

***Monacha cantiana* s.l. (Montagu, 1803) (Gastropoda: Hygromiidae) – mitochondrial lineage occurring in Austria**

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Abstract: *Monacha cantiana* s.l., a species not native to Austria, was first recorded in northern lower Austria in 1995 and later in Vienna and surroundings. Its distribution continues to spread. To clarify the origins of this introduced species, some Viennese populations of *M. cantiana* s.l. were compared with other *M. cantiana* lineages using the mitochondrial cytochrome c oxidase subunit 1 (COI) and 16S rRNA (16S) gene sequences. Genital structures were dissected to find anatomical traits that could distinguish different species or genetic lineages. A Maximum Likelihood tree placed the Austrian specimens in a clade of north Italian populations. Specimens from both areas showed identical morphology of anatomical structures. Phylogenetic analysis showed that the Austrian-north Italian clade was closely related to the French *Monacha cemelelea*, but not to *M. cantiana* s.str.. More comprehensive studies using molecular genetic markers and anatomical traits may be able to determine whether or not the Austrian-north Italian clade is conspecific with *M. cemelelea*.

Key words: *Monacha cantiana*, Austria, COI, 16S, molecular features, genital structure, species distribution

Zusammenfassung: *Monacha cantiana* s.l., ein sich nach wie vor in Österreich ausbreitendes Neozoon, wurde das erste Mal 1995 im nördlichen Niederösterreich und in weiterer Folge auch in Wien und in der Umgebung nachgewiesen. Um mehr über den Ursprung dieser eingeschleppten Art zu erfahren, wurden einige Populationen aus Wien mit anderen Linien von *M. cantiana* s.l. durch phylogenetische Analysen des mitochondrialen Gens für die Cytochrom-c-Oxidase Untereinheit 1 (COI) und des mitochondrialen Gens für die ribosomale 16S RNA (16S) verglichen. Die Maximum-Likelihood-Analyse zeigte eine enge Verwandtschaft der österreichischen Populationen mit den aus Norditalien. Einzelne untersuchte Exemplare aus beiden Gebieten zeigten auch übereinstimmende genitalanatomische Strukturen. Außerdem sind die österreichisch-norditalienischen Populationen näher mit dem Taxon *M. cemelelea* aus Frankreich als mit *M. cantiana* s. str. verwandt. Ob *M. cantiana* s.l. aus Österreich und Norditalien dieser Art zuzurechnen sind oder nicht, könnte durch zukünftige Untersuchungen geklärt werden, die molekularbiologische und anatomische Methoden integrativ verbinden.

Introduction

Monacha cantiana s.l. (Montagu, 1803) is widely distributed in Europe (Welter-Schultes 2012). Examination of several English, Italian, Austrian and French populations showed that they consisted of at least six cryptic lineages (CAN-1 – CAN-6), some of which might deserve distinct taxonomic status (Pieńkowska et al. 2018, 2019a).

In Austria, *M. cantiana* was first discovered in 1995 in Horner Becken (Waldviertel, Lower Austria) (Reischütz & Zeitlberger 1996). New populations were recently found not far from Vienna. In the present study (Pieńkowska et al. 2019b), based on an extended sample, we tested the morphological identification of the Viennese populations in a molecular analysis using mitochondrial marker sequences and compared them with other *M. cantiana* lineages.

Material and methods

New material from two populations of *Monacha cantiana* s.l. was included in our analysis of DNA sequences and morphological features (genitalia structure). Geographic information and coordinates of the two localities, collector, date, number of specimens and GenBank accession numbers are presented in Table 1. The material is kept in the Department of Cell Biology Collection (DCBC), Adam Mickiewicz University, Poznań.

