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The first *Dugesia* species (Platyhelminthes, Tricladida, Dugesiidae) documented for Saudi Arabia: an integrative description

ABDEL HALIM HARRATH[1*], LAMJED MANSOUR[1], RONALD SLUYS[2], WALEED ALDAHMICH[1], MARTA RIUTORT[3] & SALEH ALWASEL[1]
[1]*Department of Zoology, College of Sciences, King Saud University, 11451 Riyadh, Saudi Arabia.*
Saleh Alwasel: salwasel@ksu.edu.sa; https://orcid.org/0000-0002-0626-2306
Waleed Aldahmich: dhmash_28@yahoo.com; https://orcid.org/0000-0002-8423-3586
Lamjed Mansour: lmansour@ksu.edu.sa; https://orcid.org/0000-0002-9415-9383
[2] *Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands.*
Ronald.Sluys@naturalis.nl; https://orcid.org/0000-0002-9776-3471
[3]*Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Av. Diagonal 643, 08028, Barcelona, Spain.*
mriutort@ub.edu; https://orcid.org/0000-0002-2134-7674
[*]*Corresponding author. h.harrath@ksu.edu.sa; https://orcid.org/0000-0002-2170-1303*

Abstract

Exploration of various freshwater bodies in the Kingdom of Saudi Arabia led to the discovery of a new species of *Dugesia*, thus representing the first documented record of freshwater planarians in this country and the second fully documented record of a naturally sexual population of a *Dugesia* species in the Arabian Peninsula. Based on morphological, karyological, anatomical, and molecular data, this *Dugesia* population from Saudi Arabia is here identified as representing a distinct, new species, *Dugesia bursagrossa* Harrath & Sluys, **sp. nov.** The species is characterized by the following anatomical features: dorsal testes; highly glandular, barrel-shaped penis papilla; vasa deferentia opening separately into the antero-dorsal portion of a small intrabulbar seminal vesicle; ejaculatory duct opening subterminally through the ventral side of the penis papilla; muscular penis bulb composed of intermingled longitudinal and circular muscles; two ovaries positioned ventrally at approximately one-third of the distance between the brain and the root of the pharynx; oviducts opening separately and at different levels into the vaginal portion of the bursal canal; very large copulatory bursa, occupying almost the entire ample space between the posterior wall of the pharyngeal pocket and the penis bulb; bursal canal lined with a nucleated epithelium and covered by subepithelial layers of longitudinal muscles, followed by layers of circular muscles. The karyotype of the new species exhibits mixoploidy, with diploid complements of 2n = 18 and triploid complements of 3n = 27 + 1B chromosomes, with all chromosomes being metacentric. Phylogenetic analysis based on the COI sequence indicates that *D. bursagrossa* is distinct from closely related species within the same clade, with strong support from the anatomical and karyological data.

Key words: freshwater flatworm, morphology, karyology, molecular phylogeny, *Dugesia bursagrossa*

Introduction

It is now widely recognized that pollution, as well as loss and degradation of habitats resulting from human activities form significant global issues that have rapidly intensified since the Industrial Revolution, causing considerable alterations in both animal and plant ecosystems (Mgbemene *et al.* 2016; Beiras 2018; Häder *et al.* 2020). As a consequence, biodiversity is declining, leading to an unprecedented rise in extinction rates (Butchart *et al.* 2010; Ceballos *et al.* 2015). This situation has drawn considerable global attention to the conservation of biological diversity, which is essential for the sustainability of natural resources (Sutherland *et al.* 2024).

The unique geographical position of Saudi Arabia, located between Africa and Eurasia, provides it with a distinct advantage in the diversity of its flora and fauna (Vincent 2008). The climate is predominantly arid, characterized by high temperatures and low annual rainfall, although some regions may experience higher levels of precipitation (Gosling *et al.* 2011).

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Nevertheless, also in Saudi Arabia, wildlife conservation and the sustainability of natural habitats are critical issues (Midfa *et al.* 2011). As a result, extensive research has been undertaken already to understand the biodiversity, ecology, and biology of local species, ensuring that effective conservation measures are implemented (e.g., Alatawi *et al.* 2020; AlRashidi *et al.* 2021). In similar vein, we undertook an inventory of the freshwater planarian fauna of Saudi Arabia.

Although epigean freshwater planarians from the genus *Dugesia* have been extensively studied across the globe (Solà *et al.* 2022), freshwater triclads have remained a relatively unexplored component of the fauna in Saudi Arabia. To investigate the diversity of freshwater planarians in this country, we explored most of the known surface water localities, as the majority of water sources in the Kingdom consists of vast underground aquifers (Al-Zahrani & Baig 2011; Al-Ghanim 2012). Our inspection of many freshwater springs and other water bodies led to the discovery of a single population of planarians, occurring in the southern region of the Kingdom near the border of Yemen, representing a new species of *Dugesia*. The present paper describes this new species based on an integrative approach, including karyological, molecular phylogenetic, morphological and anatomical analyses, thus, representing the first documentation of a freshwater planarian for the country.

Materials and methods

Sample collection. Different bodies of freshwater (streams, springs, reservoirs, rivers) within the Kingdom of Saudi Arabia were checked for the presence of freshwater planarians (Table S1). Thus far, only one sexual planarian population was found at the Ain Dhiba spring (18°16’44.4”N 42°23’18.6”E) in Abha province, located in the south of the country. Specimens were collected with a paintbrush and then placed in a glass container with water from the habitat. Some specimens were preserved in 10% neutral buffered formalin (NBF) for histological examination, while others were preserved in pure alcohol for molecular analysis. Other animals were brought to the laboratory alive and used for karyological analysis and for breeding.

Histology. Animals fixed in 10% NBF were dehydrated through an ascending series of ethanol solutions and, thereafter, cleared in xylene and then embedded in paraffin. Histological sections were made at intervals of 7 µm and stained in Cason’s Mallory-Heidenhain (Yang *et al.* 2020). Drawings of the copulatory apparatus were made using the Adobe Illustrator program (version 2023) installed on a computer and linked to a wireless scroll tablet (XP-PEN5), as described in Harrath *et al.* (2013). The material examined is deposited in the collections of Naturalis Biodiversity Center, Leiden, The Netherlands (RMNH code).

Karyology. The karyological study was done as described previously (Harrath *et al.* 2013). Briefly, each of three live specimens were cut into two pieces and kept individually in a Petri dish at room temperature. During the third day of regeneration, for 6 hours the animals were put into water to which was added colchicine to achieve a final concentration of 0.05%. Three-days-old regenerating blastemas were removed from the regenerating parts and were placed on clean slides and then fixed in a solution of methanol-acetic acid (3:1) for 10 minutes. After fixation and staining in Giemsa, blastemas of 8 specimens were observed using a compound light microscope and photographed with a camera linked to a computer program for imaging (NIS Elements imaging software, Nikon). Other blastemas of 5 specimens were stained with Hoechst 33342 (diluted 1:15000, Life Technologies, USA), and sections were observed and imaged with a spinning disk confocal microscope from Zeiss (Model CSUX1FW-06P-01, Germany). For the karyometric analysis, the chromosomes were arranged and measured using Adobe Photoshop CS5.1 software. Relative length was calculated as the chromosome length divided by the total length of the haploid genome, multiplied by 100. The centromeric index was calculated as the length of the short arm divided by the total length of the chromosome, multiplied by 100. The histogram was generated using GraphPad Prism version 10.

