MONACHA CLAUSTRALIS (ROSSMÄSSLER 1834) NEW TO POLISH AND CZECH MALACOFAUNA (GASTROPODA: PULMONATA: HYGROMIIDAE)

JOANNA R. PIEŃKOWSKA[1], GIUSEPPE MANGANELLI[2], FOLCO GIUSTI[2], ANDRZEJ LESICKI[1]

[1]Department of Cell Biology, Institute of Experimental Biology, Faculty of Biology, Adam Mickiewicz University in Poznań; Umultowska 89, 61–614 Poznań, Poland
[2]Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Universitá di Siena, Via Mattioli 4, 53100 Siena, Italy

Abstract *A morphological and molecular (nucleotide sequences of mitochondrial* COI *and* 16SrDNA *as well as nuclear ITS2 gene fragments) study confirms the occurrence of a second species of the genus* Monacha *Fitzinger 1833 in Poland. This species is identified as* Monacha claustralis *(Rossmässler 1834) by comparison of specimens from Polish, Czech, Bulgarian and Georgian populations. This is also the first identification of* M. claustralis *in the Czech Republic.*

Key words Monacha cartusiana, Monacha claustralis, *Poland, Czech Republic, geographic distribution*

INTRODUCTION

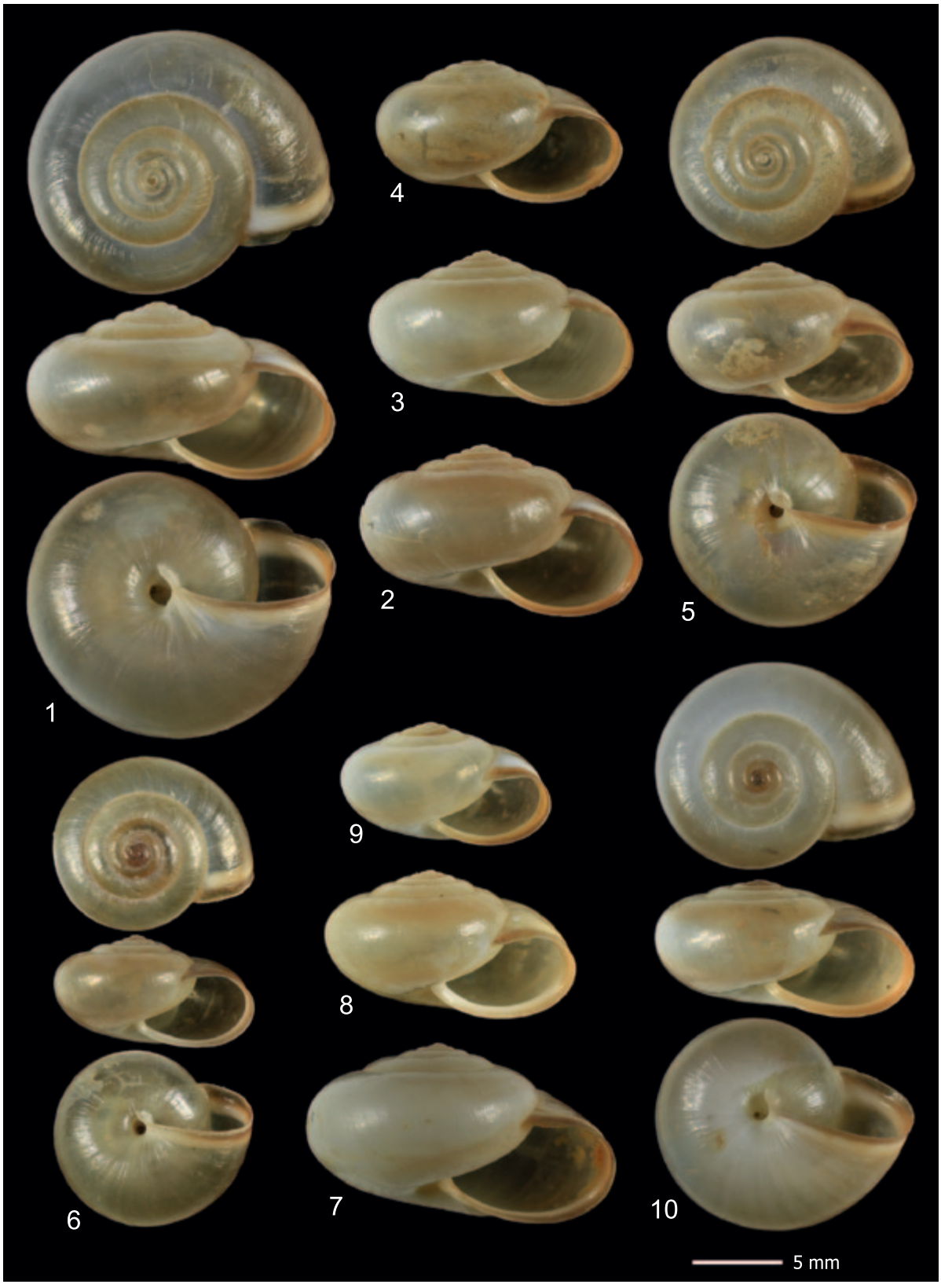
Monacha Fitzinger, 1833 (type species *Helix cartusiana* Müller 1774) is a speciose genus of the hygromiids, widespread in the western Palaearctic from western Europe to north Africa, Iran and Arabia (Hausdorf, 2000a; Welter-Schultes, 2012). It includes a large number of nominal species showing its highest diversity in the eastern sector of southern Europe and in Turkey (Hausdorf, 2000a, 2000b; Welter-Shultes, 2012).

Anthropochorous dispersal has been a major process shaping the geographic distribution of certain species of southern European origin, which reached northern Europe where they were hitherto absent. This is true for several *Monacha* species, e.g. *M. cantiana* (Montagu 1803), *M. cartusiana* and *M. claustralis* (Rossmässler 1834). The former first arrived in southern Britain in Roman times (Evans, 1972) and later also reached northwest Britain, Scotland, Belgium, the Netherlands, northern Germany and southern Sweden (Kerney & Cameron, 1979; Welter-Schultes, 2012). Step by step, *M. cartusiana* colonized various countries of northwest, central and eastern Europe (Welter-Schultes, 2012), including Poland, where it was first reported in 1973 by Kosińska (1973). Since then, this species, considered the only member of the genus in Poland, has been reported from an increasing number of sites (Cholewa *et al.*, 2003; Górka, 2005; Lesicki & Koralewska-Batura, 2007; Stworzewicz & Górka, 2008; Dembińska & Gołdyn, 2011). *M. claustralis* has probably an eastern Mediterranean origin (type locality: Kérkira, Greece) since it is widespread in the southern sector of the Balkan and Anatolian peninsulas, occurring in the southern sector of eastern Europe (Bulgaria, Albania, Macedonia, Greece and European Turkey), Crimea and Anatolian Turkey (Welter-Schultes, 1996, 2012; Hausdorf, 2000a; Irikov & Mollov, 2006; Irikov, 2008; Dhora, 2009). It has frequently been confused with the often sympatric *M. cartusiana*, due to shell similarity (Figs 1–10) (see also: Hausdorf, 2000a, 2000b; Welter-Schultes, 2012). Indeed, when first recognized anatomically by Pinter (1968) in Bulgaria, the species was described as new and named "*dissimulans*", a specific epithet indicative of its being cryptic with respect to *M. cartusiana*.

Polish populations assigned to *M. cartusiana* showed high variability in shell size (Figs 1–3, 6) and body colour, so that two of us (JP and AL) undertook detailed anatomical investigation, discovering that part of specimens belonged to a second *Monacha* species (Pieńkowska & Lesicki, 2011). Based on data in the literature (Pinter, 1968; Hausdorf, 2000a; Irikov, 2008), this species was preliminarily identified as *M. claustralis* (Pieńkowska *et al.*, 2012).

