Integrative delineation of species of Mediterranean freshwater planarians (Platyhelminthes: Tricladida: Dugesiidae)

R. Sluys (Ronald), E. Solà, K. Gritzalis, M. Vila-Farré, E. Mateos, M. Riutort

The LINNEAN SOCIETY of London ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2013, **169**, 523–547. With 18 figures

Integrative delineation of species of Mediterranean freshwater planarians (Platyhelminthes: Tricladida: Dugesiidae)

RONALD SLUYS[1,2]*, EDUARD SOLÀ[3], KONSTANTINOS GRITZALIS[4], MIQUEL VILA-FARRÉ[3,5], EDUARDO MATEOS[3] and MARTA RIUTORT[3]

[1]*Naturalis Biodiversity Center, PO Box 9514, 2300 RA Leiden, The Netherlands*
[2]*Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands*
[3]*Departament de Genètica, Facultat de Biologia and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, Catalonia, Spain*
[4]*Hellenic Centre for Marine Research, Institute of Inland Waters, Anavyssos, Greece*
[5]*Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany*

Received 10 April 2013; revised 1 August 2013; accepted for publication 1 August 2013

The paper presents an integrative taxonomic study on dugesiid freshwater flatworms from the north-eastern Mediterranean region by applying both morphological and molecular criteria in the formulation of stable species hypotheses. The morphological information obtained for the specimens was used in a traditional way by comparing the organismal traits of the various populations and candidate species with those of known species, as documented in the taxonomic literature and as revealed by examination of histological sections of museum specimens. In the molecular species delimitation the General Mixed Yule-Coalescent method (GMYC) was used. Results of this study (1) supported the presence of 13 *Dugesia* species in the Hellenic area (including *D. sicula* Lepori, 1948, a pan-Mediterranean species), (2) culminated in the description of four new *Dugesia* species, (3) suggested the presence of two Confirmed Candidate Species, (4) pointed to 12 GMYC-delimited units in Greece and two in Slovakia as Unconfirmed Candidate Species and (5) revealed the presence of an entirely new genus, represented by two newly described species and a third Unconfirmed Candidate Species. Our results revealed a high diversity of dugesiid species in this relatively small region. It is concluded that the morphological features used by taxonomists in comparative studies of dugesiid flatworms generally result in reliable identifications and delineations of species taxa, except in the case of cryptic species.

doi: 10.1111/zoj.12077

ADDITIONAL KEYWORDS: Aegean – candidate species – cryptic species – *Dugesia* – Dugesiidae – GMYC – integrative taxonomy – ***Recurva*** Sluys **gen. nov.** – species delimitation.

INTRODUCTION

The freshwater planarian genus *Dugesia* Girard, 1850 (Platyhelminthes, Tricladida, Dugesiidae) currently comprises about 80 nominal species that are distributed in the Afrotropical, Palearctic, Oriental and Australian biogeographical regions (cf. Sluys, Kawakatsu & Winsor, 1998). More than 20 species occur in Europe, particularly in the Mediterranean region. Generally, identification of species of *Dugesia* is difficult because they are externally very similar. The traditional source of taxonomic characters concerns features of their reproductive complex, notably their copulatory apparatus. But even in their reproductive system species may be very similar, making proper identification a time-consuming and painstaking enterprise, in addition to the fact that the

*Corresponding author. E-mail: ronald.sluys@naturalis.nl

necessary taxonomic characters can be observed only in histological sections. Another complication with identification concerns the fact that many Mediterranean populations reproduce asexually by fission and usually do not develop a copulatory apparatus, thus preventing taxonomic assignment to a particular known species or to a new species.

In our view, therefore, the genus *Dugesia* represents a highly suitable model group to explore an integrative approach to delimiting species. For this, we have obtained both molecular and morphological information for a large number of *Dugesia* and other dugesiid populations distributed in the eastern Mediterranean region that we used as data sources to formulate and test species boundary hypotheses. The phylogeographical history of most of these populations has been analysed in a companion paper (Solà *et al*., 2013).

A consensus is emerging that species are segments of separately evolving lineages of populations (cf. De Queiroz, 2007; Frankham *et al*., 2012), albeit that the problem remains of establishing where during this process the diverging groups reach species status. In some cases morphological, behavioural or ecological differences represent unequivocal signals that speciation has occurred. In other cases, only analyses based on population genetics and coalescent theory suggest lack of gene flow, thus evidencing the presence of cryptic species (Bickford *et al*., 2007; Fontaneto *et al*., 2007; Burbrink *et al*., 2011; cf. Olson, Goodman & Yoder, 2004; Carew, Pettigrove & Hoffmann, 2005; Vieites *et al*., 2009; Fujita *et al*., 2012). Genetic distances (cf. Memon *et al*., 2006; Fouquet *et al*., 2007; Vieites *et al*., 2009) and other non-coalescent molecular-based species delimitation methods are not suitable for species delimitation because they rely on highly subjective criteria (Hey, 2009).

Our methodology in species delimitation consisted of three main steps. First, hypotheses on *candidate* species were formulated based on the examination of morphological features. Second, agreements and divergences between these candidate species and *putative* species delineated by a coalescent-based molecular method were identified. Third, during an iterative process reciprocal illumination of morphological and molecular results eventually resulted in the formulation of stable species hypotheses.

The morphological information obtained for the dugesiid flatworms was used in a traditional way by comparing the organismal traits of the various populations and candidate species with those of known species, as documented in the taxonomic literature and as revealed by our examination of histological sections of relevant museum specimens. Conformity of the relevant characters with those of known species enabled taxonomic assignment of the populations sampled, while divergences of organismal attributes suggested the presence of a new species.

As our species concept for the delimitation of candidate species we have chosen the phylogenetic species concept as formulated by Cracraft (1983, 1987; see also Sluys, 1991). In practice this means that a species boundary is hypothesized when a population of organisms is characterized by the presence of one or more unique characters or by a unique combination of characters, each of which may be plesiomorphic. For Mediterranean dugesiids morphological characters were used for postulating such phylogenetic species hypotheses, which were compared with the molecular, coalescent-based delineations of putative species. In other words, our delimitation criterion for candidate species status was morphological diagnosability or distinctness, with many of the characters being derived from the reproductive system.

As our molecular species delimitation method we have applied the Yule-Coalescent transition analysis as implemented in the General Mixed Yule-Coalescent (GMYC) method (Pons *et al*., 2006; Fontaneto *et al*., 2007), using cytochrome oxidase I (COI) sequences. It has been shown that Yule-Coalescent model analysis with a single mitochondrial gene can be a meaningful and rapid approach to assess species diversity within a group of organisms (Monaghan *et al*., 2009; Talavera, 2012). This coalescent-based method allows species delimitation by distinguishing branching patterns between interspecific (Yule model; speciation and extinction) and intraspecific (coalescence of alleles) processes on a phylogenetic tree. It draws a threshold between these two processes, thus delimiting clades of individuals representing putative species. It is useful even in situations (a) with high numbers of singletons, (b) with low taxon level (3–5 species) or (c) without intraspecific coverage (Talavera, 2012). The efficiency of the GMYC method is mostly due to the fast evolving nature of the mitochondrial genes, which are presumed to coalesce faster than nuclear genes because of their smaller effective population sizes (Moore, 1995; Avise, 2000). However, there are some drawbacks in using only one marker. For example, one runs the risk of equating the gene tree with the species tree, and consequently cases of reticulated evolution, introgression or incomplete lineage sorting can mislead phylogenies and thus lead to incorrect species hypotheses (cf. Edwards, 2009; Lohse, 2009).

Our study revealed also the presence of a new dugesiid genus. The erection and description of this new genus is based on the presence of differential morphological traits and on a phylogenetic analysis of 18S rRNA and COI gene sequences. The resulting phylogenetic tree clearly demonstrates that the new

genus consitutes a monophyletic lineage separate from all other dugesiid genera.

Evidently, in any integrative study there may be a discordance between morphologically determined or candidate species taxa on the one hand and putative molecular species on the other hand. Although discordance between morphological and molecular data may be a nuisance from a taxonomic perspective, it is interesting from a biological or evolutionary point of view (Yeates *et al*., 2011). Discordances cannot always be resolved. For situations in which not all data coincide or just one kind of data is available, Vieites *et al*. (2009) proposed three different categories to describe the taxonomic status of the biological units under study. The first category concerns Unconfirmed Candidate Species (UCS), including those genealogical lineages that can be delineated by a molecular method but for which other data are not available. The second is the Confirmed Candidate Species (CCS), comprising those units that can be delimited by molecular data and are supported also by other data, such as morphology, but have not yet been formally described and named. The third category concerns Deep Conspecific Lineages (DCL), referring to lineages that have reached a certain molecular threshold but present the same or a very similar morphology. We have applied this system to indicate the taxonomic status of those biological units that do not have the status of described species (DS).

In this study we will not describe new species solely on the basis of molecular divergence and in the absence of morphological species markers. In this way we avoid the danger of overestimating the number of species as a consequence of possible oversplitting by the GMYC method, although we run the risk of overlooking morphologically cryptic species. We have chosen this taxonomic practice in view of (1) compatibility with past taxonomic practice, and (2) the situation that the current International Code of Zoological Nomenclature (ICZN, 1999) requires the description of a new species taxon to be accompanied with a description that clearly differentiates the taxon (see also Bauer *et al*., 2011), and by the deposition of type specimen(s). Formally, molecular data may be presented in a way that fulfils the requirements of the ICZN (1999) and resembles traditional descriptions (cf. Nygren & Pleijel, 2011). However, in our view a DNA barcode does not provide the in-depth information on organismal divergence that allows one to formulate and test scientifically interesting hypotheses on the evolution of structures, adaptations, functional morphology, life history and behaviour (Sluys, 2013). Therefore, here we refrain from describing new species solely on the basis of their DNA barcode.

MATERIAL AND METHODS

Collection of specimens

Freshwater planarians were collected from the type localities of eight Greek *Dugesia* species (cf. De Vries, 1984, 1988) and from other localities on the mainland as well as some islands during the spring seasons of 2009 and 2010 (cf. Solà *et al*., 2013). All individuals used in the molecular analyses, as well as information on their sampling localities, are listed in Supporting Tables S1 and S2. Specimens used for morphological studies are listed in the relevant Material Examined sections of the Systematic and Integrative Section and/or are deposited in the collections of the Naturalis Biodiversity Center, Leiden, the Netherlands.

Morphological analysis and species hypotheses

Animals for morphological studies were fixed in Steinmann's fluid and, subsequently, transferred to 70% ethanol. Specimens that had been preserved for anatomical analysis were cleared in clove oil and then embedded in paraffin wax, sectioned at intervals of 6 or 8 μm (depending on the size of the animals) and mounted on albumen-coated slides. Sections were stained in Mallory-Cason/Heidenhain (Humason, 1967; Romeis, 1989) and mounted in DPX. Reconstructions of the copulatory complex were obtained by using a camera lucida attached to a compound microscope. All material has been deposited in the collections of the Naturalis Biodiversity Center, Leiden, the Netherlands.

The species status of the animals from the various localities was assessed by applying the phylogenetic species concept as formulated by Cracraft (1983, 1987; see also Sluys, 1991) and by comparing qualitative features of their reproductive complex, in particular their copulatory apparatus, with those of known species, as documented in the taxonomic literature and revealed by examination of histological sections of relevant museum specimens housed in the collections of the Naturalis Biodiversity Center. Detailed discussions of relevant characters used to differentiate the new species are presented in the Systematic and Integrative Section. Conformity of the relevant characters with those of known species enabled taxonomic assignment of the populations sampled, while divergences of organismal attributes suggested the presence of a candidate new species.

DNA sequencing and alignment

In addition to the mitochondrial COI sequences obtained for a companion phylogeographical study (Solà *et al*., 2013), sequences of 1–3 individuals per locality were obtained, when possible, and included in

the alignments (Table S1), following the same procedure described in that paper. Furthermore, 18S rDNA nuclear gene sequences (18S) were obtained for 11 individuals (Table S4). Sequences and annealing temperatures for each pair of primers, both for COI and for 18S, are given in Table S3. 18S was aligned by using online software MAFFT, version 6 (Katoh & Toh, 2008), while ambiguous positions were removed with the program GBlocks with default settings, except the minimum number of sequences for a conserved position (set at 16) and with half of the allowed gap positions (Talavera & Castresana, 2007). The level of sequence saturation for COI sequences of different genera was analysed under the TN93 nucleotide substitution pattern model with the program DAMBE (Xia & Xie, 2001). The three positions were analysed at the same time and independently.

Phylogenetic analyses of dugesiid genera

To analyse the genetic differentiation of a candidate new genus, which we happened to encounter among our Greek material, as well as to determine its relationship to other European members of the Dugesiidae and also to the Australian species *Cura pinguis* (Weiss, 1909) (which shares some morphological similarities with the new genus), we performed phylogenetic analyses using two datasets. One dataset consisted of a concatenated set including 18S and COI. The second dataset concerned only COI because this enabled us to include *Cura pinguis*, for which 18S sequences are not available. In the concatenated analysis we compared 21 species of five genera by taking one specimen of each (Table S4). This dataset lacks the 18S for *Dugesia naiadis* Sluys sp. nov. and the COI for *Recurva conjuncta* Sluys sp. nov. because we were unable to amplify these sequences. In the COI analysis we also compared 21 species, but excluded *Recurva conjuncta* and included *Cura pinguis*. The land flatworm species *Bipalium adventitium* Hyman, 1943 (Tricladida, Geoplanidae, Bipaliinae) was used as outgroup.

All phylogenetic analyses were performed using two inference methods, namely maximum-likelihood (ML) and Bayesian inference (BI). We used jModelTest 2.1.1 (Darriba *et al*., 2012) to test which evolutionary model fitted best with our data. We used GTR + I + Γ for 18S and HKY + I + Γ for COI, excluding third positions, and set the parameter estimation as unlinked among genes in the concatenated analysis. ML analysis was run with the program RaxML 7.0.0 (Stamatakis, 2006). To obtain bootstrap support (BS), 1000 replicates were calculated. We used MrBayes (v. 3.2: Ronquist *et al*., 2012) to perform the BI analysis. In total, 1000 000 generations were run, saving a tree every 100 generations. Convergence of topologies and model parameters of both runs was surveyed by checking whether the standard deviation of the split frequencies reached a value below 0.01 (default burn-in = 25%). We also checked that likelihood values had stabilized by plotting them against the number of generations. To infer the topology and posterior probability (PP) values we used the default burn-in.

