FOLIA MALACOLOGICA
Folia Malacol. 24(4): 223–237
ISSN 1506-7629
The Association of Polish Malacologists
Faculty of Biology, Adam Mickiewicz University
Bogucki Wydawnictwo Naukowe
Poznań, December 2016
http://dx.doi.org/10.12657/folmal.024.019

NEW DATA ON THE DISTRIBUTION AND MOLECULAR DIAGNOSTICS OF *MONACHA CLAUSTRALIS* (ROSSMÄSSLER, 1834) AND *M. CARTUSIANA* (O. F. MÜLLER, 1774) (GASTROPODA: EUPULMONATA: HYGROMIIDAE) IN POLAND, BOSNIA AND SERBIA

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ABSTRACT: The study provides new distributional data on *Monacha claustralis* (Rossmässler) and *M. cartusiana* (O. F. Müller) which invaded Poland in the last five decades. Both species were identified based on two mitochondrial gene sequences (*COI* and *16SrDNA*) and the genital structure. *M. cartusiana,* discovered in Poland in the early 1970s, is still limited to a few localities in Wrocław (Dolnośląskie Province), but the recent records from the environs of Kielce (Świętokrzyskie Province) suggest that the species is expanding its range. The occurrence of *M. cartusiana* in Bosnia and Herzegovina and in Serbia was confirmed by molecular analysis. *M. claustralis*, discovered in Poland in the early 2000s, but only very recently correctly identified, turned out to be very invasive. Its new populations were discovered in southern (Świętokrzyskie Province), western (Wielkopolskie Province), central (Kujawsko-Pomorskie Province) and northern (Pomorskie Province) Poland. Distribution maps of the two species in Poland are provided, and the new sequences of their *COI* and *16SrDNA* genes have been deposited in GenBank.

KEY WORDS: mtDNA, *COI*, *16SrDNA*, co-occurrence, invasive species, terrestrial snails

INTRODUCTION

Species of the genus *Monacha* Fitzinger, 1833 (type species: *Helix cartusiana* O. F. Müller, 1774) are widespread in the western Palaearctic, from Western Europe to North Africa, the Asian part of Turkey, Lebanon, the Caucasus, Iran and Arabia (KERNEY et al. 1983, HAUSDORF 2000a, b, HAUSDORF & PÁLL-GERGELY 2009, WELTER-SCHULTES 2012, NEUBERT & BARICHE 2013).

In Poland, *M. cartusiana* was first recorded in 1971 in Wrocław (KOSIŃSKA 1973, 1979) and for a long time the locality was regarded as unique (RIEDEL 1988, WIKTOR 2004, SULIKOWSKA-DROZD 2008). Some new localities were added in the 21st century (CHOLEWA et al. 2003, GÓRKA 2005, LESICKI & KORALEWSKA-BATURA 2007, STWORZEWICZ & GÓRKA 2009, DEMBIŃSKA & GOŁDYN 2012), but many of those actually pertained to another species of *Monacha* (PIEŃKOWSKA & LESICKI 2012, PIEŃKOWSKA et al. 2013). A detailed analysis of the structure of its copulatory organs and the nucleotide sequences of its *COI* and *16SrDNA* gene fragments (PIEŃKOWSKA et al. 2015) made it possible to identify it as *Monacha claustralis* (Rossmässler, 1834), another invasive species which originally occurred in south-eastern Europe.

In this paper we present new data on the distribution of *M. claustralis* and *M. cartusiana* in Poland. Besides, we provide new information on molecular diagnostics for Polish, Bosnian and Serbian specimens.

Ministry of Science and Higher Education
Republic of Poland
Folia Malacologica is funded by the Ministry of Science and Higher Education, Republic of Poland under agreement no. 646/P-DUN/2016 allocated to the activities of disseminating science

MATERIAL AND METHODS

One hundred and twelve specimens of *M. claustralis* and *M. cartusiana* were collected in the localities listed in Table 1 and used for the study. Their identification was confirmed based on their copulatory organs (Pieńkowska et al. 2015).

For the analysis of two mitochondrial gene sequences (*COI* and *16SrDNA*) total genomic DNA was extracted from 20 mg of foot tissue of each specimen using Tissue Genomic DNA extraction MiniKit (Genoplast). The rest of the body (including the genitalia) was preserved individually in 75% alcohol as voucher specimens in the Department of Cell Biology Collection, Adam Mickiewicz University, Poznań, Poland (DCBC).