Two mitochondrial gene fragments were analysed, namely cytochrome c oxidase subunit 1 (COI; partial sequence of 636 bp) and 16S ribosomal RNA gene (16S, partial sequence of 315–316 bp). All new sequences were deposited in GenBank (Tab. 1). The COI and 16S sequences obtained from GenBank for comparisons are listed in Table 2. For the phylogenetic analysis, mitochondrial COI and 16S gene fragments were concatenated into com-

Table 1. List of new localities of the specimens of *Monacha cantiana* s.l. used in the present study

No.	Localities			<i>M. cantiana</i> lineage	COI			16S		
	coordinates	country and site	collector / date / No. of specimens (collection)		haplotype (No. sps)	voucher designation	GenBank ##	haplotype (No. sps)	voucher designation	GenBank ##
1.	48°13'04.6"N 16°25'23.4"E	Austria, Vienna: Donauinsel: between Reichsbrücke and Praterbrücke, 165 m a.s.l.	M. Duda / 26.08.2018 / 10 (DCBC0020104AT02)	COI 1A (1)	DCBC0020104AT02/SP01	MN107010	16S 1A (1)	DCBC0020104AT02/SP01	MN100590	
				COI 2A (1)	DCBC0020104AT02/SP02	MN107011	16S 2A (1)	DCBC0020104AT02/SP02	MN100591	
				COI 3A (1)	DCBC0020104AT02/SP03	MN107012	16S 1A (3)	DCBC0020104AT02/SP03	MN100592	
				COI 4A (2)	DCBC0020104AT02/SP04	MN107013		DCBC0020104AT02/SP04	MN100593	
					DCBC0020104AT02/SP05	MN107014		DCBC0020104AT02/SP05	MN100594	
2.	48°08'04.7"N 16°17'12.8"E	Austria, Vienna: Liesing: East of Railway station, 222 m a.s.l.	M. Duda / 29.08.2018 / 12 (DCBC0020104AT03)	COI 5A (2)	DCBC0020104AT03/SP01	MN107015	16S 2A (5)	DCBC0020104AT03/SP01	MN100595	
					DCBC0020104AT03/SP02	MN107016		DCBC0020104AT03/SP02	MN100596	
				COI 6A (1)	DCBC0020104AT03/SP03	MN107017		DCBC0020104AT03/SP03	MN100597	
				COI 5A (2)	DCBC0020104AT03/SP04	MN107018		DCBC0020104AT03/SP04	MN100598	
					DCBC0020104AT03/SP05	MN107019		DCBC0020104AT03/SP05	MN100599	

No. sps – number of specimens with particular haplotypes; DCBC – Department of Cell Biology Collection, Adam Mickiewicz University, Poznań

