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## Research article

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# Two new genera and three new subterranean species of Hydrobiidae (Caenogastropoda: Truncatelloidea) from Tunisia

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**Abstract.** The aquatic biodiversity of springs and groundwater systems of North Africa remains largely unexplored. In an earlier field survey of Tunisian springs, a new gastropod genus, *Bullaregia*, was discovered as a phylogenetically independent lineage of uncertain position within the family Hydrobiidae. Here, we provide taxonomic and phylogenetic assignments for three newly collected populations of hydrobiids from springs in northern Tunisia based on morphological, anatomical and genetic (mtCOI and 18S) data. Among these and specimens of *Bullaregia*, major differences were observed in male and female genitalia as well as in mtCOI sequences (divergence 8.0–9.1%). Based on these findings, we describe two new genera and three new species: *Belgrandiellopsis chorfensis* gen. et sp. nov., *Belgrandiellopsis secunda* gen. et sp. nov. and *Biserta putealis* gen. et sp. nov. In all our phylogenetic analyses, these three new species were well resolved as a monophyletic group together with *Bullaregia tunisiensis*. Unexpectedly, this clade emerged as sister to the European valvatiform genera *Corbellaria* and *Kerkia* and not to the recently discovered clade of groundwater, conchologically similar, species living in Bulgaria (Balkan Peninsula). These Tunisian species are each locally endemic and form part of a newly discovered clade which in future systematic studies could eventually be identified as a distinct hydrobiid subfamily.

**Keywords.** Taxonomy, phylogeny, springsnails, North Africa, *Bullaregia*.

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## Introduction

Vast expanses of carbonate rocks in several countries of North Africa (Morocco, Algeria and Tunisia) give rise to aquifer systems under conditions of an arid or semi-arid climate (Woodward 2009). Despite

these favourable conditions, northern African ground waters are currently under considerable stress due to climate change and/or anthropogenic factors such as land use or water pollution (Ouedraogo *et al.* 2016). In Tunisia, human populations have long used groundwater resurgences (springs and wells) as drinking and irrigation water. As a result, wells and captured springs can be found across the country, and these are also home to highly adapted, subterranean organisms. While more than 100 stygobiont (i.e., obligate subterranean) species have been discovered in North Africa (Gibert & Culver 2009), information on the subterranean biodiversity of Tunisia, including those species living in springs of underground waters, is still very limited (García *et al.* 2010). The restricted geographic distribution of these species, often of difficult access, coupled to their small body size and convergent structures (Culver 2012) make biodiversity inventories in these ecosystems a real challenge.

Molluscs are an important component of spring and groundwater systems of North Africa (García *et al.* 2010). For more than a decade now, surveys of continental underground ecosystems (e.g., streams, springs and wells) in Tunisia have revealed that the most abundant and frequent mollusc species belong to the gastropod family Hydrobiidae Stimpson, 1865 (Khalloufi & Boumaiza 2007; Khalloufi *et al.* 2017). Even when applying the *sensu stricto* definition based on molecular evidence (Wilke *et al.* 2001, 2013), hydrobiids are generally considered one of the most species-rich families of freshwater molluscs, and have been described as a hotspot of diversity in Northern Africa (Strong *et al.* 2008; Miller *et al.* 2018). However, the status of most Tunisian hydrobiid species is unknown, and the species richness picture of this family in Tunisia is still incomplete. Minute-sized snails from this region were previously discovered mainly from surface fresh- and brackish-waters. Based on shell morphology, most species were tentatively assigned to the non-hydrobiid genera *Amnicola* Gould & Haldeman, 1840, *Bythinella* Moquin-Tandon, 1856 and *Paludestrina* D’Orbigny, 1840 (Bourguignat 1860, 1864; Debeaux 1863; Issel 1880; Letourneux & Bourguignat 1887; Pallary 1921, 1923; Seurat 1921, 1934). Later on, according to new anatomical evidence, Boeters (1976) and Glöer *et al.* (2010) transferred some of these taxa to the hydrobiid genera *Pseudamnicola* Paulucci, 1878, *Mercuria* Boeters, 1971 and *Hydrobia* Hartmann, 1821. In the latter study (Glöer *et al.* 2010), new species of *Pseudamnicola* and *Mercuria* from Tunisia were also identified based on samples from museum collections. Recent molecular phylogenies (Delicado *et al.* 2015, 2018) have also unveiled unknown lineages within *Pseudamnicola* which may represent new species to science.

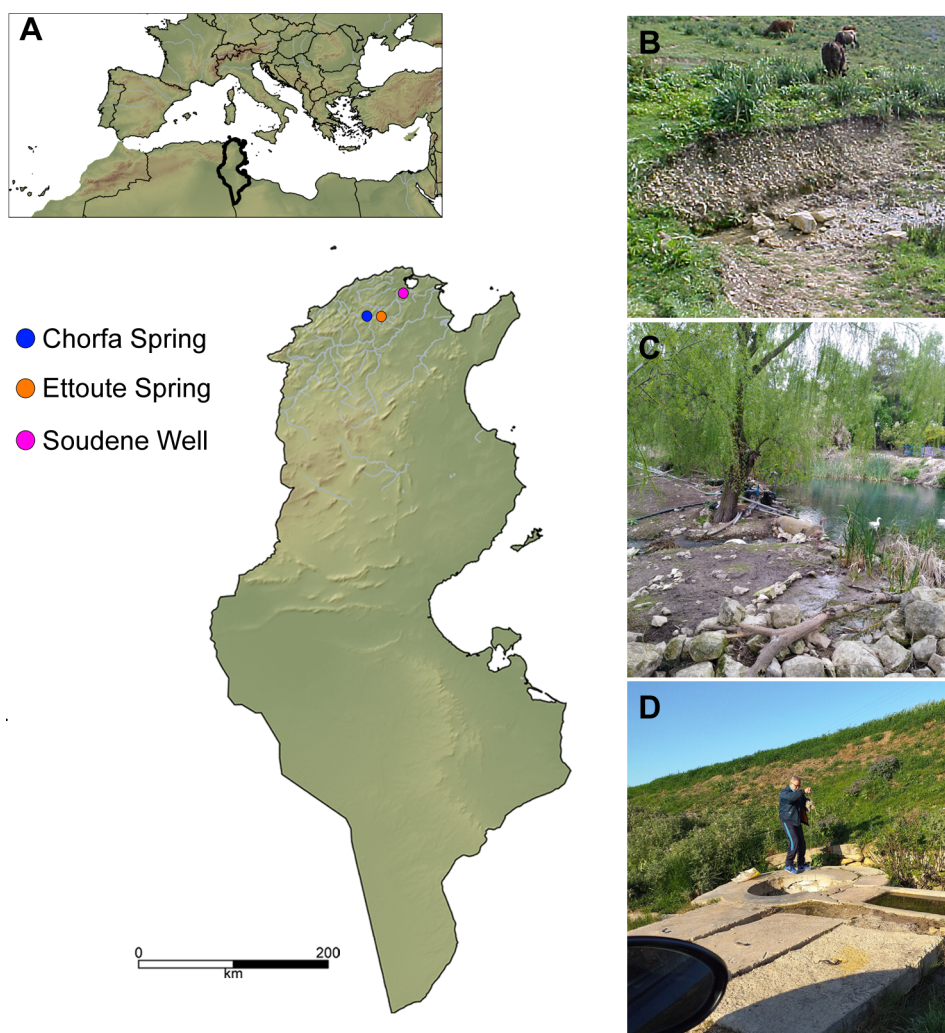
Sampling efforts in northern Africa, especially Tunisia, have focused little on underground water ecosystems. In Algeria and northern Tunisia, a few subterranean species with very small, conic shells (e.g., *Bythinella* (?) *limnopsis* Letourneux & Bourguignat, 1887, *B.* (?) *mauritanica* Bourguignat, 1887, *B.* (?) *microcochlia* Letourneux & Bourguignat, 1887, *B.* (?) *punica* Letourneux & Bourguignat, 1887 and *Hydrobia* (?) *nana* (Terver, 1839)) were collected and described by early authors (e.g., Letourneux & Bourguignat 1887; Pallary 1921; Terver 1839). The latter species was also reported from Zaghouan and Bulla Régia (in Tunisia). Pallary (1923) retained only this species, whose genus remains unspecified between *Hydrobia* and *Bythinella*. However, studies on these taxa have not advanced until recently. Khalloufi *et al.* (2017) erected a new genus, *Bullaregia* Khalloufi, Béjaoui & Delicado, 2017, for the snail population living in Bulla Régia and two additional springs. Specimens of this genus resemble *Bythinella* and the hydrobiid genus *Belgrandiella* Wagner, 1928 in shell shape but bear other very distinctive anatomical features. Besides this, the sister taxa and phylogenetic position of this new genus within Hydrobiidae remain unclear (Khalloufi *et al.* 2017). Additional surveys are therefore necessary to discover their current diversity and evolutionary history, which are crucial to protect the snails’ fragile spring habitats (Hershler *et al.* 2014).

The first two authors of the present paper recently collected hydrobiid snails from three subterranean water springs in northern Tunisia. Here, we provide morphological and anatomical descriptions of these three populations along with taxonomic assignments. To assess their taxonomic status, phylogenetic

relationships and position within Hydrobiidae, we also add genetic (mitochondrial and nuclear) data for these and other recently discovered subterranean hydrobiid species from Europe (Rysiewska *et al.* 2016; Osikowski *et al.* 2017) to the previous molecular phylogeny (Khalloufi *et al.* 2017). This work forms part of a series of upcoming papers that will describe hydrobiid diversity in neglected and highly stressed aquatic ecosystems in Tunisia.

