The taxonomic status of *Dugesia biblica* from Israel and Turkey (Platyhelminthes, Tricladida, Dugesiidae)

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Abstract

The taxonomic status of *Dugesia biblica* (Platyhelminthes, Tricladida, Dugesiidae) from Israel and Turkey is problematic due to its morphological similarity with *D. sicula* since these nominal species present overlapping characters. In this study we analyzed histological preparations of specimens of these two nominal species and also compared mitochondrial *COI* gene sequences from Israeli populations to the already known haplotype composition of *D. sicula*. We concluded that these animals belong to the same species and therefore we consider *D. biblica* to be a junior synonym of *D. sicula*. This implies that the distribution range of *D. sicula* is even wider than previously thought, and that the species is present all around the Mediterranean Basin and on many of its islands.

Keywords

Platyhelminthes, Tricladida, *Dugesia*, taxonomy, synonymy, biogeography, Israel, *COI*, haplotype, karyology, morphology, Turkey

Introduction

The freshwater planarian fauna of Israel has been relatively well studied (Benazzi and Banchetti 1973; Bromley 1974, 1979, 1980; Bromley and Benazzi 1991). Hitherto, six species of triclad flatworms have been formally described for this country: two...
species of *Phagocata*, one *Atrioplanaria*, one *Dendrocoelum*, and two *Dugesia* species, most of them inhabiting the northern part of the State (Bromley 1980; Bromley and Benazzi 1991). The two species of *Dugesia* concern *D. golanica* Bromley & Benazzi, 1991 and *D. biblica* Benazzi & Banchetti, 1973. However, so far it has remained uncertain as to whether *D. biblica* is really a species different from *D. sicula* Lepori, 1948 (De Vries 1988).

*Dugesia biblica* was originally described from fissiparous specimens collected from the Jordan River in Israel (Benazzi and Banchetti 1973). Some of these specimens developed a copulatory apparatus under laboratory conditions. Later, Bromley carried out further studies (e.g. karyological and ecological) on this species by analyzing specimens collected from several springs and streams in the Jordan Rift Valley and from the Nahal Qishon water system (Bromley 1974, 1977, 1979). Bromley also found natural sexually reproducing populations (Bromley 1977, 1980). About a decade later, De Vries (1988) described *D. biblica* from two localities in the Mediterranean region of Turkey and noted that the original morphological description of *D. biblica* matches that of *D. sicula*, due to their partially overlapping diagnoses.

In the course of our studies on the evolution and diversification of the genus *Dugesia* in the Mediterranean region (cf. Lázaro et al. 2009; Lázaro and Riutort 2013; Solà et al. 2013; Sluys et al. 2013), we encountered a similar problem when we found many populations throughout Israel to be molecularly identical to *D. sicula*, a species that has never been described from Israel. This induced us to re-evaluate all currently available information. We re-examined the material studied by De Vries (1988) and also specimens from other populations of *D. sicula* that have become available to us over the past few years. Further, we have made extensive samplings throughout Israel in order to determine through DNA sequence analyses and, if possible, by morphological studies, which species are present in the area. On the basis of this integrative approach we were able to evaluate the taxonomic status of nominal *Dugesia biblica*.

**Materials and methods**

**Sampling**

New samples of *Dugesia* from Israel were obtained during winter, spring and summer seasons in 2009 and 2010. We visited 32 localities (Table 1, Suppl. material 1).

**DNA extraction and sequencing**

Total genomic DNA was extracted by using the commercial reagent DNAzol (Molecular Research Center Inc., Cincinnati, OH), following the manufacturer’s instructions. A fragment of the cytochrome c oxidase subunit I (COI) was amplified using specific primers. Sequences and annealing temperatures for the pair of primers are
Table 1. Israeli sampling localities from where Dugesia specimens were collected. The species have been identified on the basis of the COI gene sequence.