Molecular species delimitation of *Dugesia* populations

We performed a GMYC approach (Pons *et al*., 2006; Fontaneto *et al*., 2007) to compare the units delimited by this method with those identified in the morphological analysis and to detect possible cryptic species. We used the partial COI sequences of 155 individuals of *Dugesia* from 34 localities (Table S1). GMYC detects the change from population processes (coalescence of alleles) to speciation and extinction processes through analysis of branching rate patterns, setting a threshold between the inter- and intraspecific relationships. To obtain the ultrametric tree necessary for this approach, we conducted a phylogenetic analysis in BEAST v1.7.3 (Drummond & Rambaut, 2007), using a fragment of COI (745 bp) from 2–5 individuals per sampling locality (Table S1). A lognormal relaxed clock with a substitution rate of 0.017 substitutions per lineage and per million years was applied (cf. Solà *et al*., 2013). The analysis was run under a GTR + I + Γ evolutionary model. Three monophyletic clades were forced: (1) *Dugesia* species, without *D. sicula* and *D. naiadis* (used as outgroup); (2) *Dugesia* species, without *D. sicula*, *D. naiadis* and *Dugesia* from Central Europe; (3) *Dugesia* species, without *D. sicula* Lepori, 1948, *D. naiadis* Sluys sp. nov., *Dugesia* from Central Europe and *D. cretica* (Meixner, 1928; Solà *et al*., 2013). Monte Carlo Markov chains were run for 150 000 000 generations, sampling every 15 000 trees. The parameters were checked to have reached an effective sampling size (ESS) value of over 100 after a 10% burn-in with Tracer v.1.5 (Rambaut & Drummond, 2007).

The BEAST tree obtained was submitted to the SPLITS (SPecies LImits by Threshold Statistics; Ezard, Fujisawa & Barraclough, 2009) package for R (available at http://r-forge.r-project.org/projects/splits/), which implements the GMYC approach. The program also performs likelihood ratio tests (LRTs) between (a) the null and GMYC models to test whether one or multiple species are involved, and (b) single and multiple threshold options.

Abbreviations used in Figures 3–18: bc, bursal canal; cb, copulatory bursa; cg, cement glands; cod, common oviduct; cs, cyanophilic secretion; dpf, dorsal penial

fold; ed, ejaculatory duct; fl, flap; go, gonopore; in, intestine; od, oviduct; pg, penial glands; ph, pharynx; pp, penis papilla; sg, shell gland; spf, spermatphore; sv, seminal vesicle; te, testis; vd, vas deferens.

RESULTS

Morphological analysis

Analysis of the qualitative features of the reproductive complex allowed us (1) to assign the Greek populations to eight of the nine species of *Dugesia* known for Greece, namely *Dugesia aenigma* De Vries, 1984, *D. arcadia* De Vries, 1988, *D. ariadnae* De Vries, 1984, *D. cretica*, *D. damoae* De Vries, 1984, *D. elegans* De Vries, 1984, *D. malickyi* De Vries, 1984 and *D. sagitta* (Schmidt, 1861) (Table 1), and (2) to identify a sexual population from Chios (Tripes-Parparia) as *D. sicula*. Further, four new species of *Dugesia* were identified by the presence of one or more unique characters or a unique combination of characters: *Dugesia naiadis* Sluys sp. nov., *Dugesia effusa* Sluys sp. nov., *Dugesia improvisa* Sluys & Solà sp. nov. and *Dugesia parasagitta* Sluys & Solà sp. nov. (see below; Table 1, units 3, 19, 20, 33). Unfortunately, we have been unable to analyse the morphological features of several populations (Table 1, units 5–9, 12, 13, 17, 22, 23, 30, 31), due to lack of (1) fixed material, (2) sexual specimens or (3) adequate histological sections. In addition, our samplings and subsequent comparative studies revealed the presence of a new dugesiid genus, *Recurva* Sluys gen. nov., represented by two species, namely *Recurva postrema* Sluys & Solà sp. nov. and *Recurva conjuncta* Sluys sp. nov. For a possible third, as yet unnamed, species of *Recurva* no morphological information was available. Detailed accounts of the relevant characters used to differentiate the candidate new species are presented in the Systematic and Integrative Section.

Phylogenetic analysis of dugesiid genera

Saturation analysis revealed that the third positions of the COI alignment including several genera were saturated; therefore, we excluded this codon position in all subsequent analyses.

The two phylogenetic methods used (MrBayes and RaxML) yielded almost identical topologies, albeit with different supports at some nodes (Fig. 1). *Recurva* is the sister group of *Schmidtea* Ball, 1974 in both analyses, and with high bootstrap (ML)/posterior probability (BI) (89/0.98) support. In turn, these two genera form the sister group of the *Dugesia* species, with maximum support (100/1). The COI analysis including *Cura pinguis* shows that the latter is not close to *Recurva* (Fig. S1). Within the *Recurva* clade we can distinguish *R. postrema*, *R. conjuncta* and a subclade formed by three sampling localities on the island of Paros. The latter three populations are likely belong to the same species, which most probably is neither *R. postrema* nor *R. conjuncta*. However, because all specimens from Paros were asexual it has not been possible to analyse them at the morphological level. There is no resolution in the relationships among these three taxa of *Recurva*.

Molecular species delimitation of *Dugesia*

The topology of the tree found in the GMYC analysis is very similar to that obtained in an earlier companion work (cf. Solà *et al*., 2013). LRT comparison between the results of the single and multiple threshold models in GMYC revealed no significant differences ($\chi^2 = 4.39$, d.f. = 6, $P = 0.63$); therefore, we present here only the results of the single threshold model. In the GMYC analysis the likelihood ratio test of the null against the mixed model was significant ($4.5e^{-08***}$).

The single analysis indicated a total of 34 entities [confidence interval (CI) = 31–42], clustered as follows: 29 ML clusters of two or more individuals (CI = 27–33), and five singletons (Fig. 2; Table 1). Eleven of these clusters match with morphologically identified *Dugesia* species, four of these newly described in this paper: *D. parasagitta* Sluys & Solà sp. nov. (entity 3); *D. aenigma* (4); *D. malickyi* (11); *D. ariadnae* (18); *D. improvisa* Sluys & Solà sp. nov. (19); *D. effusa* Sluys sp. nov. (20); *D. damoae* (21); *D. elegans* (24); *D. gonocephala* (Dugès, 1830) (32); *D. naiadis* Sluys sp. nov. (33); and *D. sicula* (34). Unfortunately, we were unable to fully analyse the taxonomic status of clusters 10 and 14 as the histological sections currently available are not of the required quality. However, even from the damaged sections it is clear that these units are morphologically different from their sister clades, *D. malickyi* (entity 11) and *D. arcadia* (entities 15 and 16), respectively. The putative new species of cluster 10 differs from *D. malickyi* in the presence of (a) a central, broad ejaculatory duct, (b) a small, ventral penial fold and (c) a highly glandular ejaculatory duct. The putative new species of entity 14 differs from *D. arcadia* in the absence of a lateral fold projecting into the atrium, a structure that is characteristic for *D. arcadia*. Because we have molecular and morphological data suggesting that clades 10 and 14 do not belong to any already known species, we consider them here as CCS.

In three cases the GMYC clusters do not match the morphologically delimited candidate species. First, although *D. sagitta* splits into two clusters (1 and 2; Fig. 2, Table 1), we were unable to find any morphological difference supporting this split.

Table 1. Clusters obtained in the GMYC analysis

Entity*	Code of Solà *et al.* (2013)	Locality†	No. of individuals in the cluster	Taxonomic category	Species‡
1	27	1. Roda, Corfu, Greece	10	UCS	*Dugesia* sp.
	29	2. Kato vrisi spring, Klimatia, Corfu, Greece			
2	33	1. North of Vouniatades, Corfu, Greece	10	DS	*D. sagitta*
	34	2. Benitses, Corfu, Greece			
3	31	1. Ermones, Corfu, Greece	9	DS	*D. parasagitta*
	32	2. Ermones, slightly higher than 31, Corfu, Greece			
4	35	1. Near Agia Eirini, Kephalonia	10	DS	*D. aenigma*
	36	2. Digaleto, Cephalonia			
5	17	Potamia, Preveza, Greece	4	UCS	*Dugesia* sp.
6	17	Potamia, Preveza, Greece	1	UCS	*Dugesia* sp.
7	14	Vafkeri, Lefkada, Greece	5	UCS	*Dugesia* sp.
8	13	Varia, Aetolia-Acarnania, Greece	4	UCS	*Dugesia* sp.
9	12	Eleonas-Gravia, Phocis, Greece	4	UCS	*Dugesia* sp.
10	20	Polidrosos, Phoci, Greece	2	CCS	*Dugesia* sp.
11	19	1. Mexiates, Phthiotis, Greece	10	DS	*D. malickyi*
	18	2. Gorgopotamos, Phthiotis, Greece			
12	16	Filiates, Thesprotia, Greece	3	UCS	*Dugesia* sp.
13	23	Dorio-Psari, Peloponnese, Greece	4	UCS	*Dugesia* sp.
14	21	Tripi, Peloponnese, Greece	5	CCS	*Dugesia* sp.
15	26	Chalandritsa, Peloponnese, Greece	5	DCL	*D. arcadia*
16	25	Sella, Peloponnese, Greece	3		*D. arcadia*
17	24	1. Theisoa-Andritsaina, Peloponnese, Greece	12	UCS	*Dugesia* sp.
	22	2. Agios Floros, Peloponnese, Greece			
	13	3. Varia, Aetolia-Acarnania, Greece			
	23	4. Dorio-Psari, Peloponnese, Greece			
18	6	Apollonas, Naxos, Greece	5	DS	*D. ariadnae*
19	7	Melanes, Naxos, Greece	5	DS	*D. improvisa*
20	9	Nagos, Chios, Greece	5	DS	*D. effusa*
	10	Nagos, before the opening to the sea, Chios, Greece			
21	8	Manolates, Samos, Greece	5	DS	*D. damoae*
22	11	Kalamoudi, Euboea, Greece	2	UCS	*Dugesia* sp.
23	11	Kalamoudi, Euboea, Greece	1	UCS	*Dugesia* sp.
24	5	Petaloudes Valley, Rhodes, Greece	2	DS	*D. elegans*
25	1	Georgioupoli, Crete, Greece	1	DCL	*D. cretica*
26	1	Georgioupoli, Crete, Greece	4		*D. cretica*
27	3	Sasalos, Crete, Greece	4		*D. cretica*
28	3	Sasalos, Crete, Greece	1		*D. cretica*
29	2	Kakopetros, Crete, Greece	5		*D. cretica*
30	–	Vernár, Slovak Republic	1	UCS	*Dugesia* sp.
31	–	Ludrová, Slovak Republic Prosiek, Slovak Republic	2	UCS	*Dugesia* sp.
32	–	Limburg, Netherlands	2	DS	*D. gonocephala*
33	–	Fita-Kimpouries, Chios, Greece	4	DS	*D. naiadis*
34	–	Tripes-Parparia, Chios, Greece	5	DS	*D. sicula*

*Includes clusters and singletons.

†Locality details may be found in Supporting information Table S1.

‡On the basis of morphology.

CCS, Confirmed Candidate Species; DCL, Deep Conspecific Lineage; DS, Described Species; UCS, Unconfirmed Candidate Species.

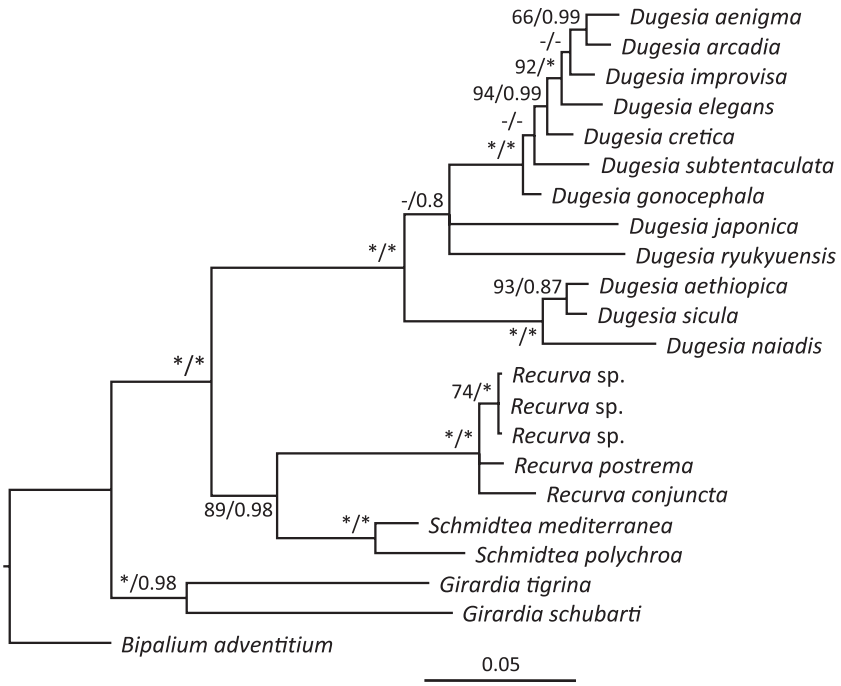


Figure 1. Bayesian tree inferred from the concatenated data set (COI + 18S). Labels correspond to species names. Node numbers correspond to bootstrap (ML)/posterior probability (BI); values are only indicated when >50/ >0.80; '*' indicates maximum support. The scale bar indicates substitutions per site.

Second, *Dugesia arcadia* was identified from Sella and Chalandritsa in the northern Peloponnisos (localities 25 and 26, or entities 15 and 16), but these two localities with morphologically identical individuals are split in the GMYC analysis. However, the divergence of these two populations almost coincides with the GMYC threshold.

A third case concerns specimens from Crete. All individuals that were examined from three sampling localities on this island presented the diagnostic features of *D. cretica*, although the coalescent-based tree splits them into no fewer than five units (entities 25–29), comprising three clusters and two singletons (Fig. 2), which we here consider DCL (Table 1).

Furthermore, 11 clusters and three singletons concern specimens that could not be checked morphologically. Among these cases is a large cluster (entity 17; 12 individuals) that includes three sampling sites from the Peloponnisos (Theisoa-Andritsaina, Agios Floros and Dorio-Psari) and also one individual from Lake Trichonida (Varia, Aetolia-Acarnania) in Central Greece. Another case is an individual from the Potamia locality in Preveza (entity 6), constituting a singleton that groups with high support with a different clade than the other four specimens from the same locality (entity 5), thus suggesting the presence of two different species at the same site. Finally, three individuals from Euboea also split in two different clusters (22 and 23). All of these clusters and singletons for which we lack morphological data are here considered as Unconfirmed Candidate Species.