The aim of this paper is to reach a definitive identification of this second Polish *Monacha* species, applying detailed comparative analysis of distal genitalia as well as molecular analysis (using nucleotide sequence polymorphism in selected mitochondrial and nuclear genes).

Contact author : pienkowj@amu.edu.pl



Figures 1–5 Shells of *Monacha claustralis*, **1–3** Poland, Poznań-Morasko, J.R. Pieńkowska & A. Lesicki leg. 5.10.2011; **4–5** Bulgaria, Plovdiv, A. Irikov leg. 19.9.2006. **6–10** Shells of *Monacha cartusiana*, **6** Poland, Wrocław, M. Proćków leg. 15.9.2010; **7** Italy, Lago Lungo (Rieti), F. Giusti leg. 10.8.1967; **8** Italy, Lago di Montepulciano, La Casetta (Siena), G. Manganelli leg. 11.10.1992; **9** Italy, Romiccioli (Grosseto), G. Manganelli leg. 25.8.1991; **10** Italy, Valdibiena (Siena), G. Manganelli leg. 26.10.2013.

Material and Methods

Morphological study

About 80 specimens were analysed from 16 sites in Poland, Czech Republic, Hungary, Bulgaria, Italy and United Kingdom (see Appendix 1). Snail bodies were dissected under the light microscope (Wild M5A, or Zeiss SteREO Lumar. V12). Anatomical details were drawn using a Wild camera lucida. Six anatomical variables were measured using a caliper under a light microscope (0.01mm) in four populations (selected as representative of *M. cartusiana* and *M. claustralis*): F flagellum, E epiphallus (from base of flagellum to beginning of penial sheath), P penis (from beginning of penial sheath to where penis enters genital atrium), DBC duct of bursa copulatrix, DV distal vagina (from outlet of digitiform glands to where penis enters genital atrium), VA vaginal appendix (also known as appendicula).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 20mg of snail's foot tissue using Tissue Genomic DNA extraction Mini Kit (*Genoplast*). Partial sequences of two mitochondrial genes were amplified: cytochrome c oxidase subunit I (*COI*: 650-bp-long "barcode sequence"), using primers bcsmF1 and bcsmR1 (Proćków *et al.*, 2013) and 16S ribosomal DNA (*16SrDNA*: about 375 bp-long), using primers after Simon *et al.* (1994) and Gantenbein *et al.* (1999), as well as one nuclear gene fragment: internal transcribed spacer 2 of rDNA (ITS2: a 660 bp fragment enclosing the *5.8SrDNA* and the ITS2), using primers NEWS2 and ITS2-RIXO (Almeyda-Arigas *et al.*, 2000). PCRs were performed: for *COI* – according to the modified protocol prepared by Biodiversity Institute of Ontario for the Consortium for the Barcode of Life (http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf), for *16SrDNA* and ITS2 – according to procedures as previously described by Manganelli *et al.* (2005) and Almeyda-Arigas *et al.* (2000), respectively.

All PCR products were run on 1% agarose gels and sequenced in both directions in an Applied Biosystems Hitachi 3130xl Genetic Analyser automated sequencer.

Phylogenetic inference

The individual sequences are deposited in GenBank, *COI*: KM247375-KM247389, *16SrDNA*: KM247390-KM247397, and ITS2: KM247398-KM247404 (Appendix 1).

Full-length sequences were aligned and edited by eye using the program BioEdit, version 7.0.5. (Hall, 1999). The alignments were performed using Prank (Löytynoja & Goldman, 2008) for *COI* and ITS2, and CLUSTAL–W (Thompson *et al.*, 1994) for *16SrDNA*. The *COI* sequences were aligned according to the translated amino acid sequences. The ends of all sequences were trimmed. The length of the sequences after cutting were 635 bp for *COI*, 267–280 bp for *16SrDNA* and about 351 bp for ITS2. Gaps and ambiguous positions were removed from alignments. The sequences were collapsed to haplotypes (*COI* and *16SrDNA*), and to common sequences (ITS2) prior to phylogenetic analysis.

The sequences were analysed using the Neighbour-Joining method (Saitou & Nei, 1987) implemented in MEGA4 (Tamura *et al.*, 2007) using the Kimura two-parameter model (K2P) for pairwise distance calculations (Kimura, 1980). NJ tree branches were supported by bootstrap analysis with 1,000 replicates (Felsenstein, 1985). Finally, Bayesian analysis of the combined *COI* and *16SrDNA* data set was conducted with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Using jModelTest (Darriba *et al.*, 2012) according to Bayesian Information Criterion (BIC), we specified a HKY model for our data set, assuming a gamma-shaped rate variation and invariant sites. Four Monte Carlo Markov chains were run for 1 million generations, sampling every 100 generations (the first 250 trees were discarded as 'burn-in'). We obtained a 50% majority rule consensus tree.

Results

Morphological study

Specimens of the second Polish *Monacha* have a whitish body and a mantle surface little pigmented or with sparse blackish brown spots, the largest of which at the mantle vertex near the opening of the pneumostome and anus. They therefore almost perfectly match the appearance of specimens of populations of *M. cartusiana* from various sites in other European countries. However, specimens of *M. cartusiana* from Wrocław (Poland) usually have a darker body, i.e. with slate grey head, neck, tail and foot, and with a well pigmented mantle bearing a blackish

brown ring along the internal surface of its border and a number of large blackish brown spots or stripes on the rest of its surface.

The two Polish *Monacha* have similar genital structure (Figs 11–23). They share: absence of penial retractor muscle; digitiform glands forming two opposite tufts, each branched into 2–5 branches; vaginal appendix annexed to final tract of distal vagina (sometimes entering genital atrium level with penis) and consisting of wide thick-walled basal portion and slender, thin-walled – apparently glandular – apical portion; rather long cylindrical penial papilla; bursa copulatrix shoe-shaped; transverse section of epiphallus at half its length with two large pilasters, separated by smaller one, opposite four small pointed pleats (the negative of this structure is the transverse section of the spermatophore: Figs 18–21). However they differ in a series of very evident characters, namely: distal vagina (tract between digitiform glands and genital atrium: short in *M. cartusiana* vs long in *M. claustralis*), vaginal sac (a lateral sac-like diverticulum of distal vagina, first identified by Giusti & Manganelli, 1987: present in *M. cartusiana* vs absent in *M. claustralis)*, vaginal appendix (with wide, short, bulbous basal portion and longer, slender apical portion in *M. cartusiana* vs evenly tapered, cone-shaped, with basal longer than apical portion in *M. claustralis*), flagellum (shorter in *M. cartusiana* vs longer in *M. claustralis*) and genital atrium (internally rather smooth or with thin, low pleats in *M. cartusiana* vs with sort of wide, spongy, slightly raised pleat separating penis from vaginal appendix in *M. claustralis*). Three of the six characters differ significantly between the two species (F, P, DV) and one (DV) shows values which do not overlap (Fig. 24, Table 1). Two anatomical characters are therefore important for distinguishing the two Polish species: the vaginal sac and length of distal vagina.