| Code | Locality    | Species     | Sampling date | Site description                              | Coordinates      |
|------|-------------|-------------|---------------|-----------------------------------------------|------------------|
| SHE  | Ein Shefa   | *D. sicula* | 06/25/2009    | Fast flowing man made spring channel          | 33°0'34.47"N, 35°8'11.15"E |
| BAN  | Nahal Banias| *D. sicula* | 08/27/2009    | Fast flowing stream                           | 33°14'47.44"N, 35°41'23.75"E |
| BET  | Nahal Betzet| *D. sicula* | 09/01/2009    | Isolated temporary pools within dry stream    | 33°4'32.84"N, 35°13'34.18"E |
| TEO  | Ein Te’o    | *D. sicula* | 02/03/2010    | Shallow spring with moderate water flow       | 33°7'55.95"N, 35°34'8.54"E |
| ENU  | Ein Nun     | *D. sicula* | 02/03/2010    | Shallow spring with moderate water flow       | 32°50'18.35"N, 35°30'39.41"E |
| EHU  | Einot Huga  | Not *D. sicula* | 05/09/2010 | Shallow spring - rather saline water ≤2000 mg Cl/l | 32°31'2.68"N, 35°32'17.27"E |
| EOV  | Ein Ovdat   | *D. sicula* | 05/09/2010    | Partly connected with slowly flowing spring pools of a desert stream | 30°49'25.07"N, 34°45'50.00"E |
| TZU  | Ein Tzuba   | *D. sicula* | 05/10/2010    | Shallow man-made spring pool                  | 31°46'58.33"N, 35°47'5.72"E |
| SAT  | Ein Sataf   | *D. sicula* | 05/10/2010    | Small spring pool inside a man-made underground cave | 31°46'15.77"N, 35°38'0.00"E |
| GED  | Ein Gedi    | *Dugesia sp.* | 08/04/2010   | Small shallow spring pool - desert area       | 31°28'0.60"N, 35°23'19.11"E |
| DAN  | Dan Springs | Not *D. sicula* | 08/18/2010   | Shallow slowly flowing stream                  | 33°14'56.82"N, 35°39'1.95"E |

Table 2. Forward (F) and Reverse (R) primers used in the amplification and sequencing of the COI mitochondrial gene sequence.

| Name | Direction | Sequence 5’−3’ | Annealing temperature (°C) | Source               |
|------|-----------|----------------|--------------------------|----------------------|
| BarT | F         | ATGACCDGSCATGGTTTAATAATGAT | 43                      | Álvarez-Presas et al. 2011 |
| COIEF3 | F     | CCWCGTGCAWAATAATTRAG      | 43                      | Solà et al. 2013    |
| COIR  | R         | CCWGTYARMCCCHCCWAYAGTAAA  | 43                      | Lázaro et al. 2009  |

given in Table 2. Final PCR reaction volume was 25 µl. To 1 µl of DNA sample to amplify we added (1) 5 µl of Promega 5X Buffer, (2) 1 µl of dNTP (10 mM), (3) 0.5 µl of each primer (25 µM), (4) 2 µl of MgCl₂ (2 mM), (5) 0.15 µl of Taq polymerase (GoTaq® Flexi DNA Polymerase of Promega). Double-distilled and autoclaved water was added to obtain the final PCR volume. The purification of the PCR products was done with the purification kit illustra™ (GFX™ PCR DNA and Gel Band of GE Healthcare) or by using a vacuum system (MultiScreen™ HTS Vacuum Manifold of Millipore). Sequencing reactions were performed by using Big-Dye (3.1., Applied Biosystems) with the same primers used to amplify the fragment, or with an inner forward
COI sequence (COIEF3), due to sequencing problems when using BarT primer. The sequencing reactions were carried out and run in an automated sequencer ABI Prism 3730 by the Unitat de Genòmica of Centres Científics i Tecnològics of the Universitat de Barcelona or by Macrogen Corporation in Europe (Amsterdam, The Netherlands). Obtained chromatograms were visually checked with the software Geneious v. 6.1.7.

Alignment and haplotype network

The number of Dugesia individuals analyzed per locality ranged between 1 and 7, depending on the available number of specimens and the success of sequencing (Table 3). The sequences were aligned online with MAFFT version 7 by setting the iterative refinement method in G-INS-i (Katoh and Standley 2013). We used the software Network version 4.613 (Bandelt et al. 1999), using Median-Joining for network calculations. Parameters were set as default.