Molecular analysis

Genomic DNA extraction. Total genomic DNA was extracted from three fresh specimen individually, using the QIAamp DNA Mini Tissue Kit (Qiagen, Germany), following the manufacturer’s recommendations. The quantity and quality (purity) of extracted genomic DNA was tested with a NanoDrop 8000 (Thermo Scientific™), and in agarose gel electrophoresis. DNA was preserved at -20^{o}C until PCR amplification.

PCR amplification and sequencing. Multiple primers were used for the cytochrome oxidase subunit 1 (COI) gene amplification, including BarS (Álvarez-Presas *et al.* 2011), COIR (Lázaro *et al.* 2009), LCO1490, HCO2198, (Folmer *et al.* 1994), dgHCO2198, and dgLCO1490 (Meyer 2003). Only the primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) designed by Bowles *et al.* (1992) generated an expected COI sequence of 450 base pairs (bp). For each PCR reaction, 20 µl final volume was used, containing 10 µl of 2X GoTaq® Master Mix (Promega, Canada), 1 µl (50-100 ng) genomic DNA, 1 µM forward and reverse primers and 8 µl DNAse/RNAse free water. The PCR reactions were performed in the thermocycler apparatus T100™ (Bio-Rad, US) in the Zoology Department, King Saud University. The PCR program included an initial denaturation stage at 95 °C for 5 minutes, followed by 35 cycles with three steps; the first step for denaturation at 95 °C for 30 seconds, the second step for annealing at 54 °C for 30 seconds, and a third step of extension at 72 °C for 90 seconds. The final extension step was set-up at 72 °C for 5 minutes. Thereafter, 3 µl of the PCR products were electrophoresed on 1 % agarose gels, then stained with ethidium bromide solution (Sigma, USA), and visualized using an UV transilluminator system. The remaining PCR amplified products were purified using a QIAquick PCR Purification kit (QIAGEN, USA), according to the manufacturer's instructions and sequenced in both directions using the same primers used for PCR by Macrogen Inc. (Seoul, South Korea).

Phylogenetic analysis. The forward and reverse sequences from each specimen were visualised, assembled, and edited using BioEdit 7.1.8.0 software (Hall 1999). For further analysis, several *Dugesia* sequences were selected based on their BLAST score and geographic locality by using the GenBank Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997). The species *Schmidtea mediterranea* Benazzi, Baguñà, Ballester, Puccinelli & Del Papa, 1975 was used as outgroup taxon. Nucleotide sequences were translated to amino acids using translation code for echinoderms and platyhelminths (translation table 9). We checked that all sequences were in frame and then aligned them, based on the amino acids sequences, by using ClustalW implemented in BioEdit 7.1.8.0 software (Hall 1999). Missing data were replaced by N's in the final alignment.

Phylogenetic relationships between the new species and selected *Dugesia* sequences were inferred with Bayesian inference (BI) and Maximum Likelihood (ML) methods. BI analysis was done in MrBayes version 3.2.6 (Ronquist *et al.* 2012) using Markov Chain Monte Carlo (MCMC) for 7,000,000 generations with two independent runs of four simultaneous MCMC chains (nchains = 4). Trees were saved every 100 generations (samplefreq = 100). A 25% burn-in (default setting) was applied. ML analysis was performed using RAxML V0.9.0 (Kozlov *et al.* 2019) with the GTR + I + G model, as indicated by evaluation with jModelTest 2.1.10 (Darriba *et al.* 2012) under Akaike Information Criterion (AIC). Nucleotide frequencies were determined based on the data. Clade support was assessed by bootstrapping with 1000 replicates. The obtained trees were initially visualised with FigTree v1.4.3 (Rambaut 2010), and thereafter edited and annotated in Adobe Illustrator (version 2023).

Abbreviations used in the figures. au, auricle; bc, bursal canal; ca, common atrium; cb, copulatory bursa; cg, cement glands; cm, circular muscle; d, diaphragm; ds, dorsal side; e, eye; ed, ejaculatory duct; ep, epithelium; g, gonopore; h, head; lm, longitudinal muscle; lu, lumen; lvd, left vas deferens; m, mouth; ma, male atrium; oc, oocyte; od, oviduct; ov, ovary; pb, penis bulb; pg, penial glands; ph, pharynx; phc, pharyngal cavity; pp, penis papilla; rvs, right vas deferens; sp, spermatophore; spv, spermiducal vesicle; sp, sperm; spz: spermatozoon; sv, seminal vesicle; t, testis; ta, tail; vd, vas deferens; vs: ventral side.

Results

Similarity search and molecular phylogeny. The obtained sequences from the three specimens were identical and a consensus one was deposited in GenBank (accession number: PP943120). After sequence analysis, BLAST search did not return any identical sequences in the database, indicating the uniqueness of the present sequences, at least among those known species for which sequences are available in GenBank. The most similar sequences belonged to the genus *Dugesia*. At the same time, the highest percentages of similarity (96.8%) were obtained with *D. arabica* Harrath & Sluys, 2013 from Yemen and *D. aethiopica* Stocchino *et al.*, 2002 from Ethiopia, and with *D. sicula* Lepori, 1948 (93.6%) from North Africa. This data combined with morphological analysis confirms the membership of our new species to the genus *Dugesia*.

The phylogenetic analysis of the COI sequences of the new species and several congeneric species resulted in identical topologies for the Bayesian inference and Maximum Likelihood methods. Still, with some differences in nodal supports (Fig. 1). Four major geographic clades could be observed: European, Eastern Asian, Malagasy, and Afrotropical-Mediterranean clades. The new species, *D. bursagrossa*, belongs with high nodal support to a subclade of the Afrotropical-Mediterranean group that includes *D. aethiopica*, *D. arabica*, and *D. sicula. Dugesia bursagrossa* is sister to *D. arabica* and *D aethiopica* again with a good support. The Afrotropical-Mediterranean group also includes *D. naiadis* Sluys, 2013 —a species described from the Greek islands but of uncertain origin (Sluys *et al.* 2013)— and *D. afromontana* Stocchino & Sluys, 2012 from South Africa.