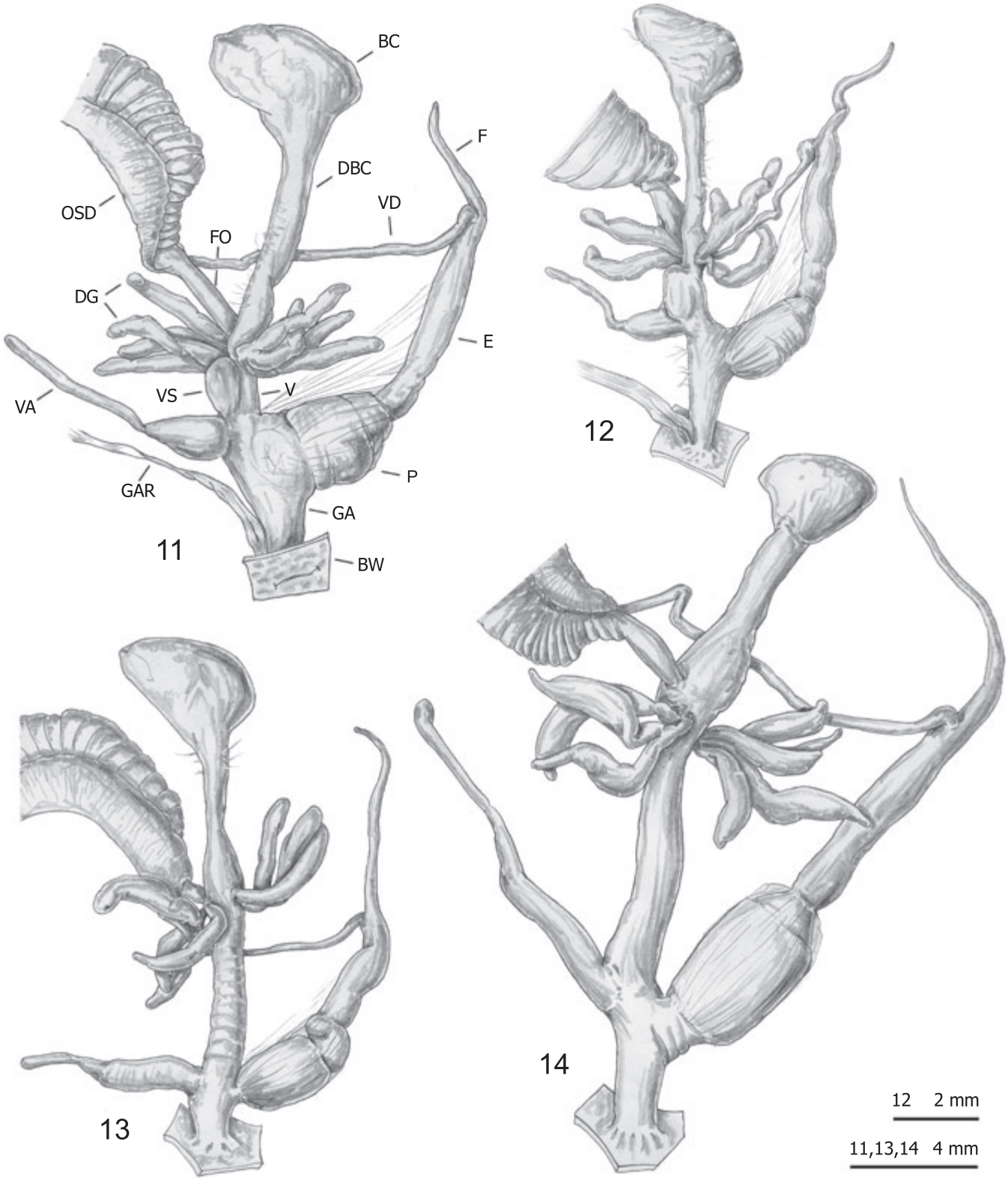
Molecular study

Fifteen haplotypes (COI 1 – COI 15) of 5'end fragments of mitochondrial *COI* gene and eight haplotypes (16S 1–16S 8) of fragments of mitochondrial *16SrDNA* gene were identified on the basis of analysis of 58 and 44 specimens, respectively. Compared by the NJ method, these sequences resulted in dendrograms with three groups of sequences (Figs 25–26). In each case, the first group included sequences (haplotypes COI 1 and 16S 1) characteristic of *Monacha cantiana* from England (loc. 1 & 2, see: Appendix 1) and sequences earlier deposited in GenBank for *M. cantiana*. The second group consisted of eight *COI* (COI 2 – COI 8, COI 15) and two *16SrDNA* (16S 2 and 16S 8) sequences, respectively, obtained from DNA isolated from specimens identified anatomically as *M. cartusiana* from Kis-Balaton (loc. 3), Wrocław (loc. 4), Prague (loc. 5–7) and Brescia (loc. 15). On the same branch of the dendrogram for *16SrDNA* (Fig. 26) a sequence deposited for *M. cartusiana* in GenBank was located, exactly the same sequence (designated by us as 16S 8) found in the specimen from Brescia (loc. 15). The remaining six *COI* (COI 9 – COI 14) and five *16SrDNA* (16S 2–16S 7) sequences created a third group composed of specimens from Czech Republic (loc. 6–8), Georgia (loc. 9), Poland (loc. 10–13) and Bulgaria (loc. 14). Among them, the specimens from Bulgaria, with sequences COI 12 – COI 14, as well as the specimen from Georgia, with haplotype COI 9, were previously assigned to *Monacha claustralis* (Irikov, 2008, Hausdorf pers. comm.), suggesting that all specimens grouping with them on the COI dendrogram belong to this species.

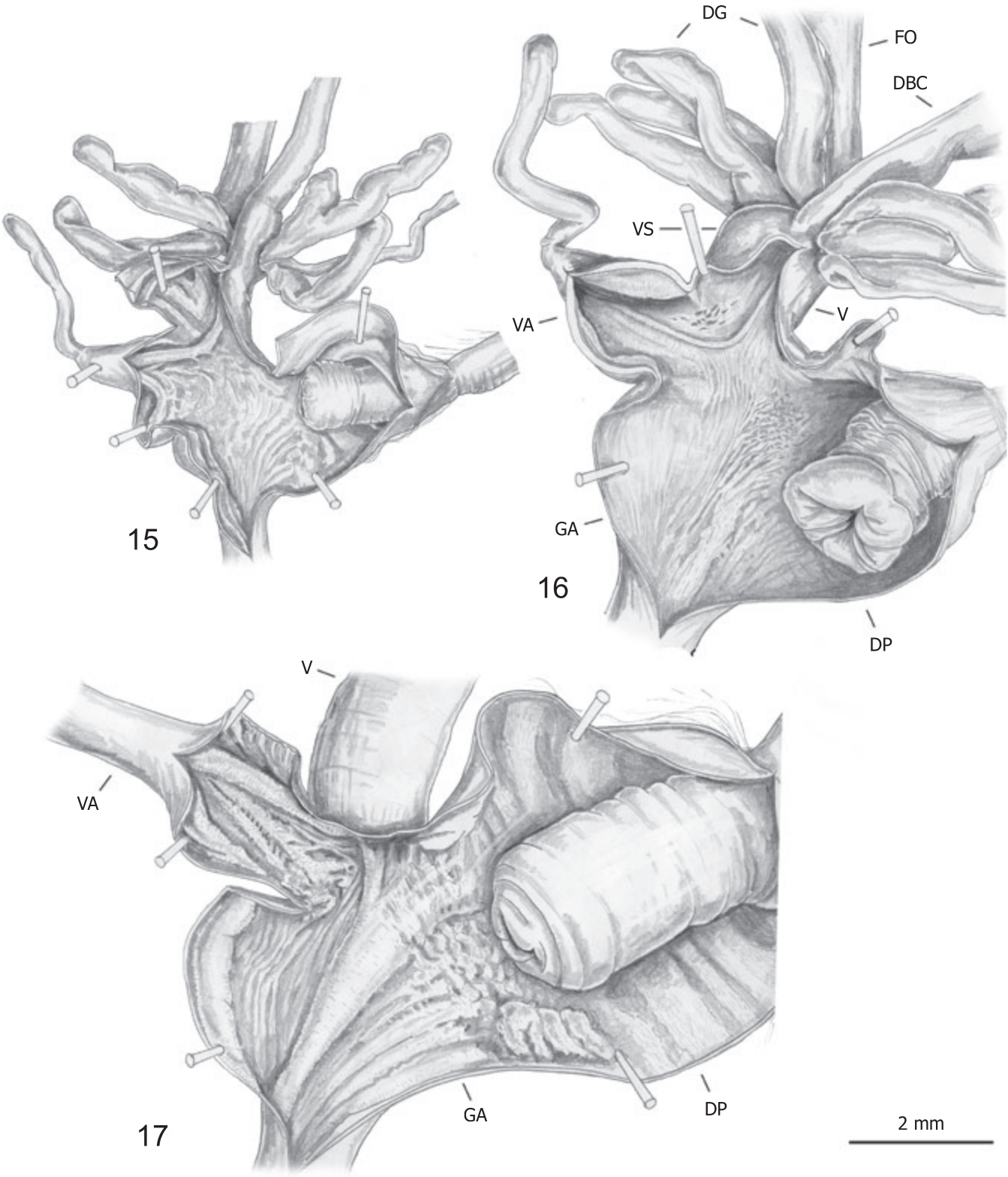
K2P distances between *COI* and *16SrDNA* for *M. cartusiana* (0.2–0.6% for *COI*, 0.4% for *16SrDNA*) and *M. claustralis* (0.2–4.1% for *COI*, 0.4–2.0% for *16SrDNA*), respectively, confirmed very similar sequences within each species (Table 2), but they differed much more (12.4–20.7% for *COI*, 9.5–24.2% for *16SrDNA*) from populations assigned to other species. The K2P distances between sequences of *COI* gene from *M. cartusiana* and *M. claustralis* populations (12.2–14.1%) may be regarded as proof that the specimens belong to separate species according to "barcode" idea (Hebert *et al*., 2003; Remigio & Hebert, 2003).

