approaches (Ball 1977, 1981; Sluys 1989*a*), it could also be interpreted as a synapomorphy, thus supporting the new Terricola–Dugesiidae clade proposed here. However, the problem is rather complex due to several differences (described below) in the eye structure of the Terricola and the dugesiids:

Connection with the central nervous system

In dugesiids, the optic nerves connect the eyes with the brain. But in land planarians, the optic nerves meet the dermal nerve plexus (von Graff 1912–1917; Sluys 1989*b*).

The orientation of the retinal clubs inside the retinal cups

In land planarians at least, a number of retinal cells face the opening of the pigment cup. However, in the dugesiids the retinal clubs face the pigment cup (Sluys 1989*b*).

The way the dendrites enter the eye cup

In the dugesiid eye, the dendrites enter the eye cup via its opening. But in land planarians, the dendrites penetrate between the pigment cells (Sluys 1989*b*).

These three differences are here considered as secondary modifications of basic eye structure (multicellular eye cup and numerous retinal cells).

Nevertheless, under this hypothesis, the Bipaliidae represent an anomaly. They bear multiple marginal eyes formed by one pigment cell, housing 1–8 retinal cells (Shirasawa & Makino 1981; Sluys 1989*b*), similar to those of the Maricola and non-dugesiid Paludicola. Pending further studies of more species within this family, the most parsimonious explanation under the molecular phylogenetic hypothesis presented here is the secondary loss of the multicellular pigment cell cup in this family.

To summarize, the three morphological synapomorphies proposed by Sluys (1989*a*) supporting the monophyly of the Paludicola appear to us either as weak positional characters (e.g. probursal condition) or insufficiently studied characters (e.g. sperm transfer through spermatophores and subepidermal musculature consisting of four layers) which need a thorough analysis using a larger set of species. In addition, the multicellular eye structure of the dugesiids and most land planarians, considered as a convergence by Sluys (1989*a*), should be reassessed in the light of our molecular data as it may represent a new important synapomorphy for the Dugesiidae–Terricola clade.

(b) *Phylogenetic relationships within the Terricola–Dugesiidae clade*

The phylogenetic relationships within the new Terricola–Dugesiidae clade are also of interest and deserve comment because it is important to establish whether the Terricola and the Dugesiidae are monophyletic groups. The NJ tree in figure 2*a* shows that, in the comparisons of the type I sequences, members of the Terricola and the Dugesiidae cluster independently as monophyletic groups, albeit with low bootstrap values (36% and 30%, respectively). Moreover, the MP analysis (figure 2*b*) does not solve the phylogenetic relationships at this level, only recognizing as monophyletic groups the genera *Dugesia*, *Cura* and *Schmidtea* for the Dugesiidae (bootstrap value of 75%), and the three representatives of the family Geoplaniidae (bootstrap value of 100%). Due to the low bootstrap value in the NJ tree, the polytomy obtained in the MP analysis, the fact that some genera of the family Dugesiidae (*Romankenkius*, *Neppia*, *Spathula* and *Reynoldsonia*) could not be studied, and the paucity of rhynchodemids and bipaliids examined, the phyletic status of the Terricola and the Dugesiidae cannot be established with the molecular approach presented here.

From a morphological point of view, the monophyletic status of the infraorder Terricola has never been questioned (Ball 1977, 1981; Sluys 1989*a*). Sluys (1989*a*) proposed three synapomorphies for the Terricola, involving pharyngeal musculature, a creeping sole, and a diploneural nervous system. Given its high variability, it is questionable that the more complex pharyngeal musculature of land planarians, as compared to those of maricolans and most paludicolans, could be taken as a good synapomorphy of the group. Instead, the diploneural