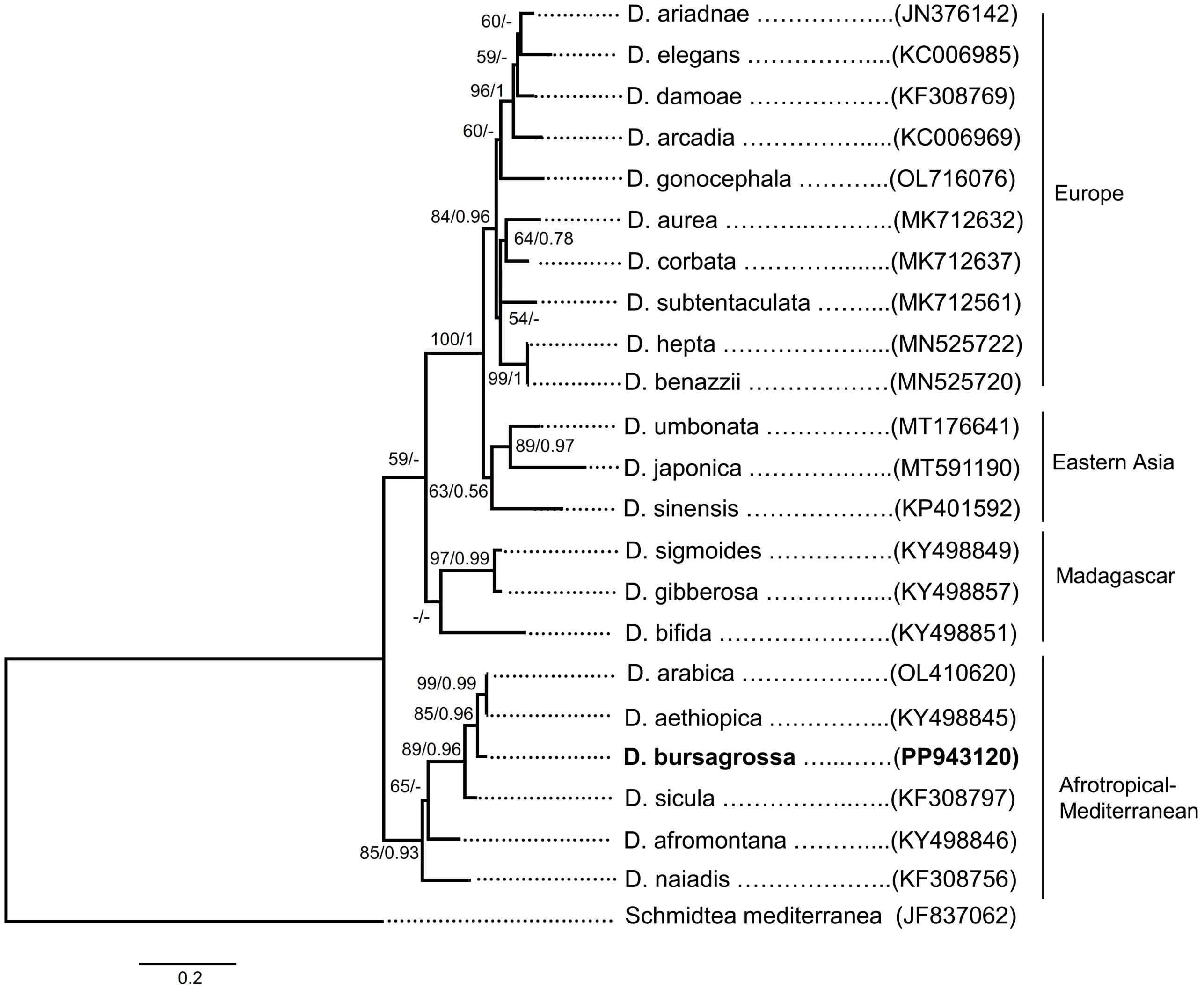

FIGURE 1. Phylogenetic tree based on Maximum likelihood inference analysis of COI, showing the position of *D. bursagrossa* (in bold) within a selected group of *Dugesia* species. Species names are accompanied by their corresponding GenBank accession numbers. Numbers at nodes indicate support values: bootstrap support (BS)/posterior probability (PP); dashes indicate values less than 50% BS or 0.5 PP. The scale bar represents substitutions per site.

Karyology. Our karyological preparations showed many over-dispersed sets of chromosomes, while others lacked well-dispersed chromosomes, both conditions making precise determination of the karyotype rather difficult. Nevertheless, we could establish that the specimens of *Dugesia bursagrossa* exhibited a mixoploid chromosome complement (Fig. 2). Among the investigated plates from the same specimen, some showed a diploid chromosome portrait of 2n = 18, with a basic number of n = 9. However, other plates from this specimen had a triploid complement of 3n = 27 chromosomes + 1B chromosome, with a basic number of n = 9 (Fig. 2E, F). The relative length, arm ratio, centromeric index and chromosome type are reported in Table 1. The karyometric data show that all chromosomes are of the metacentric type (Fig. 2D). When meiotic chromosomal plates in the gonads (testes and ovaries) of sexual individuals with a mixoploid karyotype (diploid and triploid) were examined, all plates studied exhibited a basic number of n = 9 (Fig. 3A, B).