## Material and methods

Live specimens were collected from three localities in northern Tunisia: Chorfa Spring, Province Béja, 36.865918° N, 9.363313° E, 430 m a.s.l.; Ettoute Spring, Province Bizerte, 36.883898° N, 9.505894° E, 415 m a.s.l.; and Soudene Well (Menzel Bourguiba city), Province Bizerte, 37.09046° N, 9.777997° E, 115 m a.s.l. (Fig. 1). This was done within the substrate with a 300 µm mesh cloth. Samples were preserved in 70% ethanol in the field and sorted in the laboratory under a MOTIC ST-39 series stereo microscope. Anatomical structures were studied under an Olympus SZX12 stereo microscope and photographed using a Keyence VHX 2000 3D Digital Microscope in combination with the program VHX-2000 Communication software ver. 2.3.5.0 (Keyence Corporation 2009–2012). The radula was



**Fig. 1.** Map and photographs of the collecting localities. **A.** Map showing the collecting localities in Tunisia. **B.** Chorfa Spring, Province Béja. **C.** Ettoute Spring, Province Bizerte. **D.** Soudene Well (Menzel Bourguiba city), Province Bizerte.

extracted from the buccal mass according to the first step of a CTAB protocol for DNA isolation (Wilke *et al.* 2006). Then, after drying, radulae were mounted on metallic stubs and sputter-coated with gold (Balter Sputter Coater SCD004) for 50 s so that photographs could be captured with a field emission scanning electron microscope (FESEM) DSM982 Gemini (Carl Zeiss GmbH, Germany). Anatomical characteristics (genitalia, stomach, radula and gill) were described, following the standard terminology of Hershler & Ponder (1998). Shell measurements were made with an eye-piece micrometer under the stereo microscope and consisted of length and width of the entire shell, body whorl and aperture, aperture high and width of antepenultimate and penultimate whorls (see abbreviations below). The number of specimens subjected to morphometry is indicated in the corresponding sections of the text. The 11 variables for shell dimensions and number of body whorls followed a normal distribution according to Kolmogorov-Smirnov tests and have equal variances among groups as indicated by Levene's test of homogeneity of variance. Consequently, an ANOVA could be used to test for statistical differences among these variables in the three species. These statistical calculations have been performed with the R 3.6.1 statistical environment (R Development Core Team 2019) and the R package car (Fox & Weisberg 2019). The sex of the holotype was not examined to keep the shell intact. The holotype and some of the paratypes were deposited in the Collection of Molluscs of the Museo Nacional de Ciencias Naturales de Madrid (MNCN), Spain; the other paratypes and DNA samples were deposited in the collection of the University of Giessen Systematics and Biodiversity (UGSB) (Diehl *et al.* 2018) in Germany and the collection of N. Khalloufi at the Faculty of Sciences of Bizerte, University of Carthage, in Tunisia.

We genetically analysed one to two specimens from each sample. GenBank sequences from another 32 hydrobiid species (Table 1) resembling the newly discovered Tunisian populations in shell shape and/or with evidence of their close relationship to them (Khalloufi *et al.* 2017) were added to the genetic data matrices to assess the phylogenetic status and position of these snails within the Hydrobiidae. Genomic DNA was extracted from whole specimens using the CTAB protocol of Wilke *et al.* (2006). Regions of the mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear ribosomal 18S rRNA (18S) were amplified and sequenced using the primer pairs LCO1490 (Folmer *et al.* 1994) and COR722b (Davis *et al.* 1998) for COI, and the universal metazoan 18S primers (Holland *et al.* 1991) for 18S. Amplification conditions for COI and 18S gene fragments were those of Delicado *et al.* (2012) and Schreiber *et al.* (2012), respectively. The amplified PCR product was sequenced in an ABI 3730 XL sequencer (Life Technologies, Carlsbad, CA, USA) using a Big Dye Terminator kit ver. 3.1 (Life Technologies). New sequences were deposited in GenBank (Table 1).

Forward and reverse sequences were assembled and edited in Sequencher 4.6 (Gene Codes, Ann Arbor, MI). Sequences obtained de novo were compiled in two datasets (one per gene partition), together with sequences of other selected hydrobiid species (Table 1). Protein-coding COI sequences were unambiguously aligned in MEGA 7.0.14 (Kumar *et al.* 2016). The 18S fragments were aligned using MAFFT 7.402 (Katoh *et al.* 2002) with default settings for gap penalties [gap opening penalty (GOP) = 1.53]. Sequence divergences (uncorrected p-distances) were calculated in MEGA. Wilke *et al.* (2013) found little saturation within the Hydrobiidae for the COI and 18S regions and we did not, therefore, perform saturation tests. The substitution models TIM2 (Kimura 1981) +I (invariable sites) +G (rate variation among sites) and TrNef (Tamura–Nei model with equal base frequencies; Tamura & Nei 1993) +I +G were selected as best fitting the COI and 18S datasets, respectively, in jModelTest 2.1.7 (Darriba *et al.* 2012) with the corrected Akaike's information criterion (AICc; Akaike 1974; Sugiura 1978; Hurvich & Tsai 1989).

Phylogenetic relationships of the hydrobiid species examined were estimated under maximum likelihood (ML) and Bayesian inference (BI) approaches. Maximum likelihood analysis was performed using RAxML-HPC2 8 (Stamatakis 2014) on the computer cluster of the Cyber Infrastructure for the Phylogenetic Research project (CIPRES; [www.phylo.org](http://www.phylo.org)) using the GTR (Tavaré 1986) +I +G model for

**Table 1** (continues on the next two pages). Taxa included in the molecular analysis with their corresponding locality names and geographical coordinates in decimal degrees, GenBank accession numbers and original references. N.D. = No data.

Taxon	Locality	GenBank # COI/18S	Original reference
Outgroup			
<i>Mercuria emiliana</i>	Spain, Mallorca, La Puebla (39.79111, 3.10472)	AF213346 AF212913	Wilke <i>et al.</i> (2000)
Ingroup			
<i>Agrafia wiktori</i>	Greece, Evrytania, Agrafa mountains, well at Sikiá (39.36861, 21.63139)	JF906762 JF906758	Szarowska & Falniowski (2011)
<i>Alzoniella fnalina</i>	Italy, Liguria, Savona, Molino, spring at the Porra River (44.219, 8.255)	AF367650 AF367686	Wilke <i>et al.</i> (2001)
<i>Avenionia brevis</i>	France, Gard, spring of the fountain of St. Victor-La Coste (44.057, 4.636)	AF367638 AF367670	Wilke <i>et al.</i> (2001)
<i>Balkanica yankovi</i>	Bulgaria, Sulari and Yantra villages, Izvora Cave (42.95638, 25.31444)	MF179879 MF179924	Osikowski <i>et al.</i> (2017)
<i>Belgrandia thermalis</i>	Italy, Tuscany, Pisa, S. Giuliano Terme, thermal channel (43.751, 10.440)	AF367648 AF367684	Wilke <i>et al.</i> (2001)
<i>Belgrandiella kusceri</i>	Slovenia, spring of Rakek (N.D.)	JX970610 JX970574	Wilke <i>et al.</i> (2013)
<i>Belgrandiellopsis chorfensis</i> gen. et sp. nov.	Tunisia, Béja Province, Chorfa Spring (36.86592, 9.36331)	MN580416 MN575710	Present study
<i>Belgrandiellopsis chorfensis</i> gen. et sp. nov.	Tunisia, Béja Province, Chorfa Spring (36.86592, 9.36331)	MN580417 –	Present study
<i>Belgrandiellopsis secunda</i> gen. et sp. nov.	Tunisia, Bizerte Province, Ettoute Spring (36.883900, 9.50589)	MN580418 MN575711	Present study
<i>Belgrandiellopsis secunda</i> gen. et sp. nov.	Tunisia, Bizerte Province, Ettoute Spring (36.883900, 9.50589)	MN580419 MN575712	Present study
<i>Biserta putealis</i> gen. et sp. nov.	Tunisia, Bizerte Province, Soudene Well (37.09046, 9.7780)	MN580420 MN575713	Present study
<i>Boetersiella sturmi</i>	Spain, Jaén, Mata Bejid, La Mata Spring (37.69503, -2.49297)	MH350199 MH348097	Delicado <i>et al.</i> (2019)
<i>Boleana umbilicata</i>	Slovenia, Močilnik, the spring of the Ljubljanica River (45.95416, 14.2925)	JX982795 JX982797	Falniowski & Szarowska (2012)
<i>Bullaregia tunisiensis</i>	Tunisia, Province Béja, a spring in Djebba (36.47125, 9.09972)	KX821683 MN575709	Khalloufi <i>et al.</i> (2017) Present study

**Table 1** (continued).

<b>Taxon</b>	<b>Locality</b>	<b>GenBank # COI/18S</b>	<b>Original reference</b>
<i>Bullaregia tunisiensis</i>	Tunisia, Province Béja, a spring in Djebba (36.47125, 9.09972)	MN580415 –	Present study
<i>Cavernisa zaschevi</i>	Bulgaria, Tserovo, Vodnata Cave (45.95417, 14.2925)	MF179895 MF179931	Osikowski <i>et al.</i> (2017)
<i>Corbellaria celtiberica</i>	Spain, Soria, Manubles River (41.60990, -1.95580)	MH350207 MH348099	Delicado <i>et al.</i> (2019)
<i>Dalmatinella fluviatilis</i>	Croatia, Jankovica Buk waterfalls, Zrmanja River (44.20272, 15.72136)	KC344541 KC344539	Falniowski & Szarowska (2013)
<i>Daphniola graeca</i>	Greece, Dafne Spring (39.89108, 22.60722)	EF070618 EF070624	Szarowska (2006)
<i>Deganta azarum</i>	Spain, Asturias, Borondes, La Fontona Spring (43.33189, -5.98506)	MH350208 MH348100	Delicado <i>et al.</i> (2019)
<i>Devetakia mandrica</i>	Bulgaria, Chavdartsi, Mandrata Cave (43.24222, 24.96917)	MF179891 MF179927	Osikowski <i>et al.</i> (2017)
<i>Devetakiola devetakium</i>	Bulgaria, Prevala, spring near Vreloto Cave (43.46944, 22.86111)	MF179901 MF179935	Osikowski <i>et al.</i> (2017)
<i>Fissuria boui</i>	France, Alpes Maritimes, Peymeinade, spring near La Prouveresse (43.64279, 6.88735)	AF367654 AF367690	Wilke <i>et al.</i> (2001)
<i>Graecoarganiella parnassiana</i>	Greece, Parnassus mountains, south of Eptalofos, small spring (38.59278, 22.5039)	JN202348 JN202341	Falniowski & Szarowska (2011)
<i>Graziana alpestris</i>	Italy, Liguria, Savona, Molino, spring at the Porra River (44.219, 8.255)	AF367641 AF367673	Wilke <i>et al.</i> (2001)
<i>Grossuana codreanui</i>	Romania, a spring close to the lake Techirghiol (43.99361, 28.54611)	EF061919 EF061916	Szarowska <i>et al.</i> (2007)
<i>Hauffenia tellinii</i>	Italy, Friuli-Venetia Julia, Gorizia, spring of the Isonzo River near Sagrado (45.8743, 13.4856)	AF367640 AF367672	Wilke <i>et al.</i> (2001)
<i>Horatia klecakiana</i>	Croatia, N of Kučiće, Studenci spring, in the valley of the Cetina River (43.44481, 16.80708)	KJ159128 KJ159127	Szarowska & Falniowski (2014)
<i>Islamia globulus</i>	Spain, Huesca, Gistain, Grima Spring (42.58660, -0.31766)	MH350222 MH348103	Delicado <i>et al.</i> (2019)