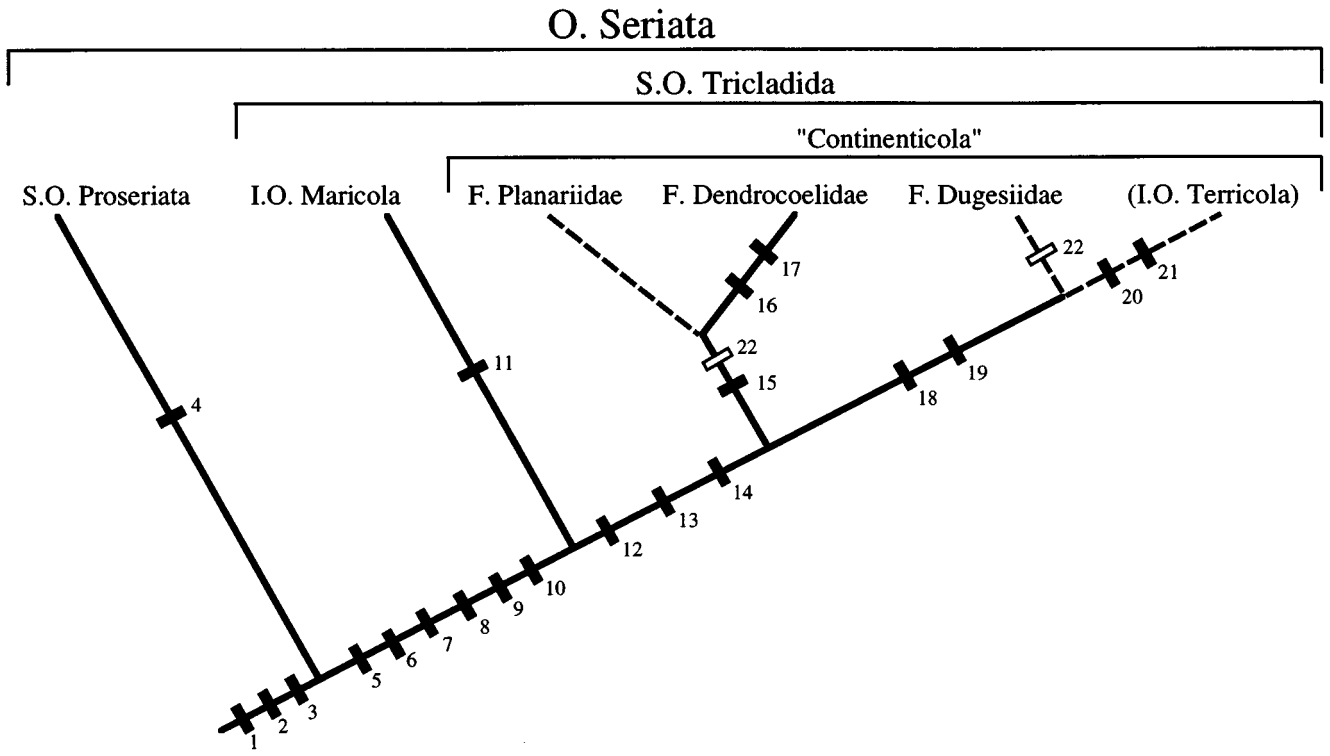

Figure 3. Phylogenetic hypothesis for the Tricladida proposed in the present study. Selected morphological characters from Ball (1981) and Sluys (1989) have been mapped onto the tree, with black rectangles referring to derived characters, and white rectangles to convergences. 1, serial arrangement of testes and vitellaria; 2, backwards directed tubiform and plicate pharynx; 3, elongated body shape; 4, loss of lamellated rhabdites; 5, tricladoid intestine; 6, crossing-over of pharynx muscles; 7, embryology; 8, cerebral position of female gonads; 9, serial arrangement of many nephridiopores; 10, marginal adhesive zone; 11, Haftpapillen in annular zone; 12, loss of Haftpapillen; 13, resorptive vesicles; 14, reduction number of longitudinal nerve cords; 15, common oviduct opening into atrium; 16, dendrocoelid pharyngeal musculature; 17, anterior adhesive organ; 18, multicellular eye cup with numerous retinal cells; 19, two types of 18S rDNA genes (type I and type II); 20, creeping sole; 21, diploneuran nervous system; 22, spermatophores. Dashed lines indicate groups that are not well-supported in the molecular phylogenetic analysis.

nervous system and the creeping sole have always been considered very important synapomorphies for the Terricola (Steinböck 1925; Ball 1974*a*; Sluys 1989*a*) as they represent adaptations to the terrestrial lifestyle. Although the low bootstraps supporting the monophyly of the Terricola in our distance and MP trees (figure 2*a*,*b*) would be congruent with a parallel independent evolution of both structures from ancestors not bearing them, a most parsimonious explanation more in agreement with the present data is a unique event followed by diversification.