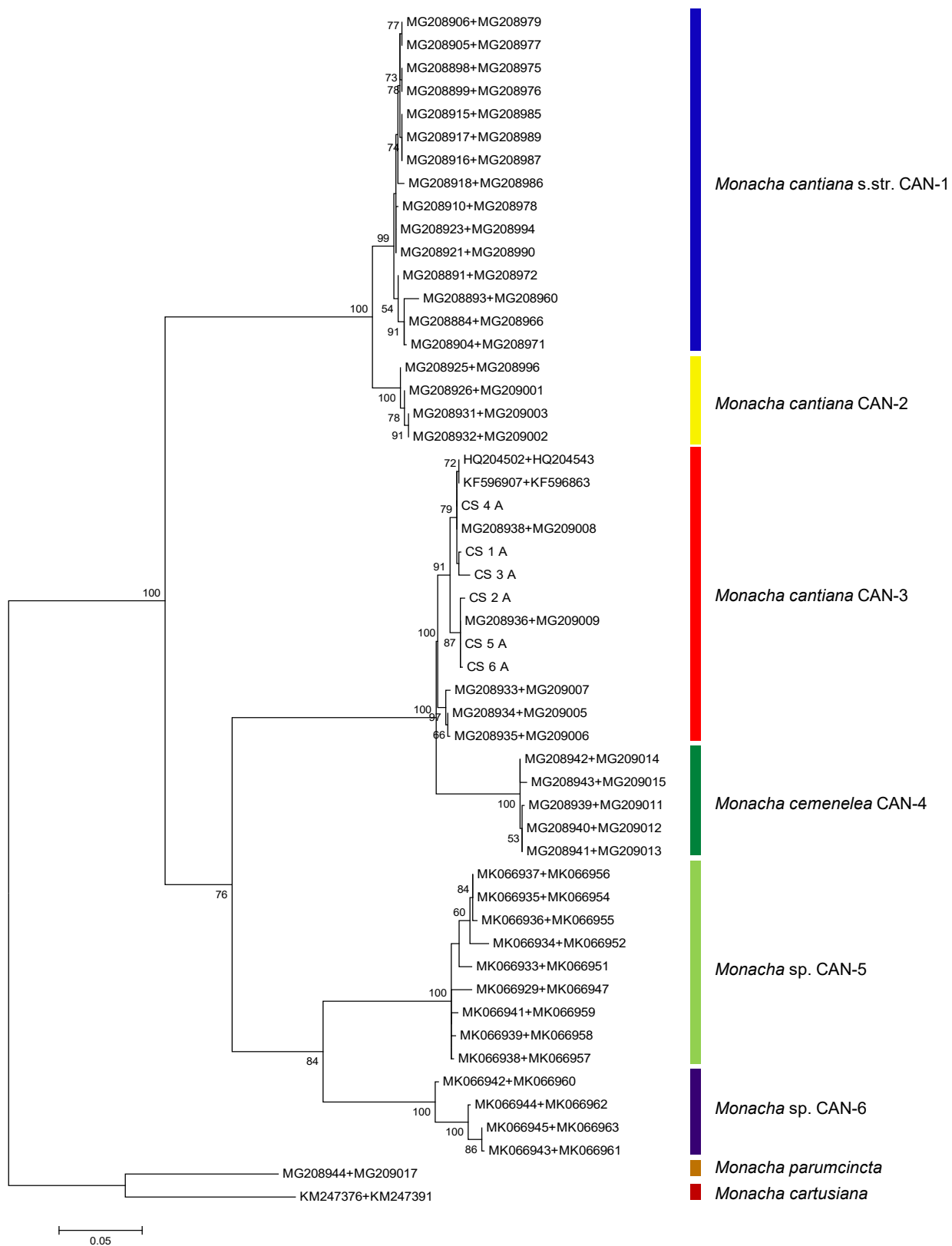


Fig. 1. Maximum Likelihood (ML) tree of combined *COI* and *16S* haplotypes of *Monacha cantiana* lineages. For geographic origin and abbreviations, see Tab. 1. The Hasegawa-Kishino-Yano (HKY) model was used for ML analysis (Hasegawa et al. 1985; Kumar et al. 2016). The rate variation model allowed some sites to be evolutionarily invariable [+I]. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with *Monacha parumincta* and *M. cartusiana* sequences (see Tab. 3).

Table 2. GenBank sequences used for comparison in the phylogenetic analysis

Species / mt lineage	COI	16S	References
<i>Monacha cantiana</i> CAN-1	KM247375	KM247390	Pieńkowska et al. 2015
	KX507234	KX495428	Neiber & Hausdorf 2015, 2017
	MG208884-MG208924	MG208960-MG208995	Pieńkowska et al. 2018
<i>Monacha cantiana</i> CAN-2	MG208925-MG208932	MG208996-MG209004	Pieńkowska et al. 2018
<i>Monacha cantiana</i> CAN-3	HQ204502	HQ204543	Duda et al. 2011, Kruckenhauser et al. 2014
	KF596907	KF596863	Cadahia et al. 2014
	MG208933-MG208938	MG209005-MG209010	Pieńkowska et al. 2018
<i>Monacha cemenelea</i> CAN-4	KF986833		Dahirel et al. 2015
	MG208939-MG208943	MG209011-MG209015	Pieńkowska et al. 2018
<i>Monacha</i> sp. CAN-5	MK066929-MK066941	MK066947-MK066959	Pieńkowska et al. 2019a
<i>Monacha</i> sp. CAN-6	MK066942-MK066946	MK066929-MK066964	Pieńkowska et al. 2019a
<i>Monacha parumcincta</i>	MG208944	MG209017	Pieńkowska et al. 2018
<i>Monacha cartusiana</i>	KM247376	KM247391	Pieńkowska et al. 2015
	KX507189	KX495378	Neiber & Hausdorf 2015, 2017

bined sequences of 843 bp (557 COI + 286 16S) (Tab. 3).

DNA extraction, amplification, sequencing methods and phylogenetic inference are described in detail in our previous papers (Pieńkowska et al. 2018, 2019a).

For anatomical analysis of genital structure, snail bodies from five specimens from each new Viennese population were dissected under a light microscope (Wild M5A or Zeiss SteREO Lumar V12). Anatomical structures were drawn using a Wild camera lucida (for details see Pieńkowska et al. 2018, 2019a).