**Table 1** (continued).

Taxon	Locality	GenBank # COI/18S	Original reference
<i>Kerkia kusceri</i>	Slovenia, Ivančna Gorica, Cave Krška jama (45.89, 14.7711)	KY087867 KY087833	Rysiewska <i>et al.</i> (2017)
<i>Pauluccinella minima</i>	Italy, S. Egidio, Lago di Piediluco (42.52925, 12.7474)	JX970612 JX970578	Wilke <i>et al.</i> (2013)
<i>Pontobelgrandiella angelovi</i>	Bulgaria, a spring in Tvarditsa (42.70556, 25.89778)	KU496965 MF179938	Rysiewska <i>et al.</i> (2016) Osikowski <i>et al.</i> (2017)
<i>Pseudamnicola lucensis</i>	Italy, Tuscany, Bagni di Lucca, Bagni Caldi, thermal spring (44.007, 10.585)	AF367651 AF367687	Wilke <i>et al.</i> (2001)
<i>Radomaniola callosa</i>	Italy, Abruzzo, Caramanico Terme, stream on the left bank of Orfento River (42.1571, 14.0167)	AF367649 AF367685	Wilke <i>et al.</i> (2001)
<i>Sadleriana fluminensis</i>	Croatia, Jadro River at Solin near Split (43.5453, 16.48780)	AF367647 AF367683	Wilke <i>et al.</i> (2001)
<i>Stoyanovia stoyanovi</i>	Bulgaria, Bezhanovo village, Parnitsite Cave (43.22889, 24.38861)	MF179902 MF179936	Osikowski <i>et al.</i> (2017)
<i>Trichonia kephalovrissonia</i>	Greece, spring in Thérmon (38.57294, 21.66622)	EF070619 EF070630	Szarowska (2006)

each partition and a rapid bootstrapping algorithm. Node support was assessed through 1000 bootstrap (BS) pseudoreplicates. Bayesian inference analysis was conducted in MrBayes 3.2.6 (Ronquist *et al.* 2012) using the substitution models selected through jModelTest. Markov chain Monte Carlo (MCMC) methods were run with random starting trees and 4 chains for 5 000 000 generations, sampling one generation every 1000 sampling points. After verifying chain convergence (i.e., average standard deviations of split frequencies < 0.01), the first 10% of the samples was discarded as burn-in. The robustness of the inferred trees was evaluated by Bayesian posterior probabilities (BPPs). Tree topologies and branch supports were visualized in FigTree 1.4.3 (Rambaut 2010).

### Abbreviations

#### Shell measurements

AH	=	aperture height
AL	=	aperture length
AW	=	aperture width
LBW	=	length of the body whorl
NSW	=	number of spire whorls
SL	=	shell length
SW	=	shell width
WAW	=	width of antepenultimate whorl
WBW	=	width of body whorl
WPW	=	width of penultimate whorl

#### Anatomy

Ag	=	albumen gland
Bc	=	bursa copulatrix
Bd	=	bursal duct
Bl	=	basal lobe
Bp	=	basal portion of penis
Cg	=	capsule gland
Ct	=	ctenidium
Dl	=	distal lobe
Dp	=	distal portion of penis
Op	=	oviduct pouch
Os	=	osphradium
Ov	=	renal oviduct
Pf	=	penial filament (i.e., structure apart from lobes)
SR1	=	distal seminal receptacle
SR2	=	proximal seminal receptacle
Tp	=	terminal papilla

#### Results

The combined alignment of the COI (648 bp) and 18S (468 bp) sequences yielded 1116 bp. Average base frequencies for COI were 30.8% A, 13.0% C, 11.2% G and 45% T. Based on the combined dataset, ML and BI trees agreed in depicting a well-supported (BS = 100%, BPP = 1) monophyletic clade, informally referred to herein as the ‘Tunisian clade’, comprising the Tunisian populations examined here and *Bullaregia tunisiensis*. Each of the Tunisian populations formed a well-resolved subclade within this clade (Fig. 2). Uncorrected pairwise sequence divergences among representatives of this clade ranged from 7.9–9.3% for COI and were 0% for 18S. These results are similar to those observed among other genera of our phylogenetic study (e.g., 6.2–10% COI and 0% 18S among the subterranean genera from Bulgaria). Phylogenetic relationships within the Tunisian clade were well supported between populations from Chorfa and Ettoute Springs (BS = 86%, BPP = 0.96). The remaining relationships within this clade were weakly supported. BI analysis resolved (BPP = 1) the Tunisian clade as sister to the species *Corbellaria celtiberica* Girardi & Boeters, 2012 from the Iberian Peninsula and *Kerkia kusceri* (Bole, 1961) from the Balkans. In both analyses, all these species weakly grouped with the subterranean hydrobiid genera from Bulgaria (Fig. 2).

Phylum Mollusca Cuvier, 1795  
Class Gastropoda Cuvier, 1795  
Superorder Caenogastropoda Cox, 1960  
Superfamily Truncatelloidea Gray, 1840  
Family Hydrobiidae Stimpson, 1865

Genus *Belgrandiellopsis* gen. nov.

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#### Type species

*Belgrandiellopsis chorfensis* sp. nov., by present designation.

#### Diagnosis

Shell small (maximum length slightly exceeding 2 mm), ovate-conic to elongate-conic, with obtuse and planispiral protoconch; whorls slightly convex, with weak sutures. Penultimate whorl tall relative



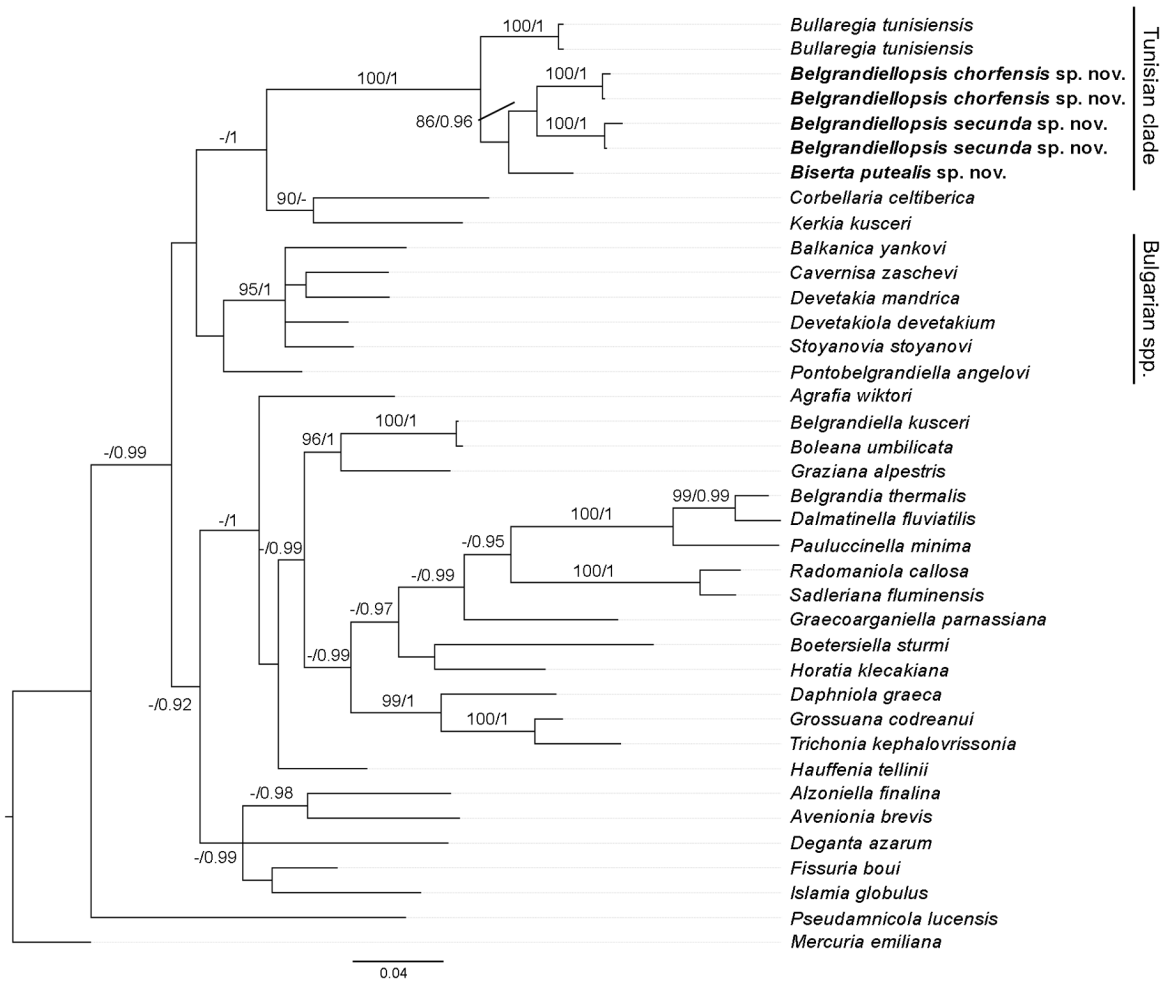
to remaining whorls. Aperture pyriform to ovate, complete, angled on top and often fused to the body whorl. Umbilicus closed by inner lip. Operculum corneous, whitish to yellowish, thin, pliable, elongate-ellipsoidal, spiral, paucispiral, with submarginal nucleus. One pair of small triangular basal cusps on radular central tooth. Ctenidium well developed. Stomach with a small gastric (posterior) caecum. Presence of bursa copulatrix; unpigmented renal oviduct with a spherical pouch at the end of the coiled section; two seminal receptacles. Penis attached to the neck behind the right eye with two glandular lobes distantly positioned, each of them bearing a terminal papilla.

**Etymology**

Refers to the shell similarities with the hydrobiid genus *Belgrandiella*; gender feminine.

**Remarks**

*Belgrandiellopsis* gen. nov. differs from the conchologically similar genus *Belgrandiella* in having a strap-like penis with two glandular lobes, two seminal receptacles and one pair of basal cusps on the central radular teeth (for comparison see Szarowska 2006); from the closely related genus *Bullaregia* according to the presence of two lobes on the inner edge of the penis, smaller gastric caecum, shorter



**Fig. 2.** Bayesian inference based on the COI and 18S datasets. Bootstrap values and BPP's for branches are given when  $\geq 70\%$  and  $\geq 0.90$ , respectively. Scale bar = expected change per site.

bursal duct, larger bursa copulatrix and in having generally a smaller number of cusps on radular teeth (see Khalloufi *et al.* 2017); and from *Biserta* gen. nov. according to its smaller shell umbilicus, longer penis with a larger distance between penial lobes, slightly smaller bursa copulatrix and longer bursal duct (Figs 4, 6, 8). *Belgrandiellopsis* gen. nov. differs in its mean COI divergence by 9.3% and 8.4% from the closely related *Bullaregia* and *Biserta* gen. nov., respectively, and by 17.6% from *Belgrandiella*.

*Belgrandiellopsis chorfensis* gen. et sp. nov.