The combined haplotypes of *COI* and *16SrDNA* (Table 3) analysed with jModeltest were used for Bayesian construction of a phylogenetic tree (Fig. 27). The outcome of this part of phylogenetic inference fully confirmed species delimitation earlier achieved by NJ method (Figs 25–26).

The last molecular analysis was performed on ITS2 dataset consisting of 7 different sequences identified in 27 individuals (Appendix 1) and two sequences derived from GenBank: one of *M. cantiana*, and another of *Trochulus hispidus* as outgroup. Phylogenetic analysis (NJ) of these sequences revealed three clades (Fig. 28). The first



Figures 11–12 Distal genitalia of *Monacha cartusiana*, **11** Italy, Lago di Montepulciano, La Casetta (Siena), G. Manganelli leg. 11.10.1992; **12** Poland, Wrocław, M. Proćków leg. 20.5.2010. **13–14** Distal genitalia of *Monacha claustralis*, **13** Bulgaria, Plovdiv, A. Irikov leg. 19.9.2006; **14** Poland, Poznań-Morasko, J. Pieńkowska leg. 5.10.2011. Key to acronyms: **BC** bursa copulatrix, **BW** body wall, **DBC** duct of bursa copulatrix, **DG** digitiform glands, **E** epiphallus, **F** flagellum, **FO** free oviduct, **GA** genital atrium, **GAR** genital atrium retractor muscle, **P** penis, **OSD** ovispermiduct, **V** vagina, **VA** vaginal appendix, **VD** vas deferens, **VS** vaginal sac



Figures 15–16 Internal structure of the distal genitalia of *Monacha cartusiana,* **15** Poland, Wrocław, M. Proćków leg. 20.5.2010; **16** Italy, Lago di Montepulciano, La Casetta (Siena), G. Manganelli leg. 11.10.1992. **17** Internal structure of the distal genitalia of *Monacha claustralis* Poland, Poznań-Morasko, J. Pieńkowska leg. 5.10.2011. Key to acronyms: **DP** distal penis, for rest of acronyms see Figs 11–14.

Figures 18, 20 Sections of spermathophores of *Monacha cartusiana,* **19, 21** sections of the epiphallus of *M. cartusiana;* **18–19** Poland, Wrocław, M. Proćków leg. 20.5.2010; **20–21** Italy, Lago di Montepulciano, La Casetta (Siena), G. Manganelli leg. 11.10.1992. **22–23** sections of the epiphallus of *Monacha claustralis,* **22** Poland, Poznań-Morasko, J. Pieńkowska leg. 5.10.2011; **23** Bulgaria, Plovdiv, A. Irikov leg. 19.9.2006

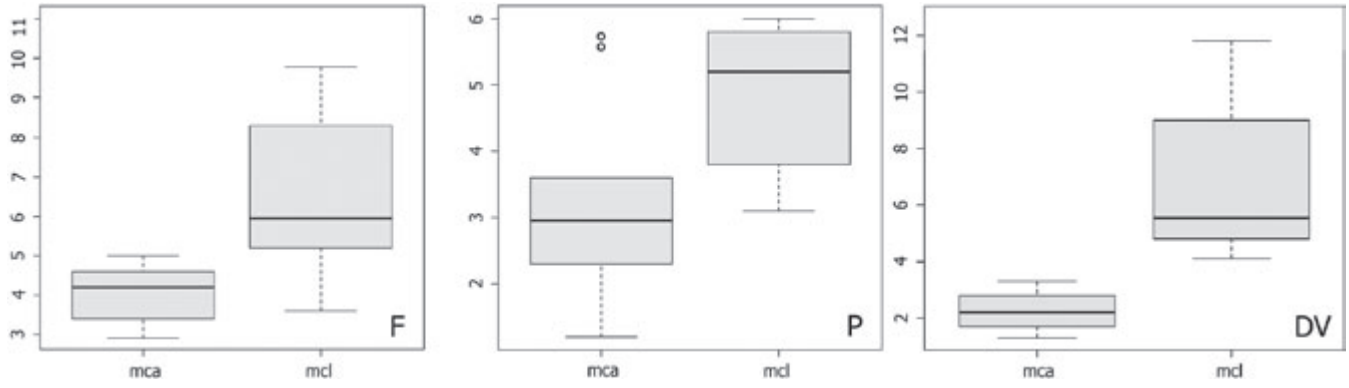


Figure 24 Box plots for DV, F and P values in *Monacha claustralis* (mcl) and *Monacha cartusiana* (mca) ($P < 0.05$, Wilcoxon rank sum test). The lower and upper limits of the rectangular boxes indicate the 25th to 75th percentile range, and the horizontal line within the boxes is the median (50th percentile).

group contained a sequence ITS2 1, different by one deletion from the GenBank sequence assigned to *M. cantiana*. The second group included three slightly different sequences (ITS2 5, ITS2 6 and ITS2 7 which differ in three nucleotides only) found in Georgian (loc. 9), Polish (loc. 11 & 12) and Bulgarian (loc. 14) specimens assigned to *M. claustralis*. This suggests that this group contains sequences unique for *M. claustralis*. The third group included three other sequences (ITS2 2 – ITS2 4) which differed in 2–10 nucleotide sites found in both specimens of *M. cartusiana* and *M. claustralis* (Appendix 1).

