A reappraisal of the phylogenetic and taxonomic position of land planarians (Platyhelminthes, Thrbellaria, Tricladida) inferred from 18S rDNA sequences

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Summary. Land planarians belong to the suborder Tricladida within which four infraorders are currently recognized: the Maricola, the Cavernicola, the Paludicola and the Terricola (land planarians). Phylogenetic analyses using morphological characters support the monophyly of all infraorders with the Maricola as the primitive sister group to the rest of the Tricladida, and Terricola and Paludicola being derived sister groups. In view of the biological importance of land planarians, a robust phylogenetic scheme of the Tricladida is required for both taxonomy and comparative purposes. Using nucleotide-sequence data of the 18S ribosomal genes it has recently been reported that the Terricola and a family of the Paludicola, the Dugesiidae, share an 18S gene duplication (types I and II) that can be explained only by accepting a common origin for the Terricola+Dugesiidae (Carranza et al. 1998). In addition, both distance and maximum parsimony analyses also support that Terricola and Dugesiidae form a monophyletic group. Here, we extend our analysis up to 18 species of paludicolans and 11 species of terricolans. Distance-based and maximum-likelihood analyses resulted in phylogenetic trees strongly supporting the Terricola and the Dugesiidae as forming a monophyletic group, though the internal phylogeny of each clade remains unresolved. These results strongly supports that landplanarians derive from freshwater ancestors and, therefore, that its taxonomic rank and position within the Tricladida has to be reconsidered.

Key words: I8S rDNA, Tricladida, planarians, Terricola, phylogeny, Platyhelminthes

Introduction

For over 100 years systematists have recognised three major groups within the Order Tricladida (Phylum Platyhelminthes, Class Turbellaria, Order Seriata), using Hallez's (1890) ecological names: Paludicola (freshwater planarians), Terricola (land planarians) and Maricola (marine planarians). A fourth clade, the Cavernicola, which groups taxa close to the Paludicola was recently proposed by Sluys (1990). The taxonomic rank of these groups has shifted between that of suborder and infraorder, as it is accepted today. The systematic and phyloge-

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netic relationships of these infraorders have been discussed, based on morphological and ultrastructural characters, by Ball (1977, 1981), Sopott-Ehlers (1985), and Sluys (1989a). Sluys' phylogenetic scheme is based on a reassessment of previously used characters and the introduction of new ones. It supports the monophyly of the Tricladida, the Terricola, the Maricola and the Paludicola, with Maricola branching first, Terricola next, and the Paludicola forming the most derived group. Under this scenario, land planarians had evolved from marine ancestors whereas freshwater planarians came either from a land planarian ancestor or from a common marine ancestor to land and freshwater planarians.

Relationships within the infraorders have been considered in some detail within the Paludicola in which three families are recognized: Dugesiidae Ball, 1974, Planariidae Stimpson, 1857 and Dendrocoelidae Hallez, 1894 (Ball 1974). Planariidae plus Dendrocoelidaeis considered the derived sister-group of the Dugesiidae (Ball 1981). There are, however, some uncertainties. Firstly, no autapomorphies defining the family Planariidae have so far been found. Secondly, 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 1989a,b). The internal phylogeny of the Maricola has been dealt with in detail by Sluys (1989b). To our knowledge no attempt has been undertaken to sort out the internal phylogeny of the Terricola. The monophyletic status of the Terricola rest on three morphological synapomorphies: pharyngeal musculature, creeping sole, and diploneural nervous system (Ball 1977, 1981; Sluys 1989a). The Terricola is presently divided into three families: Bipaliidae von Graff, 1899, Geoplanidae Stimpson, 1857 and Rhynchodemidae von Graff, 1899. Biogeographical distribution and morphological features suggests Rhynchodemidae, and especially the subfamily Microplaninae, as the most primitive family, with the Bipaliidae and Geoplanidae being derived and geographically more restricted (Marcus 1953; Froehlich 1967).