Partial sequences of two mitochondrial gene fragments were amplified: cytochrome c oxidase subunit 1 (*COI*, 650 bp long barcode sequence), using primers bcsmF1 and bcsmR1 (Proćków et al. 2013), and 16S ribosomal DNA (*16SrDNA*, ca. 375 bp long), using primers recommended by Simon et al. (1994) and Gantenbein et al. (1999). Amplifications were performed following the procedure previously described for *COI* or *16SrDNA* by Manganelli et al. (2005). The PCR conditions were as follows: for *COI* – 3 min at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, followed by final elongation of 7 min at 72°C; for *16SrDNA* – 2 min at 94°C followed by 25 cycles of 20 s at 92°C, 30 s at 54°C, and 30 s at 72°C, followed by final elongation of 5 min at 72°C. The PCR products were visualised on 1% agarose gels and sequenced in an Applied Biosystems Hitachi 3130x1 Genetic Analyser automated sequencer.

The individual sequences were deposited in GenBank: *COI* – KX258308-KX258418, *16SrDNA* – KX258234-KX258307 (Table 1). Full-length sequences were aligned and edited by eye, using BioEdit ver. 7.0.5 software (Hall 1999). The alignments were performed using Prank (Löytynoja & Goldman 2008) for *COI* and CLUSTAL-W (Thompson et al. 1994) for *16SrDNA*. Possibly uncertain areas were removed from the *16SrDNA* alignment with GBlocs 0.91b (Castresana 2000, Talavera & Castresana 2007). The *COI* sequences were aligned according to translated amino acid sequences. For *16SrDNA* alignment parameters allowing relaxed selection of blocs were used. The ends of all the sequences were trimmed to the length of 584 bp for *COI* and 262 bp for *16SrDNA*. The sequences were also collapsed to combined haplotypes (*COI* and *16SrDNA*).

The sequences were analysed using the Neighbour-Joining (NJ) method (Saitou & Nei 1987) implemented in MEGA ver. 6 software (Tamura et al. 2013) using the Kimura two-parameter (K2P) model for pair-wise distance calculations (Kimura 1980). NJ tree branches were supported by bootstrap analysis with 1,000 replicates (Felsenstein 1985). Finally, Bayesian analysis of the combined haplotype (*COI* and *16SrDNA*) dataset was conducted with MrBayes 3.1.2 software (Ronquist & Huelsenbeck 2003). A Hasegawa, Kishino and Yan (HKY) model for our dataset, assuming a gamma-shaped rate variation and invariant sites, was specified using jModelTest (Darriba et al. 2012) according to the Bayesian Information Criterion (BIC). Four Monte Carlo Markov chains were run for 1 million generations, sampling every 100 generations (the first 250 trees were discarded as 'burn-in'). Consequently, we obtained a 50% majority rule consensus tree.

RESULTS

Seventeen new sequences of mitochondrial *COI* (COI 1 – COI 17) and twelve *16SrDNA* (16S 1 – 16S 12) gene fragments (Table 1) were found. The sequences were clustered in two groups (Figs 1, 2) using the NJ method. They were grouped together with sequences deposited in GenBank for *M. claustralis* or alternatively with those deposited for *M. cartusiana*, both for *COI* (Fig. 1) and *16SrDNA* (Fig. 2). We could identify the specimens with sequences COI 1 to COI 8 and 16S 1 to 16S 6 as *M. claustralis*, while the specimens with sequences COI 9 to COI 17 and 16S 7 to 16S 12, as *M. cartusiana*. Kimura K2P distances used for the NJ analysis are presented in Appendices 1 & 2. They confirm the specific distinctness of *M. claustralis* and *M. cartusiana* (Table 2).

The combined haplotypes of *COI* and *16SrDNA* sequences (Table 3) were used for construction of a Bayesian phylogenetic tree (Fig. 3) which confirmed the identification of *M. claustralis* and *M. cartusiana* among the studied specimens.

DISCUSSION

Molecular techniques, especially the analysis of nucleotide sequences of selected genes, are now frequently used for species classification, delimitation and identification (Hebert et al. 2003a, b, Tautz et

Table 1. List of localities of *M. claustralis* (Rossmässler, 1834) and *M. cartusiana* (O. F. Müller, 1774) used for molecular analyses and summary of results. All the specimens are deposited in the Department of Cell Biology Collection (DCBC), Adam Mickiewicz University, Poznań, Poland