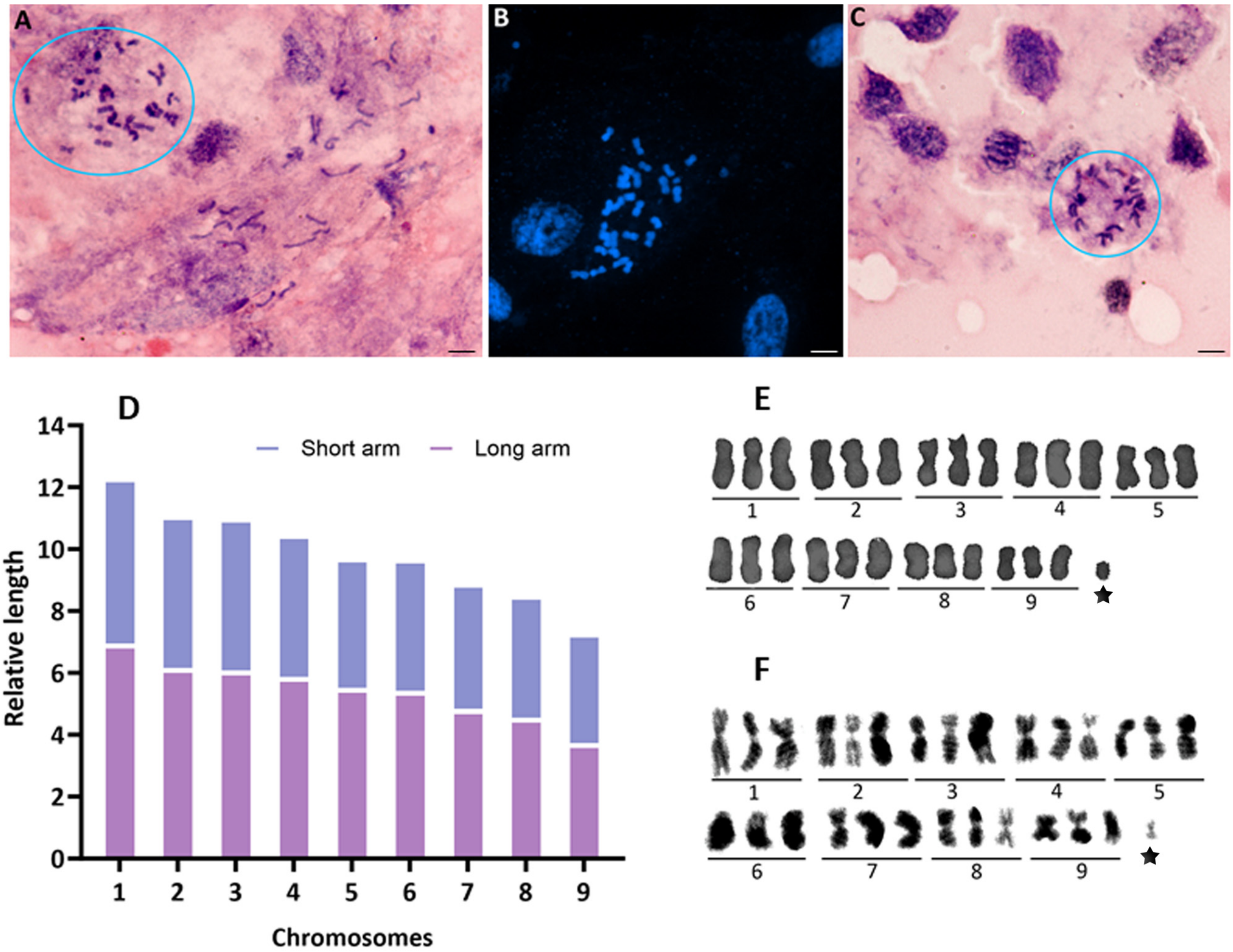

FIGURE 2. Karyology of *Dugesia bursagrossa*. **A.** Photomicrograph of metaphasic plate (blue circle) of a triploid cell from a sexual specimen (Giemsa staining); **B**. Photomicrograph of metaphasic plate of a triploid cell from a sexual specimen (Hoechst 33342 staining); **C**. Photomicrograph of metaphasic plate (blue circle) of a diploid cell from a sexual specimen (Giemsa staining); **D**. Idiogram of the chromosomes; **E.** Chromosome complement obtained in (**A**) arranged in triplets; **F.** Chromosome complement obtained in (**B**) arranged in triplets. B-chromosomes indicated by asterisks. Scale bars: 5 µm.

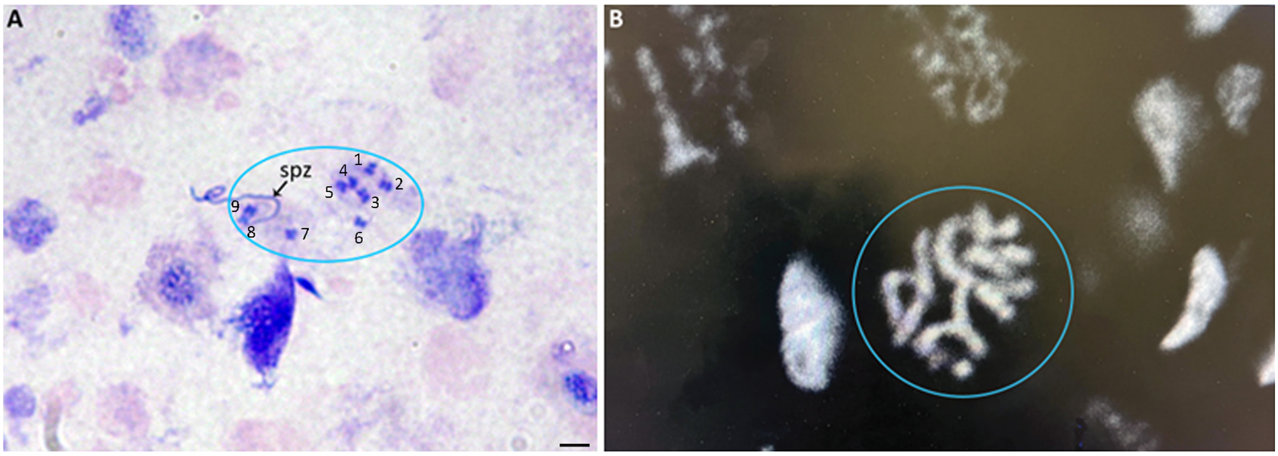

FIGURE 3. A. Photomicrograph of metaphasic plate (blue circle) of a testis cell from a sexual specimen of *Dugesia bursagrossa*, showing a haploid complement of n = 9 after Giemsa staining (The numbers indicated do not correspond to the ones in FIGURE 2). Scale bar: 10 µm. **B**. Photomicrograph of metaphasic plate (blue circle) of an ovarian cell from sexual specimen of *D. bursagrossa* showing a haploid complement of n = 9 after Hoechst 33342 staining. Scale bar not available.

TABLE 1. Karyotype parameters of *Dugesia bursagrossa* (mean ±SD).

Chromosome	Relative length	Centromeric index	Arm ratio	Chromosome type
1	12.32 ± 1.15	43.57 ± 0.34	1.31 ± 0.03	Metacentric
2	11.10 ± 0.57	44.53 ± 2.68	1.25 ±0.14	Metacentric
3	11.02 ± 0.02	44.78 ± 3.74	1.24 ± 0.19	Metacentric
4	10.48 ± 0.21	43.90 ± 5.83	1.32 ± 0.33	Metacentric
5	9.73 ± 0.14	43.28 ± 2.66	1.33 ± 0.14	Metacentric
6	9.70 ± 1.27	44.26 ± 2.20	1.27 ± 0.12	Metacentric
7	8.92 ± 0.68	45.79 ± 1.62	1.20 ± 0.08	Metacentric
8	8.53 ± 0.61	46.71 ± 0.87	1.14 ± 0.04	Metacentric
9	7.32 ± 0.35	48.99 ± 0.82	1.04 ± 0.03	Metacentric

Systematic account

Order Tricladida Lang, 1884

Suborder Continenticola Carranza, Littlewood, Clough, Ruiz-Trillo, Baguñà & Riutort, 1998

Family Dugesiidae Ball, 1974

Genus *Dugesia* Girard, 1850

Dugesia bursagrossa Harrath & Sluys, sp. nov.

Material examined. Holotype: RMNH.VER.21535.1, Ain Dhiba, Abha province, Saudi Arabia (18°16’44.4”N 42°23’18.6”E), 16th August 2020, coll. Waleed Aldahmash and Abdel Halim Harrath, sagittal sections on 42 slides. Paratypes: RMNH.VER.21535.2, ibid., sagittal sections on 44 slides; RMNH.VER.21535.3, ibid., horizontal sections on 42 slides, RMNH.VER.21535.4, ibid., sagittal sections on 61 slides; RMNH.VER.21535.5, ibid., horizontal sections on 27 slides.

Etymology. The specific epithet is based on the Latin noun *bursa*, alluding to the copulatory bursa in planarians, and the Latin adjective *grossus*, big, thick; thus, the name refers to the very large copulatory bursa in the new species.

Diagnosis. *Dugesia bursagrossa* is characterized by the following anatomical features: dorsal testes; symmetrical and highly glandular, barrel-shaped penis papilla; vasa deferentia opening separately into the antero-dorsal portion of a small intrabulbar seminal vesicle; ejaculatory duct opening subterminally through the ventral side of the penis papilla; muscular penis bulb composed of intermingled longitudinal and circular muscles; two ovaries positioned ventrally at approximately one-third of the distance between the brain and the root of the pharynx; oviducts opening separately and at different levels into the vaginal portion of the bursal canal; shell glands opening into the proximal, vaginal portion of the bursal canal; very large copulatory bursa, occupying almost the entire ample space between the posterior wall of the pharyngeal pocket and the penis bulb; bursal canal lined with a nucleated epithelium and covered by subepithelial layers of longitudinal muscles, followed by layers of circular muscles; mixoploid karyotype, with diploid complements of 2n = 18 and triploid complements of 3n = 27 + 1B chromosomes, with all chromosomes being metacentric.