The monophyletic status of the Dugesiidae is supported from a morphological point of view by the character 'peculiar eye structure consisting of a multicellular eye cup and numerous retinal cells' (Sluys 1989*a*). We propose that character is a synapomorphy for the new Terricola–Dugesiidae clade. Although there are several differences between the eye structures of the Dugesiidae and the land planarians (see § 4*a*(iv)), most of these are based on much earlier studies (von Graff 1912–1917; Hesse 1902, p. 635) and should be analysed again under the new hypothesis before we consider any of them as valid phylogenetic characters.

The phylogenetic relationships within the family Dugesiidae presented in figure 2*a*,*b* contradict a previous, well-established hypothesis about the phylogenetic relationships within this family (De Vries & Sluys 1991). In their phylogenetic analysis of the nine extant genera of Dugesiidae using previously used and new morphological characters, the genera *Schmidtea*, *Girardia* and *Cura* were grouped according to the presumed synapomorphy 'angled bursal canal'. The genus *Dugesia* instead clustered outside with the related genus *Neppia*, whereas the rest of the genera fell between them. The NJ and MP analyses described here clearly contradict the close affinity of the three genera *Schmidtea*, *Girardia* and *Cura*, as proposed by De Vries & Sluys (1991). We found that *Schmidtea* and *Cura* cluster together with high bootstrap support (74% and 79%, respectively), forming a sister group to *Dugesia*, with *Girardia* falling clearly outside them. This clustering agrees with a previous analysis using enzyme data and 18S rRNA sequences (Riutort *et al*. 1992). Whereas the 'angled bursal canal' is present in all *Girardia* species examined, it is not present in any of the three *Schmidtea* species examined (Benazzi *et al*. 1975) nor in *Cura pinguis* (Ball 1974*b*). Instead, several morphological characters (large oocytes, structure of yolk inclusion, presence of preovarial vitellaria, and extremely well-developed shell glands and cement glands) are shared by *Cura* and *Schmidtea* species, although some are plesiomorphies or characters whose polarity has not been determined.

(c) *Taxonomic remarks*

In the light of new molecular evidence the infraorder Maricola remains monophyletic, but the Paludicola

appears to be paraphyletic. Instead, the families Dugesiidae, Planariidae, Dendrocoelidae and the land planarians form a new clade. Perhaps a suitable name for this grouping would be the Continenticola. The dugesiids and the terrestrial planarians (Terricola) form a monophyletic group. Further evidence is required to test the monophyly of the dugesiids and the Terricola before a systematic revision of the group can be formalized. A scheme of the sister-group relationships that these new molecular data support is shown in figure 3, where we also map morphological character changes which may guide future morphological investigations.

We thank Hugh Jones, Ronald Sluys and an anonymous referee for critically reviewing earlier drafts of the manucript. We are indebted to the following individuals for providing us with fresh and fixed specimens: Peter Anderson, Mario Benazzi, Marco Curini-Galletti, Peter M. Johns, Hugh Jones, Klaus Rohde, Boris Joffe, and Oleg Timoshkin. Funding was provided by a DGICYT grant PB90-0477 and a CIRIT grant GRQ93-1044 to J.B. D.T.J.L. and K.A.C. were funded by a Wellcome Trust Senior Research Fellowship in Biodiversity to D.T.J.L. (043965/Z/95/Z). David Swofford kindly provided a pre-release version of PAUP* (v. 4.0d55).

REFERENCES

Adoutte, A. & Philippe, H. 1993 The major lines of metazoan evolution: summary of traditional evidence and lessons from ribosomal RNA sequence analysis. In *Comparative molecular neurobiology* (ed. Y. Pichon), pp. 1–30. Basel: Birkhäuser Verlag.