Localities			*Monacha* species	*COI*			*16SrDNA*		
coordinates	short description	collector name, date (no. of specimens)		new sequence	no. of specimens	GenBank ##	new sequence	no. of specimens	GenBank ##
S Poland, Świętokrzyskie Province									
50°51'02.7"N 20°33'44.1"E	**Kielce-Na Ługach** St., ruderal-xerothermic herbs along railway tracks	M. GÓRKA, 18.09.2013 (6)	*M. claustralis*	COI 1	6	KX258308–KX258313	16S 1	1	KX258234
50°51'41.2"N 20°33'00.7"E	**Kielce-Białogon**, Siedmiu Źródeł St., herbaceous vegetation on roadside	M. GÓRKA, 11.09.2013 (6)	*M. claustralis*	COI 1	6	KX258314–KX258319	16S 1	1	KX258235
50°51'31.5"N 20°30'10.6"E	**Jaworznia** near Kielce, environs of quarry	M. GÓRKA, 28.07.2011 (6)	*M. claustralis*	COI 1	6	KX258320–KX258325	16S 1	5	KX258236–KX258240
50°44'55.3"N 20°37'44.2"E	**Morawka** near Kielce, herbs along road on Morawka River (Morawica reservoir)	M. GÓRKA, 19.07.2013 (6)	*M. claustralis*	COI 1	6	KX258326–KX258331	16S 1	1	KX258241
50°44'13.6"N 20°36'52.6"E	**Morawica** near Kielce, field/xerothermic sward, along road near quarry	M. GÓRKA, 01.05.2014 (3) M. GÓRKA, 29.08.2015 (2)	*M. claustralis*	COI 1	5	KX258332–KX258336	16S 1	3	KX258242–KX258244
50°41'50.1"N 20°27'36.7"E	**Sobków** near Jędrzejów, herbs and grasses on meadow near cemetery and Lewiatan Market Centre	M. GÓRKA, 08.09.2013 (5)	*M. claustralis*	COI 1	3	KX258337–KX258339	16S 1	1	KX258245
				COI 2	1	KX258351	16S 2	1	KX258258
				COI 4	1	KX258361			
50°48'28"N 20°15'55"E	**Małogoszcz** herbaceous vegetation by estate road along garages	M. GÓRKA, 11.09.2015 (6)	*M. claustralis*	COI 4	5	KX258362–KX258366	16S 3	6	KX258260–KX258265
				COI 5	1	KX258369			
50°39'04.5"N 20°17'15.3"E	**Jędrzejów**, along embankment of railway route Kielce-Cracow	M. GÓRKA, 08.09.2013 (11)	*M. claustralis*	COI 2	8	KX258352–KX258359	16S 2	1	KX258259
				COI 3	1	KX258360	16S 3	1	KX258266
				COI 4	2	KX258367–KX258368	16S 4	1	KX258267

Table 1 continued

Localities			*Monacha* species	*COI*			*16SrDNA*		
coordinates	short description	collector name, date (no. of specimens)		new sequence	no. of specimens	GenBank ##	new sequence	no. of specimens	GenBank ##
50°51'31.2"N 20°35'48.9"E	**Kielce-Grzybowa** St., scrubs along dirt road near railway	M. GÓRKA, 19.07.2013 (5)	*M. claustralis* and	COI 1	1	KX258340	16S 1	1	KX258246
			M. cartusiana	COI 9	4	KX258393–KX258396	16S 7	1	KX258288
50°51'17.0"N 20°38'24.4"E	**Kielce-Wietrznia**, old quarry on Wietrznia hill	M. GÓRKA, 30.05.2012 (1)	*M. cartusiana* (*)	COI 9	1	KX258397	16S 7	1	KX258289
50°56'03.9"N 21°22'39.7"E	**Ostrowiec Świętokrzyski**, Zagłoby St., near railway station, lawn footbridge over railway	M. GWARDJAN, 03.07.2015 (5)	*M. cartusiana*	COI 10	5	KX258398–KX258402	16S 8	5	KX258292–KX258296
W Poland, Wielkopolskie Province									
52°25'34.0"N 16°51'24.8"E	**Poznań-Wola**, Pilotów/ Startowa St., weedy plot	A. WIKTOR, A. LESICKI, 29.06 & 27.07.2015 (5)	*M. claustralis*	COI 1	5	KX258341–KX258345	16S 1	5	KX258247–KX258251
52°14'59.7"N 16°53'35.4"E	**Rogalinek** near Poznań, herbs along Warta River	J. R. PIEŃKOWSKA, 06.07.2015 (5)	*M. claustralis*	COI 1	4	KX258346–KX258349	16S 1	5	KX258252–KX258256
52°17'35.9"N 16°51'40.0"E	**Puszczykowo** near Poznań, garden vegetation	J. R. PIEŃKOWSKA, 29.07.2015 (5)	*M. claustralis*	COI 1	1	KX258350	16S 1	1	KX258257
				COI 6	4	KX258370–KX258373	16S 4	4	KX258268–KX258271
N Poland, Pomorskie Province									
54°49'54.3"N 18°19'27.8"E	**Jastrzębia Góra**, pathway at entrance to holiday resort Meduza (Różewska 7)	M. GÓRKA, 19.08.2013 (1)	*M. claustralis*	COI 7	1	KX258374	16S 5	1	KX258275
54°34'35"N 18°03'00"E	**Nadole**, sparse vegetation in gravel pit near Lake Żarnowieckie	M. GÓRKA, 06.09.2011 (6)	*M. claustralis*	COI 7	6	KX258375–KX258380	16S 5	4	KX258276–KX258279
54°48'15"N 18°22'14"E	**Chłapowo** near Władysławowo, scrubs near road at entrance to Rudnik ravine	M. GÓRKA, 08.09.2011 (6)	*M. claustralis*	COI 7	6	KX258381–KX258386	16S 5	4	KX258280–KX258283
							16S 6	1	KX258287
54°46'40"N 18°09'40"E	**Krokowa**, herbaceous vegetation along cobblestone road near petrol station	M. GÓRKA, 18.09.2015 (6)	*M. claustralis*	COI 7	3	KX258387–KX258389	16S 4	3	KX258272–KX258274
				COI 8	3	KX258390–KX258392	16S 5	3	KX258284–KX258286