Discussion

The structure of the distal genitalia confirms that the *Monacha* specimens from the Polish localities 10–13 (see Appendix 1) correspond to a species different from *M. cartusiana,* as suggested by previous preliminary reports (Pieńkowska & Lesicki, 2011; Pieńkowska *et al*., 2012). Two characters – absence of vaginal sac and length of distal vagina – immediately distinguish this *Monacha* from *M. cartusiana*. They also suggest a closer relationship with a group of species from SE Europe and Turkey, including *M. carascaloides* (Bourguignat 1855; type locality: Gallipoli, Turkey), *M. solidior* (Mousson 1863: type locality: Bursa, Turkey), *M. venusta* Pinter 1969 (type locality: "am rechten Ufer des Flusses Ropotamo, etwa 1.5km östlich von der unteren Brücke", Bulgaria) and *M. claustralis* (Rossmässler 1834; type locality: Kérkira, Greece). *M. carascaloides* and *M. solidior* differ by virtue of vaginal

Table 1 Mean, standard deviation and statistical significance ($p < 0.05$) of six anatomical variables in two populations of *Monacha claustralis* and *Monacha cartusiana*. The Wilcoxon rank sum test was used to evaluate significant differences between the two species with respect to each variable. Data was log-transformed before performing statistical analysis. Acronyms: DBC duct of bursa copulatrix, DV distal vagina, E epiphallus, F flagellum, P penis, VA vaginal appendix (for details see text)

	Monacha claustralis (Rossmässler 1834) Locality no. 12: W Poland, Poznań-Morasko, A. Lesicki & J.R. Pieńkowska leg. 5.10.2011	*Monacha claustralis* (Rossmässler 1834) Locality no. 14: Bulgaria, Plovdiv, A. Irikov leg. 19.9.2006	*Monacha cartusiana* (Müller 1774) Locality no. 4: SW Poland, Wrocław, M. Proćków leg. 15.9.2010	*Monacha cartusiana* (Müller 1774) Locality no. 16: Central Italy, Lago di Montepulciano, G. Manganelli leg. 11.10.1992	Student t-test (significant p values in bold)
F	7.8±1.6 mm	4.9±1.2 mm	3.8±0.7 mm	4.4±0.7 mm	**0.007**
E	7.6±1.4 mm	3.8±0.6 mm	4.2±0.9 mm	5.2±1.0 mm	0.54
P	5.8±0.2 mm	3.9±0.7 mm	2.4±0.8 mm	4.1±1.5 mm	**0.010**
DBC	6.8±2.2 mm	4.1±1.5 mm	3.7±1.1 mm	5.2±0.8 mm	0.29
DV	8.9±2.1 mm	4.6±0.4 mm	2.1±0.5 mm	2.5±0.9 mm	**0.0002**
VA	10.4±1.5 mm	5.2±0.7 mm	4.3±1.1 mm	6.7±2.2 mm	0.07

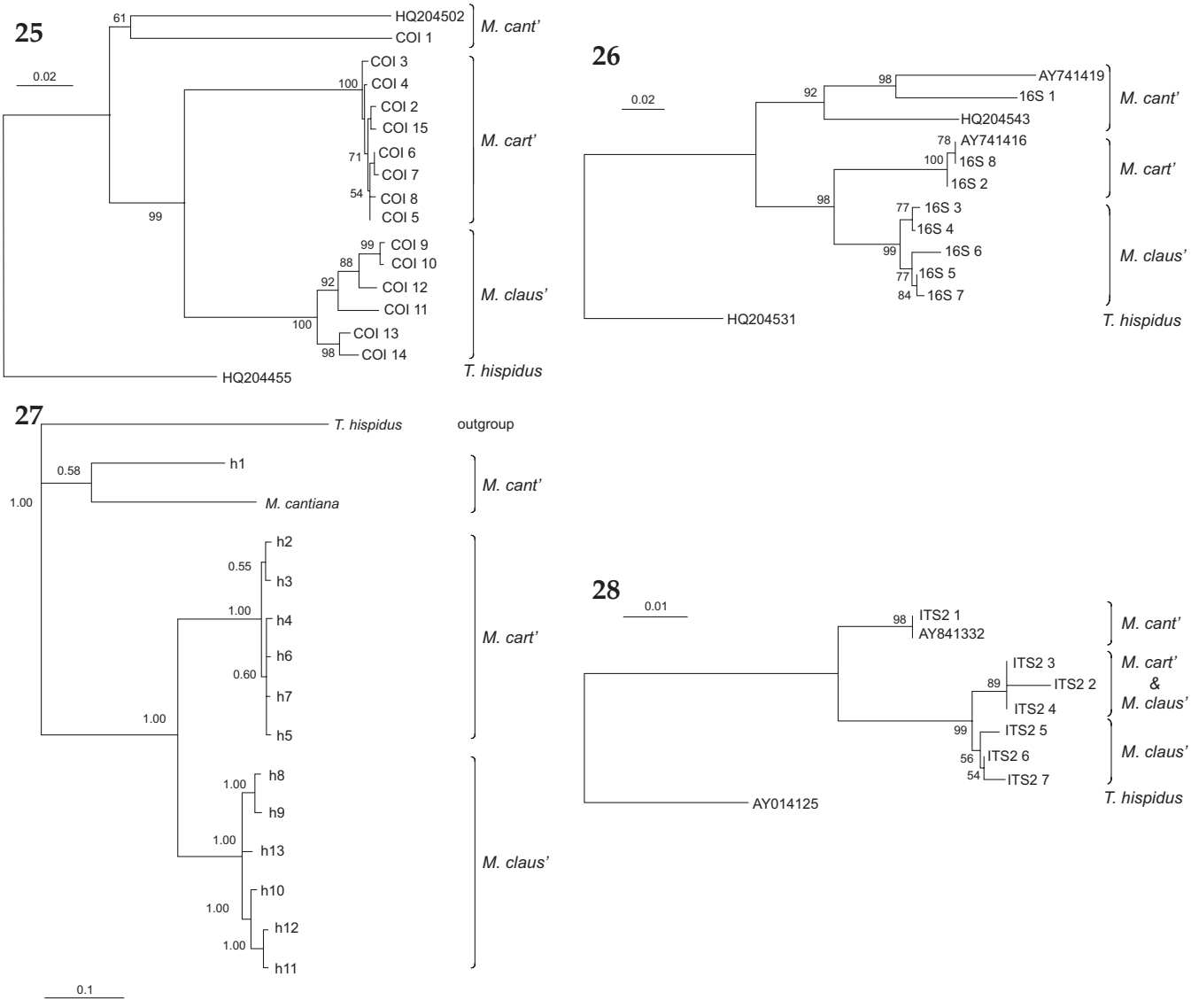


Table 2 Ranges of K2P genetic distances for *COI* and *16SrDNA* sequences analysed (mean values in parentheses)

Comparison	*COI* (%)	*16SrDNA* (%)
Within *M. cartusiana*	0.2–0.6 (0.4)	0.4 (0.4)
Within *M. claustralis*	0.2–4.1 (2.1)	0.4–2.0 (1.2)
Within *M. cantiana*	0.0	0.0
Between *M. cartusiana* and *M. claustralis*	12.2–14.1 (13.1)	9.5–10.4 (9.9)
Between *M. cartusiana* and *M. cantiana*	18.8–19.8 (19.3)	21.0–21.5 (21.2)
Between *M. claustralis* and *M. cantiana*	18.1–20.7 (19.4)	21.0–24.2 (22.6)

Table 3 Combined *COI* and *16SrDNA* data sets (locality numbers in parenthesis)

combined haplotypes	*COI*	*16SrDNA*	Distribution
Monacha cantiana			
h1	COI 1	16S 1	England: East Acton (1) & Barrow (2)
Monacha cartusiana			
h2	COI 3	16S 2	Hungary: Kis-Balaton (3)
h3	COI 4	16S 2	Hungary: Kis-Balaton (3)
h4	COI 15	16S 8	Italy: Brescia (15)
h5	COI 7	16S 2	Poland: Wrocław (4)
h6	COI 5	16S 2	Poland: Wrocław (4)
h7	COI 8	16S 2	Czech Republic: Prague (5, 6, 7)
Monacha claustralis			
h8	COI 14	16S 6	Bulgaria: Plovdiv (14)
h9	COI 13	16S 6	Bulgaria: Plovdiv (14)
h10	COI 12	16S 5	Bulgaria: Plovdiv (14)
h11	COI 9	16S 4	Czech Republic: Prague (7, 8); Georgia: Saguramo (9)
h12	COI 10	16S 3	Poland: Janikowo (10)
h13	COI 11	16S 7	Poland: Poznań-Cybina (11), Poznań-Morasko (12), Wietrznia (13)