SYSTEMATIC AND INTEGRATIVE SECTION

Order Tricladida Lang, 1884

Family Dugesiidae Ball, 1974

Genus *Dugesia* Girard, 1850

Dugesia effusa Sluys **sp. nov.** (Figs 3–5)

Material examined: Holotype: ZMA V.Pl. 7114.1, river just before opening into the sea, Nagos, Chios, Greece, 38°33′32.31″N, 26°4′59.42″E, 30 April 2010, coll. M. Vila-Farré, sagittal sections on seven slides.

Paratypes: ZMA V.Pl. 7114.2, ibid., sagittal sections on six slides; V.Pl. 7114.3 (RS 221-3), ibid., horizontal sections on three slides.

Other material: ZMA V.Pl. 7115.1, river, Nagos, Chios, Greece, 38°33′27.57″N, 26°4′51.61″E, 30 April 2010, coll. M. Vila-Farré, sagittal sections on five slides; V.Pl. 7115.2, ibid., sagittal sections on five slides; V.Pl. 7115.3, ibid., horizontal sections on three slides.

Etymology: The specific epithet is derived from the Latin adjective *effusus*, generous, abundant, and alludes to the highly glandularized penis papilla.

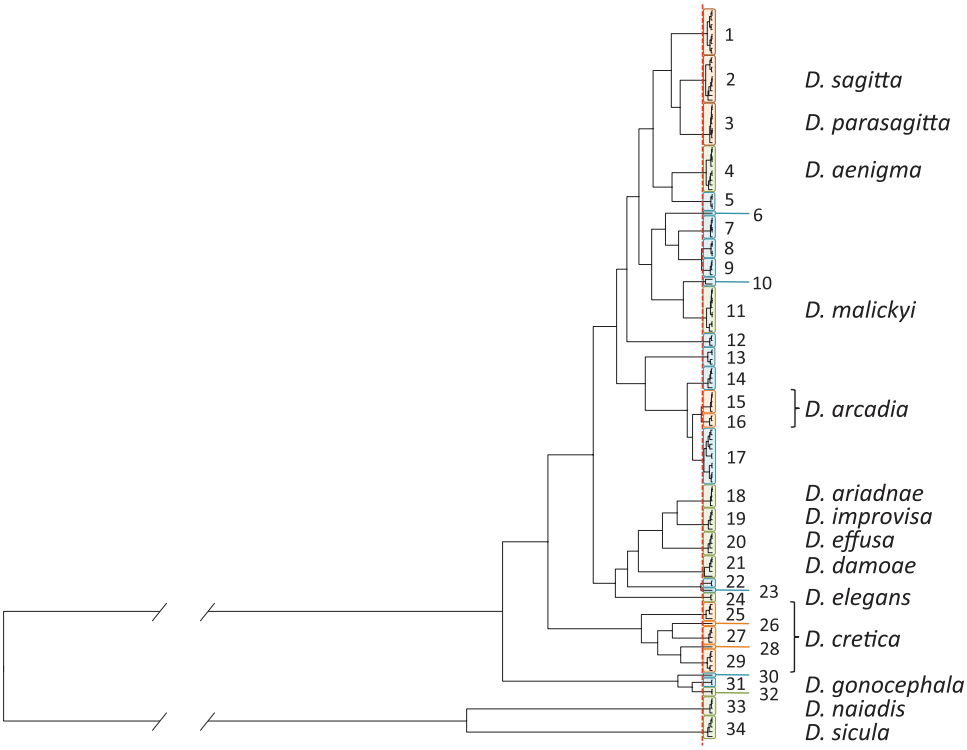


Figure 2. Result of the GMYC analysis. Threshold-delimiting speciation and coalescent processes plotted as a broken line. Numbers indicate molecular-based entities; labels correspond to species names. Entities in green show correspondence between the molecular species delimitation method and the morphologically identified species. In orange are shown groupings where there is conflict between morphological and molecular methods. In blue are shown the groupings for which only molecular data are available.

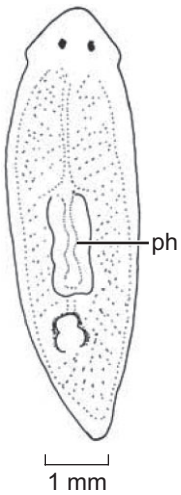


Figure 3. ***Dugesia effusa*** Sluys **sp. nov**. Dorsal view of preserved specimen.

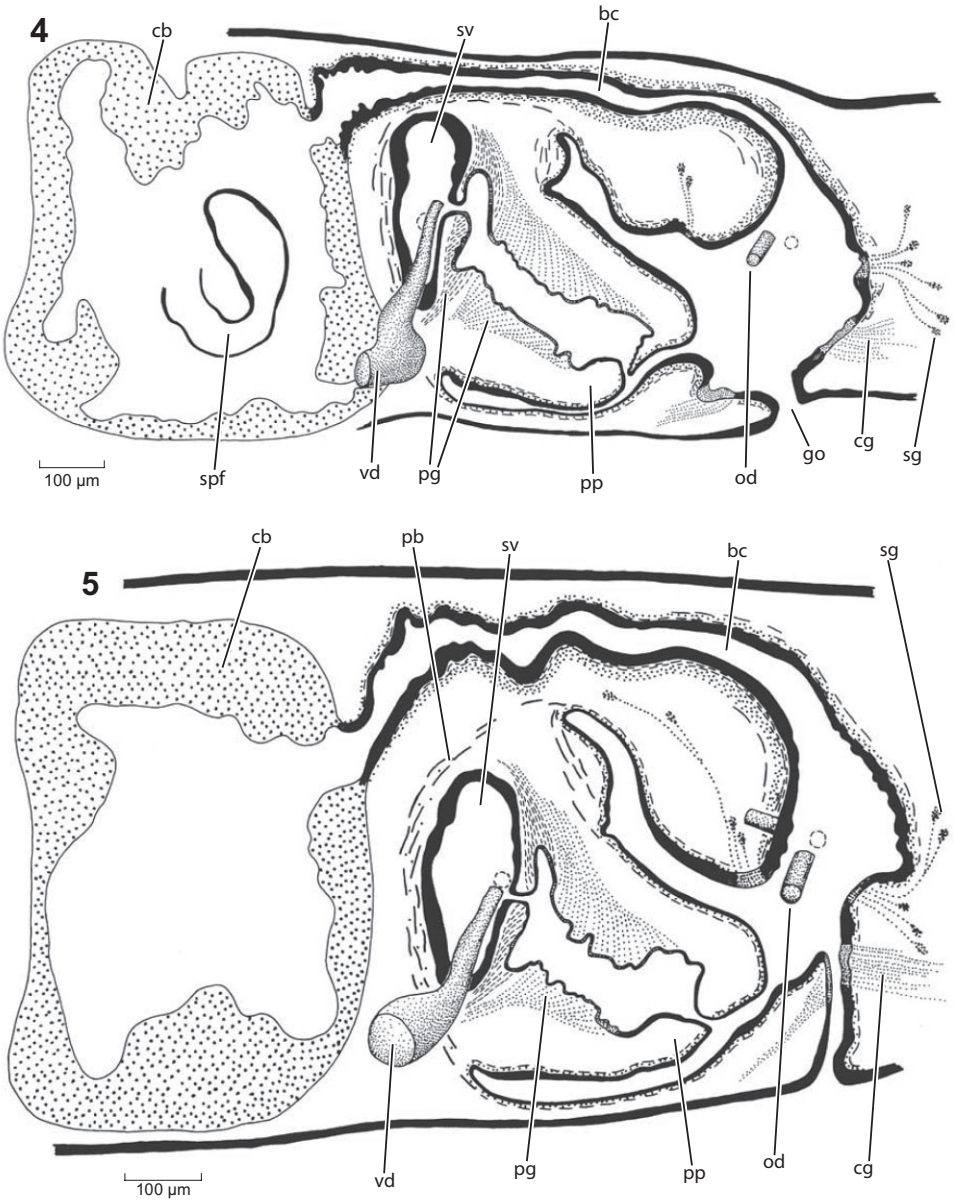
Diagnosis: Dugesia effusa is characterized by the combination of the following features: presence of a small, dorsal penial fold; central ejaculatory duct; short, valve-like diaphragm; large, intrabulbar seminal vesicle; highly glandularized penis papilla; a bursal canal that widens considerably at its communication with the atrium; ectal reinforcement of the bursal canal confined to the vaginal region.

Ecology and distribution: The species is known only from two sites in the same river, i.e. the type locality close to the opening into the sea and another site further upstream.

Description: Preserved specimens up to 9 × 2.25 mm, with low-triangular head with rounded auricles; tail obtusely pointed (Fig. 3). Dorsal surface pale brown; ventral surface pale. Two eyes, situated in pigment-free patches.

Pharynx situated in the mid-region of the body, measuring between one-quarter and one-sixth of the body length. Mouth opening located at the posterior end of the pharyngeal pocket.

The testes are located dorsally and extend from the level of the ovaries into the posterior end of the body. The vasa deferentia penetrate the ventro-lateral wall of the penis bulb and open into the seminal vesicle at a point very close to the diaphragm. The ovoid or



Figures 4, 5. ***Dugesia effusa*** Sluys **sp. nov. 4.** ZMA V.Pl. 7114.2. Sagittal reconstruction of the copulatory apparatus. **5.** ZMA V.Pl. 7114.1. Sagittal reconstruction of the copulatory apparatus.

pear-shaped seminal vesicle fills the major part of the penis bulb and is lined with a columnar, nucleated epithelium. Through a very narrow diaphragm this seminal vesicle opens into the funnel-shaped, proximal section of the ejaculatory duct (Fig. 4). The short, stubby lips of the valve-like diaphragm, as well as the funnel-shaped section of the ejaculatory duct, receive the finely granular and dark red staining secretion of erythrophil penis glands. The broad ejaculatory duct follows a slightly ventrally displaced course through the penis papilla and opens at the blunt tip of the penis papilla, the actual opening being rather narrow. Along the major part of its length the lining epithelium of the ejaculatory duct is pierced by the numerous openings of abundant penis glands that produce an orange–brown secretion.

The plug-shaped penis papilla is lined with a nucleated epithelium and is provided with a subepithelial layer of circular muscles, followed by a layer of longitudinal muscles. A penial fold is located symmetrically at the dorsal base of the penis papilla; the fold is traversed by some longitudinal muscle fibres.

The ovaries are situated directly medially to the ventral nerve cords and are located at one-third to

one-quarter of the distance between the brain and the root of the pharynx. The oviducts are lined with an infranucleated epithelium and are surrounded by a well-developed coat of circular muscles. The oviducts open separately into the ventral-most, widened section of the bursal canal, close to the point where the canal communicates with the atrium. Shell glands discharge their secretion into the bursal canal ventrally to the oviducal openings.

The bursal canal is lined with a nucleated, cuboidal-columnar epithelium. The diameter of the bursal canal increases considerably near its point of communication with the atrium. Notably the most ventral section of the canal, at the level of the oviducal openings, shows a widening into posterior direction (Fig. 5). The bursal canal is overlain with a thin layer of circular muscles, the latter being particularly developed in the vaginal region. Ectal reinforcement in the form of outer longitudinal muscle fibres is present in the vaginal area and extends towards the point where the bursal canal bends forwards. The copulatory bursa is a voluminous sac-shaped structure that fills the entire dorsoventral space of the body. In several specimens remnants of a spermatophore are present in the bursa.

Discussion

A dorsal penial fold of similar size and location as in this species *D. effusa* is present also in *D. sagitta* (some specimens have only one, dorsal fold), *D. malickyi*, *D. benazzii* Lepori, 1951, *D. elegans* and *D. leporii* Pala, Stocchino, Corso & Casu, 2000. In *D. elegans* the openings of the vasa deferentia into the seminal vesicle are far removed from the diaphragm, contrasting with the location of the openings immediately anterior to the diaphragm in all other species mentioned. In addition, the penial fold of *D. elegans* is more developed and more strongly muscular than in *D. effusa*. (cf. De Vries, 1984).

Dugesia leporii differs from *D. effusa* in the presence of a pointed diaphragm and small intrabulbar seminal vesicle, and in the fact that its ectal reinforcement extends from the vaginal area far anterior along the bursal canal (cf. Pala *et al*., 2000). In contrast to *D. effusa*, *D. benazzii* is provided with a small intrabulbar seminal vesicle and a pointed diaphragm (cf. Lepori, 1951; De Vries, 1984).

The gross morphology of *D. effusa* is very similar to that of *D. malickyi* and *D. sagitta*. But *D. malickyi* differs from *D. effusa* in the presence of (1) a considerably bigger penial fold that also has a distinctly lateral position, and (2) a much narrower and distinctly ventrally displaced ejaculatory duct, the latter being devoid of the high glandularization that occurs in *D. effusa*. Such a highly glandular papilla, however, is also characteristic of *D. sagitta* (cf. De Vries, 1984) and also of *D. improvisa* Sluys & Solà sp. nov. The last-mentioned species lacks the penial fold as well as the widening of the bursal canal in the vaginal area, while its seminal vesicle is highly glandular, in contrast to the conditions in *D. effusa*.

The GMYC analysis supports *D. effusa* as a different species (Fig. 2, Table 1), clearly delimitating the specimens from Chios as entity 20. Furthermore, *D. effusa* is not close to *D. sagitta* in the phylogenetic tree of Solà *et al*. (2013). Nevertheless, *D. effusa* shares with *D. sagitta* the 'V-shaped' glandular zone that surrounds the ejaculatory duct (cf. De Vries, 1984: 106). In *D. sagitta* there are usually two penial folds, the ventral one being smaller than the dorsal one; the ventral fold may also be completely absent. However, in relation to the size of the penis papilla, the penial fold of *D. sagitta* is considerably bigger than that in *D. effusa*. Furthermore, the dorsal penial fold of *D. sagitta* is traversed by a cyanophilic secretion, which is discharged through its lining epithelium; such is not the case in *D. effusa*.

DUGESIA IMPROVISA SLUYS & SOLÀ SP. NOV. (FIGS 6–9)

Material examined: Holotype: ZMA V.Pl. 7116.1, Melanes, Naxos, Greece, 37°5′3.38″N, 25°26′59.40″E, alt. 199 m, 9 April 2009, coll. Eduardo Mateos & Eduard Solà, sagittal sections on nine slides.

Paratypes: ZMA V.Pl. 7116.2, ibid., sagittal sections on ten slides; V.Pl. 7116.3, ibid., horizontal sections on four slides; V.Pl. 7116.4, ibid., sagittal sections on eight slides.