Results

The Maximum Likelihood (ML) tree obtained for the combined sequences (Fig. 1) showed that the new Viennese samples are closely related to the Austrian haplotypes included in the CAN-3 lineage (Pieńkowska et al. 2018). They are distinct from populations of *M. cantiana* s.str. which occur in the UK and in Italy (Latium region) (CAN-1). They are also distinct from other Italian populations in Venetum, Lombardy (CAN-2) and the Apuan Alps (CAN-5 and CAN-6) (see Pieńkowska et al. 2018, 2019a). However, haplotypes similar to Austrian CAN-3 were found in two north Italian localities – one along the river Setta (Emilia Romagna), another in Passo di Monte Croce Carnico (Friuli-Venezia Giulia) (Pieńkowska et al. 2018).

K2P distances for COI nucleotide sequences between the CAN-3 lineage and other Italian (CAN-1, CAN-2, CAN-5, CAN-6) *M. cantiana* lineages were rather high (13.1–19.5 %) (Tab. 4). However although the sequences of the CAN-3 lineage are separated on the ML tree, they were rather close to CAN-4 (Fig. 1): K2P distances (4.2–7.2 %) between *M. cantiana* CAN-3 and CAN-4 lineages were smaller than between CAN-3 and other groups (Tab. 4).

The CAN-4 lineage, occurring in southern France (Sainte Thècle near Nice), matches *M. cemenelea* (Risso, 1826).

The additionally analysed distal genitalia of the three specimens from the Viennese populations (Figs. 2–3) do not differ from those previously found in the *M. cantiana* CAN-3 lineage (see Pieńkowska et al. 2018: figs 40–46).

Discussion

While the shells of CAN-1, CAN-2 and CAN-3 are very much alike, they are only marginally distinct from those of CAN-4 (Pieńkowska et al. 2018). The same is true of the distal genitalia (Pieńkowska et al. 2018). Since shell and distal genitalia characters have poor discriminating value and only one population from the CAN-4 lineage was examined, this result should be investigated further.

Our molecular results confirm the close relationships between Austrian and Italian CAN-3 and French CAN-4 populations (Pieńkowska et al. 2019b). Unfortunately, no material has yet been examined from the population from Horner Becken (Waldviertel, Lower Austria) (Reischütz & Zeitlberger 1996). Thus it is still unclear which mitochondrial lineage occurs in northern Austria. The question arises whether CAN-3 should be assigned to *M. cemenelea*. However it should be taken into account that species delimitation is complex and DNA sequence data alone may not be used to distinguish species or to make decisions on taxonomy and nomenclature (Pieńkowska et al. 2018, 2019a). Ideally any decision should be supported by morphological analysis (of shells and/or genital anatomy) of a comprehensive geographic sample, especially in the case of CAN-4.