Ax, P. 1961 Verwandtschaftsbeziehungen und Phylogenie der Turbellarien. *Ergeb. Biol.* **24**, 1–68.

Ax, P. 1996 *Multicellular animals: a new approach to the phylogenetic order in nature, vol. 1.* Berlin: Springer-Verlag.

Baguñà, J., Romero, R., Saló, E., Collet, J., Auladell, C., Ribas, M., Riutort, M., Garcia-Fernández, J., Burgaya, F. & Bueno, D. 1990 Growth, degrowth and regeneration as developmental phenomena in adult freshwater planarians. In *Experimental embryology in aquatic plants and animals* (ed. H. J. Marthy), pp. 129–162. New York: Plenum Press.

Baguñà, J., Saló, E., Romero, R., Garcia-Fernández, J., Bueno, D., Muñoz-Marmol, A. M., Bayascas-Ramirez, J. R. & Casali, A. 1994 Regeneration and pattern formation in planarians: cells, molecules and genes. *Zool. Sci.* **11**, 781–795.

Ball, I. R. 1974*a* A contribution to the phylogeny and biogeography of the freshwater triclads (Platyhelminthes: Turbellaria). In *Biology of the Turbellaria* (ed. N. W. Riser & M. P. Morse), pp. 339–401. New York: McGraw-Hill.

Ball, I. R. 1974*b* A new genus of freshwater triclad from Tasmania, with reviews of the related genera *Cura* and *Neppia* (Turbellaria, Tricladida). *Life. Sci. Contr. R. Ont. Mus.* **99**, 1–48.

Ball, I. R. 1977 On the phylogenetic classification of aquatic planarians. *Acta Zool. Fenn.* **154**, 21–35.

Ball, I. R. 1981 The phyletic status of the Paludicola. *Hydrobiologia* **84**, 7–12.

Ball, I. R. & Reynoldson, T. B. 1981 *British planarians*. Cambridge University Press.

Ball, I. R & Sluys, R. 1990 Turbellaria: Tricladida: Terricola. In *Soil biology guide* (ed. D. Dindal), pp. 137–153. New York: Wiley.

Bayascas, J. R., Castillo, E., Muñoz-Mármol, A. M. & Saló, E. 1997 Planarian *Hox* genes: novel patterns of expression during regeneration. *Development* **124**, 141–148.

Benazzi, M. & Giannini, V. 1973 Developmental biology of triclad turbellarians. In *Developmental biology of freshwater invertebrates* (ed. F. W. Harrison & R. C. Cowden), pp. 151–211. New York: Alan R. Liss.

Benazzi, M., Baguñà, J., Ballester, R., Puccinelli, I. & Del Papa, N. 1975 Further contribution to the taxonomy of the '*Dugesia lugubris–polychroa* group' with the description of *Dugesia mediterranea* n. sp. (Tricladida, Paludicola). *Boll. Zool.* **42**, 81–89.

Boag, B., Evans, K. A., Neilson, R., Yeates, G. W., Johns, P. M., Mather, J. G., Chrsitensen, O. M. & Jones, H. D. 1995 The potential spread of terrestrial planarians *Artioposthia triangulata* and *Australoplana sanguinea* var. *alba* to continental Europe. *Ann. Appl. Biol.* **127**, 385–390.

Carranza, S., Giribet, G., Ribera, C., Baguñà, J. & Riutort M. 1996 Evidence that two types of 18S rDNA coexist in the genome of *Dugesia* (*Schmidtea*) *mediterranea* (Patyhelminthes, Turbellaria, Tricladida). *Molec. Biol. Evol.* **13**, 824–832.

Carranza, S., Baguñà, J. & Riutort, M. 1997 Are the Platyhelminthes a monophyletic primitive group? An assessment using 18S rDNA sequences. *Molec. Biol. Evol.* **14**, 485–497.

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J. & Rutter, W. 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

De Vries, E. J. & Sluys, R. 1991 Phylogenetic relationships of the genus *Dugesia* (Platyhelminthes, Tricladida, Paludicola). *J. Zool.* **223**, 103–116.