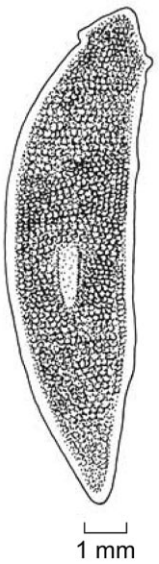
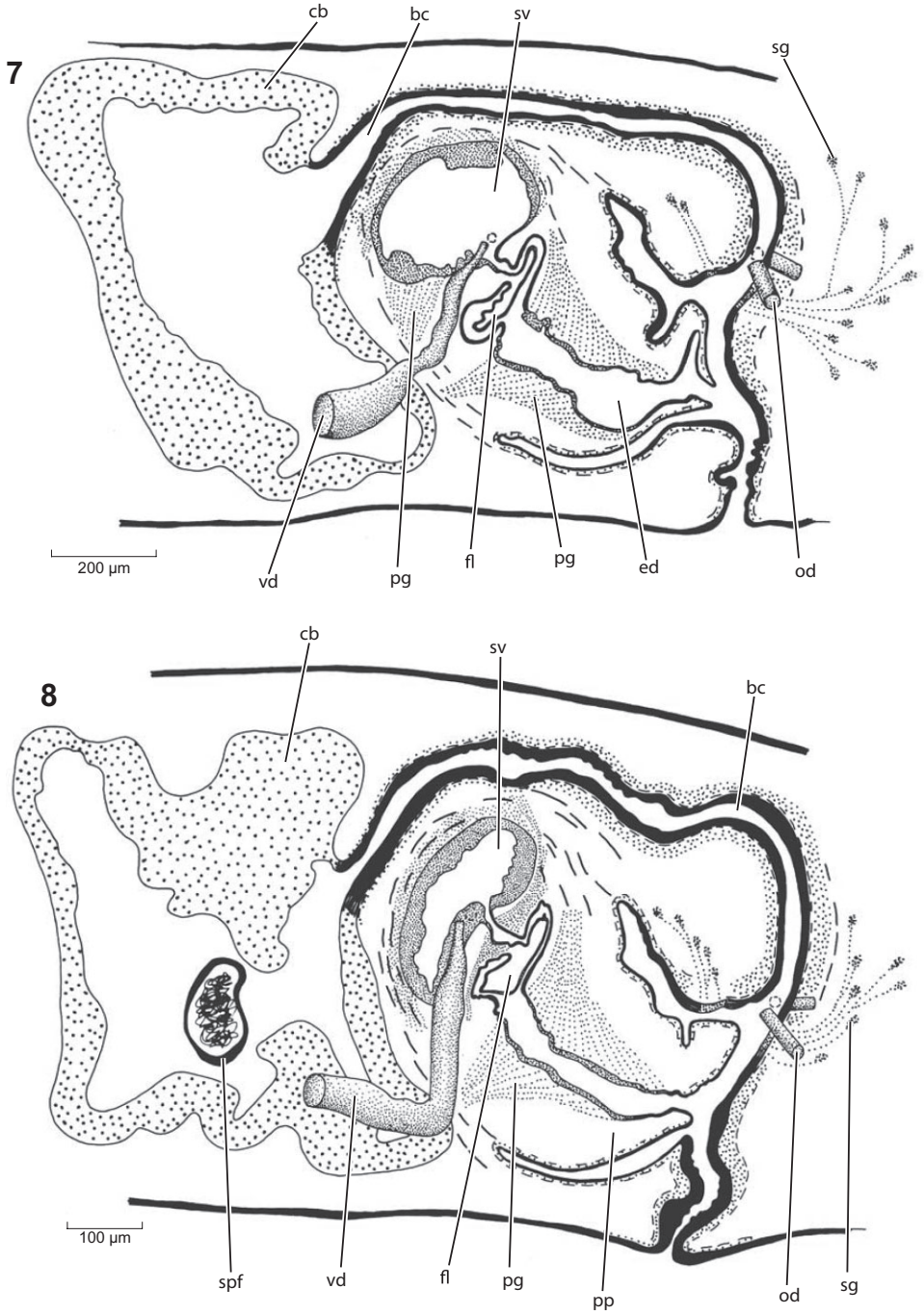


Figure 6. ***Dugesia improvisa*** Sluys & Solà **sp. nov.** Dorsal view of preserved specimen.



Figures 7, 8. ***Dugesia improvisa*** Sluys & Solà **sp. nov. 7.** ZMA V.Pl. 7116.2. Sagittal reconstruction of the copulatory apparatus. **8.** ZMA V.Pl. 7116.1. Sagittal reconstruction of the copulatory apparatus.

Etymology: The specific epithet is derived from the Latin adjective *improvisus*, unexpected, and alludes to our surprise in finding a second and new species of *Dugesia* on such a small island as Naxos.

Diagnosis: Dugesia improvisa is characterized by: an acentral, ventrally displaced ejaculatory duct, opening at the tip of the penis papilla; a short diaphragm; ectal reinforcement being confined to the posterior wall of the ascending portion of the bursal canal; vasa deferentia separately opening into the anterior section of the seminal vesicle, at a point close to the diaphragm; broad zone of abundant penis glands traversing the penial papilla and opening into the ejaculatory duct.

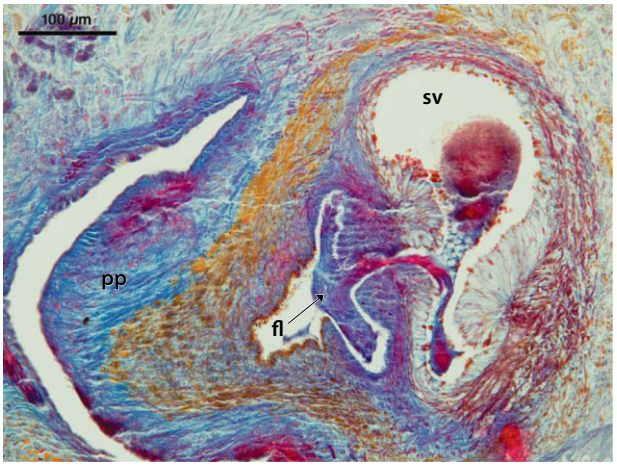


Figure 9. ***Dugesia improvisa*** Sluys & Solà **sp. nov.** Photomicrograph of penial complex of specimen ZMA V.Pl. 7116.4, showing the sickle-shaped flap of tissue or secretion.

Ecology and distribution: Specimens were collected from under stones in a small, shallow pool, receiving the outflow of water from a concrete pipe. The species is known only from this type locality.

Description: Preserved specimens up to about 12.5 × 3 mm. Triangular head with distinct, blunt auricles. Posterior end obtusely pointed. Dorsal surface pale brown, with the pigment arranged in a finely reticulated pattern and with a concentration of pigment following the outline of the pharyngeal pocket (Fig. 6). Dorsal body margin and ventral surface pale. The two eyes are situated in conspicuous pigment-free patches.

The pharynx is located in the posterior half of the body and measures about 1/8th of the body length in preserved specimens. The mouth opening is located at the posterior end of the pharyngeal pocket.

The testes are located dorsally and extend from the level of the ovaries to the posterior end of the body.

The vasa deferentia penetrate the antero-lateral wall of the intrabulbar seminal vesicle; the ducts open separately into the vesicle at a position very close to the diaphragm (Fig. 7). The intrabulbar seminal vesicle is lined with an epithelium, consisting of columnar cells, that is pierced by the numerous openings of penis glands, the latter producing a granular, erythrophil secretion. At the free end of the lining epithelium of the seminal vesicle this secretion projects into the lumen as relatively large, pear-shaped, granular drops. Through a short, stubby diaphragm the seminal vesicle opens into the proximal, funnel-shaped section of the ejaculatory duct.

The diaphragm is short. The proximal funnel-shaped section of the ejaculatory duct, immediately adjacent to the diaphragm, houses a sickle-shaped flap of tissue or secretion (Figs 7–9). This flap seems to be attached to the rest of the diaphragm by only a minute piece of tissue. The lining epithelium of the flap is pierced by the openings of the erythrophil penis glands that open into the seminal vesicle and also penetrate the epithelium of the rest of the diaphragm. The flap was observed in all four specimens examined and its histology suggested true mesenchyme, surrounded by an epithelium.

The ejaculatory duct runs slightly acentrally, i.e. ventrally displaced, through the penis papilla, opening at its tip. The major portion of the ejaculatory duct receives the conspicuous, abundant and granular secretion of erythrophil penis glands, which are located outside of the penis.

The penis papilla is a broad, pointed or blunt cone. The papilla is covered with a nucleated epithelium and is underlain with a subepithelial layer of circular muscles, followed by a layer of longitudinal muscles. The penis bulb is well developed and muscular.

The small, paired ovaries are situated at about 1/3rd of the distance between the brain and the root of the pharynx and are positioned directly medially to the ventral nerve cords. The oviducts arise from the dorsal surface of the ovaries and run backwards immediately dorsally to the ventral nerve cords. At the level of the copulatory apparatus the oviducts curve dorso-medially to open separately into the most proximal, posterior, section of the bursal canal, i.e. close to the point where the duct communicates with the atrium. Erythrophil shell glands open into the bursal canal immediately ventrally to the openings of the oviducts.

The bursal canal is lined with a cuboidal, nucleated epithelium and is surrounded by a reversed musculature: a thin subepithelial layer of longitudinal muscle, followed by a thicker layer of circular muscle. Around the proximal, posterior, section of the bursal canal this circular muscle layer is rather thick, but it becomes gradually thinner towards the copulatory bursa. Ectal reinforcement of the bursal canal musculature is only present along the proximal section of the canal, i.e. from its opening into the atrium to about the point where the duct curves anteriad. However, this ectal reinforcement is only present as a single layer of longitudinal muscle along the posterior wall of the ascending portion of the bursal canal; it was not observed along the anterior wall of this part of the canal. The bursal canal communicates with a large, sac-shaped copulatory bursa, which occupies most of the dorso-ventral space of the body. In two specimens the bursa contained remnants of a sclerotic spermatophore.

Discussion

The presence of a peculiar flap of tissue on the diaphragm sets *D. improvisa* immediately apart from

any of the known species of *Dugesia*. However, in specimens of other species of *Dugesia* a more or less crescent-shaped stretch of secretion may be present in precisely the same position, albeit less clearly attached to the epithelium, while in these specimens its staining properties clearly suggest a glandular origin. In these animals, and also in *D. improvisa*, this flap or stretch of secretion may be related to the formation of the spermatophore (which is formed in the ejaculatory duct) or to the transfer of sperm into the latter. However, in *D. improvisa* the flap did not resemble a spermatophore *in statu nascendi* but suggested true mesenchyme surrounded by an epithelium. We are hesitant to consider this feature as a diagnostic character of *D. improvisa*, but would first prefer to check the presence of this flap in another series of individuals of *D. improvisa*. Unfortunately, additional material is not presently available. However, *D. improvisa* also presents a combination of other characters that makes it different from its congeners.

In the fact that the vasa deferentia open into the seminal vesicle at a point close to the diaphragm, *D. improvisa* resembles a good number of other species of *Dugesia* (cf. Sluys *et al*., 1998, table II). However, in other features these species differ much from *D. improvisa*, for example in the presence of penial or atrial folds, except *Dugesia subtentaculata* (Draparnaud, 1801) and *D. burmaensis* (Kaburaki, 1918). However, the atrium of *D. subtentaculata* shows a distinct musculo-glandular area (cf. De Vries, 1986), which is absent in *D. improvisa*. Furthermore, *D. subtentaculata* also possesses a ring of spongiose mesenchymatic tissue in the penis papilla that is absent in *D. improvisa*. In addition, in *D. subtentaculata* the ectal reinforcement along the bursal canal is much more developed and extends much farther anteriad.

The gross morphology of the copulatory apparatus of *D. burmaensis* is very similar to that of *D. improvisa*. However, for *D. burmaensis* it has been reported that the oviducts arise from the anterolateral wall of the ovaries, contrasting with their dorsal origin in *D. improvisa*. *Dugesia burmaensis* resembles *D. improvisa* in the presence of highly developed penis glands, discharging their abundant secretion into the ejaculatory duct. Such a broad zone with abundant secretion traversing the penis papilla is also characteristic of *D. sagitta* from Corfu. However, there are a number of clear differences between *D. sagitta* and *D. improvisa*.

In *D. sagitta* the penis papilla is blunt and provided with distinct, asymmetric penial folds at both the dorsal and the ventral side of its base (cf. De Vries, 1984), which are absent in *D. improvisa*. Furthermore, in *D. sagitta* the ejaculatory duct follows a central course through the penis papilla, whereas it has a ventrally displaced trajectory in *D. improvisa*. In addition, the ectal reinforcement of the bursal canal extends much farther anterior in *D. sagitta*.

In all molecular analyses *D. improvisa* is the sister species of *D. ariadnae* (Fig. 2; Solà *et al*., 2013), the latter also restricted in its distribution to the island of Naxos. However, the two species are clearly delimited in the GMYC analysis, while morphologically *D. ariadnae* is very different from *D. improvisa*. In particular, *D. ariadnae* is characterized by two well-developed adenodactyls that are suspended from the dorsal atrial wall, one on either side of the base of the penis. On the basis of our comparative and integrative analysis, as presented above, we conclude that *D. improvisa* concerns a new species.

DUGESIA NAIADIS SLUYS **SP. NOV.**
(FIGS 10–12)

Material examined: Holotype: ZMA V.Pl. 7117.1, 650 m before Kipouries (coming from Fita), Chios, Greece, 38°30′43.31″N, 25°59′55.06″E, 30 April 2010, coll. M. Vila-Farré, sagittal sections on 12 slides.

Paratype: ZMA V.Pl. 7117.2, ibid., sagittal sections on nine slides.

Etymology: The specific epithet is derived from the Latin *naias*, water nymph, and alludes to the small freshwater stream from which the specimens were collected.