[urn:lsid:zoobank.org:act:2C5704A1-BDFC-41BA-86C6-CF17A999BBC8](https://zoobank.org/act:2C5704A1-BDFC-41BA-86C6-CF17A999BBC8)

Figs 3–4

### Diagnosis

Shell 1.45 to 1.65 mm high, elongate-conic, brown-yellowish; central radular tooth formula (5)4–C–4(5)/1–1; bursa copulatrix ovoid, with the same length as the duct; two seminal receptacles, SR1 more developed and pedunculated, SR2 small, globular, located near loop before the pouch; prostate gland bean shaped, ovate in section, folded; penis cylindrical, slender, distal end tapered, unpigmented, with two lobes distantly positioned; basal lobe more developed. Distinguished from other closely related species by > 8.0% for COI.

### Etymology

A geographic epithet referring to Chorfa Spring, where the species was found.

### Type material

#### Holotype

TUNISIA • 1 spec. (sex unknown, preserved in ethanol 80%); Béja Province, ca 15 km west of Joumine, Chorfa Spring; 36.865918° N, 9.363313° E; 430 m a.s.l.; 15 Mar. 2015; N. Khalloufi leg.; MNCN 15.05/200097H

#### Paratypes

TUNISIA • 5 specs (preserved in ethanol 80%); same collection data as for holotype; MNCN 15.05/200097P • 40 specs (preserved in ethanol 80%, 2 ♀♀, 2 ♂♂ dissected and 2 specs processed for DNA sequencing); same collection data as for holotype; UGSB 17663 • 150 specs (preserved in ethanol 95%, ca 70 individuals dissected); same collection data as for holotype; Khalloufi's collection.

### Type locality

TUNISIA: Béja Province, ca 15 km west of Joumine, Chorfa Spring, 36.865918° N, 9.363313° E, 430 m a.s.l.

### Description

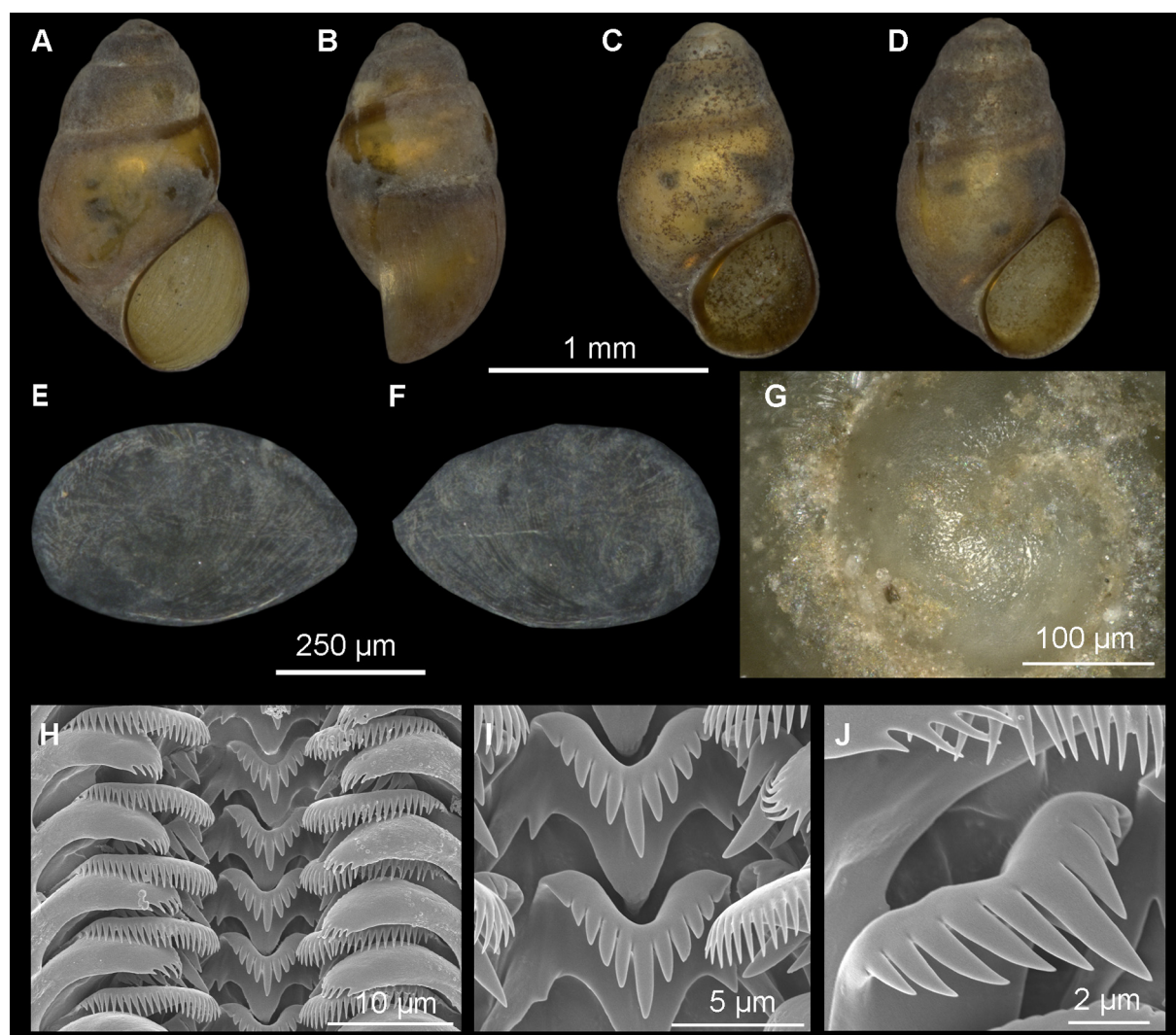
MEASUREMENTS. Holotype: SL = 1.61 mm, SW = 0.88 mm, SL/SW = 1.83, AH = 0.66 mm, SL-LBW = 0.49 mm, WBW = 0.84 mm, AL = 0.78 mm, AW = 0.53 mm, WPW = 0.64 mm, WAW = 0.10 mm.

SHELL. Elongate-conic, brown to yellowish, with 4–4.5 whorls (Fig. 3A–D; Table 2). Protoconch (Fig. 3G) obtuse, near planispiral, about 1.2 whorls, diameter about 350 µm; protoconch microsculpture granulated. Teleoconch whorls weakly convex, separated by deep sutures; body whorl occupying  $\frac{2}{3}$  of total shell length. Aperture about 40% of total shell length, pyriform, strongly angled on the top and narrowly disjunct from parietal wall of body whorl; inner lip thin but thicker than outer lip; peristome margin (Fig. 3B) straight. Umbilicus narrow and closed by inner lip.

OPERCULUM. As for genus, thin, flat, whitish (nuclear region slightly yellowish), with ca 2 whorls (Fig. 3E–F); attachment scar area, oval and located near nucleus.

RADULA. Length intermediate (20% total maximum shell length), having about 80 rows of teeth. Central tooth (Fig. 3H–I) formula (5)4–C–4(5)/1–1, cutting edge concave; basal tooth face M-shaped and relatively narrow wings; basal tongue broadly V-shaped, about equal to lateral margins; a single small pair of basal cusps, triangular. Lateral teeth (Fig. 3J) formula (4)3–C–5; central cusp large, narrow, pointed. Inner marginal teeth (Fig. 3H) having 25–27 sharp cusps. Outer marginal teeth having 20–22 sharp cusps.

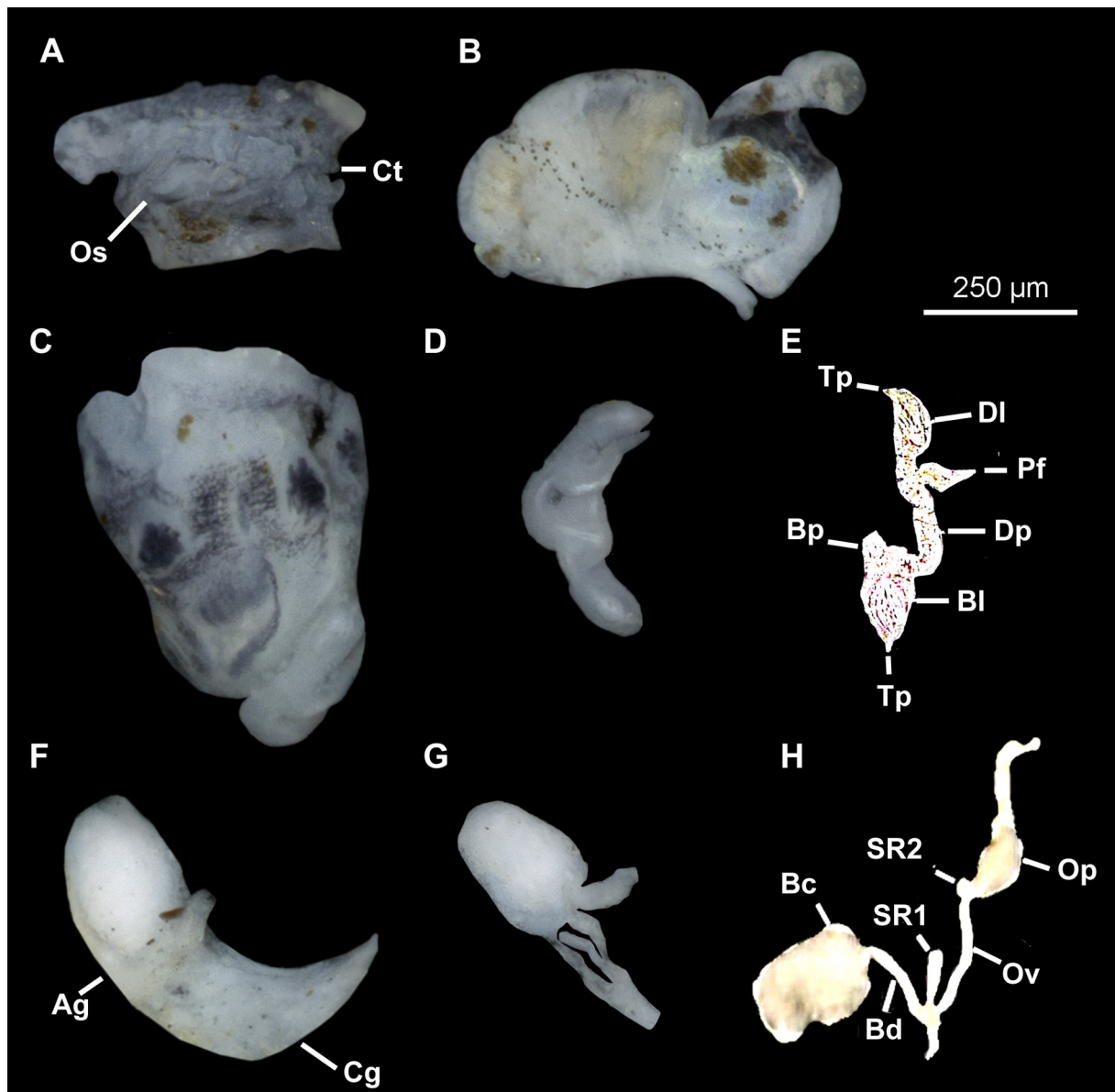
PIGMENTATION AND ANATOMY. Visceral coil brown-yellowish, showing by transparency the gonad; pallial coil nearly pale with traces of pigment on the roof, anteriorly to edge of mantle; foot and area between tentacles slightly pigmented (Fig. 4C). Black small eyes surrounded by brownish pigment. Snout with



**Fig. 3.** Shells, opercula and radulae, *Belgrandiellopsis chorfensis* gen. et sp. nov. **A–D.** Shells. **A–B.** Holotype, MNCN 15.05/200097H. **C–D.** Paratypes, MNCN 15.05/200097P. **E–F.** Operculum (E = inner side; F = outer side). **G.** Detailed microsculpture of protoconch. **H–J.** Radulae. **H.** Portion of radula ribbon. **I.** Central radular teeth. **J.** Lateral teeth.

medium distal lobation. Tentacles parallel-side, distal end nonexpanded. Ctenidium filaments about 12, small, taller than wide. Osphradium whitish, ellipsoidal, positioned centrally along ctenidium (Fig. 4A). Stomach slightly longer than wide with a small gastric (posterior) caecum (Fig. 4B); rectum wide, folded and S-shaped.