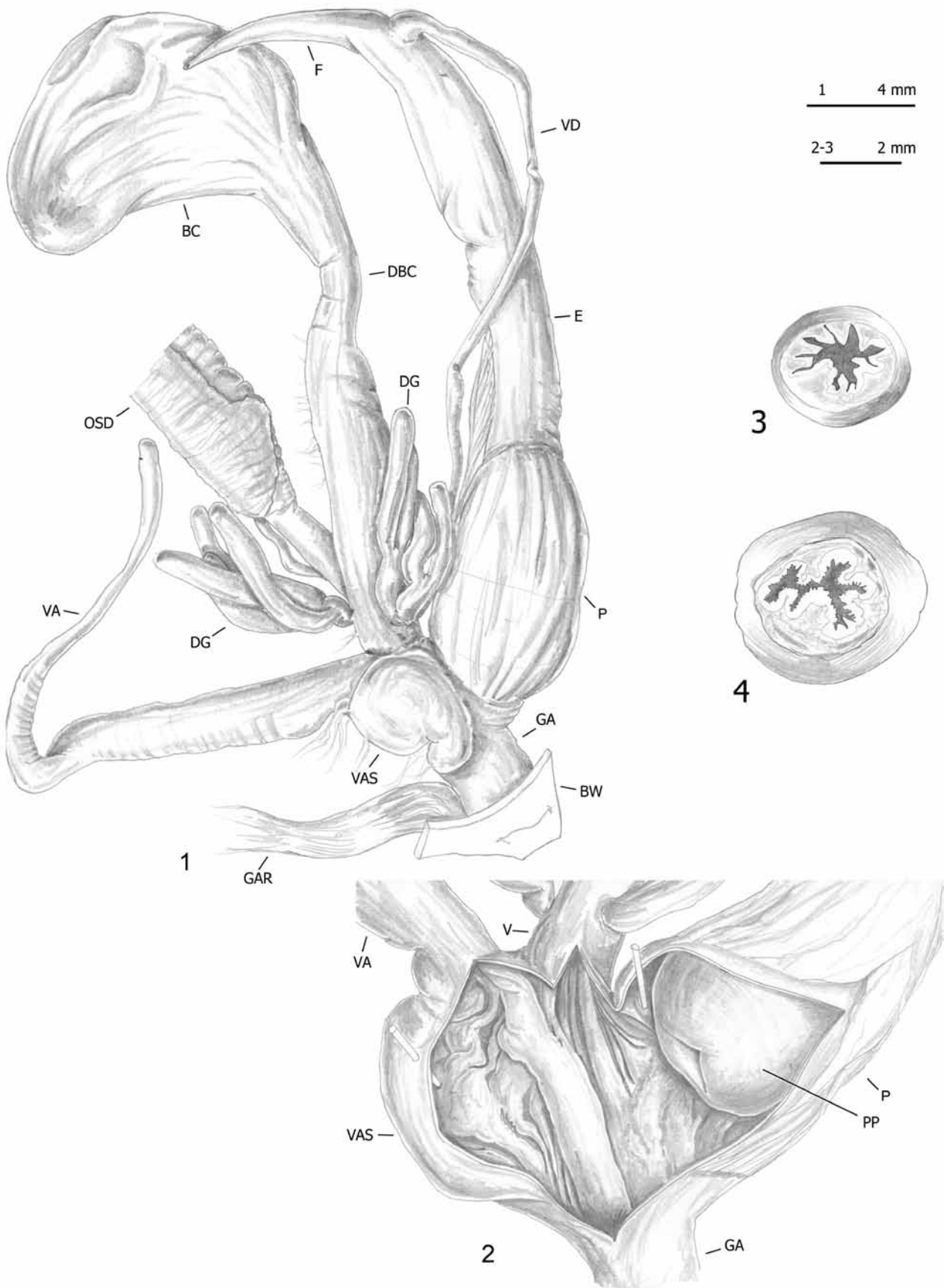


Fig. 2. Genitalia (proximal parts excluded) (1), internal structure of distal genitalia (2), transverse section of medial epiphallus (3) and penial papilla (4) of *Monacha cantiana* s.l., CAN-3 from Vienna, Donauinsel (1 specimen: DCBC0020104AT02/SP05). Acronyms: BC bursa copulatrix, BW body wall, DBC duct of bursa copulatrix, DG digitiform glands, E epiphallus (from base of flagellum to beginning of penial sheath), F flagellum, GA genital atrium, GAR genital atrium retractor, OSD ovispermiduct, P penis, PP penial papilla, V vagina, VA vaginal appendix, VAS vaginal appendix basal sac, VD vas deferens.