Dover, G. 1982 Molecular drive—a cohesive mode of species evolution. *Nature* **299**, 111–117.

Ehlers, U. 1985 *Das Phylogenetische System der Plathelminthes*. Stuttgart: Gustav Fisher Verlag.

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R. & Raff, R. R. 1988 Molecular phylogeny of the animal kingdom. *Science* **239**, 748–753.

Gremigni, V. 1988 Planarian regeneration—an overview of some cellular mechanisms. *Zool. Sci.* **5**, 1153–1163.

Gunderson, J. H., Sogin, M. L., Wollett, G., Hollingdale, M., De La Cruz, V. F., Waters, A. P. & McCutchan, T. F. 1987 Structurally distinct, stage-specific ribosomes occur in plasmodium. *Science* **238**, 933–937.

Hallez, P. 1890 Catalogue des Turbellariés (Rhabdocoelides, Triclades et Polyclades) du Nord de la France et de la Côte Boulonnaise. *Revue. Biol. Nord de la France* **2**, 1–179.

Hallez, P. 1894 *Catalogue des Rhabdocoelides, Triclades et Polyclades du nord de la France*. Lille: L. Daniel.

Heinzel, L. 1929 Zur Kenntis der Ryhnchodemiden. *Zool. Jahrb. (Syst.)*, **56**, 425–462.

Hesse, R. 1902 Untersuchungen über die Organe der Lichtempfindung bei niederen Tieren VIII. *Z. Wiss. Zool.* **72**, 565–656.

Hillis, D. M. & Dixon, M. T. 1991 Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66**, 411–453.

Holmquist, C. & Karling, T. G. 1972 Two new species of interstitial marine triclads from the North American Pacific coast, with comments on evolutionary trends and systematics in Tricladida (Turbellaria). *Zool. Scr.* **1**, 157–184.

Huelsenbeck, J. P., Bull, J. J., Cunningham, C. W. 1996 Combining data in phylogenetic analysis. *Trends Ecol. Evol.* **11**, 152–158.

Hyman, L. H. 1944 Marine Turbellaria from the Atlantic coast of North America. *Am. Museum Novitates* **1266**, 1–15.

Jones, H. D. & Boag, B. 1996 The distribution of New Zealand and Australian terrestrial flatworms (Platyhelminthes, Turbellaria, Tricladida, Terricola) in the British Isles—the Scottish Survey and MEGALAB worms. *J. Nat. Hist.* **30**, 955–975.

Karling, T. G. 1974 On the anatomy and affinities of the Turbellarian orders. In *Biology of the Turbellaria* (ed. N. W. Riser & M. P. Morse), pp. 1–16. New York: McGraw-Hill.

Kishino, H. & Hasegawa, M. 1989 Evaluation of the maximum likelihood estimates of the evolutionary tree topologies from sequence data, and the branching order in Hominoidea. *J. Molec. Evol.* **29**, 170–179.

Lake, J. A. 1991 Reconstructing evolutionary trees from DNA and protein sequences: paralinera distances. *Proc. Natn. Acad. Sci. USA* **91**, 1455–1459.

Littlewood, D. T. J. & Smith, A. B. 1995 A combined morphological and molecular phylogeny for sea urchins (Echinoidea: Echinodermata). *Phil. Trans. R. Soc. Lond.* B **347**, 213–234.

Lockhart, P. J., Steel, M. A., Hendy, M. D. & Penny, D. 1994 Recovering evolutionary trees under a more realistic model of sequence evolution. *Molec. Biol. Evol.* **11**, 605–612.

Meixner, J. 1928 Der Genitalapparat der Tricladed und seine Beziehungen zu ihrer allgemeinen Morphologie, Phylogenie, Ökologie und Verbreitung. *Z. Morph. Ökol. Tiere* **11**, 570–612.

Meixner, J. 1938 Turbellaria (Strudelwürmer). Allgemeiner Teil. *Die Tierwelt d. Nord-u. Ostsee* **IVb**. 146 pp.