Table 1 continued

Localities			*Monacha* species	*COI*			*16SrDNA*		
coordinates	short description	collector name, date (no. of specimens)		new sequence	no. of specimens	GenBank ##	new sequence	no. of specimens	GenBank ##
SW Poland, Dolnośląskie Province									
51°08'30.5"N 16°56'55.9"E	**Wrocław-Pilczyce** (Mączna St.), 80 m E of Ślęza River bank, dry meadow with grassy vegetation	M. Proćków, 31.07.2015 (5)	*M. cartusiana*	COI 11	2	KX258403–KX258404	16S 8	5	KX258297–KX258301
				COI 12	3	KX258407–KX258409			
Bosnia and Herzegovina									
43°54'24.2"N 17°36'23.3"E	**Gornji Vakuf-Uskoplje**, roadside parking lot, 852 m a.s.l.	J. R. Pieńkowska, 18.08.2014 (5)	*M. cartusiana*	COI 13	1	KX258410	16S 9	2	KX258302–KX258303
				COI 14	4	KX258411–KX258414			
Serbia									
44°49'26.6"N 20°31'12.6"E	**Belgrade**, herbaceous plants on Danube banks	M. Proćków, 24.07.2014, (6)	*M. cartusiana*	COI 11	2	KX258405–KX258406	16S 7	2	KX258290–KX258291
				COI 15	2	KX258415–KX258416	16S 10	1	KX258304
				COI 16	1	KX258417	16S 11	2	KX258305–KX258306
				COI 17	1	KX258418	16S 12	1	KX258307

(*) Co-occurrence with *M. claustralis* reported by Pieńkowska et al. (2015).