Figure 25 Neighbour-Joining tree based on the 635-nt-long fragment of *COI* sequences of three *Monacha* species: *M. cantiana*, *M. cartusiana* and *M. claustralis*. The *COI* sequence of *Trochulus hispidus* HQ204455 (Duda *et al.*, 2011) was used as an outgroup, and *M. cantiana* HQ204502 (Duda *et al.*, 2011) as a reference sequence. Numbers on branches represent bootstrap support above 50%. The evolutionary distances were computed using the Kimura two-parameter method and are in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Figure 26 Neighbour-Joining tree of *16SrDNA Monacha* sequences. *Trochulus hispidus* HQ204531 (Duda *et al.*, 2011) was chosen as an outgroup. *M. cantiana* AY741419 (Manganelli *et al.*, 2005), HQ204543 (Duda *et al.*, 2011) and *M. cartusiana* AY741416 (Manganelli *et al.*, 2005) sequences were used as references. Calculation parameters were the same as for Fig. 25.

Figure 27 Majority-rule consensus tree obtained from Bayesian inference analysis of the combined data set of *COI* and *16SrDNA* sequences. Posterior probabilities are marked at the nodes. The tree was rooted with *T. hispidus* HQ20455 & HQ204531, and *M. cantiana* HQ204502 & HQ204543 (Duda *et al.*, 2011) was used as a reference sequence.

Figure 28 NJ tree of *Monacha* ITS2 sequences, using *Trochulus hispidus* AY014125 (Wade *et al.*, 2001) as an outgroup, and *M. cantiana* AY841332 (Wade *et al.*, 2001) as a reference sequence. The method used for calculation of evolutionary distances and tree reliability were the same as in Fig. 25.

appendix entering distal vagina medially (*M. carascaloides*, see: Hausdorf, 2000a: Fig. 17) or proximally (*M. solidior*, see: Irikov, 2008: Figs 1, 5), whereas *M. venusta* has an even longer vagina and a very slender vaginal appendix, without widened basal portion (Irikov, 2008: Fig. 6) and the fourth species, *M. claustralis*, is identical to the *Monacha* species new for Poland.

Direct examination of specimens of *M. claustralis* from Plovdiv (Bulgaria) confirmed this: although the study was based on specimens preserved directly in spirit (the various genital tracts may have undergone contraction and shortening), all the data, qualitative and quantitative, was consistent (Figs 1–5, 13–14, 17; Table 1). This only partly confirms what Hausdorf (2000a: 80; 2000b: 1586) and Welter-Schultes (2012: 505) stated, i.e. that *M. claustralis* is distinct from *M. cartusiana* by virtue of a shorter epiphallus (penis ratio 2.0–2.3in *M. claustralis*, 2.3–3.5in *M. cartusiana*; vagina ratio 0.6–1.8in *M. claustralis*, 1.5–2.6in *M. cartusiana*). Indeed, we found an E/P ratio of 1.3±0.2in *M. claustralis* and 1.6±0.4in *M. cartusiana* and an E/DV ratio of 0.8±0.1in *M. claustralis* and 2.1±0.3in *M. cartusiana*. Incidentally, we agree with Hausdorf (2000a, 2000b) when he stated that Figs 374–376 by Schileyko (1978) concern *M. claustralis* not *M. cartusiana*.

Identification of the *Monacha* species new for Poland as *M. claustralis* was also confirmed by molecular studies. Our molecular results make it possible to report *M. claustralis* from the Czech Republic where it was formerly misidentified as *M. cantiana* (Hlaváč & Peltanová, 2010). The nucleotide sequences of mitochondrial (*COI*, *16SrDNA*) and nuclear (ITS2) gene fragments (Figs 25–28) from specimens of Polish localities created common groups with specimens from Plovdiv (Bulgaria), Saguramo (Georgia) and some localities in Prague (Czech Republic). While in the case of *COI* and *16SrDNA* fragment analysis, these groups were clearly distinguished from the groups composed of sequences from specimens identified as *M. cartusiana* (from Polish, Czech, Hungarian and Italian localities as well as from GenBank) in both dendrograms, analysis of ITS2 sequences did not allow clear separation of *M. cartusiana* and *M. claustralis*. This is probably connected with lower variability of nuclear gene sequences than mitochondrial ones what may result from faster evolution of mitochondrial genome than the nuclear one (Remigio & Hebert, 2003). Similar suggestions were previously given in the studies of species complexes in *Conus orbignyi* Audouin 1831 (Puillandre *et al.*, 2011) or *T. hispidus* (Linnaeus 1758) (Kruckenhauser *et al.*, 2014) as well as of species delimitation in the genus *Stagnicola* Jeffreys 1830 (Pieńkowska *et al.*, 2015).

M. claustralis, widespread in the southern sector of the Balkan and Anatolian peninsulas and apparently related (at least from a morphological point of view) to a group of species with a similar distribution (Hausdorf, 2000a; Irikov & Mollov, 2006; Irikov, 2008), is recognized by Hausdorf (2000a: 82) as "especially synanthropical". It can be considered certain that its distribution has been progressively enlarged by anthropochorous dispersal. This was made evident by its recent arrival in Poland and especially by the fact that the populations from widely distant sites, examined here, revealed remarkable molecular affinity.

This molecular affinity and the constancy of morphological characters of the *M. claustralis* specimens from widely distant sites also diminish the importance of studying fresh topotypical materials which were presently unavailable to us. Future research will nevertheless tackle this problem to exclude any doubt as to species identity. The study of topotypical material of *M. subobstructa* Bourguinat 1855 (locus typicus: "Beicos", Turkey) and *M. oshanovae* Pinter & Pinter 1970 (locus typicus: Skrât, westllich von Petrič, Struma Gebiet, Bulgaria) is also necessary to confirm the synonymization with *M. claustralis* proposed by Hausdorf (2000a), and by Irikov (2008) and Welter-Schultes (2012), respectively.

ACKNOWLEDGEMENTS

We thank Robert Cameron (University of Sheffield, UK), Marcin Górka (University of Warsaw, Poland), Atanas Irikov (University of Plovdiv, Bulgaria) and Małgorzata Proćków (University of Wrocław, Poland) for providing specimens. We also thank Marco Neiber and Dr. Bernhard Hausdorf (Zoologisches Museum der Universität Hamburg, Germany) for kindly offering sequences obtained in their laboratory and Valerio Ketmaier (University of Rome, Italy) for his useful comments on the molecular analysis.

References

Almeyda-Artigas RJ, Bargues MD & Mas-Coma S 2000 ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human gnathostomiasis in the Americas. *Journal of Parasitology* **86**: 537–544.

Cholewa S, Koralewska-Batura E & Batura M 2003 A new locality of *Monacha cartusiana* (O. F. Müller, 1774) (Gastropoda: Pulmonta: Helicidae) in Poland. *Folia Malacologica* **11**: 59–61.

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

Dembińska A & Gołdyn B 2011 Nowe stanowiska *Monacha cartusiana* w okolicach Poznania. *In* T Kałuski, J Kobak & P Cieluch (eds) *Problemy Współczesnej Malakologii 2011 – XVII Krajowe Seminarium Malakologiczne, Toruń – Tleń 6–8.04.2011* 21. Bogucki Wydawnictwo Naukowe, Poznań.

Dhora D 2009 Molluscs of Albania. *Archives of Biological Science (Belgrade)* **61**: 537–553.