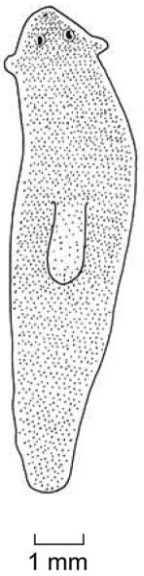
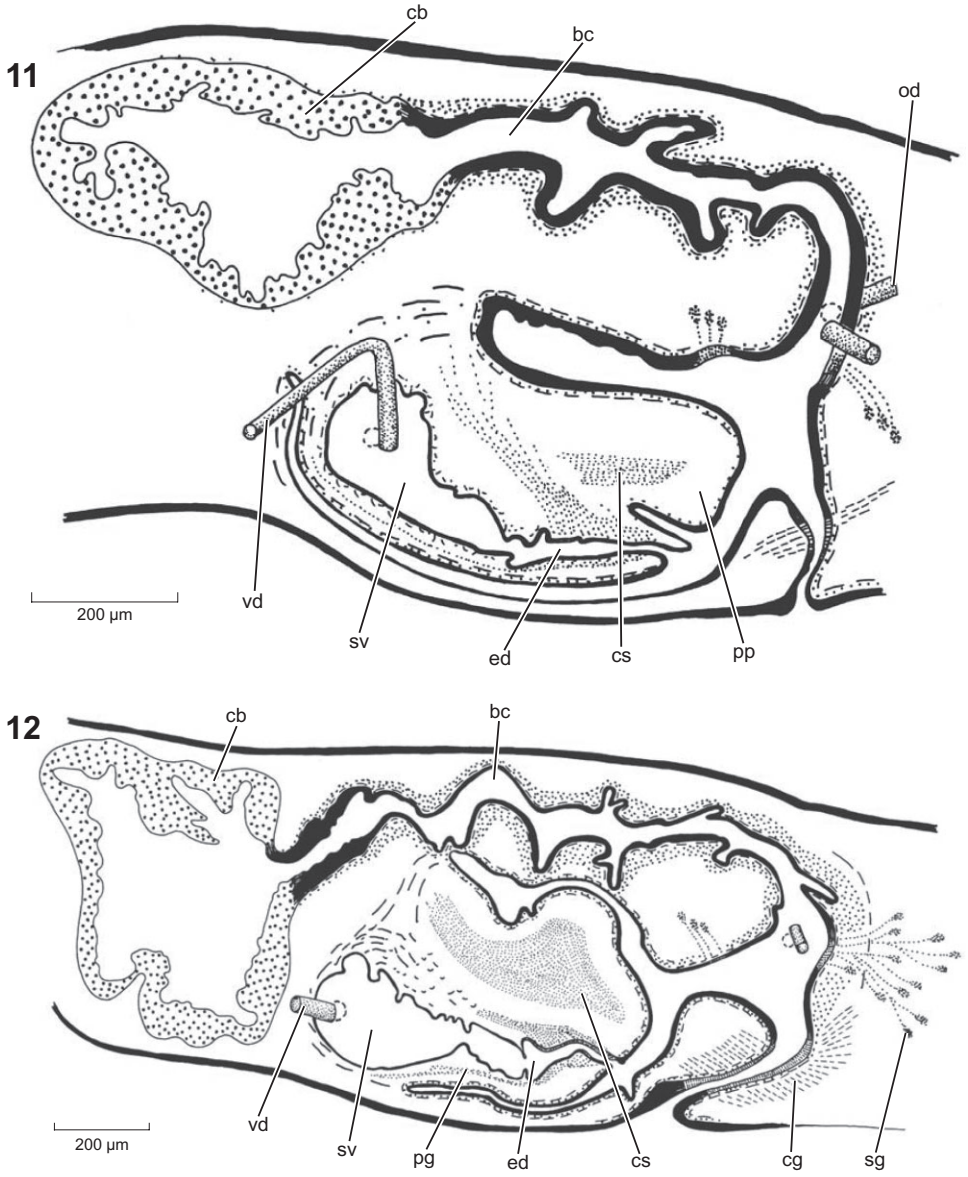


Figure 10. ***Dugesia naiadis*** Sluys **sp. nov.** Dorsal view of preserved specimen.



Figures 11, 12. ***Dugesia naiadis*** Sluys **sp. nov. 11.** ZMA V.Pl. 7117.2. Sagittal reconstruction of the copulatory apparatus. **12.** ZMA V.Pl. 7117.1. Sagittal reconstruction of the copulatory apparatus.

Diagnosis: Dugesia naiadis is characterized by: vasa deferentia that open into the proximal, anterior section of the seminal vesicle; a short diaphragm; an acentral, ventrally displaced ejaculatory duct, opening terminally at the tip of a blunt penis papilla; a broad zone of cyanophilic secretion in the dorsal section of the penis papilla; oviducts that open symmetrically into the most proximal section of the bursal canal; a bursal canal provided with many irregular pleats and folds, surrounded by a well-developed coat of circular muscle and a zone of mesenchymatic, erythrophil gland cells; hyperplasic ovaries; lack of testes.

Ecology and distribution: Specimens were collected from a small creek; the species is known only from the type locality.

Description: Preserved specimens with low triangular head and rounded auricles (Fig. 10), measuring up to 11 mm in length and 2.5 mm in width. Dorsal body surface pale brown; ventral surface pale. A pair of eyes is present and somewhat smaller additional eyes are present also in the sectioned specimens.

Pharynx located in the middle of the body, measuring about 1/6th of the body length. The mouth opening is located at the posterior end of the pharyngeal pocket.

Testes are completely absent. The ovaries are hyperplasic: ovarian tissue fills the entire dorso-ventral space over a distance of about 750 μm. The midpoint of the hyperplasic ovaries is located at about 1/4th the distance between the brain and the root of the pharynx.

The oviducts open separately and symmetrically into the most proximal section of the bursal canal, i.e. close to the point where the canal communicates with the atrium (Fig. 11). Erythrophil shell glands discharge their secretion into the bursal canal, immediately ventrally to the oviducal openings.

The bursal canal is lined with a nucleated epithelium; it follows a somewhat undulating course towards the copulatory bursa, while giving rise to a number of irregular pleats or folds that project into the surrounding mesenchyme (Fig. 12). The canal is surrounded by a very thin, subepithelial layer of longitudinal muscle, followed by a thick layer of circular muscle. Ectally to its surrounding coat of muscles the bursal canal is surrounded by a zone of mesenchymatic, erythrophil gland cells, which discharge their secretion into the lining epithelium of the canal. Only in specimen ZMA V.Pl. 7117.1 (Fig. 12) could ectal reinforcement by some longitudinal muscles be detected on the posterior wall of the bursal canal, in the region of the oviducal openings.

In specimen ZMA V.Pl. 7117.1 (Fig. 12) the copulatory bursa is a large sac-shaped structure that fills the entire dorso-ventral space, but in ZMA V.Pl. 7117.2 (Fig. 11) the bursa is much smaller and also lined with cells with a more densely stained content.

Although the oviducts run from the level of the copulatory apparatus to the ovaries, vasa deferentia could be traced only in the vicinity of the penis bulb. After having penetrated the ventro-lateral wall of the penis bulb, the vasa deferentia open separately into the proximal, anterior section of the seminal vesicle. The latter gradually narrows towards a small diaphragm, through which it communicates with the ejaculatory duct. Seminal vesicle and ejaculatory duct are positioned in the ventral region of the penis papilla, which therefore is asymmetrical: its dorsal section is much larger that the ventral section. The ejaculatory duct receives the secretion of numerous erythrophil penis glands and opens terminally at the blunt tip of the penis papilla. The latter is a plug-shaped structure that fills most of the male atrium. The penis papilla is covered with a nucleated epithelium that is underlain by a thin layer of circular muscle, followed by an equally thin layer of longitudinal muscle. The dorsal section of the penis papilla is traversed by a broad zone of strands of cyanophilic secretion that does not seem to open into the ejaculatory duct or through the covering epithelium of the papilla. The spaces present in the penial mesenchyme, near the tip of the papilla, seem to result from clefts in torn tissue.

Discussion

Presence of hyperplasic ovaries and complete absence of testes are signs that these animals probably concern sexualized specimens from an otherwise asexually reproducing population. Such sexualization may be induced either spontaneously (as was the case with these animals from Chios) or experimentally and has been reported for 11 species of *Dugesia* (cf. Charni *et al*., 2004 and references therein; Stocchino, Sluys & Manconi, 2012; Harrath *et al*., 2013). Furthermore, hyperplasic ovaries and poorly developed testes have been found also in ex-fissiparous specimens of *Phagocata morgani* (Stevens & Boring, 1906; Benazzi & Ball, 1972).

The fortunate circumstance that animals of an otherwise asexually reproducing population sometimes develop reproductive organs enables taxonomic identification of such specimens. In that context, the animals from Chios should be compared with other species for which a ventrally displaced ejaculatory duct has been reported, forming a presumably monophyletic subset within the genus *Dugesia* (Sluys *et al*., 1998). This comparison should be restricted to those species in which the ventrally displaced ejaculatory duct opens terminally at the tip of the penis papilla, thus excluding species with a subterminal opening. This immediately excludes *D. sicula*, *D. aethiopica* Stocchino *et al*., 2013 and *Dugesia arabica* Harrath & Sluys, 2013 as candidate species because these have a subterminal opening of the ejaculatory duct. However, both *D. aethiopica* and *D. arabica* resemble the Chios specimens in the presence of a bursal canal with many elaborate folds, a feature that has been reported also for *D. biblica* (cf. Benazzi & Banchetti, 1972), albeit that in the latter it is much less developed in comparison with *D. aethiopica*, *D. arabica* and the Chios specimens of *D. naiadis*. For *D. biblica* Benazzi & Banchetti (1972) describe the bursal canal as having '. . . un diametro alquante irregolare . . .' [a considerably irregular diameter], which agrees with our observations on specimens from Israel (ZMA V.Pl. 698.1, V.Pl. 699.1).

Another difference between the Chios animals and *D. aethiopica* and *D. sicula* concerns the openings of the oviducts into the bursal canal. In both *D. sicula* and *D. arabica* the oviducal openings are highly asymmetrical, in contrast to the symmetrical openings in *D. naiadis* (cf. Sluys, 2007; Harrath *et al*., 2013). In the specimens of *D. aethiopica* from Ethiopia the situation is different in that the oviducts open symmetrically into the ventral part of the horizontally running section of the bursal canal. In these

type specimens the proximal section of the bursal canal approaches the atrium by running more or less parallel to the body surface, thus contrasting with the course of the canal in *D. naiadis*.

In the presence of mesenchymal glands around the bursal canal and the patch of cyanophilic secretion in the penis papilla *D. naiadis* resembles *D. sicula*, *D. biblica* and the presumed *biblica* specimens from Bucak, Turkey (ZMA V.Pl. 813). However, in other features *D. naiadis* differs from these taxa.

The phylogenetic analysis (Fig. 1) shows that *D. naiadis* belongs to the *sicula–aethiopica* clade (as defined in Lázaro *et al*., 2009) with maximum support (100/1), being the sister group of *D. aethiopica* and *D. sicula*. The fact that the GMYC method (Fig. 2, Table 1, entity 33) delimits the four specimens of *D. naiadis* as a differentiated species supports the description of this new species. Interestingly, *D. naiadis* does not present the duplication in the nuclear ribosomal internal transcribed spacer-1 (ITS-1) molecule that *D. aethiopica* and *D. sicula* share (data not shown; cf. Baguñà *et al*., 1999; Lázaro *et al*., 2009).

On the basis of their gene identity we have been able to assign several asexual *Dugesia* populations from Chios to either *D. naiadis* or *D. sicula* (Table S5).

DUGESIA PARASAGITTA SLUYS & SOLÀ **SP. NOV.** (FIG. 13)

Material examined: Holotype: ZMA V.Pl. 7118.1, Ermones, Corfu, Greece, 39°36′37.98″N, 19°46′41.64″E, somewhat higher upstream than ZMA V.Pl. 7119, 20 April 2009, coll. R. Sluys, sagittal sections on 13 slides.

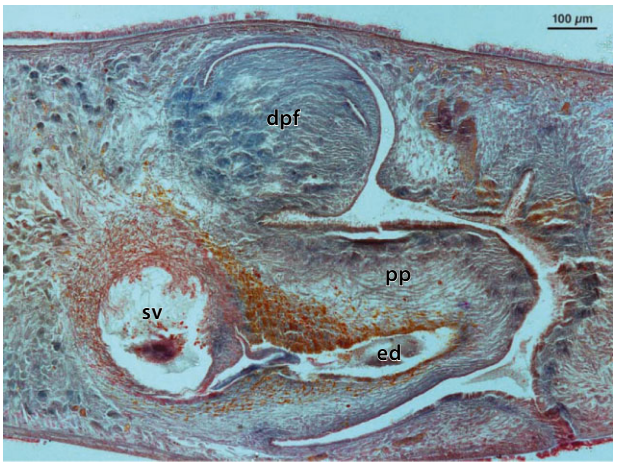


Figure 13. ***Dugesia parasagitta*** Sluys & Solà **sp. nov.** Photomicrograph of large dorsal penial fold in specimen ZMA V.Pl. 7118.1.

Paratypes: ZMA V. Pl. 7118.2, ibid., horizontal sections on eight slides; V.Pl. 7118.3, ibid., sagittal sections on six slides.

Other material examined: ZMA V.Pl. 7119.1, Ermones, Corfu, Greece, 39°36′41.93″N, 19°47′1.40″E, outflow of river into the sea, 20 April 2009, coll. R. Sluys, sagittal sections on five slides; V.Pl. 7119.3, ibid., horizontal sections on six slides; V.Pl. 7119.4, ibid., sagittal sections on 18 slides, V.Pl. 7119.5, ibid., sagittal sections on 14 slides; V.Pl. 7119.6, ibid., sagittal sections on 17 slides.

Etymology: The specific epithet is based on the prefix *para* (somewhat resembling, related to) and the specific epithet of the species *D. sagitta*.

Diagnosis: The species differs morphologically from its closest relative, *D. sagitta*, in the presence of a very large dorsal penial fold, very small ventral fold and a ventrally displaced ejaculatory duct.

Ecology and distribution: The species is known only from two sites in the same river. One site is close to the opening of this river into the sea, while the type locality is located slightly farther upstream.

Comparative discussion: The taxonomic status of *D. sagitta* (Schmidt, 1861) from Corfu as a valid and separate species was clarified by De Vries (1984). Prior to her study, the *Dugesia* populations from Corfu were usually considered to be conspecific with *D. gonocephala*, following a conclusion reached by Komárek (1925). To avoid future taxonomic confusion, De Vries (1984) fortunately designated a series of neotypes for *D. sagitta*. Although the International Code of Zoological Nomenclature (ITZN, 1985; ICZN, 1999) restricts designation of a neotype to only one specimen that forms the new name-bearing type of a nominal species and thus does not allow it to be a *series* of animals, the neotype specimens specified by De Vries (1984: 104) represent a morphologically homogeneous set of animals. As neotype locality was chosen Messonghi River, just west of Messonghi.

The Ermones population was first mentioned by Ball (1979), who attributed it to *D. gonocephala*. In the same paper the karyotype of presumed *D. gonocephala* from Corfu was analysed but it is not clear which population was studied, either the one from Ermones or the animals from Messonghi River. However, De Vries (1984) writes that animals from the neotype locality of *D. sagitta*, i.e. Messonghi River, were analysed.