Preparations

Material examined (collections Naturalis Biodiversity Center, Leiden):

Dugesia biblica:
ZMA V.Pl. 698.1, Banias Waterfall, Israel, transverse sections on 6 slides, V.Pl. 698.2, ibid., sagittal sections on 8 slides.
ZMA V.Pl. 699.1, Ein El Hanea, Israel, January 1972, sagittal sections on 8 slides; V.Pl. 699.2., ibid., transverse sections on 12 slides.
ZMA V.Pl. 813.1, spring, 5 km NW of Bucak, Turkey, sagittal sections on 2 slides; V.Pl. 813.2, ibid., sagittal sections on 3 slides; V.Pl. 813.3, ibid., frontal sections on 2 slides.
ZMA V.Pl. 814.1, stream near Yerkopru, Hadim, Turkey, sagittal sections on 4 slides; V.Pl. 814.2, ibid., sagittal sections on 3 slides; V.Pl. 814.3, ibid., frontal sections on 3 slides.

Dugesia sicula:
ZMA V.Pl. 7152.1, Tripes, Chios, Greece, 2 May 2010, sagittal sections on 10 slides.

Results

Samples

Out of the 32 localities that we visited in Israel, about one-third (11) yielded specimens of Dugesia (Fig. 1, Table 1, Suppl. material 1). At two of these localities we found some Dugesia specimens that were molecularly different from D. biblica or D. sicula. One of these two populations, from Dan Springs (Table 1), might be D. go-
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...lanica, which was originally described from Dan Springs and also from Banyas Springs, in the vicinity of Dan Springs. Our second series of specimens, from Einot Huga, may represent a different species, according to its very distant phylogenetic position (data not shown). Perhaps specimens from the latter locality represent *Dugesia salina* (Whitehouse, 1913), currently a *species inquirenda*. According to Bromley (1980), the chromosomal complement for *D. salina* is 2n = 16 and is different from *D. golanica*, although she did not describe the chromosomes from the latter species. Whitehouse

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**Table 3.** Details on the Israeli individuals sequenced for the present work.

| Individual | Locality       | Polymorphic | Haplogroup | Haplotype in Figure 2 | GenBank Acc. Number |
|------------|----------------|-------------|------------|-----------------------|---------------------|
| D01TEO     | Ein Te’o       | No          | A          | 7                     | KR140038            |
| D01BAN     | Nahal Banias   | No          | B          | 2                     | KR140035            |
| D02BAN     | Yes            | –           | –          |                       | KR140040            |
| D03BAN     | Yes            | –           | –          |                       | KR140045            |
| D04BAN     | No             | B           | 2          |                       | KR140049            |
| D02SHE     | No             | B           | 3          |                       | KR140043            |
| D03SHE     | No             | B           | 3          |                       | KR140047            |
| D04SHE     | Yes            | –           | –          |                       | KR140052            |
| D05SHE     | Yes            | –           | –          |                       | KR140056            |
| D06SHE     | No             | B           | 3          |                       | KR140059            |
| D01BET     | Nahal Betzet   | No          | B          | 8                     | KR140036            |
| D02BET     | No             | B           | 8          |                       | KR140041            |
| D03BET     | No             | B           | 8          |                       | KR140046            |
| D04BET     | No             | B           | 3          |                       | KR140050            |
| D05BET     | No             | B           | 3          |                       | KR140053            |
| D01TZU     | Ein Tzuba      | No          | B          | 4                     | KR140039            |
| D02TZU     | No             | B           | 4          |                       | KR140044            |
| D03TZU     | No             | B           | 4          |                       | KR140048            |
| D07TZU     | No             | B           | 4          |                       | KR140062            |
| D08TZU     | No             | B           | 4          |                       | KR140063            |
| D09TZU     | No             | B           | 4          |                       | KR140066            |
| D10TZU     | No             | B           | 4          |                       | KR140067            |
| D04SAT     | Ein Sataf      | No          | B          | 5                     | KR140051            |
| D05SAT     | No             | B           | 5          |                       | KR140055            |
| D06SAT     | No             | B           | 5          |                       | KR140058            |
| D07SAT     | No             | B           | 5          |                       | KR140061            |
| D11SAT     | Yes            | –           | –          |                       | KR140068            |
| D06EOV     | Ein Ovdat      | No          | B          | 1                     | KR140057            |
| D07EOV     | No             | B           | 1          |                       | KR140060            |
| D09EOV     | No             | B           | 6          |                       | KR140065            |
| D01ENU     | Ein Nun        | Yes         | –          | –                     | KR140037            |
| D02ENU     | Yes            | –           | –          |                       | KR140042            |
| D05ENU     | Yes            | –           | –          |                       | KR140054            |
| D09ENU     | Yes            | –           | –          |                       | KR140064            |
| D16ENU     | Yes            | –           | –          |                       | KR140069            |
Figure 1. Map of Israeli localities sampled for this study: 1 Nahal Banias 2 Ein Te’o 3 Nahal Betzet 4 Ein Shefa 5 Ein Nun 6 Ein Tzuba 7 Ein Sataf 8 Ein Ovdat. For locality details, see Table 1.
(1913) reported *D. salina* from near et-Tabghah (= En Sheva), while Bromley (1974, 1980) reported populations from En Sheva, En Soda, and from River Jordan at its outlet from Lake Kinneret. Our locality of Einot Huga is actually very close to En Soda. However, as these two species, *D. golanica* and *D. salina*, fall outside of the scope of the present study, we did not include the specimens in our analyses.