MALE GENITALIA. Penis cylindrical (Fig. 4D–E), medium sized, unpigmented, slender, slightly folded; filament short, tapering; basal portion intermediate in width, shorter than distal portion, attached centrally to the head well behind the eyes; penis having two developed lobes located relatively far from one another on opposite sides of penis; both lobes with distal papilla, showing by transparency



**Fig. 4.** Anatomy of *Belgrandiellopsis chorfensis* gen. et sp. nov. (paratypes, UGSB 17663). **A.** Ctenidium and osphradium. **B.** Stomach. **C.** Head of male and penis. **D.** Detail of penis and penis lobes (observed by transmitted light in E). **F.** Pallial oviduct. **G.** Bursa copulatrix and seminal receptacles. **H.** Ditto, observed by transmitted light. Anatomical abbreviations explained in the text.

**Table 2.** Shell measurements (in mm) for the species. 1. *Belgrandiellopsis chorfensis* gen. et sp. nov. from Chorfa Spring. 2. *B. secunda* gen. et sp. nov. from Ettoute Spring. 3. *Biserta putealis* gen. et sp. nov. from Soudene Well. \*\*\* P<0.001; \*\* P<0.01; \* P<0.05; ns, not significant.

	1	2	3	ANOVA
	Mean ± SD; CV (Min–Max) (n = 13)	Mean ± SD; CV (Min–Max) (n = 12)	Mean ± SD; CV (Min–Max) (n = 10)	
SL	1.54 ± 0.05; 0.04 (1.45–1.63)	1.92 ± 0.12; 0.06 (1.73–2.11)	1.74 ± 0.10; 0.06 (1.60–2.00)	F <sub>2,32</sub> = 49.55***
SW	0.84 ± 0.03; 0.04 (0.79–0.89)	1.10 ± 0.07; 0.06 (1.02–1.28)	0.97 ± 0.05; 0.05 (0.87–1.04)	F <sub>2,32</sub> = 82.64***
SL/SW	1.84 ± 0.06; 0.04 (1.75–1.95)	1.74 ± 0.06; 0.04 (1.65–1.87)	1.79 ± 0.10; 0.05 (1.63–1.95)	F <sub>2,32</sub> = 4.76*
AH	0.66 ± 0.04; 0.07 (0.59–0.72)	0.87 ± 0.06; 0.07 (0.75–0.95)	0.80 ± 0.09; 0.11 (0.69–0.98)	F <sub>2,32</sub> = 34.85***
SL-LBW	0.46 ± 0.04; 0.08 (0.40–0.53)	0.53 ± 0.06; 0.10 (0.45–0.64)	0.53 ± 0.08; 0.15 (0.42–0.70)	F <sub>2,32</sub> = 5.85**
WBW	0.80 ± 0.03; 0.04 (0.75–0.85)	1.03 ± 0.05; 0.05 (0.94–1.14)	0.90 ± 0.04; 0.05 (0.83–0.97)	F <sub>2,32</sub> = 97.23***
AL	0.70 ± 0.03; 0.05 (0.68–0.78)	0.93 ± 0.03; 0.03 (0.87–0.99)	0.84 ± 0.05; 0.06 (0.77–0.93)	F <sub>2,32</sub> = 106.4***
AW	0.50 ± 0.03; 0.07 (0.44–0.56)	0.68 ± 0.06; 0.09 (0.60–0.80)	0.60 ± 0.04; 0.07 (0.54–0.70)	F <sub>2,32</sub> = 44.17***
WPW	0.60 ± 0.03; 0.05 (0.57–0.66)	0.72 ± 0.04; 0.06 (0.65–0.78)	0.67 ± 0.06; 0.09 (0.60–0.78)	F <sub>2,32</sub> = 19.69***
WAW	0.10 ± 0.02; 0.19 (0.06–0.13)	0.11 ± 0.02; 0.20 (0.08–0.14)	0.11 ± 0.03; 0.24 (0.08–0.17)	F <sub>2,32</sub> = 1.22 <sup>ns</sup>
NSW	4.13 ± 0.17; 0.04 (4.00–4.50)	4.29 ± 0.23; 0.05 (4.00–4.50)	4.18 ± 0.17; 0.04 (4.00–4.50)	F <sub>2,32</sub> = 2.19 <sup>ns</sup>

tubular glands distal; basal lobe larger, thumb-like, not pedunculated; distal lobe smaller, pyriform, pedunculated. Prostate gland small, close, bean-shaped, longer than wide (0.2 mm/0.10 mm). Posterior vas deferens developed in its median portion with several loops (about 4 mm in length and 0.01 mm in section); seminal vesicle yellowish. Pallial vas deferens straight, narrow, whitish, unciliated, entering the posteroventral portion of gland and exiting from its anterior end.

FEMALE GENITALIA. Ovary simple, with stalked lobes, occupying near 40% of visceral coil. Distal female genitalia occupying near 70% of pallial roof; albumen gland approximately as long as capsule gland (Fig. 4F). Bursa copulatrix ovoid to pyriform, having a duct as long as bursal length. Renal oviduct unpigmented, coiled, having a spherical pouch at the end of the coiled section. Two seminal receptacles; SR1 medium sized, elongate, pedunculate, joining renal oviduct just above the insertion point with bursal duct; SR2 small, globular, sessile, located on renal oviduct near loop before pouch (Fig. 4G–H).

### Ecology and distribution

*Belgrandiellopsis chorfensis* gen. et sp. nov. was found in a small spring near a rural settlement called Chorfa between the cities of Mateur and Beja, in northern Tunisia. It is known only from the type locality and some springs in this region (e.g., Ain Elgaid Spring). The specimens were found on the bottom and attached to the underside (algae-side) of gravel and stones. Density decreased drastically downstream from the water resurgence. *Pseudamnicola* sp. was the only co-occurring gastropod species.

### Remarks

Differentiated from its congener according to its smaller shell size (Table 2), smaller number of lateral cusps on the central radular teeth, larger penis and slightly smaller bursa copulatrix. *Belgrandiellopsis chorfensis* gen. et sp. nov. also differs in its mean COI divergence by 8.4% from *B. secunda* gen. et sp. nov.

### *Belgrandiellopsis secunda* gen. et sp. nov.

[urn:lsid:zoobank.org:act:D3A45301-6EBE-4086-A740-FEFBE9328FB5](https://zoobank.org/urn:lsid:zoobank.org:act:D3A45301-6EBE-4086-A740-FEFBE9328FB5)

Figs 5–6

### Diagnosis

Shell 1.7 to 2.1 mm high, elongate-conic, brown-yellowish; central radular tooth formula (6)5–C–5(6)/1–1; bursa copulatrix ovoid with short duct; two seminal receptacles, SR1 more developed and pedunculated, SR2 very small, globular, located near loop before the pouch; prostate gland bean shaped, ovate in section, folded; penis cylindrical, slender, distal end tapered, unpigmented, with two lobes relatively distant positioned; basal lobe more developed and middle positioned. Distinguished from other closely related species by >8.4% for COI.

### Etymology

Referring to the second species of the genus *Belgrandiellopsis* gen. nov. discovered so far.

### Type material

#### Holotype

TUNISIA • 1 spec. (sex unknown, preserved in ethanol 80%); Bizerte Province, 40 km south of Mateur, Ettoute Spring; 36.883898° N, 9.505894° E; 415 m a.s.l.; 15 Mar. 2015; N. Khalloufi leg.; MNCN 15.05/200098H.

#### Paratypes

TUNISIA • 5 specs (preserved in ethanol 80%); same collection data as for holotype; MNCN 15.05/200098P • 26 specs (preserved in ethanol 80%, 2 ♀♀, 2 ♂♂ dissected and 2 specs processed for DNA sequencing); same collection data as for holotype; UGSB 17666 • 250 specs (preserved in ethanol 95%, ca 70 individuals dissected); same collection data as for holotype; Khalloufi's collection.