Morphological description. Live animals measuring up to 20 mm in length and 3 mm in width. Low triangular head provided with two blunt auricles and two eyes set in pigment-free patches (Fig. 4A). Dorsal surface pigmented light brown, while the ventral surface is paler. The pharynx is situated in the middle of the body and measures about 1/6 of the body length (Fig. 4A, B). It is covered with an epithelium, which is underlain by a subepithelial layer of longitudinal muscle followed by layers of circular muscles. The inner pharynx epithelium is underlain by subepithelial layers of circular muscles, followed by a layer of longitudinal muscle (Fig. 4B, C–D)

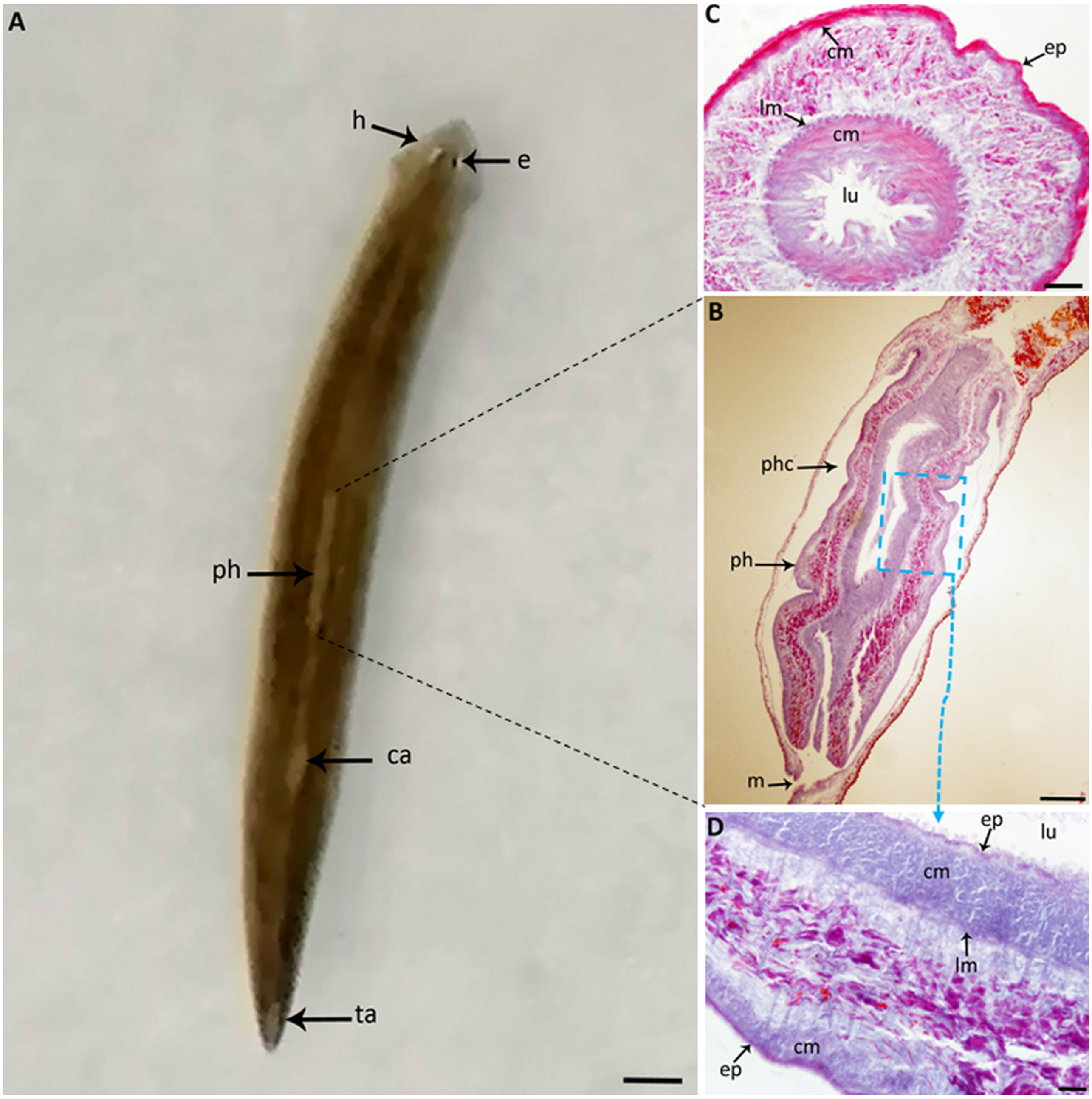

FIGURE 4. *Dugesia bursagrossa*. **A.** Photograph of live specimen in dorsal view. **B.** Paratype RMNH.VER.21535.4. Sagittal section showing the pharynx musculature. **C.** Holotype RMNH.VER.21535.1. Transverse section of the pharynx, showing its musculature. **D.** Paratype RMNH.VER.21535.4. Detail at higher magnification of the rectangular inset in panel B. Scale bars: A: 1 mm; B: 500 µm; C: 300 µm; D: 100 µm.

The numerous testes are located dorsally, extending from the posterior level of the ovaries into the posterior part of the body (Fig. 5A); the follicles show various stages of spermatogenesis (Fig. 5B). On either side of the body, a large sperm duct or vas deferens runs ventrally towards the posterior end of the body. At the level of the penis papilla, the sperm ducts turn dorso-medially and then narrow before individually, but symmetrically, opening into the intrabulbar seminal vesicle (Fig. 5C–F). This elongated seminal vesicle is lined with a columnar and nucleated epithelium and is surrounded by a well-developed coat of intermingled muscles. A small diaphragm is situated at the distal, posterior portion of the seminal vesicle and opens into the ejaculatory duct (Fig. 5D). The latter is lined with a columnar and nucleated epithelium, but any surrounding musculature was not observed. From its point of origin near the diaphragm, the ejaculatory duct at first follows a central course through the penis papilla for a short distance, but then turns towards the ventral side of the papilla where it opens subterminally to the exterior (Figs 5E, 6A, 7).