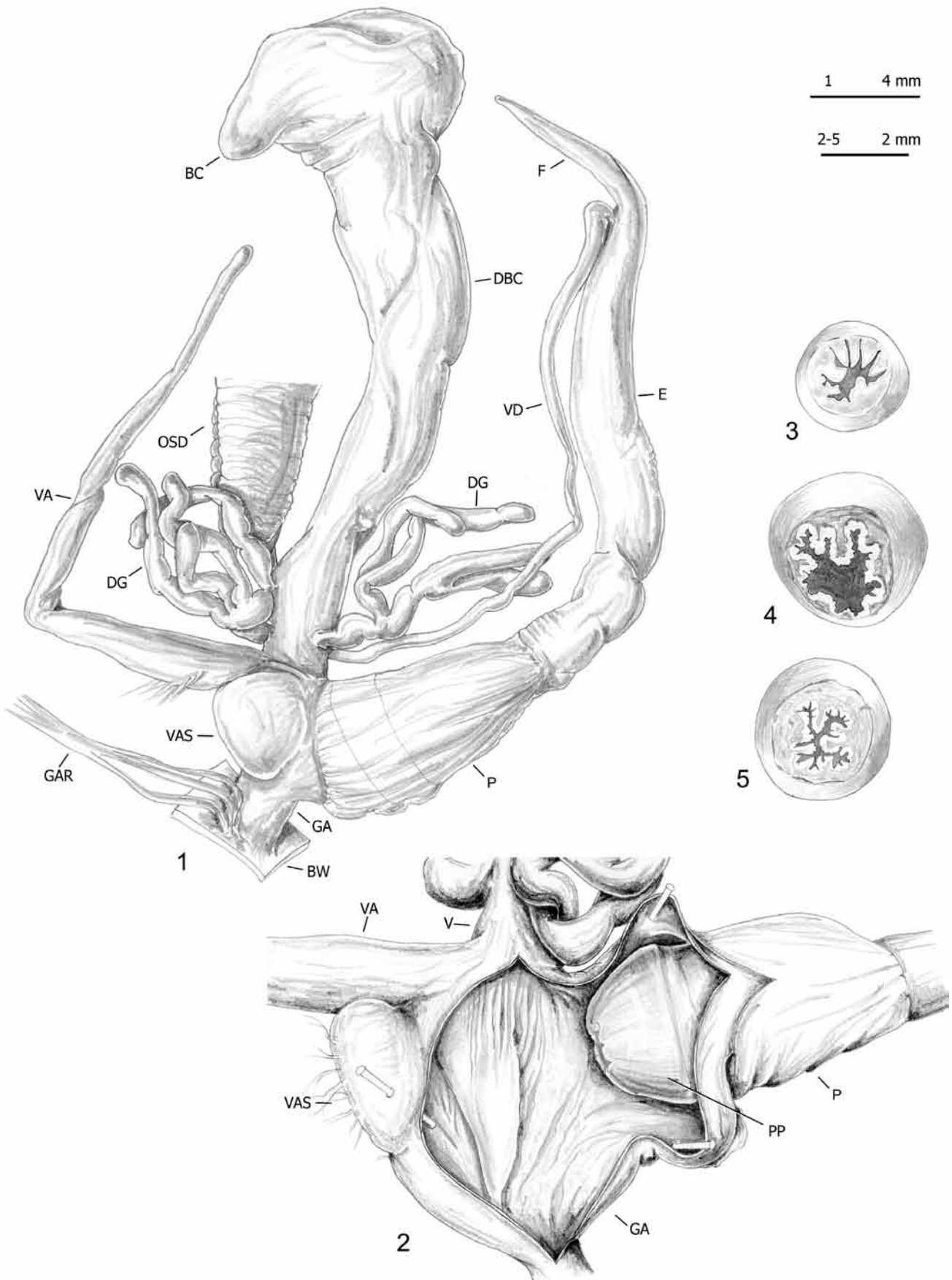


Fig. 3. Genitalia (proximal parts excluded) (1), internal structure of distal genitalia (2), transverse section of medial epiphallus (3) and penial papilla (4, 5) of *Monacha cantiana* s.l., CAN-3 from Vienna, Liesing (2 specimens: DCBC0020104AT03/SP06 & SP07). Acronyms as in Fig. 2.