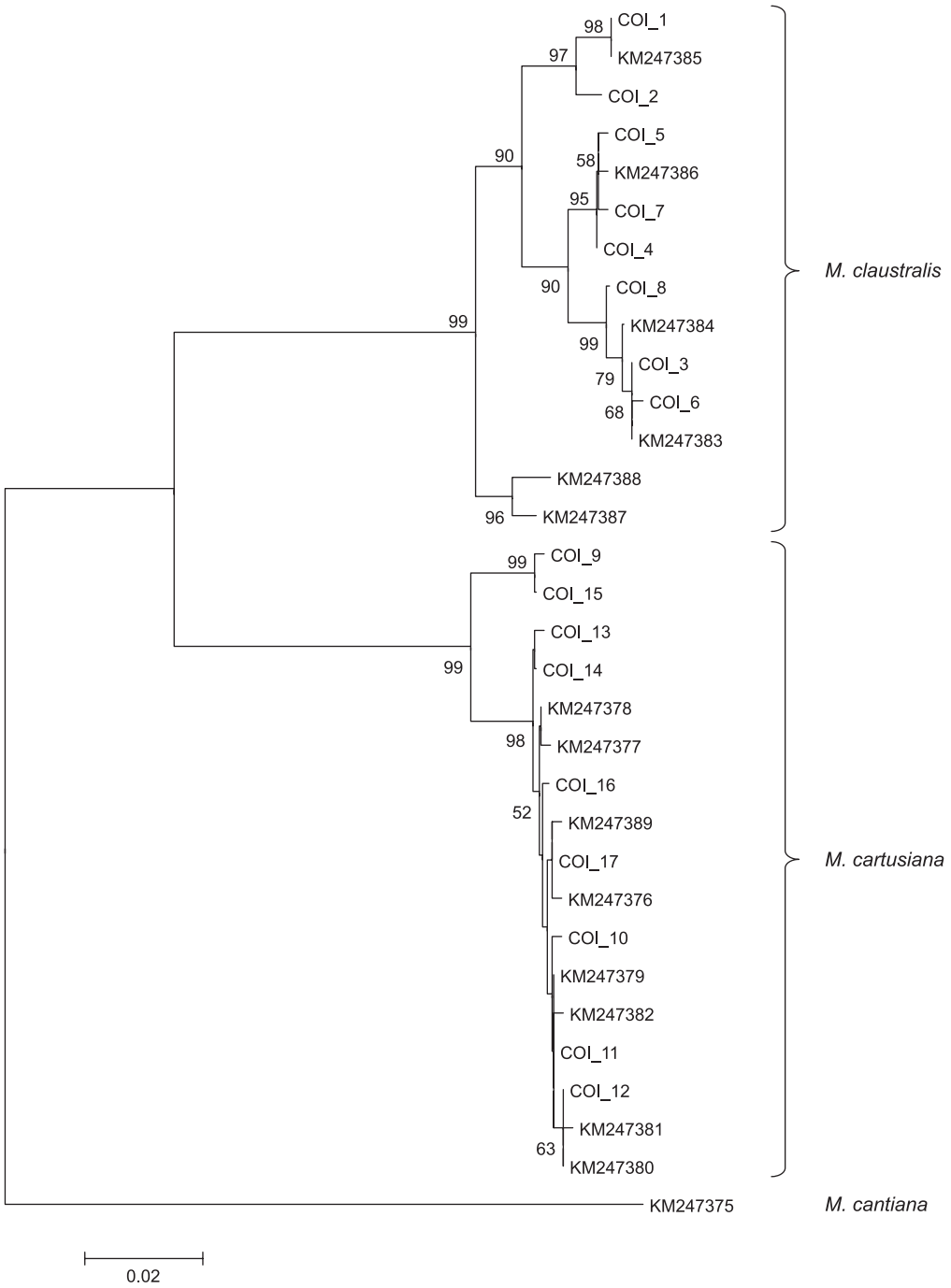

Fig. 1. Neighbour-Joining tree based on the 584-nt-long fragment of new *COI* sequences of *M. claustralis* and *M. cartusiana*. The *COI* sequence of *M. cantiana* KM247375 (Pieńkowska et al. 2015) was chosen as outgroup, and sequences of *M. claustralis* KM247383-KM247388 and of *M. cartusiana* KM247376-KM247382 & KM247389 (Pieńkowska et al. 2015) were used as references. The figures on branches represent bootstrap support above 50%. The evolutionary distances expressed as the number of base substitutions per site were computed using the Kimura two-parameter method. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option)

al. 2003, Packer et al. 2009, Goldstein & Desalle 2010). Among the gene fragments often used to solve taxonomic problems in Gastropoda, there are two mitochondrial ones, namely *COI*, coding for the 5'-end of cytochrome c oxidase subunit 1 (Falniowski & Wilke 2001, Szarowska 2006, Haase et al. 2007, Falniowski et al. 2009, Sauer & Hausdorf 2009, Proćków et al. 2013, 2014, Szarowska et al. 2014, 2015, 2016) and *16SrDNA*, coding for the 16S part of ribosomal DNA (Elejalde et al. 2005, Geenen et al. 2006, Wethington et al. 2009, Sauer & Hausdorf 2012, Rowson et al. 2014). The cited authors, aware

Table 2. Ranges of K2P genetic distances for analysed *COI* and *16SrDNA* sequences

Comparison	*COI* (%)	*16SrDNA* (%)
Within *M. claustralis*	0.0–4.3	0.0–3.1
Within *M. cartusiana*	0.0–3.0	0.0–2.3
Within *M. cantiana*	0.0	0.0
Between *M. claustralis* and *M. cartusiana*	12.3–15.1	9.3–11.9
Between *M. claustralis* and *M. cantiana*	19.2–21.5	21.3–23.8
Between *M. cartusiana* and *M. cantiana*	19.6–20.7	20.3–21.3

of the risk of application of molecular features alone (Gregory 2005, Goldstein & Desalle 2010, Sauer & Hausdorf 2012), solved taxonomic problems by combining analyses of morphological and anatomical characters with results of molecular analyses.

Similarly, for identification of the second *Monacha* species in Poland, Pieńkowska et al. (2015), based on the differences in the distal genital structures and the nucleotide sequences of *COI* and *16SrDNA* gene fragments, recognised *M. claustralis* as a species well distinct from *M. cartusiana* which had been earlier known from Poland (Kosińska 1973, 1979).

Sequences COI 1 and 16S 1, found in several specimens from Kielce-Na Ługach, Kielce-Białogon, Kielce-Grzybowa, Jaworznia, Morawka, Morawica, Sobków, Poznań-Wola, Rogalinek and Puszczykowo (Table 1) were identical to the sequences KM247385 and KM247396 deposited in GenBank for *COI* and *16SrDNA*, respectively, which had been found in *M. claustralis* from Poznań-Cybina and Poznań-Morasko (Pieńkowska et al. 2015). Sequence COI 3 from specimens collected in Jędrzejów (Table 1) was the same as sequence KM247383 deposited in GenBank for *M. claustralis* from Prague (Czech Republic), Saguramo (Georgia) and Plovdiv (Bulgaria) (Pieńkowska et al. 2015). Sequence 16S 4 from specimens from Jędrzejów and Puszczykowo was the same as GenBank sequence KM247393 (*16SrDNA*) for spec-