An independent method to test the current phylogeny of the Tricladida and of their infraorders is to employ molecular systematics. Molecular data obtained from 18S and 28S 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). Using sequences of the 18S gene from several species of Maricola, Paludicola and Terricola and maximum-parsimony and neighbor-joining methods it was shown (Carranza et al. 1998) that the Maricola is monophyletic and is the primitive sister-group to the rest of the Tricladida (Paludicola+ Terricola). The Paludicola, however, were found to be paraphyletic since the Terricola and one paludicolan family, the Dugesiidae, clustered together, with very high bootstrap support, to the rest of the Paludicola (Planariidae+Dendrocoelidae). The monophyly of Terricola+Dugesiidae was reinforced by the finding that they share an 18S gene duplication (type I and type II genes), first found in the genome of the dugesiid *Schmidtea mediterranea* (Carranza et al. 1996), that can be explained only by accepting a common origin for the Terricola+Dugesiidae. This also indicated an origin of land planarians from a freshwater ancestor and not from a marine ancestor as implied in Sluys' phylogenetic hypothesis (Sluys 1989a).

We extend here the analysis of both type I and type II 18S rDNA genes, namely the type II, in a larger set of species from both the Dugesiidae and the Terricola. Published 18S rDNA sequences from six Terricola, five Dugesiidae and eight Planariidae+Dendrocoelidae species were also included in the phylogenetic analysis. The aims of this paper are: I) to further test the paraphyletic status of the Paludicola and the monophyly of Terricola+Dugesiidae; and 2) to analyze the monophyletic or paraphyletic status of Terricola and Dugesiidae and its internal phylogeny.

Materials and Methods

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

Table 1. Classification of the 29 species used in this study, type of 18S sequence obtained (+), and Gen-Bank accession numbers. Sequences of Planariidae and Dendrocoelidae are not indicated since they bear a single l8S sequence which differs to the Type I and Type II sequences of Dugesiidae and Terricola. New sequences reported in this paper are marked #

	Type 1	Type II	Accession number
Phylum Platyhelminthes			
Order Seriata			
Suborder Tricladida			
Infraorder Terricola			
Family Geoplanidae			
Newzealandia sp. #		+	AF050431
Artioposthia triangulata	+	+	AF033038, Z99945
Caenoplana caerulea	+		AF033040
Caenoplana sp. #		+	AF048765
Australoplana sanguinea	+		AF033041
Australoplana sp. #		+	AF050434
Family Sipaliidae			
Bipalium kewense	+		AF033039
Bipalium sp.	+		X91402
Family Rhynchodemidae			
Microplana nana	+		AF033042
Microplana scharffi #		+	AF050435
Platydemus manokwari #		+	AF048766
Infraorder Paludicola			
Family Planariidae			
Polycelis nigra			AF013151
Polycelis tenuis			Z99949
Crenobia alpina			M58345
Phagocata ullala			AF0I3149
Phagocata sibirica			Z99948
Phagocata sp. #			AF0I3150
Family Oendrocoelidae			
Dendrocoelum lacteum			M58346
Baikalobia guttata			Z99946
Dendrocoelopsis lactea			085087
Family Dugesiidae			
Schmidtea mediterranea	+	+	U31084, U31085
Schmidtea polychroa	+	+	AF013152, AF013154
Cura pinguis	+		AF033043
Dugesia subtentaculata	+	+	M58343, AF013155
Dugesia japonica #	+		AF013153
Dugesia ryukyuensis #		+	AF050433
Neppia montana #	+		AF050432
Romankenkius libidinosus #		+	Z99951
Girardia tigrina	+	+	AF013157, AF013156

Sequencing of the i8S molecule

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 new species as indicated in Table l. 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). Amplification products were sequenced directly. Sequencing of the clones and the PCR products was performed using an automated sequencer ASl Prism 377, following manufacturer's protocols.

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 1622 unambiguosly aligned positions were used in the phylogenetic analyses, 610 being variable and 309 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 neighbor-joining (NJ) utilising the algorithms in PAUP* (Swofford in press), and maximum likelihood (ML) using fastDNAml. For MP we determined most parsimonious solutions with the heuristic option. Both MP and NJ trees were bootstrap resampled *(n* = 1,000) to indicate branch support; and for the ML tree a value of confidence for the branches was calculated using the PUZZLE program (Strimmer & von Haesler 1996). To root the trees, representatives of families Dendrocoelidae and Planariidae were chosen because they are known to be the sister group of the Dugesiidae+ Terricola clade (Carranza et al. 98).

Results

Two types of l8S 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 so far (Carranza et al. 1996, 1998). 18S type I sequences were obtained when PCR amplification and sequencing were carried out from 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.