Minelli, A. 1974 Primi reperti di planarie terrestri autoctone in Italia con descrizione di *Geobenazzia tyrrhenica* n. gen., n. sp. *Mem. Mus. Civ. St. Nat. Verona* **20**, 553–548.

Minelli, A. 1977 A taxonomic review of the terrestrial planarians of Europe. *Boll. Zool.* **44**, 399–419.

Riedl, R. 1978 *Order in living organisms: a systems analysis of evolution.* (English edition 1978, translated by R. P. S. Jefferies). New York: Wiley.

Riutort, M., Field, K. G., Turbeville, J. M., Raff, R. A. & Baguñà, J. 1992 Enzyme electrophoresis, 18s ribosomal-RNA sequences, and levels of phylogenetic resolution among several species of freshwater planarians (Platyhelminthes, Tricladida, Paludicola). *Can. J. Zool.* **70**, 1425–1439.

Riutort, M., Field, K. G., Raff, R. R. & Baguñà, J. 1993 18S rRNA sequences and phylogeny of Platyhelminthes. *Biochem. Syst. Ecol.* **21**, 71–77.

Rohde, K. 1990 Phylogeny of Platyhelminthes, with special reference to parasitic groups. *Int. J. Parasitol.* **20**, 979–1007.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular cloning—a laboratory manual*. 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Shirasawa, Y. & Makino, N. 1981 Light and electron microscopic studies on the normal and regenerating photoreceptor of a land planarian (*Bipalium fuscatum*). *Bull. Tokyo Med. Coll.* **7**, 35–50.

Sluys, R. 1989*a* Phylogenetic relationships of the triclads (Platyhelminthes, Seriata, Tricladida). *Bijd. Dierk.* **59**, 3–25.

Sluys, R. 1989*b A monograph of the marine triclads.* Rotterdam: A. A. Balkema.

Sluys, R. 1990 A monograph of the Dimarcusidae (Platyhelminthes, Seriata, Tricladida). *Zool. Scr.* **19**, 13–29.

Sluys, R. & De Vries, E. J. 1988 The aquatic triclads of the Crozet Islands (Platyhelminthes Tricladida). *Zool. J. Linn. Soc.* **94**, 203–217.

Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W. & Gillevet, P. M. 1994 The genetic data environment: an expandable GUI for multiple sequence-analysis. *Comp. Appl. Biosci.* **10**, 671–675.

Sogin, M. L. 1991 Early evolution and the origin of eukaryotes. *Curr. Opin. Gen. Devl.* **1**, 451–456.

Sopott-Ehlers, B. 1985 The phylogenetic relationships within the Seriata (Platyhelminthes). In *The origin and relationships of lower invertebrates* (ed. S. Conway Morris, J. D. George, R. Gibson & H. M. Platt). Systematics Association Special Volume **28**, pp. 159–167. Oxford: Clarendon Press.

Steinböck, O. 1925 Zur Systematik der Turbellaria metamerata, zugleich ein Beitrag zur Morphologie des Tricladen-Nervensystems. *Zool. Anz.* **64**, 165–192.

Stimpson, W. 1857 Prodromus descriptionis animalium evertebratorum quae in Expeditione ad Oceanum, Pacificum Septentrionalem a Republica Federata missa, Johanne Rodgers Duce, observavit et descripsit. *Proc. Acad. Nat. Sci. Phil.* 1857, 19–31.

Swofford, D. L. 1998 *PAUP*: Phylogenetic analysis using parsimony (and other methods), v. 4.0.* Sunderland, MA: Sinauer. (In the press.)

Telford, M. J. & Holland, P. W. H. 1997 Evolution of 28S ribosomal DNA in chaetognaths: duplicate genes and molecular phylogeny. *J. Molec. Evol.* **44**, 135–144.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994 CLUSTAL-W—improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**, 4673–4680.

von Graff, L. 1912–1917 Tricladida. In *Klassen und Ordnungen des Thier-Reichs IV* (*Vermes*) (ed. H. G. Bronn), pp. 2601–3369. Leipzig: C. F. Winter'sche Verlagshandlung.

Woese, C. R. 1987 Bacterial evolution. *Microbiol. Rev.* **51**, 221–271.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.