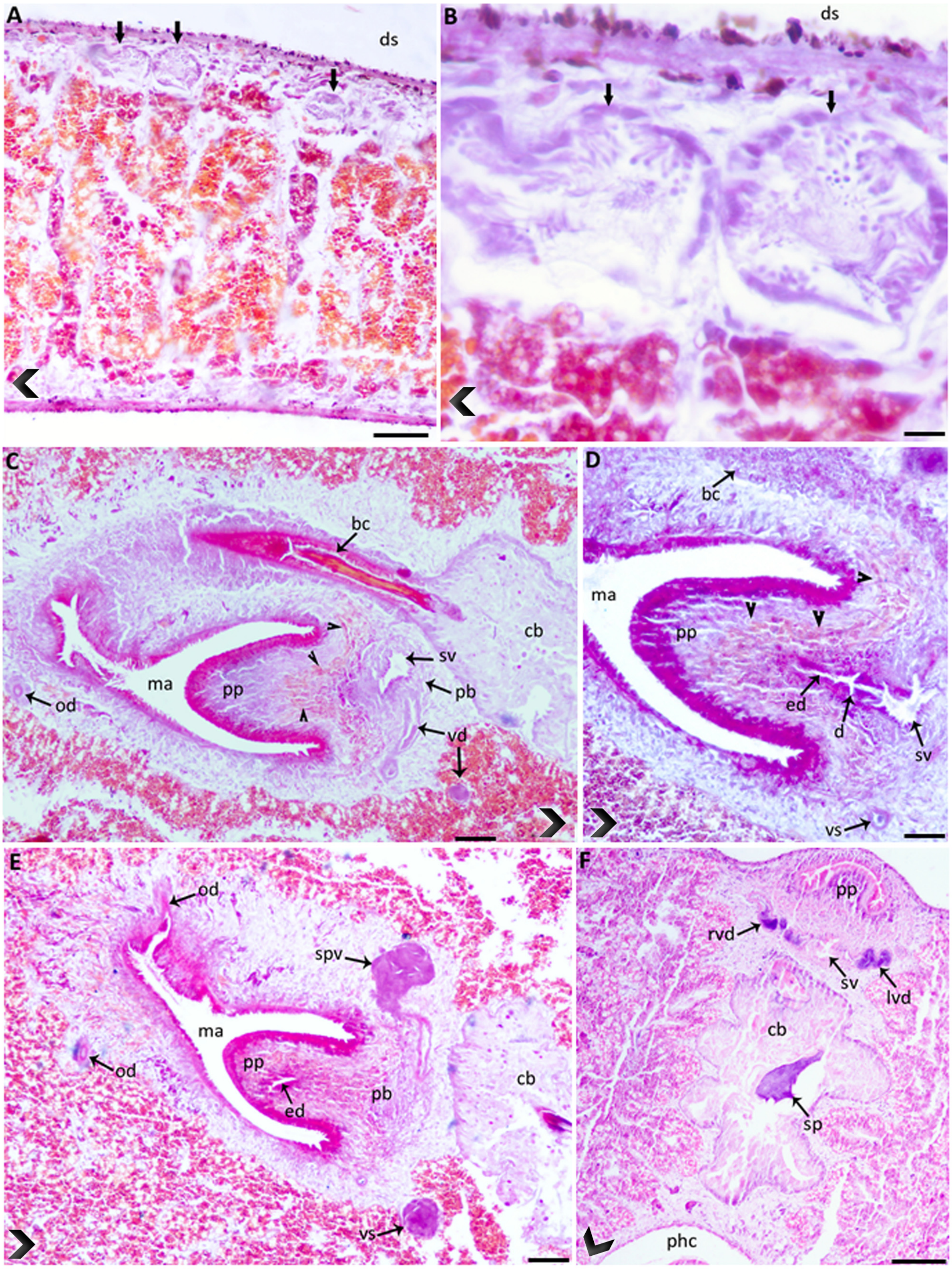

FIGURE 5. Male reproductive system of *D. bursagrossa* **A.** Paratype RMNH.VER.21535.4. Sagittal section showing the small, dorsally situated testes (indicated by arrows). **B.** Paratype RMNH.VER.21535.4. Testicular follicles showing the characteristics of normal spermatogenesis. **C.** Paratype RMNH.VER.21535.5. Horizontal section of the copulatory apparatus showing the vas deferens (vd) that recurves before opening into the seminal vesicle. **D.** Paratype RMNH.VER.21535.5. Horizontal section, showing the small diaphragm between the seminal vesicle and the ejaculatory duct. **E.** Paratype RMNH.VER.21535.5. Horizontal section of the copulatory apparatus showing the spermiducal vesicle narrowing to form the portion of the sperm duct that opens into the seminal vesicle. **F.** Paratype RMNH.VER.21535.3. Horizontal section, showing the large copulatory bursa, containing remnants of a spermatophore, and the symmetrical openings of the sperm ducts into the seminal vesicle. Scale bars: A: 500 µm; B: 100 µm; C, E and F: 300 µm; D: 250 µm. (Arrowhead indicates the anterior side of the animal).

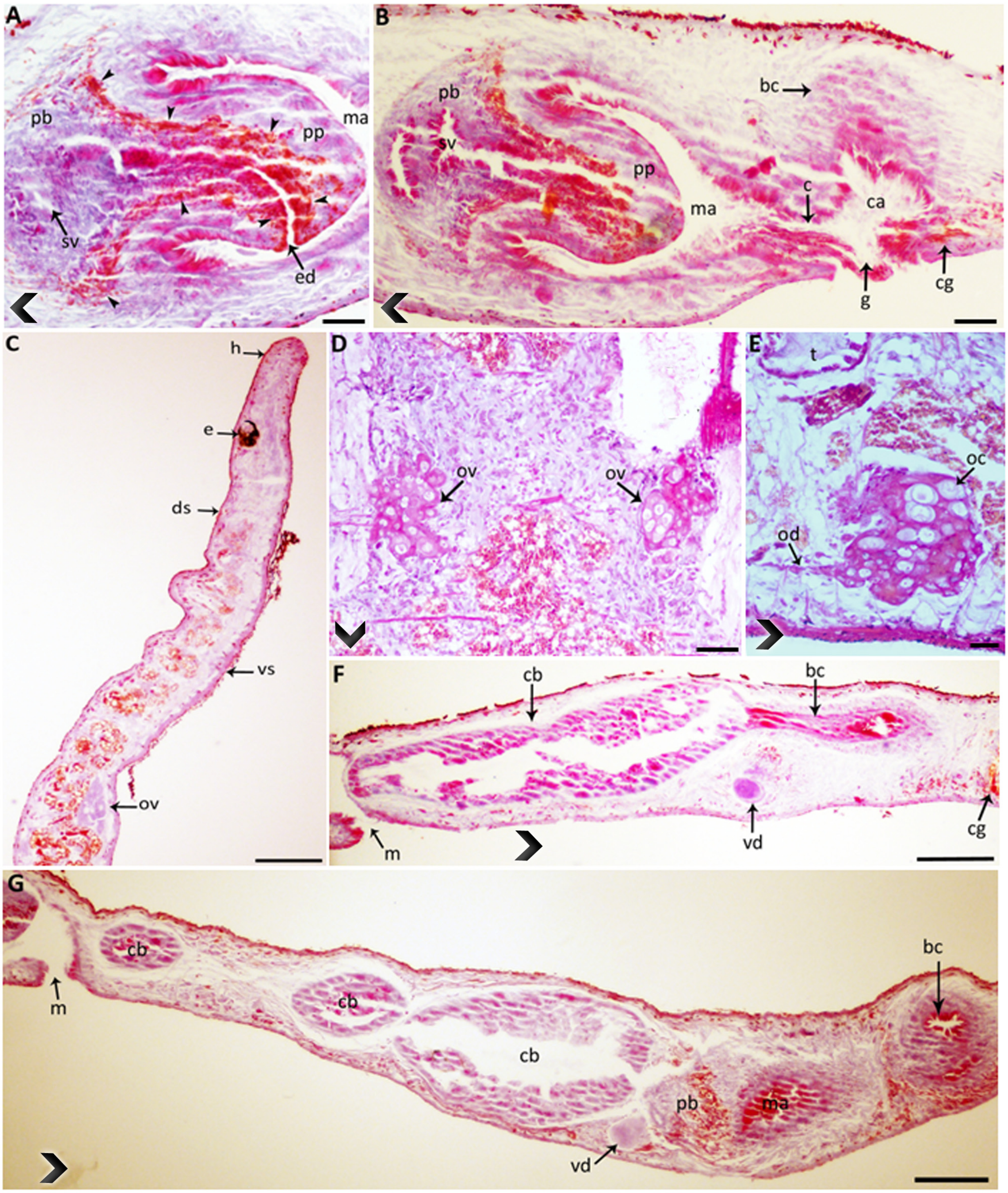

FIGURE 6. *Dugesia bursagrossa*. Photomicrographs. **A.** Holotype RMNH.VER.21535.1. Sagittal section of the penis papilla, showing the subterminal opening of the ejaculatory duct. Note the abundance of penial glands, discharging their secretion (arrowheads) into the ejaculatory duct. **B.** Holotype RMNH.VER.21535.1. Sagittal section of the copulatory apparatus showing the penis papilla, the constriction in the male atrium, the common atrium, and the gonopore. **C.** Paratype RMNH.VER.21535.4. Sagittal section showing the form and position of ovary in relation to the anterior end and the eyes. **D.** Paratype RMNH.VER.21535.5. Horizontal section showing the two ovaries. **E.** Paratype RMNH.VER.21535.5. Horizontal section of ovary, showing the characteristics of normal oogenesis. **F.** Holotype RMNH.VER.21535.1. Sagittal section of the very large and elongated copulatory bursa. **G**. Paratype RMNH.VER.21535.4. Sagittal section of the very large and elongated copulatory bursa. Scale bars: A, B and D: 200 µm; C: 400 µm; E: 150 µm; F and G: 300 µm (Arrowhead indicates the anterior end of the animal).