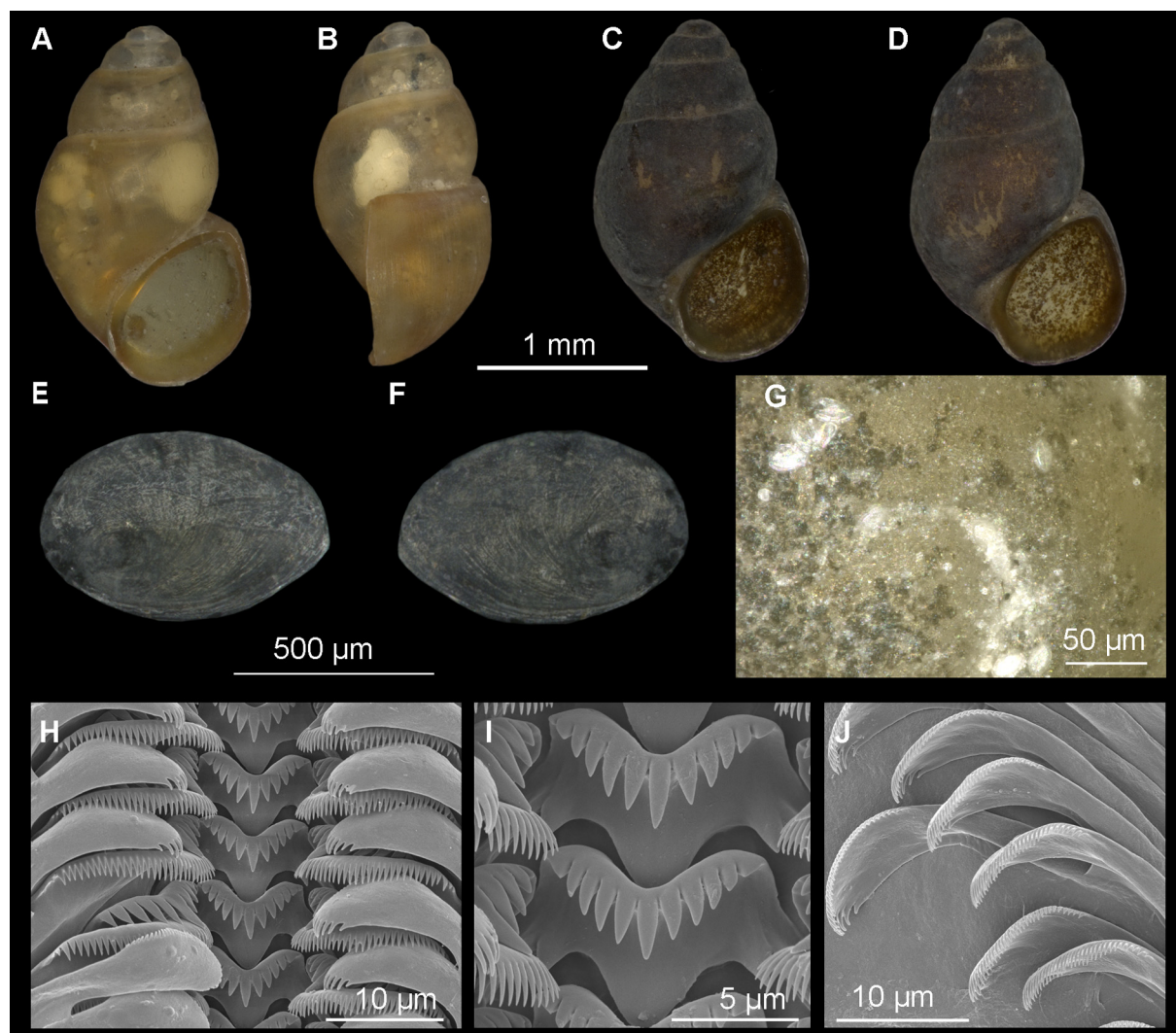
**Type locality**

TUNISIA: Bizerte Province, 40 km south of Mateur, Ettoute Spring; 36.883898° N, 9.505894° E; 415 m a.s.l.

**Description**

MEASUREMENTS. Holotype: SL = 2.09 mm, SW = 1.16 mm, SL/SW = 1.80, AH = 0.90 mm, SL-LBW = 0.59 mm, WBW = 1.09 mm, AL = 0.99 mm, AW = 0.70 mm, WPW = 0.78 mm, WAW = 0.09 mm.

SHELL. Elongate-conic, brown to yellowish, with 4–4.5 whorls (Fig. 5A–D; Table 2). Protoconch (Fig. 5G) obtuse, near planispiral, about 1.3 whorls, diameter about 350 µm; protoconch microsculpture granulated. Teleoconch whorls weakly convex, separated by deep sutures; body whorl occupying  $\frac{2}{3}$  of total shell length. Aperture about 50% of total shell length, pyriform, strongly angled on the top and



**Fig. 5.** Shells, opercula and radulae, *Belgrandiellopsis secunda* gen. et sp. nov. A–D. Shells. A–B. Holotype, MNCN 15.05/200098H. C–D. Paratypes, MNCN 15.05/200098P. E–F. Operculum (E = inner side; F = outer side). G. Detailed microsculpture of protoconch. H–J. Radulae. H. Portion of radula ribbon. I. Central radular teeth. J. Outer marginal teeth.



narrowly disjunct from parietal wall of body whorl; inner lip thin but thicker than outer lip; peristome margin (Fig. 5B) straight. Umbilicus narrow and closed by inner lip.

OPERCULUM. As for genus, thin, flat, whitish (nuclear region slightly yellowish), with ca 2 whorls (Fig. 5E–F); attachment scar area oval and located near nucleus.

RADULA. Length intermediate (25% total maximum shell length), having about 80 rows of teeth. Central tooth (Fig. 5H–I) formula (6)5–C–5(6)/1–1, cutting edge slightly concave; basal tooth face M-shaped and relatively narrow wings; basal tongue broadly V-shaped, about equal to lateral margins; a single small pair of basal cusps, triangular. Lateral teeth (Fig. 5H) formula 5–C–3(4); central cusp large, narrow, pointed. Inner marginal teeth having 32–35 sharp cusps. Outer marginal teeth having 30–33 sharp cusps (Fig. 5J).

PIGMENTATION AND ANATOMY. Visceral coil pale-yellowish, showing by transparency the gonad. Pallial coil with uniform and dense brown pigmentation on roof (left side), anterior edge of mantle, foot and between tentacles (Fig. 6C). Black small eyes surrounded by brownish pigment. Snout with medium distal lobation. Tentacles parallel-side, distal end nonexpanded. Ctenidium filaments about 12, small, taller than wide. Osphradium whitish, ellipsoidal, positioned centrally along ctenidium (Fig. 6A). Stomach slightly longer than wide with a small gastric (posterior) caecum (Fig. 6B); rectum wide, folded and S-shaped.

MALE GENITALIA. Penis cylindrical (Fig. 6D), medium sized, unpigmented, slender, slightly folded; filament short, tapering; basal portion intermediate in width, longer than distal portion, attached centrally to the head well behind the eyes; penis having two developed lobes, both with distal papilla, showing by transparency tubular glands; lobes located relatively distant from one another on opposite sides of penis; basal lobe large, conic, sessile, positioned centrally along penis; distal lobe smaller, pyriform, pedunculated. Prostate gland small, close, bean-shaped, longer than wide (0.2 mm/0.10 mm). Posterior vas deferens whitish, forming the seminal vesicle and developed in its median portion with several loops (about 3.5 mm in length and 0.05 mm in section). Pallial vas deferens straight, narrow, whitish, unciliated, entering the posteroventral portion of gland and exiting from its anterior end.

FEMALE GENITALIA. Ovary simple, with stalked lobes, occupying near 40% of visceral coil. Distal female genitalia occupying near 50% of pallial roof; albumen gland less developed than capsule gland (Fig. 6E). Bursa copulatrix ovoid to pyriform, having a duct slightly shorter than bursal length. Renal oviduct unpigmented, coiled, having a very small, ovoid to spherical pouch at the end of the coiled section. Two seminal receptacles; SR1 medium sized, elongate, pedunculate, joining renal oviduct just above the insertion point with bursal duct; SR2 small, globular, sessile, located on renal oviduct near loop before pouch (Fig. 6F–G).

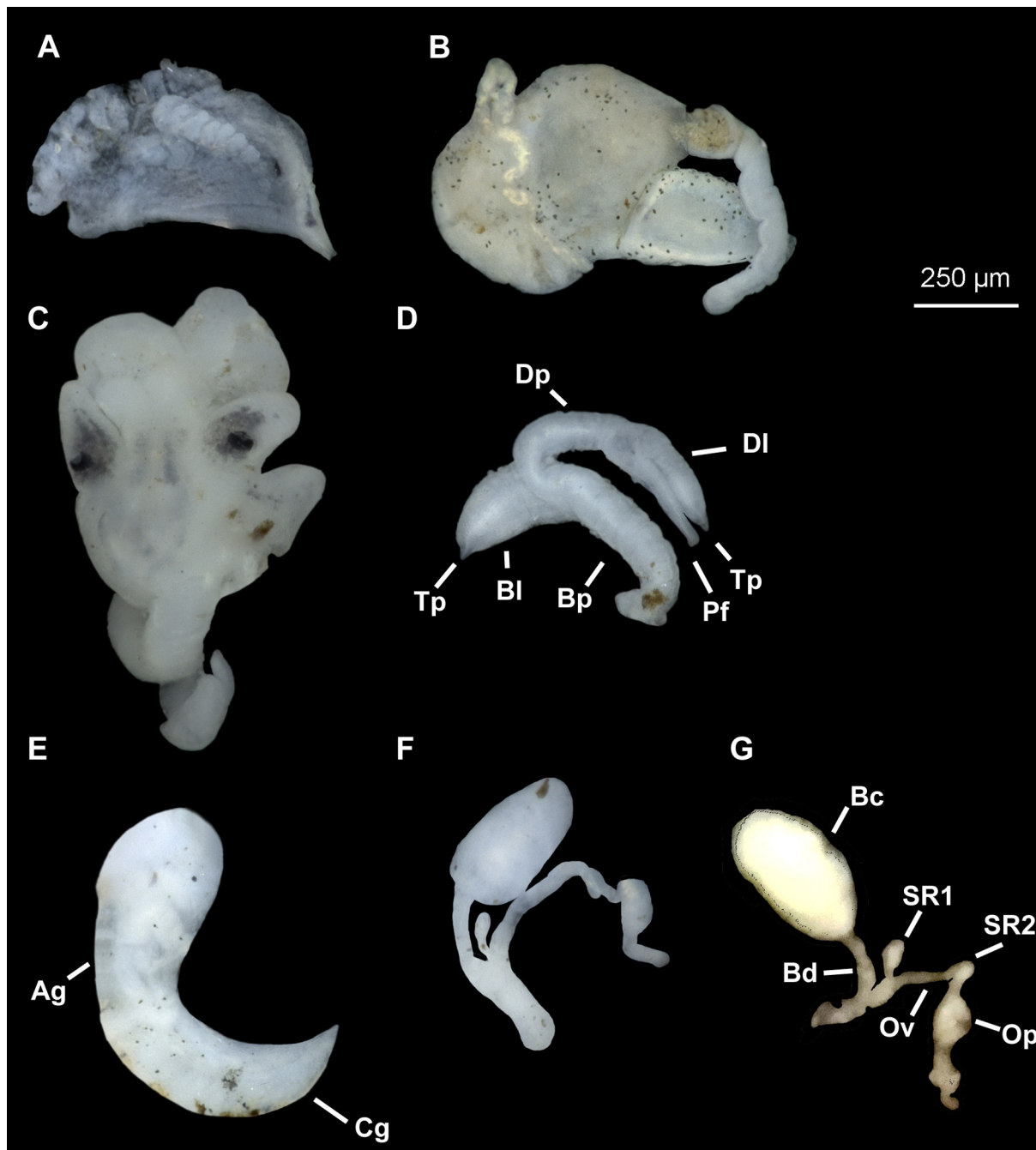
### Ecology and distribution

This species was found in a spring and its outflow in the rural village Ettoute between the cities of Mateur and Beja, in northern Tunisia. Specimens were attached to the underside of gravel and stones covered by algae. In the small stream flowing out of the spring, the snails were found in the central section featuring clean and oxygenated waters and a silty substrate. The density of this species decreased drastically downstream from the water resurgence. Co-occurring gastropod species were *Pseudamnicola* sp., *Galba truncatula* (O.F. Müller, 1774), *Bulinus truncatus* (Audouin, 1827) and *Physella acuta* Draparnaud, 1805.