Duda M, Sattmann H, Haring E, Bartel D, Winkler H, Harl J & Kruckenhauser L 2011 Genetic differentiation and shell morphology of *Trochulus oreinos* (Wagner, 1915) and *T. hispidus* (Linnaeus, 1758) (Pulmonata: Hygromiidae) in the northeastern Alps. *Journal of Molluscan Studies* **77**: 30–40.

Evans JG 1972 *Land Snails in Archeology. With special reference to the British Isles.* Seminar Press Inc., London, 436 pp.

Felsenstein J 1985 Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Gantenbein B, Fet V, Largiadèr CR & Scholl A 1999 First DNA phylogeny of *Euscorpius* Thorrell, 1876 (Scorpiones: Euscorpiidae) and its bearing on taxonomy and biogeography of this genus. *Biogeographica (Paris)* **75**: 49–65.

Giusti F & Manganelli G 1987 Notulae Malacologicae, XXXVI. On some Hygromiidae (Gastropoda: Helicoidea) living in Sardinia and in Corsica. (Studies on the Sardinian and Corsican Malacofauna VI). *Bollettino Malacologico* **23**: 123–206.

Górka M 2005 The invasion continues – a new locality of *Monacha cartusiana* (O. F. Müller) (Gastropoda: Pulmonata: Helicidae) in the Świętokrzyskie Mts. (Central Poland). *Folia Malacologica* **13**: 153–155.

Hall TA 1999 BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hausdorf B 2000a The genus *Monacha* in Turkey (Gastropoda: Pulmonata: Hygromiidae). *Archiv für Molluskenkunde* **128**: 61–151.

Hausdorf B 2000b The genus *Monacha* in the Western Caucasus (Gastropoda: Hygromiidae). *Journal of Natural History* **34**: 1575–1594.

Hebert PDN, Cywinska A, Ball SL & deWaard JR 2003 Biological identifications through DNA bardcodes. *Proceedings of the Royal Society B: Biological Sciences* **270**: 313–321.

Hlaváč JČ & Peltanová A 2010 First occurrence of the Kentish Snail *Monacha cantiana* (Mollusca: Gastropoda: Hygromiidae) in the Czech Republic. *Malacologica Bohemoslovaca* **9**: 11–15.

Irikov A 2008 Genus *Monacha* Fitzinger 1833 in Bulgaria (Gastropoda, Pulmonata, Hygromiidae). *Linzer Biologische Beitraege* **40**: 785–811.

Irikov A & Mollov I 2006 Terrestrial gastropods (Mollusca: Gastropoda) of the Western Rhodopes (Bulgaria). *In* P Beron (ed) *Biodiversity of Bulgaria. 3. Biodiversity of Western Rhodopes (Bulgaria and Greece)* 753–832. Pensoft & National Museum of Natural History, Sofia.

Kerney MP & Cameron RAD 1979 *A field guide to the land snails of Britain and North-West Europe*. Collins, London, 288 pp.

Kimura M 1980 A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

Kosińska M 1973 *Monacha cartusiana* (Müller) i *Milax budapestensis* (Hazay) – dwa interesujące gatunki w faunie Polski. *Przegląd Zoologiczny* **17**: 58–62.

Kruckenhauser L, Duda M, Bartel D, Sattman H, Harl J, Kirchner S & Haring E 2014 Paraphyly and budding speciation in the hairy snail (Pulmonata, Hygromiidae). *Zoologica Scripta* **43**: 273–288.

Lesicki A & Koralewska-Batura E 2007 *Monacha cartusiana* (O. F. Müller, 1774) (Gastropoda: Pulmonata: Hygromiidae) becomes more frequent in Poland. *Folia Malacologica* **15**: 181–184.

Löytynoja A & Goldman N 2008 Phylogeny – aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science* **320**: 1632–1635.

Manganelli G, Salomone N & Giusti F 2005 A molecular approach to the phylogenetic relationships of the western palaearctic Helicoidea (Gastropoda: Stylommatophora). *Biological Journal of the Linnean Society* **85**: 501–512.

Pieńkowska JR & Lesicki A 2011 Representatives of the genus *Monacha* in Poland. In: BM Pokryszko (ed.) *The 27th Polish Malacological Seminar – seminar report. Folia Malacologica* **20**: 62.

Pieńkowska JR, Giusti F, Manganelli G & Lesicki A 2012 A second *Monacha* species (Pulmonata: Hygromiidae) from Poland. *In* BM Pokryszko (ed.) *The 28th Polish Malacological Seminar – seminar report*. *Folia Malacologica* **21**: 45.

Pieńkowska JR, Rybska E, Banasiak J, Wesołowska M & Lesicki A 2015 Taxonomic status of *Stagnicola palustris* (O. F. Müller, 1774) and *S. turricula* (Held, 1836) (Gastropoda: Pulmonata: Lymnaeidae) in the view of new molecular and chorological data. *Folia Malacologica* **23**: 3–18.

Pinter L 1968 Über bulgarischen Mollusken. *Malakologische Abhandlungen* **2**: 209–230.

Proćków M, Mackiewicz P & Pieńkowska JR 2013 Genetic and morphological studies of species status for poorly known endemic *Trochulus phorochaetius*

(Bourguignat, 1864) (Gastropoda: Pulmonata: Hygromiidae), and its comparison with closely related taxa. *Zoological Journal of the Linnean Society* **169**: 124–143.

PUILLANDRE N, MEYER CP, BOUCHET P & OLIVERA BM 2011 Genetic divergence and geographic variation in the deep-water *Conus orbignyi* complex (Mollusca: Conoidea) *Zoologica Scripta* **40**: 350–363.

REMIGIO EA & HEBERT PDN 2003 Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. *Molecular Phylogenetics and Evolution* **29**: 641–647.

RONQUIST F & HUELSENBECK JP 2003 MRBAYES **3**: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

SAITOU N & NEI M 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

SCHILEYKO AA 1978 Molljuski, III, 6. Nazemnye molljuski nadsemejstva Helicoidea [Land molluscs of the superfamily Helicoidea]. *Fauna SSSR Novaja Serija* 117. Nauka, Leningrad, 384 pp.

SIMON C, FRATI F, BECKENBACH AT, CRESPI B, LIU H & FLOOK P 1994 Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**: 651–701.

STWORZEWICZ E & GÓRKA M 2008 Ślimak kartuzek *Monacha cartusiana* (O. F. Müller, 1774) *In* Z Głowaciński, H Okarma, J Pawłowski & W Solarz (eds) *Księga gatunków inwazyjnych obcych faunie Polski.* Online edition, Institute of Nature Conservation SAS, Cracow: http://www.iop.krakow.pl/gatunkiobce/

TAMURA K, DUDLEY J, NEI M & KUMAR S 2007 MEGA**4**: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 *Molecular Biology and Evolution* **24**: 1596–1599.

THOMPSON JD, HIGGINGS DG, & GIBSON TJ 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

WADE CM, MORDAN PB & CLARKE B 2001 A phylogeny of the land snails (Gastropoda: Pulmonata). *Proceedings of the Royal Society B: Biological Sciences* **268**: 1465 413–422.

WELTER-SCHULTES FW 1996 List of species and atlas of the non-marine molluscs of Albania. *Schriften zur Malakozoologie* **9**: 90–197.

WELTER-SCHULTES FW 2012 *European non-marine molluscs, a guide for species identification.* Planet Poster Editions, Göttingen, 679 pp.