The highly glandular, more or less barrel-shaped penis papilla is situated within the male atrium, which communicates with a small common atrium via a constriction (Fig. 6B). The papilla is covered by a columnar, nucleated epithelium (Fig. 6A), under which lies a layer of circular muscles along most of its extent. Numerous ducts from abundant erythrophilic bulbar and extra-bulbar glands traverse a wide zone within the penis papilla, eventually releasing their secretions into the posterior section of the ejaculatory duct (Fig. 6A, B). Additionally, some other penial glands are located in the parenchymal region of the penis papilla and discharge their secretions mainly through the epithelium near the tip of the papilla.

The ovaries are situated ventrally, immediately above the nerve cords at approximately one-third of the distance between the brain and the root of the pharynx and about 120 µm behind the eyes (Fig. 6C); the gonads show various stages of normal oogenesis (Fig. 6D, E). The oviducts run dorsally to the ventral nerve cords until arriving shortly posterior to the gonopore, where they turn dorso-medially to open separately into the vaginal section of the bursal canal. The oviducal openings are asymmetrical in that the left oviduct opens into the ventral portion of the bursal canal, near its communication with the atrium, whereas the right oviduct opens much higher into the bursal canal. The shell glands discharge their secretion into this portion of the bursal canal.

From its vaginal region, the bursal canal curves forwards, meanwhile diminishing in diameter. The canal is lined with a nucleated epithelium composed of cells of variable height, while it is coated with a subepithelial layer of longitudinal muscles, followed by a thick layer of circular muscles. At a level approximately at the anterior portion of the penis bulb, the bursal canal opens through the postero-dorsal wall of the copulatory bursa. This copulatory bursa is a very large and particularly elongated, sac-shaped structure that is located immediately anterior to the penis bulb and occupies almost the entire ample space between the posterior wall of the pharyngeal pocket and the penis bulb (Figs 6F, G, 7); a muscle layer around the copulatory bursa was not detected.

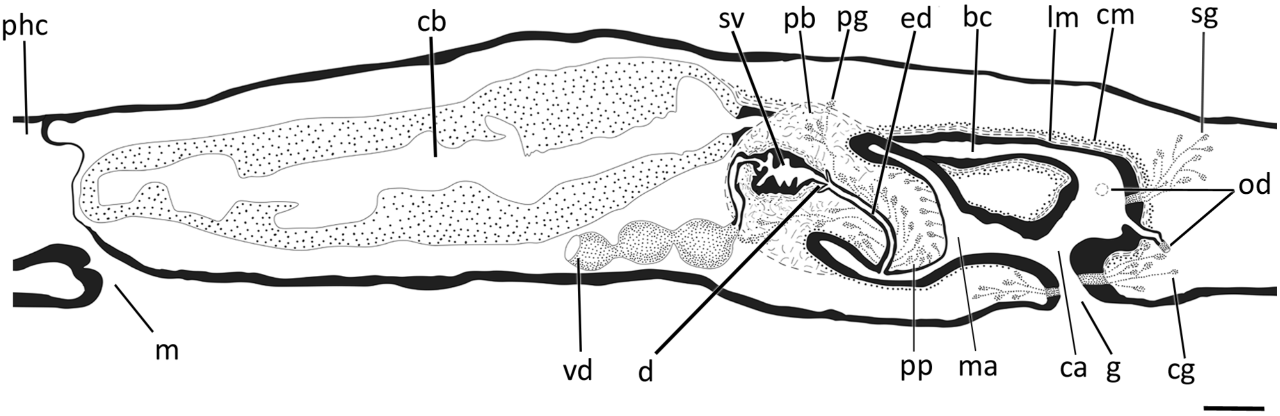

FIGURE 7. *Dugesia bursagrossa*, Sagittal reconstruction of the copulatory apparatus of the holotype. Scale bar: 100 µm. Anterior to the left.

Discussion

Phylogeny. Based on the results of the COI phylogenetic analysis, the new species *Dugesia bursagrossa* is positioned within the Afrotropical-Mediterranean clade. In this clade are included species of geographically close regions, such as *D. arabica* from Yemen, or *D. aethiopica* and *D. sicula* from Northern Africa. The latter species presents, nonetheless, a broad distribution in the Mediterranean area because of human activities (Lázaro & Riutort 2013). Therefore, the phylogenetic position of *D. bursagrossa* fits the well-established phylogeographic pattern of affiliation between the Mediterranean and some African members of this genus in a manner consistent with earlier multi-locus phylogenetic analyses that proposed multiple independent pathways for freshwater planarians in their dispersal from Africa to other regions of the world (Solà *et al.* 2022).

Morphology. The new species, *D. bursagrossa*, is mainly characterized by its very large, elongated copulatory bursa, asymmetrical oviducal openings into the bursal canal, and the subterminal opening of the ejaculatory duct through the ventral wall of the penis papilla. The latter condition has caused some problems in earlier papers on species of *Dugesia*, which concluded in similar cases that the penis papilla was asymmetrical and that the ejaculatory duct followed a ventrally displaced course through the papilla. However, when the various character states at stake are precisely defined (cf. Stocchino *et al.* 2017) it must be concluded that in *D. bursagrossa* the ejaculatory duct follows a central course and that the penis papilla is not asymmetrical, albeit provided with a subterminal opening of the ejaculatory duct.

A subterminal, ventral opening of the ejaculatory duct is present also in no less than 21 other species of *Dugesia*. Among these 21 species, there are only six that also exhibit asymmetrical oviducal openings into the bursal canal, as is the case also in *D. bursagrossa*, viz., *D. adunca* Chen & Sluys, 2022, *D. arabica*, *D. astrocheta* Marcus, 1953, *D. neumanni* (Neppi, 1904), *D. sicula*, and *D. superioris* Stocchino & Sluys, 2013. Apart from certain anatomical differences, the species *D. adunca* and *D. superioris* differ from *D. bursagrossa* in that they exhibit a basal number of 8 chromosomes.

Unfortunately, the karyotypes of *D. astrocheta* and *D. neumanni* are unknown. However, in *D. astrocheta* the bursal canal musculature includes an ectal reinforcement layer of longitudinal muscles that extends from the vaginal area to about halfway along the bursal canal, while in *D. neumanni* this muscle layer reaches the copulatory bursa (Sluys 2007). In contrast, ectal reinforcement is completely absent in *D. bursagrossa*.