### Remarks

Despite similarities in mean COI divergence between this species and the closely related species *Bullaregia tunisiensis* Khalloufi, Béjaoui & Delicado, 2017, *Biserta putealis* gen. et sp. nov. and

*Belgrandiellopsis chorfensis* gen. et sp. nov. (9.1%, 8.9% and 8.4%, respectively), *B. secunda* gen. et sp. nov. was assigned to *Belgrandiellopsis* gen. nov. based on the combination of two glandular lobes located relatively distant from one another on opposite sides of the penis [one lobe in *Bullaregia* (Khalloufi *et al.* 2017), two lobes closely positioned to one another in *Biserta* gen. nov.], small bursa copulatrix with a long duct (larger bursa copulatrix with a shorter duct in *Biserta* gen. nov.), small gastric caecum (larger in *Bullaregia*) and small shell umbilicus (larger in *Biserta* gen. nov.).



**Fig. 6.** Anatomy of *Belgrandiellopsis secunda* gen. et sp. nov. (paratypes, UGSB 17666). **A.** Ctenidium and osphradium. **B.** Stomach. **C.** Head of male and penis. **D.** Detail of penis and penis lobes. **E.** Pallial oviduct. **F.** Bursa copulatrix and seminal receptacles. **G.** Ditto, observed by transmitted light. Anatomical abbreviations explained in the text.

Genus *Biserta* gen. nov.

[urn:lsid:zoobank.org:act:11183287-E4AD-4FBB-A304-E7BB699133AA](https://zoobank.org/urn:lsid:zoobank.org:act:11183287-E4AD-4FBB-A304-E7BB699133AA)

### Type species

*Biserta putealis* sp. nov., by present designation.

### Diagnosis

Shell small (maximum length 2 mm), ovate-conic to elongate-conic, with obtuse and planispiral protoconch; whorls convex, with deep sutures. Penultimate whorl tall relative to remaining whorls. Aperture pyriform to ovate, complete, slightly angled on top and often fused to the body whorl. Umbilicus closed by inner lip. Operculum corneous, whitish to yellowish, thin, pliable, elongate-ellipsoidal, spiral, paucispiral, with submarginal nucleus. One pair of small triangular basal cusps on radular central tooth. Ctenidium well developed. Presence of bursa copulatrix; unpigmented renal oviduct with a spherical pouch at the end of the coiled section; two seminal receptacles. Penis attached to the neck behind the right eye with two glandular lobes closely positioned to one another, each of them bearing a terminal papilla.

### Etymology

*Biserta* is one of the Latin names of Bizerte, name of the province where the genus was discovered; gender feminine.

### Remarks

*Biserta* gen. nov. differs from the closely related genera *Bullaregia* and *Belgrandiellopsis* gen. nov. according to its larger shell umbilicus, two glandular lobes closely positioned to one another on the penis, slightly larger bursa copulatrix, shorter bursal duct and to its 8–8.9% COI sequence divergence.

*Biserta putealis* gen. et sp. nov.

[urn:lsid:zoobank.org:act:AB67B9A3-8BA6-4AC2-97E3-97C61E6A4C32](https://zoobank.org/urn:lsid:zoobank.org:act:AB67B9A3-8BA6-4AC2-97E3-97C61E6A4C32)

Figs 7–8

### Diagnosis

Shell 1.6 to 2.0 mm high, elongate-conic, brown-yellowish; central radular tooth formula (5)4–C–4(5)/1–1; bursa copulatrix pyriform, with a short duct; two seminal receptacles, SR1 more developed and pedunculated, SR2 small, globular, located near loop before the pouch; prostate gland bean shaped, ovate in section, folded; penis gradually tapering, unpigmented, with two sessile lobes closely positioned to one another. Distinguished from other closely related species by >8.0% for COI.

### Etymology

The species epithet is from the Latin adjective ‘*putealis*’, meaning ‘from a well’.

### Type material

#### Holotype

TUNISIA • 1 spec. (sex unknown, preserved in ethanol 80%); Bizerte Province, 7 km south of Menzel Bourguiba, Soudene Well; 37.09046° N, 9.777997° E; 115 m a.s.l.; 15 Mar. 2015; N. Khalloufi leg.; MNCN 15.05/200099H.

#### Paratypes

TUNISIA • 5 specs (preserved in ethanol 80%); same collection data as for holotype; MNCN 15.05/200099P • 32 specs (preserved in ethanol 80%, 2 ♀♀, 2 ♂♂ dissected and 2 specs processed

for DNA sequencing); same collection data as for holotype; UGSB 17664 • 70 specs (preserved in ethanol 95%, ca 40 individuals dissected); same collection data as for holotype; Khalloufi's collection.

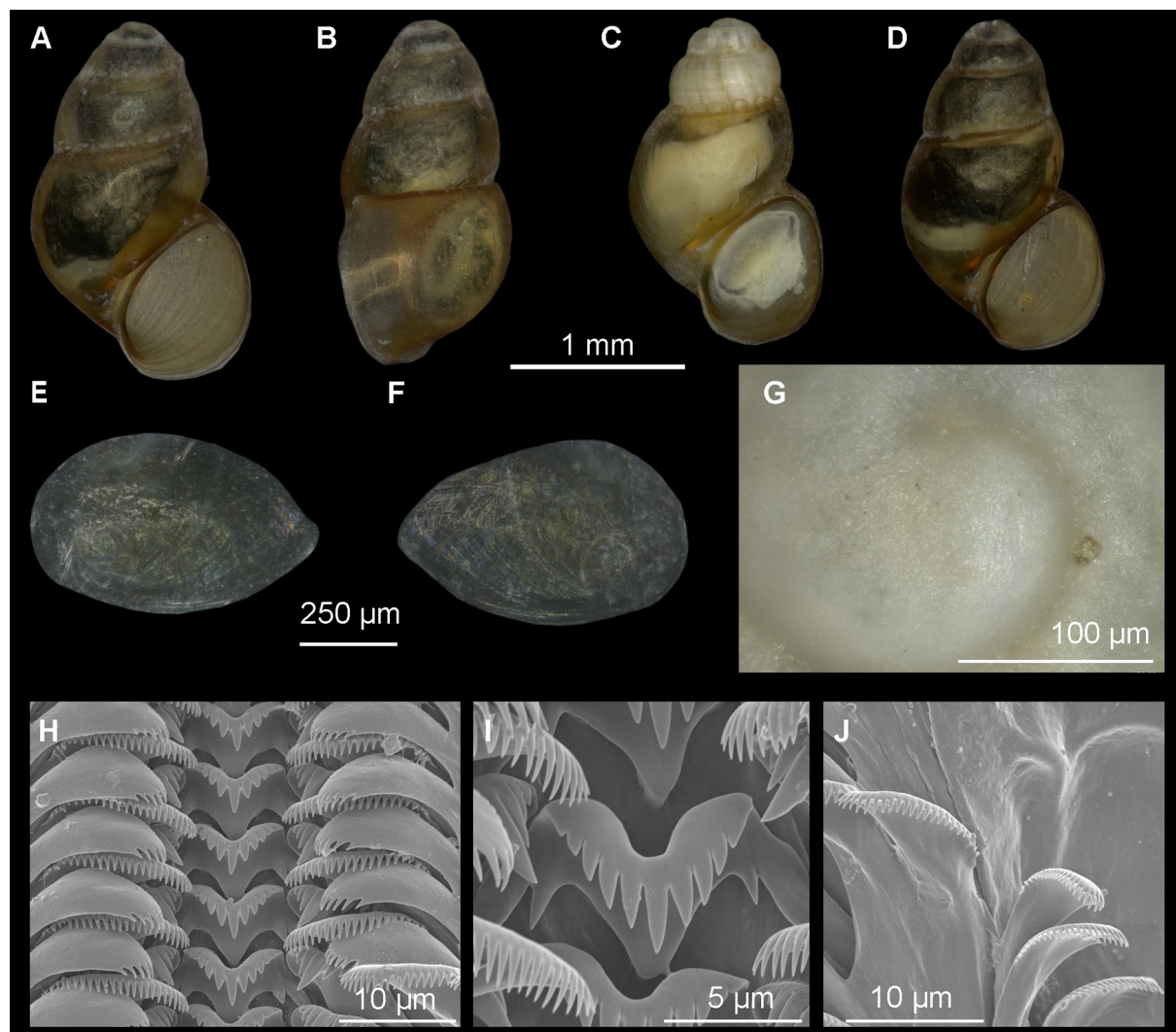
**Type locality**

TUNISIA: Bizerte Province, 7 km south of Menzel Bourguiba, Soudene Well; 37.09046° N, 9.777997° E; 115 m a.s.l.

**Description**

MEASUREMENTS. Holotype: SL = 2.00 mm, SW = 1.04 mm, SL/SW = 1.92, AH = 0.98 mm, SL-LBW = 0.70 mm, WBW = 0.97 mm, AL = 0.93 mm, AW = 0.70 mm, WPW = 0.78 mm, WAW = 0.17 mm.

SHELL. Elongate-conic, brown to yellowish, with 4–4.5 whorls (Fig. 7A–D; Table 2). Protoconch (Fig. 7G) obtuse, near planispiral, about 1.25 whorls, diameter about 300 µm; protoconch microsculpture



**Fig. 7.** Shells, opercula and radulae, *Biserta putealis* gen. et sp. nov. **A–D.** Shells. **A–B.** Holotype, MNCN 15.05/200099H. **C–D.** Paratypes, MNCN 15.05/200099P. **E–F.** Operculum (E = inner side; F = outer side). **G.** Detailed microsculpture of protoconch. **H–J.** Radulae. **H.** Portion of radula ribbon. **I.** Central radular teeth. **J.** Marginal teeth.

granulated. Teleoconch whorls convex, separated by deep sutures; body whorl occupying  $\frac{2}{3}$  of total shell length. Aperture about 50% of total shell length, pyriform, slightly angled on the top and narrowly disjunct from parietal wall of body whorl; inner lip thin but thicker than outer lip; peristome margin (Fig. 7B) straight. Umbilicus narrow and closed by inner lip.

OPERCULUM. As for genus, thin, flat, whitish (nuclear region slightly yellowish), with ca 2 whorls (Fig. 7E–F); attachment scar area oval and located near nucleus.