Full length sequences of both type I and type II I8S molecule were obtained for four dugesiids *(S. polychroa,* S. *mediterranea,* D. *subtentaculata* and G. *tigrina);* for the other five species only type I or type II could be sequenced (see Table I and below). For the Terricola full length sequences of both type I and type II were only recovered for *Artioposthia triangulata*. Type I sequences were obtained in other four species and type II in another six. This is because type II is easily amplified by PCR from DNA of ethanol fixed samples whereas live individuals are needed to sequence type I which is easier to recover from RNA.

Neighbor-joining, maximum parsimony, and maximum likelihood trees gave a similar topology, only the later being here represented (Figure 1). It shows, in agreement with a previous report (Carranza et al. 1998), but including a larger set of species: 1) that the Paludicola are paraphyletic, since the Terricola and the Dugesiidae share, with a very high bootstrap support (98 %), a more recent common ancestor than the Dugesiidae with the other representatives of the Paludicola (dendrocoelids and planariids), and 2) that the 18S duplication event giving the type I and type II 18S sequences took place in the common ancestor of the clade Dugesiidae+ Terricola.

The larger set of species of Dugesiidae and Terricola studied here and the resulting NJ and ML trees show: 1) in both NJ and ML trees the clade Planariidae+Dendrocoelidae is monophyletic with high bootstrap support; 2) the two families, Planariidae and Dendrocoelidae appear as monophyletic groups in the NJ tree (80 and 100 % bootstrap support respectively; data not shown) while in the ML analysis the Dendrocoelidae is strongly supported (94 % bootstrap value) whereas Planariidae appears only weakly so (51 % bootstrap value); 3) in both NJ and ML trees, the monophyly of the Terricola (inferred from both type I and type II sequences) is not supported because some dugesiids, such as G. *tigrina* and *Romankenkius libidinosus* cluster with some Terricola; even so, type I sequences show all Geoplaniidae studied clustering with very high support; 4) the monophyly of the family Dugesiidae inferred from the type I sequences is supported in the NJ trees (not shown) but it is not supported in the ML analysis. In the later, G. *tigrina* appears as the sister taxon of a clade formed by *Bipalium kewense* and *Microplana nana;* 5) from the type II sequences, Dugesiidae does not also appear as a monophyletic taxon in both NJ and ML trees because G. *tigrina* clusters with *Microplana scharffi* and not with the rest of the Dugesiidae. Moreover, another dugesiid, *R. libidinosus,* appears as the sister group of most Terricola with the sole exception of *Bipalium* sp and
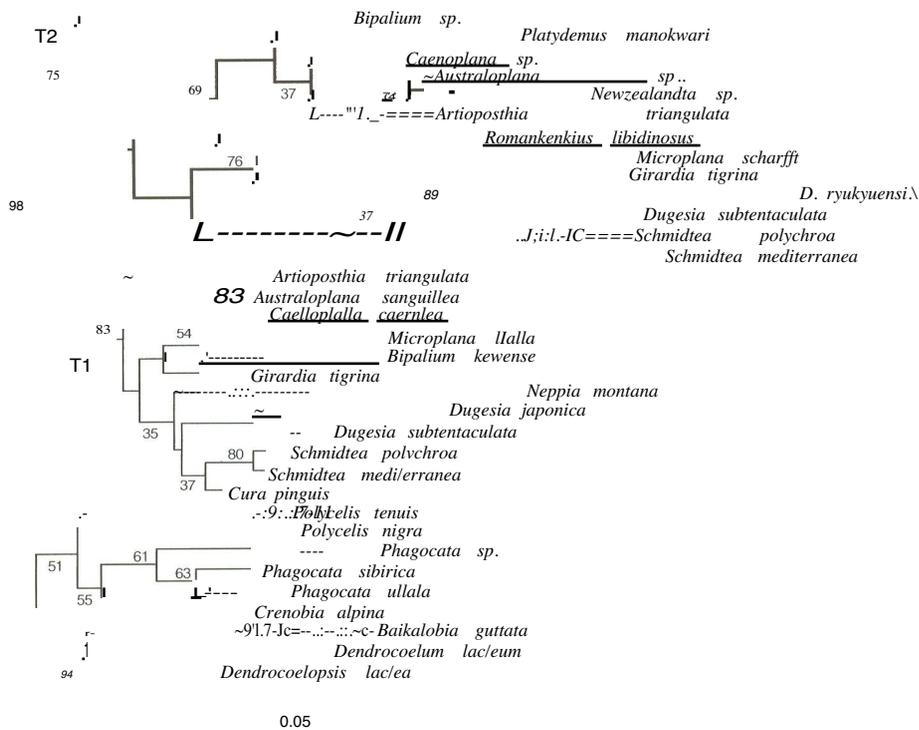
Fig. 1. Maximum-likelihood tree of the Terricola and the Dugesiidae. Numbers at the nodes represent branch support as calculated by the quartet puzzling method. Type II (T2) clade branches are represented by thick lines. Terricola species are in bold