Unfortunately, preservation and histological problems eventually prevented us of carrying out detailed morphological analyses on the reproductive apparatus of Israeli *Dugesia* specimens from the various newly sampled populations (Table 1, specimens from localities EOV, EHU, TZU, DAN).

### Alignment and haplotype networks

We were successful in obtaining COI sequences for 8 out of the 9 sampling localities; 25 out of the 35 sequences obtained for the present study presented no polymorphism, while the remaining sequences showed between 1 and 12 polymorphic positions. We used both the 25 COI non-polymorphic sequences from presumed Israeli *D. biblica* obtained for this study (Table 3), as well as those of *D. sicula*, as obtained in a previous phylogeographic study of this species (95 sequences; GenBank Acc. number: KC536630–KC536644 and KC577271–KC577350; Lázaro and Riutort 2013) in order to carry out a haplotype network analysis. The alignment contained 120 COI sequences, included 604 nucleotides, and presented 15 polymorphic positions.

Most of the Israeli COI haplotypes are identical or are only 1–4 positions removed from the major *D. sicula* COI haplotype B (Fig. 2). One individual sequence (D01TEO) belongs to the other major COI haplotype, viz haplotype A (cf. Lázaro and

![Figure 2](image-url). Haplotype network of *Dugesia sicula* and presumed *D. biblica* COI sequences. Filled red circles correspond to haplogroup A, filled blue circles correspond to haplogroup B, and filled brown circles correspond to haplogroup C of *D. sicula* (as defined in Lázaro and Riutort 2013). The size of the coloured circles is proportional to the haplotype representation. Small black dots indicate intermediate haplotypes (not-obtained). Numbers indicate the identity of Israeli haplotypes; for further details see Table 3.
Riutort 2013; Fig. 2). The geographical extension of the B haplogroup in the present study widens its known distributional range to the coast of Israel. The A haplogroup ranges from Morocco to Israel on both sides of the Mediterranean Sea.

Additionally, we compared the polymorphic sequences of the Israeli Dugesia not included in the haplotype network (Table 3) with the sequences of D. sicula COI haplotypes already defined (Lázaro and Riutort 2013; present work). We found that the polymorphic positions corresponded with those that are variable between haplotypes, indicating that these organisms were heteroplasmic for various known haplotypes.

The results of our molecular analyses suggest a wide distribution of D. sicula throughout Israel (Fig. 1), as well as the absence of any other molecularly related species in this area.

**Morphological and karyological comparison between Dugesia biblica and D. sicula**

We have been unable to find any stable structural morphological difference between sicula populations and presumed biblica populations. All of these animals are characterized by distinctly acentral opening of the ejaculatory duct; asymmetrical oviducal openings into the bursal canal; rather thick layer of circular muscles around bursal canal; bursal canal that runs somewhat laterally to the penis; zone of mesenchymatic gland cells around bursal canal; somewhat bilobed seminal vesicle; somewhat irregularly running bursal canal, with irregular diameter; distinct patch of cyanophil secretion in dorsal section of penis papilla. Benazzi and Banchetti (1973) described for D. biblica an outer pharynx musculature consisting of three layers. However, De Vries (1988) already correctly observed that such an extra, third layer is not present in biblica specimens from Israel. Bromley (1979) described atrial folds for D. biblica, but such structures were not observed by us in the available material from Israel. The vacuolated tissue that Bromley (1979) described for the penis of D. biblica in our opinion merely concerns tears in the mesenchyme of the penis papilla. Such tears or spaces in the dorsal part of the penis papilla, near its tip, were observed in histological preparations of specimens from several populations of D. sicula, e.g. specimen ZMA V.Pl. 7152.1 from Chios.