In conformity with *D. bursagrossa*, the species *D. arabica* also exhibits a mixoploid karyotype with a basic number of 9 chromosomes. Furthermore, these two species group into a clade of four species (Fig. 1). Nevertheless, *D. bursagrossa* can be distinguished from *D. arabica* in that in the latter the lining of the bursal canal is highly folded along most of its length (Sluys 2007; Harrath *et al.* 2013). Furthermore, in *D. arabica* the bursal canal is surrounded by only a layer of circular muscles, whereas in *D. bursagrossa* the canal is covered with a subepithelial layer of longitudinal muscles, followed by layers of circular muscles.

In view of the fact that *D. sicula* is a species with a rather wide distribution in the Mediterranean region, it is opportune to closely evaluate the anatomical similarities and differences between these two species, although our molecular analysis revealed that *D. bursagrossa* is genetically different from *D. sicula* (Fig. 1). In this context, it should be noted that *D. biblica* is a junior synonym of *D. sicula* (Solà *et al.* 2015). Major differences between the two species, *D. sicula* and *D. bursagrossa*, concern the anatomy of the bursal canal and the glandularization of the penial papilla. In *D. sicula* the bursal canal is surrounded by a zone of mesenchymal cells that discharge their secretion into the lining epithelium of the canal (De Vries 1988a, b); this zone of glands is absent in *D. bursagrossa*. Furthermore, in *D. sicula* there is an ectal reinforcement layer, either restricted to the vaginal region of the bursal canal or extending almost to the copulatory bursa (De Vries 1988a, b); ectal reinforcement is completely absent in *D. bursagrossa*.

Apart from the usual erythrophilic secretion of the penis glands running through the parenchyma of the papilla and opening into the ejaculatory duct, the penis papilla of *D. sicula* is characterized by the presence of a broad zone of cyanophilic secretion in the dorsal portion of the papilla (Solà *et al.* 2015, Fig. 3).

Another difference between the two species concerns the size and shape of the intrabulbar seminal vesicle. In *D. sicula* there is a rather elongated vesicle that frequently has a somewhat bifid appearance, as it may show a shallow constriction at about halfway its length. In *D. bursagrossa*, however, the seminal vesicle is a more balloon-shaped structure.

Additionally, the position of the ovaries differs between the two species. In the sexual animals of *D. sicula*, the ovaries are ventrally located immediately behind the brain, whereas in *D. bursagrossa*, they are situated also ventrally but at approximately one-third of the distance between the brain and the root of the pharynx.

Notably for specimens of *D. sicula* from Algeria it was reported that the copulatory bursa is coated with a layer of longitudinal muscles (Harrath *et al.* 2012). In contrast, the copulatory bursa of *D. bursagrossa* is devoid of any surrounding musculature.

The very large and particularly elongated copulatory bursa of *D. bursagrossa* is rather unique among species of *Dugesia* as it is only paralleled in some specimens of *D. artesiana* (Sluys *et al.* 2007, fig. 3). However, in the case of the last-mentioned species such an elongated shape of the bursa may well be due to a preservation artefact, whereas in individuals of *D. bursagrossa* the elongated shape is a constant feature.

Karyology. A mixoploid chromosome complement with a basic number of n = 9 chromosomes has been reported for three other species of *Dugesia*, viz., *D. aethiopica*, *D. maghrebiana*, and *D. arabica*. With respect to the last-mentioned species, from Yemen, the karyotype of these populations (diploid: 2n = 18 + 0–1 B-chromosome; triploid complement: 3n = 27 + 1–2 B-chromosomes) is composed of a single submetacentric and eight metacentric chromosomes (Harrath *et al.* 2013). Despite the fact that the karyological differences between *D. bursagrossa* and *D. arabica* are limited to just one chromosome —chromosome 5 in *D. arabica*— this suggests, nevertheless, a case of speciation, as chromosomal variations may accompany speciation processes (Benazzi 1982). However, the metaphase plates of *D. bursagrossa* closely resemble those of *D. aethiopica* and *D. maghrebiana*, as karyometric data show that both triploid and tetraploid sets are characterized by metacentric chromosomes (Stocchino *et al.* 2004; Stocchino *et al.* 2009). Despite these karyological similarities, *D. bursagrossa* is distinct from these species due to significant morphological differences and clear genetic differentiation in the phylogenetic trees.

Upon examining the meiotic plates of the gonads (testes and ovaries) in sexually reproducing individuals of *D. bursagrossa* with a mixoploid karyotype, the animals consistently exhibited a basic number of n = 9 in all chromosomal plates studied. This suggests that during gonadogenesis the diploid karyotype can be reestablished from the original mixoploid state, thus enabling successful sexual reproduction. These findings align with previous reports on the species *D. aethiopica* in Africa (Stocchino *et al.* 2004) and *D. japonica* (Tamura *et al.* 1995). It has been proposed that a mixoploid karyotype serves as an adaptive mechanism to withstand harsh environmental conditions, such as high temperatures and intense solar radiation (Stocchino *et al.* 2004). Evidently, this may hold true for the sexually reproducing species *D. bursagrossa* in Saudi Arabia, where high temperatures and intense solar radiation are prevalent.

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TABLE S1. Sampling localities in Saudi Arabia and their GPS coordinates.

SN	Region	Location	GPS	Date
1	Najran	Almaghrah	17°30’29.3”N 44°06’00.3”E	10/08/2020
2		Najran Valley	17°25’32.7”N 44°02’01.1”E	11/08/2020
3	Khamis Mushayt	Wadi Bishah	18°09’33.7”N 42°50’55.2”E	15/08/2020
4		Wadi Al-Jawf- Ahd Rafida	18°11’35.4”N 42°53’51.5”E	15/08/2020
5		The highest of Wadi Bishah	18°13’49.0”N 42°48’48.9”E	17/08/2020
6		End of Wadi Al-Jawf- Ahd Rafida	18°12’23.4”N 42°50’09.0”E	17/08/2020
7		Wadi Hafech Tandah	18°25’12.1”N 42°59’22.8”E	17/08/2020
8	Jizan	Aqaba Dhla	17°56’56.6”N 42°28’07.9”E	17/08/2020
9		Wadi Reem	17°52’37.2”N 42°16’42.7”E	18/08/2020
10		Wadi Ramlan	17°41’17.1”N 42°19’16.5”E	19/08/2020
11		Alhaqu	17°29’31.6”N 42°41’29.7”E	19/08/2020
12		Wadi Shahdan- Alhaqu	17°27’06.1”N 42°42’53.4”E	19/08/2020
13		The highest of Wadi Shahdan- Alhaqu	17°28’47.2”N 42°51’32.2”E	19/08/2020
14		Fifa	17°14’06.3”N 43°03’21.8”E	20/08/2020
15		Fifa	17°16’21.2”N 43°06’49.7”E	20/08/2020
16		Wadi bashe	17°34’45.1”N 42°37’59.3”E	20/08/2020
17	Abha	Ain Al-Dhiba – Al-Soudah (*)	18°16’44.4”N 42°23’18.6”E	23/08/2020
18		Rijal Al-Maa	18°11’27.9”N 42°16’46.1”E	23/08/2020
19	Tabuk	Ain musa- Al Maknah	28°23’49.3”N 34°45’05.8”E	12/01/2021

(*) Indicates the locality where *Dugesia bursagrossa* was collected.