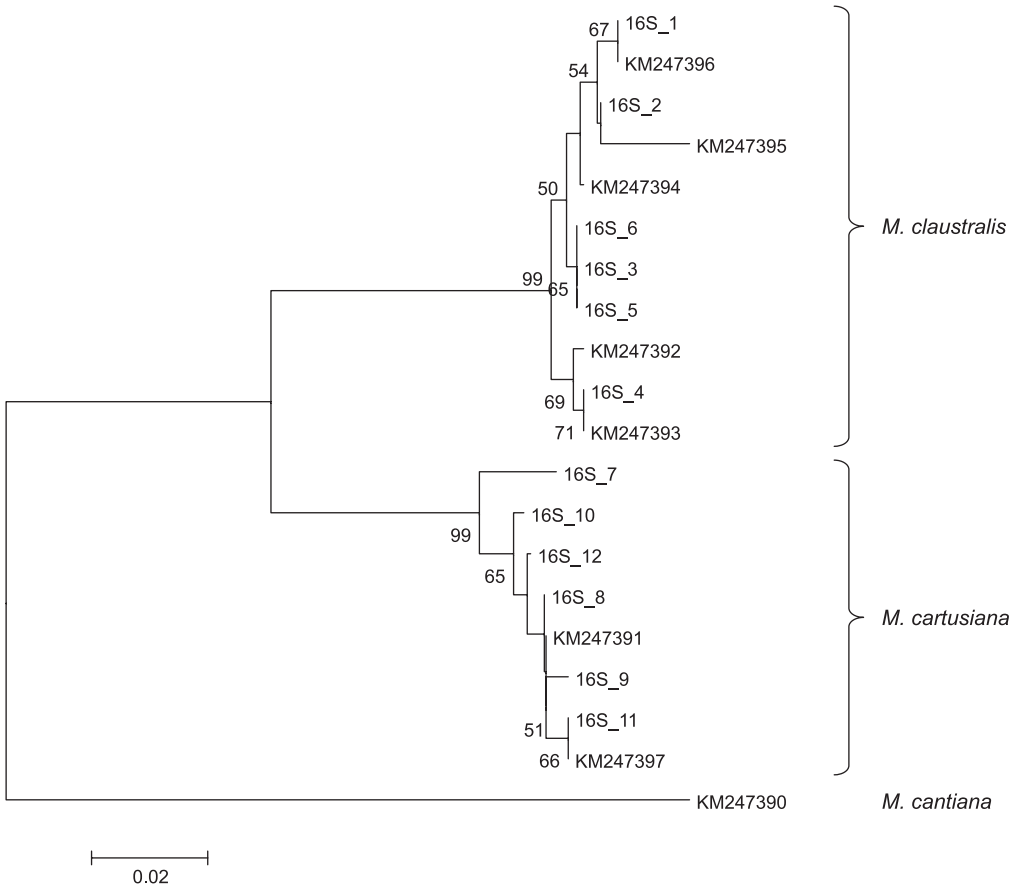

Fig. 2. Neighbour-Joining tree of new *16SrDNA* sequences of *M. claustralis* and *M. cartusiana*. The *16SrDNA* sequence of *M. cantiana* KM247390 (Pieńkowska et al. 2015) was chosen as outgroup. *M. claustralis* KM247392-KM247396 and *M. cartusiana* KM247391 & KM247397 (Pieńkowska et al. 2015) sequences were used as references. Calculation parameters were the same as for Fig. 1

Table 3. Combined *COI* and *16SrDNA* datasets for the analysed *Monacha* species