Characteristic of D. biblica is the occurrence in the field of a sexually reproducing diploid form with a chromosome complement of 2n = 18, and a triploid form that reproduces asexually by fission with a set of 3n = 27 + 1−5 supernumerary chromosomes. Under laboratory conditions, the normally fissioning animals can be induced to develop reproductive organs. The structure of the copulatory organs of these sexualized animals is identical to that of the normally sexually reproducing diploid forms. However, in the diploid forms, testes and ovaries show their normal dimensions and development, whereas in the sexualized animals the testes are underdeveloped and the ovaries hyperplasic (cf. Bromley 1974, 1977, 1979). The difference in karyology between the asexual individuals and the naturally sexual animals induced Bromley (1979, 1980) to coin the subspecies Dugesia biblica biblica Benazzi & Banchetti, 1973 and D. biblica monticola Bromley, 1980, respectively.
The situation that (1) in the field some populations may reproduce asexually and show a triploid set of $3n = 27 + 2−3$ B chromosomes, (2) others reproduce sexually and show a complement of $2n = 18$ gradually decreasing, metacentric chromosomes, and (3) sexualized, triploid specimens show hyperplasic ovaries and poorly developed testes is well-known for *D. sicula* (cf. Charni et al. 2004 and references therein). Thus, also from this perspective, there seems to be no difference between *D. sicula* and *D. biblica*.

**Conclusion: the taxonomic status of *Dugesia biblica***

In addition to the morphological and karyological similarities between nominal *Dugesia biblica* and *D. sicula* (see above), our molecular analysis shows presumed *biblica* populations to be molecularly indistinguishable from *sicula* populations. The Israeli haplotypes obtained are either identical to previously obtained *sicula* or present few differences from these. Therefore, on the basis of our integrative analysis, we consider *D. biblica* to be a junior synonym of *D. sicula*.

This conclusion holds true for one of the two Turkish populations of presumed *biblica* described by De Vries (1988), viz. ZMA V.Pl. 814 from Yerkopru. But the

![Figure 3](image.png)

*Figure 3.* Presumed *Dugesia sicula* from Bucak, Turkey (ZMA V.Pl. 813.2), showing the presence of the zone of cyanophil secretion in the penis papilla. Abbreviations: d diaphragm ed ejaculatory duct pp penis papilla sv seminal vesicle zcs zone of cyanophil secretion.
other population (ZMA V.Pl. 813 from 5 km NW of Bucak) concerns animals that are morphologically somewhat different from *D. sicula*. Foremost, the ejaculatory duct does not have a subterminal opening (cf. De Vries 1988, Fig. 2). Other differences concern the position of the ovaries at 1/3rd – 1/4th of the distance between the brain and the root of the pharynx (1/4th – 1/5th in *D. sicula*), the much wider bursal canal, which is surrounded by a much thinner layer of circular muscle (depicted far too thick in De Vries 1988, Fig. 2), and the smaller copulatory bursa in the specimens from Bucak. The animals from Bucak agree with *D. sicula* in the presence of numerous mesenchymal glands discharging their erythrophil secretion into the lining epithelium of the bursal canal, the presence of the zone of cyanophil secretion in the penis papilla (Fig. 3), and the asymmetrical openings of the oviducts into the bursal canal. In several respects the animals from Bucak remind one of *D. naiadis* Sluys, 2013 from Chios, albeit that in the latter the oviducts open symmetrically into the bursal canal, in contrast to the asymmetrical oviducal openings in the Bucak specimens (cf. De Vries 1988, Fig. 2). However, for the moment we refrain from assigning the animals from Bucak to a different and possibly new species of *Dugesia* and postpone any taxonomic decision until more material has become available for both morphological and molecular analyses.

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Supplementary material 1

Supplementary Table 1
Authors: Eduard Solà, Ronald Sluys, Ori Segev, Leon Blaustein, Marta Riutort
Data type: occurrence
Explanation note: Localities in Israel from which no specimens of *Dugesia* could be obtained during our samplings.
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