M. scharfii which branch first; 6) within the Terricola and at variance with the relative clustering of geoplanids, the rhynchodemidae (here represented by *M. nana, M. scharffi* and *Platydemus manokwari)* do not cluster together; and 7) within the family Dugesiidae, the genera *Schmidtea, Cura* and *Dugesia* cluster together with a moderate bootstrap support. Instead, *Girardia, Neppia* and *Romankenkius* fall clearly outside them.

Discussion

The Terricola and the Dugesiidae cluster in NJ, MP and ML analyses (inferred either from the type I or from the type II 18S sequences) as a monophyletic group with very high bootstrap support (see Carranza et al. 1998 for the NJ and MP trees; and Fig. 1 here for the ML tree). In addition, the Terricola and the Dugesiidae share an 18S gene duplication that can be explained only by accepting a common origin for the Terricola+Dugesiidae (see Carranza et al. 1998, and Fig. I here). Both sets of data provide very strong evidence for the paraphyly of the Paludicola and support for the Dugesiidae and the Terricola forming a monophyletic clade.

The conclusions drawn from previously published molecular data, namely the paraphyly of the Paludicola and the new clade Dugesiidae+ Terricola, were found inconsistent (Carranza et al. 1998) with current phylogenetic analyses of the suborder Tricladida based on morphological characters (Ball 1981 ; Sluys 1989a). As Hedges and Sibley (1994) state, when molecular data produce a robust phylogeny that conflicts with morphology, it is probable that morphological characters presumed to be shared-derived are shared-primitive or convergent. In

Sluys' analysis, the monophyly of the Paludicola is based on three main morphological characters: probursal condition, sperm transfer through spermatophores, and subepidermal musculature consisting of four layers. A closer inspection of these characters led Carranza et al (1998) to consider them as either week positional characters (e.g. probursal condition) or insufficiently studied characters (e.g. sperm transfer through spermatophores and subepidermal musculature consisting of four layers) and to call for a deeper analysis using a larger set of species. Moreover, another character, the dugesiid eye structure, was considered in Ball's and Sluys' analyses a derived feature constituting a synapomorphy for the Dugesiidae (Ball 1977, 1981; Sluys 1989a,b). However, and with the exception of bipaliids, this character is also a feature of land planarians, though some differences in the internal structures have been noted and need to be considered. The finding of multicellular eye cups in both dugesiids and land planarians was considered a convergence in the morphological approaches (Ball 1977, 1981; Sluys 1989a). However, the strong support from molecular data for a clade Dugesiidae+ Terricola allows to reinterpret the multicellular eye cup as a potential synapomorphy supporting the new clade (Carranza et al. 1998). Nevertheless, under this hypothesis Bipaliidae represent an anomaly. They bear multiple marginal eyes formed by one pigment cell, housing one to eight retinal cells (Shirasawa & Makino 1981; Sluys 1989b), similar to those of Maricola and non-dugesiids Paludicola. Pending further studies of more species of this family, the most parsimonious explanation under the molecular phylogenetic hypothesis presented herein is the secondary loss of the multicellular pigment cell cup in this family.

The strong links of land planarians with the freshwater dugesiids suggests that invasion and colonization of land by triclads occurred from freshwater ancestors and not from marine triclads as previously hypothesized (Ball 1981; Sluys 1989a). In other animal groups, colonization of land has usually passed through some freshwater intermediates. There are arguments from biogeography (e.g. the cosmopolitan distribution of dugesiids) and physiology (e.g. the osmoregulatory preadaptation of freshwater organisms to inhabit moist land habitats) which also backs this trend in triclad evolution.

Are the Terricola and the Dugesiidae monophyletic?