Combined haplotypes	*COI*	*16SrDNA*	Locality (reference)
Monacha claustralis			
Mclau-PL-h1	KM247384	KM247392	Poland: Janikowo (Pieńkowska et al. 2015)
Mclau-PL-h2	KM247385	KM247396	Poland: Poznań-Cybina, Poznań-Morasko, Kielce-Wietrznia (Pieńkowska et al. 2015)
Mclau-PL-h3	COI 1	16S 1	Poland: Kielce-Białogon, Kielce-Na Ługach, Kielce-Grzybowa, Morawica, Morawka, Jaworznia, Sobków, Poznań-Wola, Rogalinek, Puszczykowo (this paper)
Mclau-PL-h4	COI 2	16S 2	Poland: Jędrzejów, Sobków (this paper)
Mclau-PL-h5	COI 3	16S 4	Poland: Jędrzejów (this paper)
Mclau-PL-h6	COI 4	16S 3	Poland: Jędrzejów, Małogoszcz (this paper)
Mclau-PL-h7	COI 5	16S 3	Poland: Małogoszcz (this paper)
Mclau-PL-h8	COI 6	16S 4	Poland: Puszczykowo (this paper)
Mclau-PL-h9	COI 7	16S 5	Poland: Jastrzębia Góra, Chłapowo, Nadole, Krokowa (this paper)
Mcalu-PL-h10	COI 7	16S 6	Poland: Chłapowo (this paper)
Mclau-PL-h11	COI 8	16S 4	Poland: Krokowa (this paper)
Monacha cartusiana			
Mcart-PL-h1	KM247379	KM247391	Poland: Wrocław-Legnicka (Pieńkowska et al. 2015)
Mcart-PL-h2	KM247381	KM247391	Poland: Wrocław-Legnicka (Pieńkowska et al. 2015)
Mcart-PL-h3	COI 9	16S 7	Poland: Kielce-Wietrznia, Kielce-Grzybowa (this paper)
Mcart-PL-h4	COI 10	16S 8	Poland: Ostrowiec Świętokrzyski (this paper)
Mcart-PL-h5	COI 11	16S 8	Poland: Wrocław-Pilczyce (this paper)
Mcart-PL-h6	COI 12	16S 8	Poland: Wrocław-Pilczyce (this paper)
Mcart-BA-h1	COI 13	16S 9	Bosnia: Gornji Vakuf-Uskoplje (this paper)
Mcart-BA-h2	COI 14	16S 9	Bosnia: Gornji Vakuf-Uskoplje (this paper)
Mcart-XS-h1	COI 11	16S 10	Serbia: Belgrade (this paper)
Mcart-XS-h2	COI 11	16S 12	Serbia: Belgrade (this paper)
Mcart-XS-h3	COI 15	16S 7	Serbia: Belgrade (this paper)
Mcart-XS-h4	COI 16	16S 11	Serbia: Belgrade (this paper)
Mcart-XS-h5	COI 17	16S 11	Serbia: Belgrade (this paper)
Monacha cantiana (as an outgroup)			
Mcan-UK-h1	KM247375	KM247390	England: East Acton & Barrow (Pieńkowska et al. 2015)

imens from Prague and Saguramo. All the other sequences presented in Table 1 (COI 2, COI 4 – COI 8, as well as 16S 2, 16S 3, 16S 5, 16S 6) were unique, never reported for *M. claustralis* previously. It is noteworthy that populations of *M. claustralis* from Kielce-Na Ługach, Kielce-Białogon, Jaworznia, Morawka, Morawica, Poznań-Wola, Rogalinek and Nadole (newly discovered) as well as those from Poznań-Cybina, Poznań-Morasko and Kielce-Wietrznia (reported earlier: Pieńkowska et al. 2015) seem to be genetically uniform, with only one *COI* and *16SrDNA* haplotype. The population from Chłapowo is uniform in respect of *COI* haplotype, but not for *16SrDNA* (two haplotypes were found in its specimens, Table 1). An opposite situation was found in the Małogoszcz population: uniform in *16SrDNA* haplotype, but with two *COI* haplotypes (Table 1). Moreover, populations of *M. claustralis* from Puszczykowo, Jędrzejów and Sobków appeared genetically differentiated, with two (COI 1 and COI 6 in Puszczykowo) or three (COI 2, COI 3, COI 4 for Jędrzejów and COI 1, COI 2, COI 4 for Sobków) *COI* sequences as well as two (16S 1 and 16S 2 for Sobków) or three (16S 2, 16S 3 and 16S 4 for Jędrzejów) *16SrDNA* sequences.

It must be stressed that some sequences of *COI* (COI 7) and *16SrDNA* (16S 5) turned out to be peculiar to *M. claustralis* from northern Poland (found only in populations from Jastrzębia Góra, Chłapowo, Nadole and Krokowa; Table 1).

Overall, all the sequences were similar, with K2P distances of 0.0–4.3% for *COI* (Appendix 1) and 0.0–3.1% for *16SrDNA* (Appendix 2) and thus conspecific and representing *M. claustralis* (Table 2).

In the dendrograms (Figs 1, 2) sequences COI 9 and 16S 7, found in populations from Kielce-Wietrznia and Kielce-Grzybowa, COI 10 and 16S 8 from Ostrowiec Świetokrzyski as well as COI 11, COI 12 and 16S 8 from Wrocław-Pilczyce (Table 1) clustered with GenBank sequences for *M. cartusiana* from Wrocław-Legnicka (Poland), Kis-Balaton (Hungary), Prague (Czech Republic) and Brescia (Italy) (Pieńkowska et al. 2015). They differed in K2P distances from those GenBank sequences by 0.0–3.0% and 0.0–2.3% for *COI* and *16SrDNA*, re-