Appendix 1 Material examined for morphological and molecular study

No.	localities				gene fragment sequences		
	coordinates	description[1]	collection data: collector / date / no. of specimens (in collection[2])	species	*COI* / GenBank ## (number of specimens)	*16SrDNA*/ GenBank ## (number of specimens)	ITS2/ GenBank ## (number of specimens)
1	51°30'30''N 0°15'38''W	United Kingdom, East Acton near London	M. Proćków / 7.6.2010 / 2 specimens (DCBC)	*Monacha cantiana* (Montagu 1803)	COI 1 / KM247375 (2)	16S 1 / KM247390 (1)	-
2	53°31'29''N 1°27'54''W	United Kingdom, Barrow near Barnsley	R.A.D. Cameron / 10.2011 / 2 specimens (FGC)	*Monacha cantiana* (Montagu 1803)	COI 1 / KM247375 (2)	16S 1 / KM247390 (3)	ITS2 1 / KM247398 (2)
3	46°42'10''N 17°14'38''E	Hungary, Kis-Balaton, about 30m from the Zala Canal on the underside of goldenrod leaves in the scrub-field	J.R. Pieńkowska / 31.7.2011 / 8 specimens (DCBC)	*Monacha cartusiana* (Müller 1774)	COI 2 / KM247376 (1) COI 3 / KM247377 (1) COI 4 / KM247378 (1)	16S 2 / KM247391 (3)	ITS2 3 / KM247400 (1)
4	51°07'03.34''N 16°59'52.83''E	SW Poland, Wrocław, the area around the railway tracks	M. Proćków / 20.5.2010, 15.9.2010, 22.9.2011 / 26 shells, 29 specimens (DCBC and FGC)	*Monacha cartusiana* (Müller 1774)	COI 5 / KM247379 (7) COI 6 / KM247380 (1) COI 7 / KM247381 (1)	16S 2 / KM247391 (7)	ITS2 2 / KM247399 (4)
5	50°07'28''N 14°37'20''E	Czech Republic, Prague, Horni Počernice district, near Sezemická Street	J.R. Pieńkowska / 3.8.2011 / 66 specimens (DCBC)	*Monacha cartusiana* (Müller 1774)	COI 8 / KM247382 (5)	16S 2 / KM247391 (2)	ITS2 3 / KM247400 (1)
6	50°07'13.86''N 14°37'47.60''E	Czech Republic, Prague, Horni Počernice district, fallow lands in the area of the National Museum	J.R. Pieńkowska / 3.8.2011 / 36 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) and *Monacha cartusiana* (Müller 1774)	COI 9 / KM247383 (1) COI 8 / KM247382 (3)	- 16S 2 / KM247391 (3)	- ITS2 3 / KM247400 (1)
7	50°07'18.65''N 14°37'50.65''E	Czech Republic, Prague, Horni Počernice district, grassy patches in the area of the National Museum	D. Pieńkowski / 4.8.2011 / 31 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) (Hlaváč & Peltanová, 2010: as *M. cantiana*) and *Monacha cartusiana* (Müller 1774)	COI 9 / KM247383 (2) COI 8 / KM247382 (2)	16S 4 / KM247393 (2) 16S 2 / KM247391 (1)	ITS2 3 / KM247400 (1) ITS2 3 / KM247400 (1)

No.	coordinates	localities description[1]	collection data: collector / date / no. of specimens (in collection[2])	species	gene fragment sequences *COI* / GenBank ## (number of specimens)	*16SrDNA* / GenBank ## (number of specimens)	ITS2 / GenBank ## (number of specimens)
8	50°07'21.72''N 14°37'44.16''E	Czech Republic, Prague, Horni Počernice district, grassy patches in the area of the National Museum	J.R. Pieńkowska / 4.8.2011 / 5 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) (Hlaváč & Peltanová, 2010: as *M. cantiana*)	COI 9 / KM247383 (2)	16S 4 / KM247393 (1)	ITS2 3 / KM247400 (2)
9	41°53'38''N 44°46'09''E	Georgia, Saguramo, Mtskheta-Mtianeti, between village and cemetery, 650m a.s.l	M.T. Neiber & F. Walther / 3.10.2011 / 1 specimen (ZMH 86012)	*Monacha claustralis* (Rossmässler 1834)	COI 9 / KM247383 (1)	16S 4 / KM247393 (1)	ITS2 6 / KM277403 (1)
10	52°46'35.44''N 18°06'52.23''E	Central Poland, Janikowo near Inowrocław, gravel pit	J.R. Pieńkowska & E. Rybska / 5.4.2011 / 32 shells, 11 specimens (DCBC) J.R. Pieńkowska / 21.9.2011 / 90 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) (Lesicki & Koralewska-Batura, 2007: as *M. cartusiana*)	COI 10 / KM247384 (6)	16S 3 / KM247392 (1)	-
11	52°24'41.11''N 16°57'03.12''E	W Poland, Poznań-Cybina, banks of the Cybina River at the mouth of the Warta River	J.R. Pieńkowska / 28.8.2011 / 10 shells, 12 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) (Cholewa *et al.*, 2003: as *M. cartusiana*)	COI 11 / KM247385 (8)	16S 4 / KM247393 (1) 16S 7 / KM247396 (1)	ITS2 6 / KM277403 (2)
12	52°27'57.35''N 16°55'22.60''E	W Poland, Poznań-Morasko, fallow lands around the bike path to academic campus	A. Lesicki & J.R. Pieńkowska / 23.8.2010–5.10.2011 / 54 shells, 30 specimens (DCBC and FGC)	*Monacha claustralis* (Rossmässler 1834) (Lesicki & Koralewska-Batura, 2007: as *M. cartusiana*)	COI 11 / KM247385 (3)	16S 7 / KM247396 (8)	ITS2 5 / KM277402 (2) ITS2 6 / KM277403 (3) ITS2 7 / KM277404 (1)
13	50°51'17.02''N 20°38'24.38''E	S Poland, Wietrznia near Kielce, old quarry on the mountainside Wietrznia	M. Górka / 30.5.2012 / 19 specimens (DCBC)	*Monacha claustralis* (Rossmässler 1834) (Górka, 2005: as *M. cartusiana*)	COI 11 / KM247385 (5)	16S 4 / KM247393 (5)	ITS2 4 / KM277401 (2)

No.	coordinates	localities description[1]	collection data: collector / date / no. of specimens (in collection[2])	species	gene fragment sequences *COI* / GenBank ## (number of specimens)	*16SrDNA*/ GenBank ## (number of specimens)	ITS2/ GenBank ## (number of specimens)
14	42°08'35.72''N 24°44'59.51''E	Bulgaria, Plovdiv	A. Irikov / 19.9.2006 / 4 specimens (AIC)	*Monacha claustralis* (Rossmässler 1834) (det. *M. dissimulans*)	COI 12 / KM247386 (1) COI 13 / KM247387 (1) COI 14 / KM247388 (1)	16S 5 / KM247394 (1) 16S 6 / KM247395 (3)	ITS2 6 / KM277403 (2)
15	45°46'38''N 10°30'12''E	N Italy Brescia, Anfo towards Ponte Caffaro, calcareous rocks at branch towards Tre Casali, 400m a.s.l.	B. Hausdorf / 19.8.2009 / 1 specimen (ZMH 51710)	*Monacha cartusiana* (Müller 1774)	COI 15 / KM247389 (1)	16S 8 / KM247397 (1)	ITS2 3 / KM247400 (1)
16	43°05'52''N 11°55'01''E	Central Italy, Lago di Montepulciano	G. Manganelli / 11.10.1992 / 9 specimens (FGC)	*Monacha cartusiana* (Müller 1774)	-	-	-

[1] Detailed description of the vegetation at some localities (nos. 7, 8, 10–13) was published earlier (Cholewa *et al*., 2003; Górka, 2005; Lesicki & Koralewska-Batura, 2007; Hlaváč & Peltanová, 2010).

[2] Acronyms of institutions where voucher specimens are kept: AIC A. Irikov coll. (University of Plovdiv, Bulgaria), DCBC Department of Cell Biology coll. (Adam Mickiewicz University, Poznań, Poland), FGC F. Giusti coll. (University of Siena, Italy), ZMH Zoologisches Museum der Universität Hamburg (Germany)