Our integrative analysis of the populations that we sampled from Corfu revealed an unexpected and interesting situation. Molecular analysis of both COI and ITS-1 grouped the various populations sampled

into three clades (cf. Solà *et al*., 2013). These three clades are also identified as separate entities in the GMYC analysis (Fig. 2, entities 1, 2 and 3). One clade was formed by populations 27, 28, 29 and 30 (i.e. north of the San Salvador mountain range). The second clade consisted of populations 33 and 34. The third clade consisted of two samples from basically the same locality, namely Ermones (localities 31 and 32) (Fig. S2).

On the basis of morphological analysis of the populations from Corfu we were able to differentiate between only two types. The majority of the populations sampled conformed to the classical diagnosis of *D. sagitta*, notably in the presence of a well-developed dorsal fold and a very small or absent ventral fold, and with a central ejaculatory duct. This also holds true for populations that we have not re-collected, but of which material is present in the collections of the NBC: Messonghi River, Marbella beach (now called Par. Ag. Ioannis Peristeron) and Mesaria. However, the population from Ermones (ZMA V.Pl. 7118 + V.Pl. 7119) is characterized by a very large dorsal penial fold, very small ventral fold and a ventrally displaced ejaculatory duct (Fig. 13). Thus, coincidence of molecular and morphological results suggests that at least the population from Ermones is well differentiated from other populations on Corfu. Therefore, we do here describe this population as the new species *D. parasagitta*.

It remains remarkable that the populations that are geographically closest to the *D. sagitta* type locality, namely ZMA V.Pl. 7120 from near Vouniatades (locality 33) and ZMA V.Pl. 7121 from near Benitses (34) (entity 2, Fig. 2), differ molecularly so much from the populations in the northern part of the island (entity 1, Fig. 2), whereas morphologically they cannot be distinguished from each other, nor from the neotype population.

After the separation and description of *D. parasagitta*, the nominal species *D. sagitta* actually forms a paraphyletic taxon, according to all molecular analyses done so far (cf. Solà *et al*., 2013; COI gene tree, Fig. 2). Furthermore, the geographical distribution of the various populations (Fig. S2) suggests that these two units form two independent lineages. In view of the definition of a species as an independently evolving lineage, this suggests that these lineages are actually two different species. We do take a conservative approach to taxonomy and do not assign formal species status to these taxa, pending the availability of further data. However, we do suggest that entity 2 (from localities 33 and 34, i.e. in the proximity of the neotype locality of *D. sagitta*) is assigned to the nominal species *D. sagitta*, and that entity 1 (from localities 27 and 29) represents a UCS (Fig. S2, Table 1).

GENUS *RECURVA* SLUYS **GEN. NOV.**

Diagnosis: Dugesiidae with very slender body and rotund head. Asymmetrical penis papilla with oblique or almost vertical orientation, when non-extended. Ejaculatory duct with a distinctly subterminal opening at the anterior or antero-ventral side of the penis papilla and surrounded by a well-developed coat of circular muscle. Testes dorsal, distributed throughout the body length. Intrabulbar seminal vesicle surrounded by well-developed coat of interwoven muscle. Common oviduct, opening onto ventral, horizontal and broadened section of the bursal canal, which receives the openings of shell glands anteriorly to the oviducal opening. Bursal canal covered with a coat of circular muscle.

Type species: *Recurva postrema* Sluys & Solà sp. nov.

Etymology: The generic name is derived from the Latin adjective *recurvus*, bent backwards, and alludes to the situation that the ejaculatory duct curves backwards to such an extent that its opening is located at the antero-ventral side of the penis papilla.

Gender: female.

RECURVA POSTREMA SLUYS & SOLÀ **SP. NOV.**
(FIGS 14–16)

Material examined: Holotype: ZMA V.Pl. 7122.1, NE Laerma, Rhodes, Greece, 36°10′6.76″N, 27°57′34.55″E, alt. 135 m, 5 April 2009, coll. Eduardo Mateos and Eduard Solà, sagittal sections on six slides.

Paratypes: ZMA V.Pl. 7122.2, ibid., sagittal sections on four slides (not fully mature specimen); V.Pl. 7122.3, ibid., sagittal sections on six slides; V.Pl.7122.4, ibid., sagittal sections on four slides; V.Pl. 7122.5, ibid., sagittal sections on seven slides; V.Pl. 7122.6, ibid., horizontal sections on four slides; V.Pl. 7122.7, ibid., sagittal sections on six slides; V.Pl. 7122.8, ibid., sagittal sections on eight slides; V.Pl. 7122.9, ibid., sagittal sections on six slides.

Etymology: The specific epithet is derived from the Latin adjective *postremus*, located posteriorly, and alludes to the far posteriorly located position of the copulatory apparatus.

Diagnosis: Animals slender, with rotund head. Pharynx and copulatory apparatus situated in the far posterior end of the body. Dorsal testes, distributed throughout the body length but anteriormost testes located at a considerable distance behind the brain. Vasa deferentia open asymmetrically into intrabulbar seminal vesicle. Penis papilla asymmetrical, with more or less vertical orientation in the male atrium.

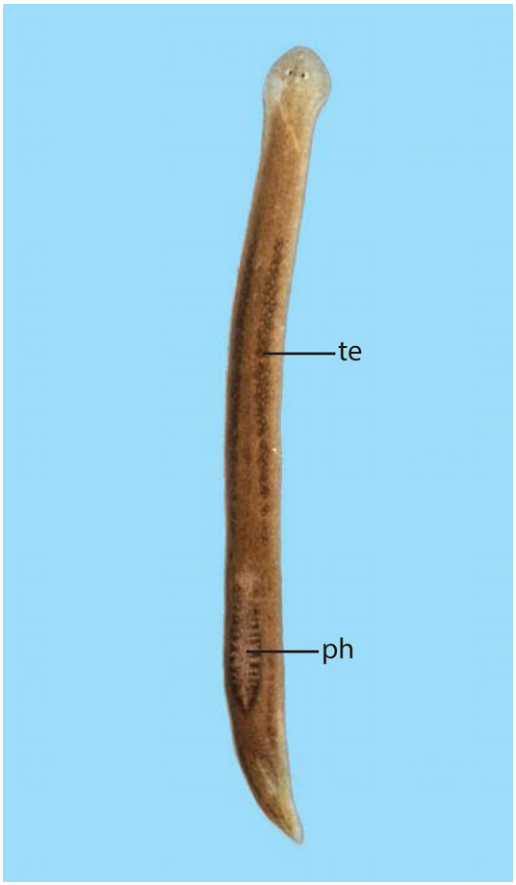


Figure 14. ***Recurva postrema*** Sluys & Solà **sp. nov.** Photograph of external features (scale bar not available).

Ejaculatory duct opening at the anterior or ventro-anterior side of the penis papilla. Ventral or ventro-anterior, muscular penial fold present at the point of insertion of the penis papilla. Ovaries located at about 1/4th the distance between the brain and the root of the pharynx. Distal, posterior parts of the oviducts increase in diameter before communicating with an equally wide common oviduct. Bursal canal is surrounded by a well-developed coat of circular muscle.

Ecology and distribution: The species is known only from the type locality, where it was collected from stagnant water in a rather dry creek. Specimens were found in high numbers, gliding on the substrate, together with other small, white flatworms of an unknown species.

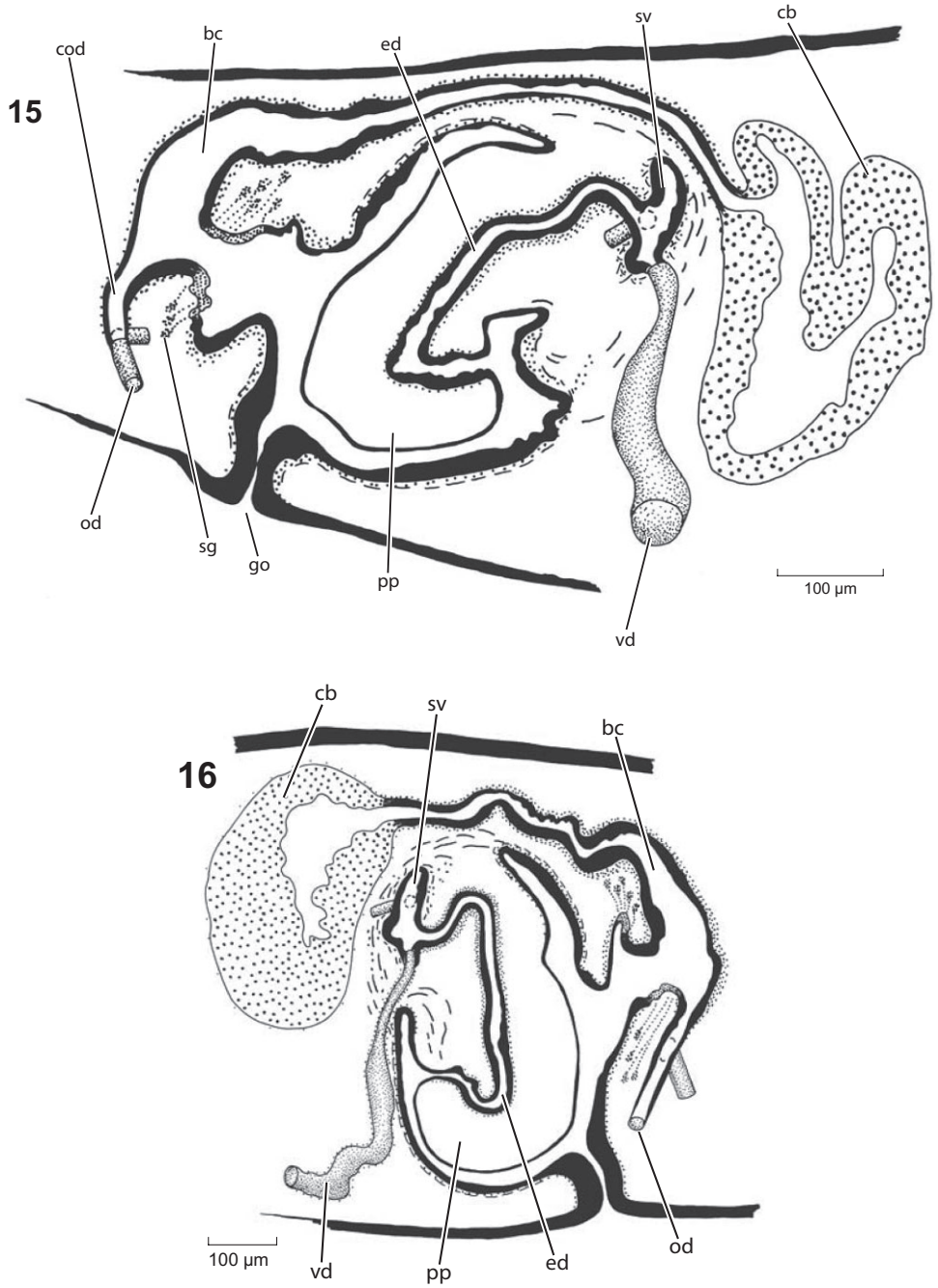
Description: Preserved specimens measure up to 9.5 mm in length and 2.25 mm in width. Notably live specimens are very slender (Fig. 14), with a rotund head that is provided with a pair of close-set eyes, situated in pigment-free patches. Each eye cup houses numerous retinal cells. Behind the eyes, along the lateral margins of the body, there is an auricular streak on either side, at the level of which the head narrows so that there is a more slender neck region. The dorsal surface is finely pigmented pale brown, with notable accumulations of pigment around the pharyngeal pocket. Ventral surface pale.

The pharynx measures between 1/6th and 1/8th of the body length and is positioned far into the posterior part of the body. The musculature of the pharynx conforms to the planariid type. This highly posterior location of the pharynx means that the copulatory apparatus is pushed far into the tail end of the animal. The mouth opening is located at the posterior end of the pharyngeal pocket.

The testes are located dorsally, extending from directly behind the ovaries to almost the posterior margin of the body. After having penetrated the penis bulb, the vasa deferentia open separately into the intrabulbar seminal vesicle. The openings of the seminal ducts are asymmetrical in that one vas deferens opens into the ventral section and the other in a more dorsal section of the seminal vesicle (Figs 15, 16). The latter, lined with a nucleated epithelium and surrounded by a coat of intermingled muscle, communicates with the ejaculatory duct, which in most of the specimens examined exhibits an S-shaped loop before curving downwards to follow its central course through the penis papilla. The papilla is more or less cylindrical in shape and has a more or less vertical orientation in the male atrium. The penis papilla is highly asymmetrical in the sense that in its distal, ventral section the ejaculatory duct shows a sharp, anteriorly directed, knee-shaped bend, after which it opens at the anterior or ventro-anterior side of the penis papilla (Figs 15, 16). This course of the ejaculatory duct results in the situation that the anterior portion or lip of the penis papilla is shorter and smaller, in some specimens much shorter and smaller, than the posterior section. At the base of this anterior or ventro-anterior lip of the penis papilla, at its point of insertion, a penial fold is present. This fold is characterized by a more or less developed outbulging and is provided with its own musculature. It is a penial fold, in contrast to an atrial fold, because it is located entally to the point of attachment of the musculature of the penis bulb. The penis papilla is covered with a thin, nucleated epithelium.

The ovaries are located at about 1/4th the distance between the brain and the root of the pharynx. This implies that also the row of testes starts at a considerable distance posterior to the brain, as may be observed even in living specimens (Fig. 14).

Directly posterior to the gonopore the oviducts turn dorso-medially, while their diameter increases



Figures 15, 16. ***Recurva postrema*** Sluys & Solà **sp. nov. 15.** ZMA V.Pl. 7122.4. Sagittal reconstruction of the copulatory apparatus. **16.** ZMA V.Pl. 7122.1. Sagittal reconstruction of the copulatory apparatus.

considerably. Subsequently, the oviducts fuse to form a common oviduct, with an equally wide diameter, that opens into the ventral section of the bursal canal. The latter starts at the copulatory bursa as a rather narrow duct that gradually widens and posterior to the gonopore makes a sharp anteriorly directed bend before opening into the rather dorsal section of the atrium. The more or less horizontally running and widened part of the bursal canal receives the openings of the shell glands anteriorly to the opening of the common oviduct. The nucleated bursal canal is surrounded by a well-developed coat of circular muscle. The copulatory bursa sits immediately anterior to the penis bulb.

RECURVA CONJUNCTA SLUYS **SP. NOV.**
(FIG. 17)

Material examined: Holotype: ZMA V.Pl. 7123.1, near Agios Georgios, Kefalonia, Greece, 38°6′0.72″N, 20°44′55.50″E, 26 April 2009, coll. R. Sluys, sagittal sections of the anterior, prepharyngeal end of the animal on six slides; V.Pl. 7123.1, ibid., sagittal sections of the posterior end (including the pharynx) of the same animal on six slides.