RADULA. Length intermediate (25% total maximum shell length), having about 80 rows of teeth. Central tooth (Fig. 7H–I) formula (5)4–C–4(5)/1–1, cutting edge strongly concave; basal tooth face M-shaped and relatively narrow wings; basal tongue broadly V-shaped, about equal to lateral margins; a single small pair of basal cusps, triangular. Lateral teeth (Fig. 7H) formula (4)3–C–5(6); central cusp large, narrow, pointed. Inner marginal teeth having 23–25 sharp cusps. Outer marginal teeth having 22–23 sharp cusps (Fig. 7J).

PIGMENTATION AND ANATOMY. Visceral coil brown-yellowish, showing by transparency the gonad; pallial coil with uniform and dense pigmentation on the roof, anteriorly to edge of mantle, foot and area between tentacles (Fig. 8C). Black small eyes surrounded by brownish pigment. Snout with medium distal lobation. Tentacles parallel-side, distal end nonexpanded. Ctenidium filaments about 13, small, taller than wide. Osphradium whitish, ellipsoidal, positioned centrally along ctenidium (Fig. 8A). Stomach slightly longer than wide with a small gastric (posterior) caecum (Fig. 8B); rectum wide, folded and S-shaped.

MALE GENITALIA. Penis gradually tapering (Fig. 8D), medium sized, unpigmented, slightly folded; filament short, tapering; basal portion intermediate in width, shorter than distal portion, attached centrally to the head well behind the eyes; penis having two developed lobes, very close to one another on opposite sides of penis; both lobes having distal papilla and showing by transparency tubular glands; ventral lobe large, pyriform, pedunculated; dorsal lobe small, pyriform, sessile. Prostate gland small, close, bean-shaped, longer than wide (0.25 mm/0.05 mm). Posterior vas deferens with several loops, yellowish and developed in its median portion (about 2 mm in length and 0.05 mm in section). Pallial vas deferens straight, narrow, whitish, unciliated, entering the posteroventral portion of gland and exiting from its anterior end.

FEMALE GENITALIA. Ovary simple, with stalked lobes, occupying near 40% of visceral coil. Distal female genitalia occupying near 50% of pallial roof; albumen gland approximately as long as capsule gland (Fig. 8E). Bursa copulatrix pyriform, having a shorter duct than bursal length. Renal oviduct unpigmented, coiled, having a spherical pouch at the end of the coiled section. Two seminal receptacles; SR1 medium sized, elongate, pedunculate, joining renal oviduct just above the insertion point with bursal duct; SR2 small, globular, sessile, located on renal oviduct near loop before pouch (Fig. 8F–G).

### Ecology and distribution

This species was found only in a small well in southern Menzel Bourguiba city, northern Tunisia. The well is 1.5 m in diameter and 4 m deep. Specimens were very abundant on the rock walls and on the rocky and sandy bottom. Water salinity was about 1.5 ppt. *Pseudamnicola* sp. was the only co-occurring gastropod species.

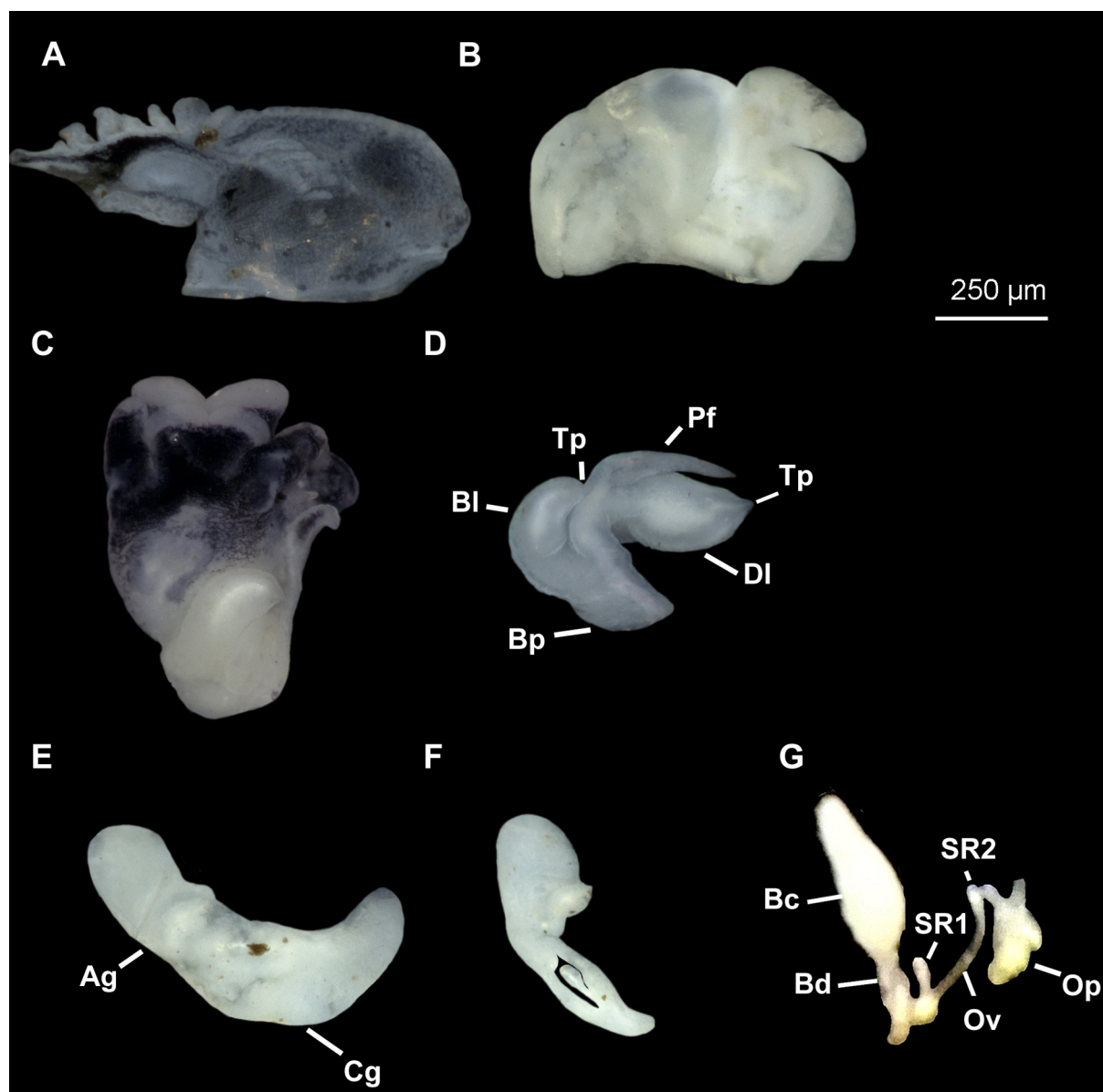
### Remarks

Some individuals of the collected population lack body pigmentation (Fig. 7C), which could indicate adaptation to subterranean conditions (Delicado 2018). Other anatomical structures resembled those of the pigmented individuals.



## Discussion

The discovery of *Bullaregia tunisiensis* by Khalloufi *et al.* (2017) prompted our intense research on groundwater snails in an effort to gain a better understanding of the species richness, distribution and evolutionary patterns of hydrobiid clades in Tunisia. In prior work (Khalloufi *et al.* 2017), we detected an uncertain phylogenetic position of this genus within the Hydrobiidae. In the present study, we provide morphological and genetic data for previously unknown populations from Tunisian groundwater systems and evidence of three new taxa resolved in a monophyletic group together with *B. tunisiensis*. In our phylogenetic analyses (Fig. 2), no well-supported relationship was found between this group and the recently discovered clade of subterranean hydrobiid species from Bulgaria



**Fig. 8.** Anatomy of *Biserta putealis* gen. et sp. nov. (paratypes, UGSB 17664). **A.** Ctenidium and osphradium. **B.** Stomach. **C.** Head of male and penis. **D.** Detail of penis and penis lobes. **E.** Pallial oviduct. **F.** Bursa copulatrix and seminal receptacles. **G.** Ditto, observed by transmitted light. Anatomical abbreviations explained in the text.

(Osikowski *et al.* 2017); instead, it formed a monophyletic group with *Corbellaria celtiberica* and *Kerkia kusceri*. The sister relationship between these two species of valvatiform snails was previously shown by Delicado *et al.* (2019). However, this grouping was not supported by morphological evidence as, for instance, the Tunisian snails feature elongate-conic shells and remarkable differences in female and male genitalia (see Girardi & Boeters 2012 and Bodon *et al.* 2001 for morphological comparison with *C. celtiberica* and *K. kusceri*, respectively). We anticipate that additional genetic data from undiscovered populations of groundwater and spring snails living in unexplored areas such as Tunisia will change this phylogenetic picture and help to further unravel the phylogenetic position of this Tunisian clade within the Hydrobiidae.

The two new genera presented in this study raises the number of genera of Hydrobiidae *sensu stricto* in Tunisia to seven (*Pseudamnicola*, *Hydrobia*, *Ecrobia* Stimpson, 1865, *Mercuria*, *Bullaregia*, *Belgrandiellopsis* gen. nov., *Biserta* gen. nov.; Wilke *et al.* 2002; Glöer *et al.* 2010; Delicado *et al.* 2015; Khalloufi *et al.* 2017; Vandendorpe *et al.*, 2019) and to eight for North Africa (García *et al.* 2010). The three subterranean genera recovered here as a monophyletic group (i.e., the Tunisian clade) are as genetically divergent (8.0–9.2% COI p-distances) as within other clades of hydrobiid subterranean genera (e.g., 7.8–11.8% COI p-distances; Osikowski *et al.* 2017). Although this genetic divergence falls into the range of intrageneric genetic variation reported for spring genera of Hydrobiidae (e.g., 5.39–11.15% COI p-distances within *Corrosella* Boeters, 1970; Delicado *et al.* 2012), we found sufficient morphological evidence to assign the three collected populations to two distinct new genera outside *Bullaregia*. The three genera comprising the Tunisian clade (i.e., *Bullaregia*, *Belgrandiellopsis* gen. nov. and *Biserta* gen. nov.) differ mainly in anatomical rather than in shell shape. For instance, *Bullaregia* presents a single medial lobe on the outer edge of the penis (two lobes on both edges of the penis in *Belgrandiellopsis* gen. nov. and *Biserta* gen. nov., more distally positioned in the former genus) and more cusps on the radular teeth.

These new genera and species show a limited distribution in Tunisia, as they are endemic to one or two localities. However, their distribution could extend to other localities in northern Tunisia and northeastern Algeria. Considering that many groundwater bodies have not yet been explored, the number of endemic taxa in Tunisia is likely larger. Thus, field surveys in these aquatic systems should be intensified to discover and inventory their faunal diversity. This point is crucial for the design of conservation measures before these local endemic species become extinct.

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