Table 3. *COI+16S* combined sequences used in the phylogenetic analysis

Combined sequences <i>COI + 16S</i> haplotypes	Locality
<i>Monacha cantiana</i> CAN-1	
MG208884 + MG208966	UK, Barrow near Barnsley
MG208904 + MG208971	UK, Sheffield
MG208899 + MG208976	
MG208893 + MG208960	UK, Rotherham
MG208898 + MG208975	
MG208891 + MG208972	UK, Cambridge
MG208915 + MG208985	Italy, Latium, Valle dell’Aniene
MG208916 + MG208987	
MG208917 + MG208989	
MG208918 + MG208986	
MG208905 + MG208977	Italy, Latium, Gole del Velino
MG208906 + MG208979	
MG208910 + MG208978	
MG208921 + MG208990	Italy, Latium, Valle del Tronto
MG208923 + MG208994	Italy, Latium, Valle del Turano
<i>Monacha cantiana</i> CAN-2	
MG208925 + MG208996	Italy, Venetum, Sorga
MG208926 + MG209001	
MG208932 + MG209002	Italy, Lombardy, Rezzato
MG208931 + MG209003	
<i>Monacha cantiana</i> CAN-3	
HQ204502 + HQ204543	Italy, Friuli-Venezia Giulia, Passo di
KF596907 + KF596863	Monte Croce Carnico
MG208936 + MG209009	Austria, Breitenlee
MG208938 + MG209008	
MG208933 + MG209007	Italy, Emilia Romagna
MG208934 + MG209005	
MG208935 + MG208006	
CS 1A (COI 1A + 16S 1A)	Austria, Vienna: Donauinsel
CS 2A (COI 2A + 16S 2A)	
CS 3A (COI 3A + 16S 1A)	
CS 4A (COI 4A + 16S 1A)	
CS 5A (COI 5A + 16S 2A)	Austria, Vienna: Liesing
CS 6A (COI 6A + 16S 2A)	
<i>Monacha cemelelea</i> CAN-4	
MG208939 + MG209011	France, Sainte Thècle
MG208940 + MG209012	
MG208941 + MG209013	
MG208942 + MG209014	
MG208943 + MG209015	
<i>Monacha</i> sp. CAN-5	
MK066929 + MK066947	Italy, Apuan Alps, Foce di Pianza
MK066933 + MK066951	
MK066938 + MK066957	Italy, Apuan Alps, Piastra
MK066939 + MK066958	
MK066941 + MK066959	
MK066934 + MK066952	Italy, Apuan Alps, Campo Cecina
MK066935 + MK066954	
MK066936 + MK066955	
MK066937 + MK066956	
<i>Monacha</i> sp. CAN-6	
MK066942 + MK066960	Italy, Campagrina
MK066943 + MK066961	
MK066944 + MK066962	
MK066945 + MK066963	
<i>Monacha parumcincta</i>	
MG208944 + MG209017	Italy, Basilicata
<i>Monacha cartusiana</i>	
KM247376 + KM247391	Hungary, Kis-Balaton

Table 4. Ranges of K2P genetic distances between analysed *COI* sequences

Comparison	<i>COI</i> (%)
Within <i>M. cantiana</i> CAN-1	0.5–0.9
Within <i>M. cantiana</i> CAN-2	0.2–1.9
Within <i>M. sp.</i> CAN-3	0.2–2.9
Within <i>M. cemelelea</i> CAN-4	0.2–2.4
Within <i>M. sp.</i> CAN-5	1.3
Within <i>M. sp.</i> CAN-6	2.4
Between <i>M. cantiana</i> CAN-1 and <i>M. cantiana</i> CAN-2	3.3–5.1
Between <i>M. cantiana</i> CAN-1 and <i>M. sp.</i> CAN-3	18.3–19.5
Between <i>M. cantiana</i> CAN-1 and <i>M. cemelelea</i> CAN-4	18.0–19.7
Between <i>M. cantiana</i> CAN-1 and <i>M. sp.</i> CAN-5	17.3–18.3
Between <i>M. cantiana</i> CAN-1 and <i>M. sp.</i> CAN-6	18.8–19.5
Between <i>M. cantiana</i> CAN-2 and <i>M. sp.</i> CAN-3	17.8–18.2
Between <i>M. cantiana</i> CAN-2 and <i>M. cemelelea</i> CAN-4	18.2–18.7
Between <i>M. cantiana</i> CAN-2 and <i>M. sp.</i> CAN-5	17.6–18.2
Between <i>M. cantiana</i> CAN-2 and <i>M. sp.</i> CAN-6	18.3–19.0
Between <i>M. sp.</i> CAN-3 and <i>M. cemelelea</i> CAN-4	4.2–7.2
Between <i>M. sp.</i> CAN-3 and <i>M. sp.</i> CAN-5	13.1–14.3
Between <i>M. sp.</i> CAN-3 and <i>M. sp.</i> CAN-6	13.7–17.2
Between <i>M. cemelelea</i> CAN-4 and <i>M. sp.</i> CAN-5	14.6–15.3
Between <i>M. cemelelea</i> CAN-4 and <i>M. sp.</i> CAN-6	15.7–16.8
Between <i>M. sp.</i> CAN-5 and <i>M. sp.</i> CAN-6	13.0–14.5

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