Etymology: The specific epithet is derived from the Latin adjective *coniunctu*s, connected, and alludes to the genito-intestinal connection present in this species.

Diagnosis: Animals slender, with rotund head. Dorsal testes, distributed throughout the body length. Vasa deferentia narrow when penetrating the ventro-lateral side of the penis bulb, subsequently expanding again and opening into the mid-lateral section of the intrabulbar seminal vesicle. Asymmetrical penis papilla, with an oblique, ventro-posterior orientation. Ejaculatory duct opening at the antero-ventral side of the penis papilla. Common oviduct surrounded by a coat of circular muscle. Copulatory bursa communicating with a branch of the intestine. Bursal canal surrounded by a layer of circular muscle.

Ecology and distribution: The species is known only from its type locality, where it was found under stones in an almost dry, muddy stream flowing beneath a concrete bridge.

Description: In the field the two specimens collected (one immature) were identified as *Schmidtea*-like animals, i.e. with a rounded head. The animals were very slender, the holotype specimen measuring up to 2 cm in length when fully stretched and moving. Dorsal surface pigmented, ventral surface pale (as deduced from examination of the sections). Each eye cup houses numerous retinal cells.

The pharynx measures about 1/9th of the body length, its root being situated about half-way along the body length. The mouth opening is located at the posterior end of the pharyngeal cavity.

The testes are situated dorsally, extending from directly behind the brain into the posterior end of the body. The vasa deferentia, which are expanded to spermiducal vesicles, narrow considerably when they penetrate the ventro-lateral side of the penis bulb. Once within the bulb, the ducts expand again in diameter and, subsequently, open into the mid-lateral section of the intrabulbar seminal vesicle. The latter is lined with a nucleated epithelium and surrounded by a rather thick coat of interwoven muscles.

The ejaculatory duct arises from the dorsal section of the seminal vesicle and immediately thereafter

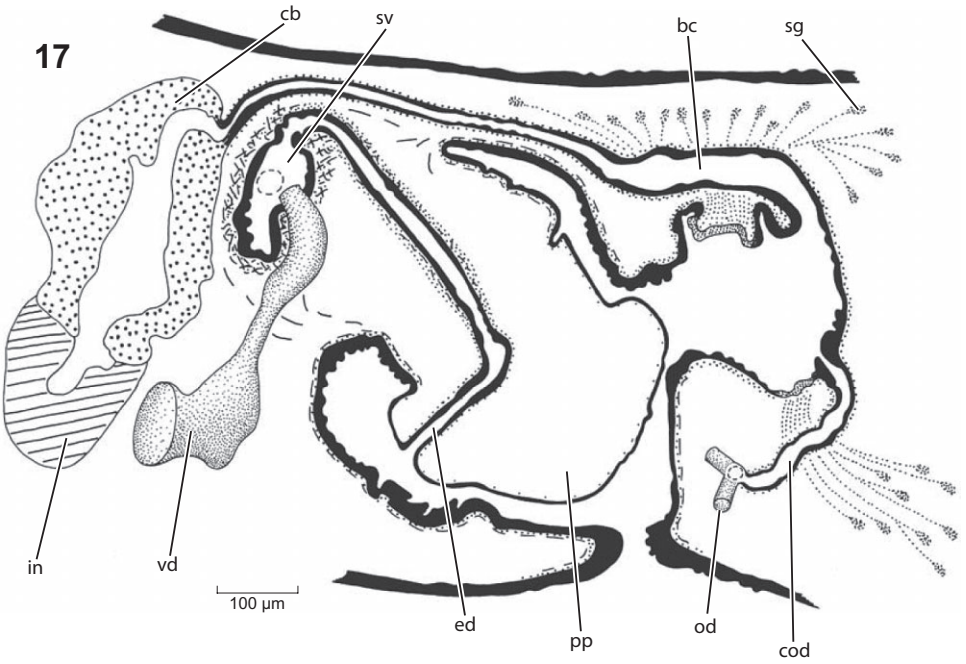


Figure 17. ***Recurva conjuncta*** Sluys **sp. nov.** ZMA V.Pl. 7123.1. Sagittal reconstruction of the copulatory apparatus.

sharply curves postero-ventrally to continue its more or less central course through the penis papilla. However, at some point the ejaculatory duct makes another sharp, hooked bend towards the antero-ventral surface of the body (Fig. 17). This results in the situation that the duct opens at the antero-ventral side of the penis papilla. The papilla has an oblique, ventro-posterior orientation and is covered with a nucleated epithelium. Because of the peculiar course of the ejaculatory duct, the distal section of the penis papilla is highly asymmetrical, with a short ventral lip and a bulky dorsal lip. In fact, the tip of the papilla is to some extent also curved towards the lateral side of the male atrium. Therefore, the opening of the ejaculatory duct is not only displaced towards the antero-ventral side of the penis papilla but also to a more lateral position. This lateral twist of the tip of the penis papilla may be due to a preservation artefact. The major portion of the ejaculatory duct is surrounded by a relatively thick layer of mostly circular muscle fibres.

The paired ovaries are situated directly behind the brain. Immediately posterior to the gonopore the oviducts turn medially and fuse to form a common oviduct, which opens at the postero-ventral section of the bursal canal. The common oviduct is surrounded by a coat of circular muscle.

The bursal canal arises as a broad duct from the mid-posterior wall of the atrium. This first, broad section of the canal runs more or less horizontally and receives the openings of the abundant shell glands, which open anteriorly to the opening of the common oviduct. This broad part of the bursal canal narrows considerably and, subsequently, curves forwards to continue its course immediately dorsally to the male atrium and the penis bulb. Half-way along its course the canal becomes even narrower before communicating with the copulatory bursa. The entire bursal canal is lined with a nucleated epithelium and is surrounded by a layer of circular muscle.

The copulatory bursa lies immediately anterior to the penis bulb, while its ventral part is connected with a branch of the intestine.

Comparative discussion of *Recurva*

The new genus *Recurva* shows a combination of morphological features that sets it apart from all dugesiid genera known at present, albeit that the rounded head, the muscular intrabulbar seminal vesicle and the muscular ejaculatory duct remind one of the genus *Schmidtea*. However, *Schmidtea* is characterized (a) by two muscular seminal vesicles, while (b) its bursal canal is surrounded by a coat of intermingled muscles (characters 18-1 and 22-2, respectively in Sluys, 2001: fig. 7.15), and (c) by separate oviducal openings into the bursal canal. *Recurva* again resembles *Schmidtea* in the dorsally displaced opening of the bursal canal into the atrium. However, such a dorsally displaced communication between bursal canal and atrium is also present in the genus *Cura* Strand, 1942. There are also some other resemblances between *Cura* and *Recurva*, notably (1) the presence of a common oviduct, and (2) the situation that the shell glands open into the section of the bursal canal that lies between its point of communication with the atrium and the point where the canal receives the opening of the common oviduct. However, in other features there is not much resemblance between *Recurva* and *Cura*.

The phylogenetic analyses based on 18S + COI (Fig. 1) and COI alone (Fig. S1) also clearly show that *Recurva* groups independently from the genera *Dugesia*, *Schmidtea*, *Girarda* and *Cura*, and that the species from Rhodes groups closely with the species from Kefalonia. Interestingly, asexual specimens from Paros form the sister group of *Recurva postrema* and *R. conjuncta*, thus constituting an independent lineage. Although we have not performed a molecular species delimitation analysis, this situation nevertheless suggests the presence of a third species of *Recurva* on this island. The external appearance of the Paros animals is very similar to *R. postrema* and *R. conjuncta* in that the animals are also very slender, with rounded head. The Paros specimens (Fig. 18) have their pharynx located in the far posterior region of the body, as is the case also in *R. postrema*. We do here consider the putative third species of *Recurva* from Paros to be a UCS.

A comparison between *Recurva postrema* and *R. conjuncta* reveals clearly that they represent different, species-specific variations on the Bauplan of the genus *Recurva*. Animals of *R. postrema* from Rhodes can be differentiated immediately by the situation that the pharynx and the copulatory apparatus are shifted very far into the posterior end of the body; such is not the case in *R. conjuncta*. Furthermore, *R. postrema* possesses a ventral penial fold, which is absent in *R. conjuncta*. Other differences between the two species concern the asymmetrical openings of the vasa deferentia into the seminal vesicle of *R. postrema*, the fact that the distal sections of its oviducts expand before communicating with the equally wide common oviduct, and the presence of a genito-intestinal connection in *R. conjuncta*.

GENERAL DISCUSSION

Although in the past several papers have been published on the biodiversity of dugesiid freshwater planarians in the Mediterranean region (see above), our study of only the north-eastern Mediterranean

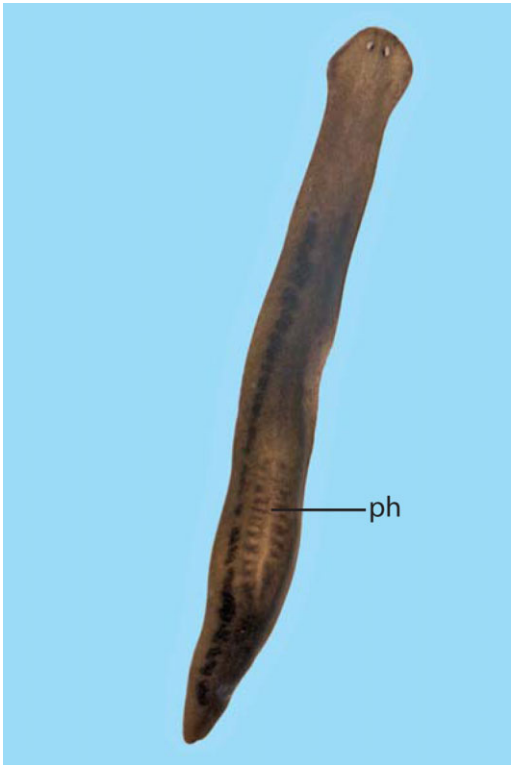


Figure 18. *Recurva* sp. Photograph of external features of specimen from Paros (scale bar not available).

raised the world total of *Dugesia* species with four newly described species, two CCS, 12 UCS from Greece and two more from Slovakia, and at the same time increased the number of dugesiid genera with one new genus, currently comprising two newly described species and one UCS.

Evidently, there is no single objective procedure to delimit higher level taxonomic groups, such as the new genus *Recurva* in the present study. However, the use of genes with a level of variability that results in well-supported and resolved phylogenetic trees (such as 18S rDNA and COI) generally suffices to detect lineage independence. Molecular monophyly combined with the presence of distinct morphological differences subsequently allows for a robust delimitation of higher taxonomic groups, as was the case with *Recurva*.

Although it was not the focus of our present study, it is noteworthy that our results suggest the presence of two UCS of *Dugesia* in Central Europe (Slovakia; entities 30 and 31) that are different from the *D. gonocephala* specimens included in our analysis (Fig. 2, Table 1). Generally, central and northern European specimens of *Dugesia* are assigned to the species *D. gonocephala* as the species has been established to occur with certainty in Denmark, Germany, the Netherlands, Belgium, France, Austria, Bulgaria and the Former Yugoslav Republic of Macedonia (De Vries, 1986). But the fact that Ude's (1908) presumed *D. gonocephala* specimens from Austria differed in certain respects from *D. gonocephala sensu stricto* may have already foreshadowed the possible presence of other species of *Dugesia* in Central Europe, as is now also suggested by our study.

Our study also shows that the planarian diversity of a rather well-researched region such as the Mediterranean remains grossly underestimated and that such must apply to an even greater degree to the global species richness of these animals.

The integrative approach detailed above revealed the beneficial effect of reciprocal illumination of morphological and genetic data in triclads. These different types of data complement each other by pointing out ambiguities or unstable hypotheses on the basis of only a single character set. For example, the gross morphology of *D. effusa* is very similar to that of *D. sagitta* and *D. improvisa*. However, the GMYC analysis delimits *D. effusa* as a different species from *D. improvisa*, while in the phylogenetic trees *D. effusa* is not closely related to *D. sagitta*. Thus, molecular information supports the presumed species status of *D. effusa* that was suggested by the morphological data.

In another case, the opposite situation applied. Molecular data suggested a separate identity for *D. parasagitta* populations on Corfu. As a consequence, more detailed morphological investigations were started, which uncovered some divergent morphological characters with *D. sagitta* as described in the literature and as revealed after examination of both new material and museum specimens. The two data sets thus reinforced each other and induced us to describe the new species *D. parasagitta*.

Although conflicts between datasets can be expected in an integrative taxonomic study because speciation is not always accompanied by simultaneous character change at all levels (Padial & de la Riva, 2009; Padial *et al.*, 2010), our analysis of *Dugesia* actually revealed in many cases a good correspondence between species boundaries hypothesized on morphological data and those suggested by molecular data. As these different lines of evidence generally converged in the delimitation of the same units of biotic diversity, the species taxa recognized can be considered stable systematic hypotheses. For example, we found full correspondence between the GMYC analysis and the morphology-based species hypotheses concerning units from the Eastern and Central Aegean region, namely *D. ariadnae*, *D. damoae*, *D. effusa*, *D. elegans* and *D. improvisa*. However, in other cases we have found DCLs (as in *D. cretica*) or potential cryptic species (as in *D. sagitta*) in which morphology and molecules do not fully correspond. The situation that GMYC can potentially overestimate the number of species (Lohse,

2009) and that we used only a single gene marker has made us refrain from proposing new species solely on the basis of molecular divergence.

Two important conclusions can be drawn from our study. First, despite the fact that we used only a single molecular marker in the present study, GMYC analysis with COI turns out to form a good strategy for detecting potentially new species and for testing the taxonomic status of known species. Second, the morphological features generally used by taxonomists in their comparative studies of dugesiid flatworms indeed result in reliable identifications and delineations of species taxa, at least when no cryptic species are involved, in which case the use of other types of data is unavoidable. This is a comforting insight because it is to be expected that morphological characters will '. . . retain an outstanding role in taxonomy . . .' (Padial & de la Riva, 2010: 753).

ACKNOWLEDGEMENTS

This research was supported by Grant BES-2009-022530 from the Ministerio de Ciencia e innovación to E.S., and by grants CGL2008-00378 and CGL2011-23466 to M.R. Completion of this study was made possible by a grant from the Naturalis Biodiversity Center to R.S. We are indebted to Núria Bonada, Joan Solà, Dora Vázquez and Caterina Rodríguez who provided material and helped with sampling. We are also indebted to Marta Álvarez-Presas for providing the 18S *D. sicula* sequence and to Gema Blasco and Eva Làzaro for laboratory assistance. Professor Dr. M. Kawakatsu (Sapporo) is thanked for nomenclatural advice. We are grateful to Mr J. van Arkel (University of Amsterdam) for the digital rendering of the figures. Mr N. Korenhof (Naturalis Biodiversity Center) is thanked for digitally finalizing the plates.