In the recently published neighbor-joining (NJ) analysis of the new clade Dugesiidae+ Terricola, members of the Terricola and Dugesiidae clustered independently as monophyletic groups albeit with very low bootstrap values (36 and 30 % respectively) (Carranza et al. 1998). Maximum Parsimony (MP) analysis, however, only recovered as monophyletic groups the genera *Dugesia, eura* and *Schmidtea* for the Dugesiidae (bootstrap value of 74 %), and the three representati ves of the family Geoplanidae (bootstrap value of 100 %). Therefore, the phyletic status of the Terricola and the Dugesiidae could not be ascertained.

The larger set of species analyzed here fall short to settle the issue. Despite 12 type II sequences (compared to the 5 studied in Carranza et al. 1998) were analyzed, Terricola and Dugesiidae do not appear monophyletic. Whereas the genera *Dugesia* (including D. *ryukyuensis)* and *Schmidtea* cluster together, G. *tigrina* and *R. libidinosus* cluster with different terricolan representatives. In addition, bipaliids, here represented by *Bipalium* sp. branches first to the rest of dugesiids+terricolans. Type I sequences, here represented by 13 species (compared to the 10 studied in Carranza et al. 1998) reproduces the clustering of most dugesiids, namely the genera *eura, Schmidtea* and *Dugesia,* and the new entry *Neppia montana* though G. *tigrina* clusters, unexpectedly, with the sole representatives of rhynchodemids and bipaliids. Again, geoplanids cluster together with a high bootstrap value (83%). Taking together both sets of sequences, geoplanids and the bulk of dugesiids form clear monophyletic groups, whereas the dugesiids G. *tigrina* and *R. libidinosus* as well as the representatives of bipaliids and rhynchodemids have at present uncertain positions which turn Dugesiidae and Terricola polyphyletic at the present state of knowledge. It is also interesting to point out that representatives of the subfamilies Rhynchodeminae and Microplaninae of the Rhynchodemidae do not cluster together (see Type II sequences in Fig. 1). This agrees with views which consider Rhynchodemidae as an artificial clade (L. Winsor and P. M. Johns pers. comm.).

Although the monophyly of the Terricola has never been questioned (Ball 1977, 1981; Sluys 1989a) it is clear from molecular evidence that the group is in need of taxonomic revision. The group as such is defined by three synapomorphies: complex pharyngeal musculature, creeping sole, and diploneural nervous system. The last two have always been considered very important synapomorphies for the Terricola (Steinbock 1925; Ball 1974; Sluys 1989a) as they represent adaptations to the terrestrial lifestyle. The diploneural nervous system is formed by the longitudinal nerve cords, which take a much more internal position, and by a thickened subcutaneous nerve plexus. There is ample morphological evidence that the ventral nerve cords of the Terricola are homologous to those of the Maricola and Paludicola, including dugesiids. Moreover, it is highly likely that the subcutaneous plexus of land planarians represent a quantitative differentiation of similar plexuses described in dugesiids (e.g. the subepidermal and submuscular plexuses; Bagufiil & Ballester 1978) related to the presence of a creeping sole. The later structure is developed to varying extents in the different families and subfamilies. The fact that the creeping sole and the diploneural nervous system are clear adaptations to the terrestrial life-style jointly with the low bootstraps or the lack of support backing the monophyly of the Terricola in NJ, MP (Carranza et al. 1998) and ML (this work) trees would be congruent with a parallel independent evolution of both structures from freshwater ancestors not bearing them. However, unless a denser sampling of species is carried out, namely of bipaliids and rhynchodemids, a unique event followed by diversification is also likely, and may be more parsimonious.

To summarize, phylogenetic analyses of 18S ribosomal sequences of-type I and type II from 18 species of freshwater planarians (including 9 dugesiids and 9 planariids+dendrocoelids) and 11 species of land planarians supports the paraphyly of the Paludicola and the monophyly of the clade Dugesiidae+ Terricola but does not lend support, so far, to the monophyly of the Terricola and the Dugesiidae. In addition, whereas Geoplanidae seems to be a clear monophyletic group, Rhynchodemidae do not, with Microplaninae and Rhynchodeminae likely being separate clades. Further evidence is required, namely a denser sampling of both Dugesiidae and Terricola, specially of Bipaliidae and Rhynchodemidae using both 18S type I and type II sequences, to test the monophyly or polyphyly of the Terricola, and their actual relationships to particular dugesiid genera. In the meantime, the infraorder Terricola seems redundant and should be better be considered, like the Dugesiidae, a family.

Acknowledgements

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