REFERENCES

Avise JC. 2000. *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

Baguñà J, Carranza S, Pala M, Ribera C, Giribet G, Arnedo M, Ribas M, Riutort M. 1999. From morphology and karyology to molecules. New methods for taxonomical identification of asexual populations of freshwater planarians. A tribute to Professor Mario Benazzi. *Italian Journal of Zoology* **66:** 207–214.

Ball IR. 1974. A contribution to the phylogeny and biogeography of freshwater triclads (Platyhelminthes: Turbellaria). In: Riser NW, Morse MP, eds. *Biology of the Turbellaria*. New York: McGraw-Hill, 339–401.

Ball IR. 1979. The karyotypes of two *Dugesia* species from Corfu, Greece (Platyhelminthes, Turbellaria). *Bijdragen tot de Dierkunde* **48:** 187–190.

Bauer AM, Parham JF, Brown RM, Stuart BL, Grismer L, Papenfuss TJ, Böhme W, Savage JM, Carranza S, Grismer JL, Wagner P, Schmitz A, Ananjeva NB, Inger RF. 2011. Availability of new Bayesian-delimited gecko names and the importance of character-based species descriptions. *Proceedings of the Royal Society B* **278:** 490–492.

Benazzi M, Ball IR. 1972. The reproductive apparatus of sexual specimens from fissiparous populations of *Fonticola morgani* (Tricladida, Paludicola). *Canadian Journal of Zoology* **50:** 703–704.

Benazzi M, Banchetti R. 1972. Descrizione di *Dugesia biblica*, nuova microspecie del 'gruppo *Dugesia gonocephala*' trovata nel fiume Giordano (Israele). *Atti della Società Toscane di Scienze Naturali, Memorie, Series B* **79:** 83–91.

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution* **22:** 148–155.

Burbrink FT, Yao H, Ingrasci M, Bryson JRW, Guiher TJ, Ruane S. 2011. Speciation at the Mogollon Rim in the Arizona Mountain Kingsnake (*Lampropeltis pyromelana*). *Molecular Phylogenetics and Evolution* **60:** 445–454.

Carew ME, Pettigrove V, Hoffmann AA. 2005. The utility of DNA markers in classical taxonomy: using cytochrome oxidase I markers to differentiate Australian *Cladopelma* (Diptera: Chironomidae) midges. *Annals of the Entomological Society of America* **98:** 587–594.

Charni M, Harrath AH, Sluys R, Tekaya S, Zghal F. 2004. The freshwater planarian *Dugesia sicula* Lepori, 1948 (Platyhelminthes, Tricladida) in Tunisia: ecology, karyology, and morphology. *Hydrobiologia* **517:** 161–170.

Cracraft J. 1983. Species concepts and speciation analysis. *Current Ornithology* **1:** 159–187.

Cracraft J. 1987. Species concepts and the ontology of evolution. *Biology and Philosophy* **2:** 329–346.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

De Queiroz K. 2007. Species concepts and species delimitation. *Systematic Biology* **56:** 879–886.

De Vries EJ. 1984. On the species of the *Dugesia gonocephala* group (Platyhelminthes, Turbellaria, Tricladida) from Greece. *Bijdragen tot de Dierkunde* **54:** 101–126.

De Vries EJ. 1986. On the taxonomic status of *Dugesia gonocephala* and *Dugesia subtentaculata* (Turbellaria, Tricladida, Paludicola). *Journal of Zoology, London* **209:** 43–59.

De Vries EJ. 1988. On the identity and occurrence of *Dugesia sicula* and *D. biblica* (Platyhelminthes, Tricladida, Paludicola) in the Mediterranean region. *Fortschritte der Zoologie* **36:** 405–411.

Draparnaud J. 1801. *Tableau des Mollusques terrestres et fluviatiles de la France*. Paris: Montpellier.

Drummond A, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7:** 214.

Dugès A. 1830. Aperçu de quelques observations nouvelles

sur les Planaires et plusieurs genres voisins. *Annales des Sciences Naturelles* **21:** 70–91.

Edwards SV. 2009. Is a new and general theory of molecular systematics emerging? *Evolution* **63:** 1–19.

Ezard T, Fujisawa T, Barraclough T. 2009. *Splits: species' limits by threshold statistics*. R package version 1.0. Available at: http://R-Forge.R-project.org/projects/splits/

Fontaneto D, Herniou EA, Boschetti C, Caprioli M, Melone G, Ricci C, Barraclough TG. 2007. Independently evolving species in asexual bdelloid rotifers. *PLoS Biology* **5:** e87.

Fouquet A, Gilles A, Vences M, Marty C, Blanc M, Gemmell NJ. 2007. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE* **2:** e1109.

Frankham R, Ballou JD, Dudash MR, Eldridge MDB, Fenster CB, Lacy RC, Mendelson III JR, Porton IJ, Ralls K, Ryder OA. 2012. Implications of different concepts for conserving biodiversity. *Biological Conservation* **153:** 25–31.

Fujita MK, Leaché AD, Burbrink FT, McGuire JA, Moritz C. 2012. Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology and Evolution* **27:** 480–488.

Girard C. 1850. A brief account of the freshwater Planariae of the United States. *Proceedings of the Boston Society of Natural History* **3:** 264–265.

Harrath AH, Sluys R. 2013. In: Harrath AH, Sluys R, Aldahmash W, Errazagui AK, Ibrahim K, Alwasel S. 2013. Reproductive strategies, karyology, parasites, and taxonomic status of *Dugesia* populations from Yemen (Platyhelminthes, Tricladida, Dugesiidae). *Zoological Science* **30:** 502–508.

Harrath AH, Sluys R, Aldahmash W, Errazagui AK, Ibrahim K, Alwasel S. 2013. Reproductive strategies, karyology, parasites, and taxonomic status of *Dugesia* populations from Yemen (Platyhelminthes, Tricladida, Dugesiidae). *Zoological Science* **30:** 502–508.

Hey J. 2009. On the arbitrary identification of real species. In: Butlin RK, Bridle J, Schluter D, eds. *Speciation and patterns of diversity*. Cambridge: Cambridge University Press, 15–28.

Humason GL. 1967. *Animal tissue techniques*, 2nd edn. San Francisco: W. H. Freeman.

Hyman L. 1943. Endemic and exotic land planarians in the United States with a discussion of necessary changes of names in Rhynchodemidae. *American Museum Novitates* **1667:** 1–21.

ICZN, International Commission on Zoological Nomenclature. 1999. *International code of zoological nomenclature*, 4th edn. London: The International Trust for Zoological Nomenclature.

ITZN, International Trust for Zoological Nomenclature. 1985. *International code of zoological nomenclature*, 3rd edn. Berkeley and Los Angeles, CA: University of California Press.

Kaburaki T. 1918. Freshwater triclads from the basin of the Inle Lake. *Records of the Indian Museum* **14:** 187–194 + 1 pl.

Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* **9:** 286–298.

Komárek J. 1925. Die O. Schmidt'schen Süsswassertricladen von Corfu und Cephalonia, gesammelt von Prof. Wilhelmi. *Zoologischer Anzeiger* **63:** 322–328.

Lang A. 1884. *Die Polycladen (Seeplanarien) des Golfes von Neapel und der angrenzenden Meeresabschnitte*. Fauna und Flora des Golfes von Neapel, Monographie 11. Leipzig: W. Engelmann.

Lázaro EM, Sluys R, Pala M, Stocchino GA, Baguñà J, Riutort M. 2009. Molecular barcoding and phylogeography of sexual and asexual freshwater planarians of the genus *Dugesia* in the Western Mediterranean (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution* **52:** 835–845.

Lepori NG. 1948. Descrizione di *Dugesia sicula*, nuova sp. di Triclade d'acqua dolce dei dintorni di Catania. *Archivio Zoologico Italia* **33:** 461–472.

Lepori NG. 1951. Sulla caratteristiche morfologiche e sulla posizione sistematica della planaria di Sardegna e Corsica già ascritta a *Dugesia gonocephala* (Dugès). *Atti della Società Toscane di Scienze Naturali, Memorie, Series B* **58:** 28–47.

Lohse K. 2009. Can mtDNA barcodes be used to delimit species? A response to Pons *et al*. (2006). *Systematic Biology* **58:** 439–442.

Meixner J. 1928. Der Genitalapparat der Tricladen und seine Beziehungen zu ihrer allgemeinen Morphologie, Phylogenie, Ökologie und Verbreitung. *Zeitschrift für Morphologie und Ökologie der Tiere* **11:** 570–612.

Memon N, Meier R, Manan A, Su KF. 2006. On the use of DNA sequences for determining the species limits of a polymorphic new species in the stink bug genus *Halys* (Heteroptera: Pentatomidae) from Pakistan. *Systematic Entomology* **31:** 703–710.

Monaghan MT, Wild R, Elliot M, Fujisawa T, Balke M, Inward DJG, Lees DC, Ranaivosolo R, Eggleton P, Barraclough TG, Vogler AP. 2009. Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology* **58:** 298–311.

Moore WS. 1995. Inferring phylogenies from mtDNA variation: mitochondrial gene trees versus nuclear-gene trees. *Evolution* **49:** 718–726.

Nygren A, Pleijel F. 2011. From one to ten in a single stroke – resolving the European *Eumida sanguinea* (Phyllodocidae, Annelida) species complex. *Molecular Phylogenetics and Evolution* **58:** 132–141.

Olson LE, Goodman SM, Yoder AD. 2004. Illumination of cryptic species boundaries in long-tailed shrew tenrecs (Mammalia: Tenrecidae; Microgale), with new insights into geographic variation and distributional constraints. *Biological Journal of the Linnean Society* **83:** 1–22.

Padial J, Miralles A, de la Riva I, Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* **7:** 16.

Padial JM, de la Riva I. 2009. Integrative taxonomy reveals cryptic Amazonian species of *Pristimantis* (Anura: Strabomantidae). *Zoological Journal of the Linnean Society* **155:** 97–122.

Padial JM, de la Riva I. 2010. A response to recent proposals for integrative taxonomy. *Biological Journal of the Linnean Society* **101:** 747–756.

Pala M, Stocchino GA, Corso G, Casu S. 2000. A new species of freshwater planarian belonging to the genus *Dugesia* (Platyhelminthes, Tricladida) from Sardinia. *Italian Journal of Zoology* **67:** 379–383.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55:** 595–609.

Rambaut A, Drummond AJ. 2007. Tracer v1.4. Available at: beast.bio.ed.ac.uk/Tracer

Romeis B. 1989. *Mikroskopische Technik*, 17th edn. München: Urban und Schwarzenberg.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61:** 539–542.

Schmidt O. 1861. Untersuchungen über Turbellarien von Corfu und Cephalonia, nebst Nachträgen zu früheren Arbeiten. *Zeitschrift für wissenschaftliche Zoologie* **11:** 1–32.

Sluys R. 1991. Species concepts, process analysis, and the hierarchy of nature. *Experientia* **47:** 1162–1170.

Sluys R. 2001. Towards a phylogenetic classification and characterization of dugesiid genera (Platyhelminthes, Tricladida, Dugesiidae): a morphological perspective. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*, Syst. Ass. Spec. Vol. ser. 60. London: Taylor & Francis, 57–73.

Sluys R. 2007. Annotations on freshwater planarians (Platyhelminthes Tricladida Dugesiidae) from the Afrotropical Region. *Tropical Zoology* **20:** 229–257.

Sluys R. 2013. The unappreciated, fundamentally analytical nature of taxonomy and the implications for the inventory of biodiversity. *Biodiversity and Conservation* **22:** 1095–1105.

Sluys R, Kawakatsu M, Winsor L. 1998. The genus *Dugesia* in Australia, with its phylogenetic analysis and historical biogeography (Platyhelminthes, Tricladida, Dugesiidae). *Zoologica Scripta* **27:** 273–289.

Solà E, Sluys R, Gritzalis K, Riutort M. 2013. Fluvial basin history in the northeastern Mediterranean Region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution* **66:** 877–888.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22:** 2688–2690.

Stevens NM, Boring AM. 1906. *Planaria morgani* n. sp. *Proceedings of the Academy of Natural Sciences of Philadelphia* **58:** 7–9 + 1 pl.

Stocchino GA, Corso G, Manconi R, Pala M. 2002. African planarians: *Dugesia aethiopica* sp. n. (Platyhelminthes, Tricladida) from Lake Tana (NW Ethiopia). *Italian Journal of Zoology* **69:** 45–51.

Stocchino GA, Sluys R, Manconi R. 2012. A new species of *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae) from the Afromontane forest in South Africa, with an overview of freshwater planarians from the African continent. *Zootaxa* **3551:** 43–58.

Strand E. 1942. Miscellanea nomenclatoria zoologica et palaeontologica. *Folia Zoologica et Hydrobiologica* **11:** 386–402.

Talavera G. 2012. Phylogenetic inference at different insect taxonomic levels. Unpublished D. Phil. Thesis, Universitat Autònoma de Barcelona.

Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56:** 564–577.

Ude J. 1908. Beiträge zur Anatomie und Histologie der Süßwassertricladen. *Zeitschrift für wissenschaftliche Zoologie* **89:** 308–370 + 3 pls.

Vieites DR, Wollenberg KC, Andreone F, Köhler J, Glaw F, Vences M. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences USA* **106:** 8267–8272.

Weiss A. 1909. Turbellaria, Rhabdocoelida and Tricladida paludicola. In: Michaelsen W, Hartmeyer R, eds. *Die Fauna Südwest-Australiens: Ergebnisse der Hamburger südwest-australischen Forschungsreise 1905*. Jena: Verlag von Gustav Fischer, 403–410.

Xia X, Xie Z. 2001. DAMBE: software package for data analysis in molecular biology and evolution. *Journal of Heredity* **92:** 371–373.

Yeates DK, Seago A, Nelson L, Cameron SL, Joseph L, Trueman JWH. 2011. Integrative taxonomy, or iterative taxonomy? *Systematic Entomology* **36:** 209–217.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sampling localities and their various codes.
Table S2. Sampling localities of specimens of ***Recurva*** Sluys **gen. nov**.
Table S3. Forward and reverse primers used in amplification and sequencing.
Table S4. Species and genes used in the phylogenetic analysis.
Table S5. Species status of asexual *Dugesia* from Chios.
Figure S1. Bayesian tree inferred from the COI data set.
Figure S2. Location of the *Dugesia* sampling sites on Corfu.