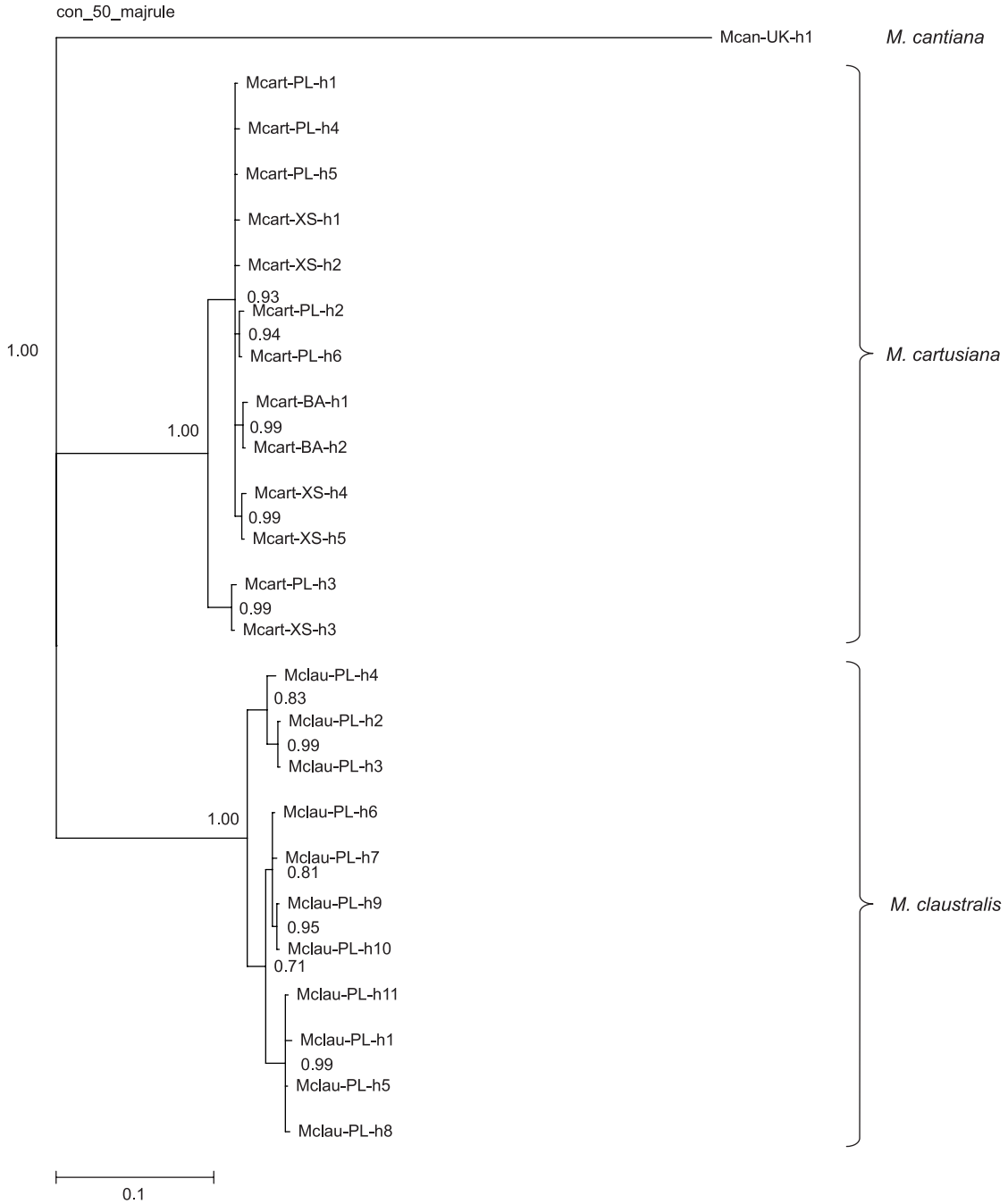

Fig. 3. Majority-rule consensus tree obtained from Bayesian inference analysis of the combined data set of *COI* and *16SrDNA* sequences of *M. claustralis* and *M. cartusiana* (Table 3). Posterior probabilities are marked at the nodes. The tree was rooted with *M. cantiana*. Two *M. claustralis* and two *M. cartusiana* sequences were used as reference (see Table 3)

spectively (Table 2, Appendices 1 & 2), which confirms their affiliation to *M. cartusiana*.

The fact that K2P distances between the sequences of *M. claustralis* and *M. cartusiana* were 12.3–15.1% and 9.3–11.9% for *COI* and *16SrDNA*, respectively (Table 2), shows that these gene fragments allow a clear distinction between the two species. It is noteworthy that the specimens from Wrocław-Pilczyce represent exactly the same population of *M. cartusiana* as that discovered in Wrocław in the 1970s (KOSIŃSKA 1973, 1979) and the sequence COI 12 is exactly the same as the GenBank sequence KM247380 for specimens from another population, Wrocław-Legnicka (PIEŃKOWSKA et al. 2015). The specimens from Wrocław-Pilczyce, like those from some other populations of *M. cartusiana*, i.e. Kis-Balaton and Wrocław-Legnicka, are differentiated in *COI* but not in *16SrDNA* sequences.

New sequences COI 13, COI 14 and 16S 9 were found in *M. cartusiana* from Gornji Vakuf-Uskoplje (Bosnia and Herzegovina). On the other hand, new sequences COI 15 – COI 17 and 16S 10 – 16S 12

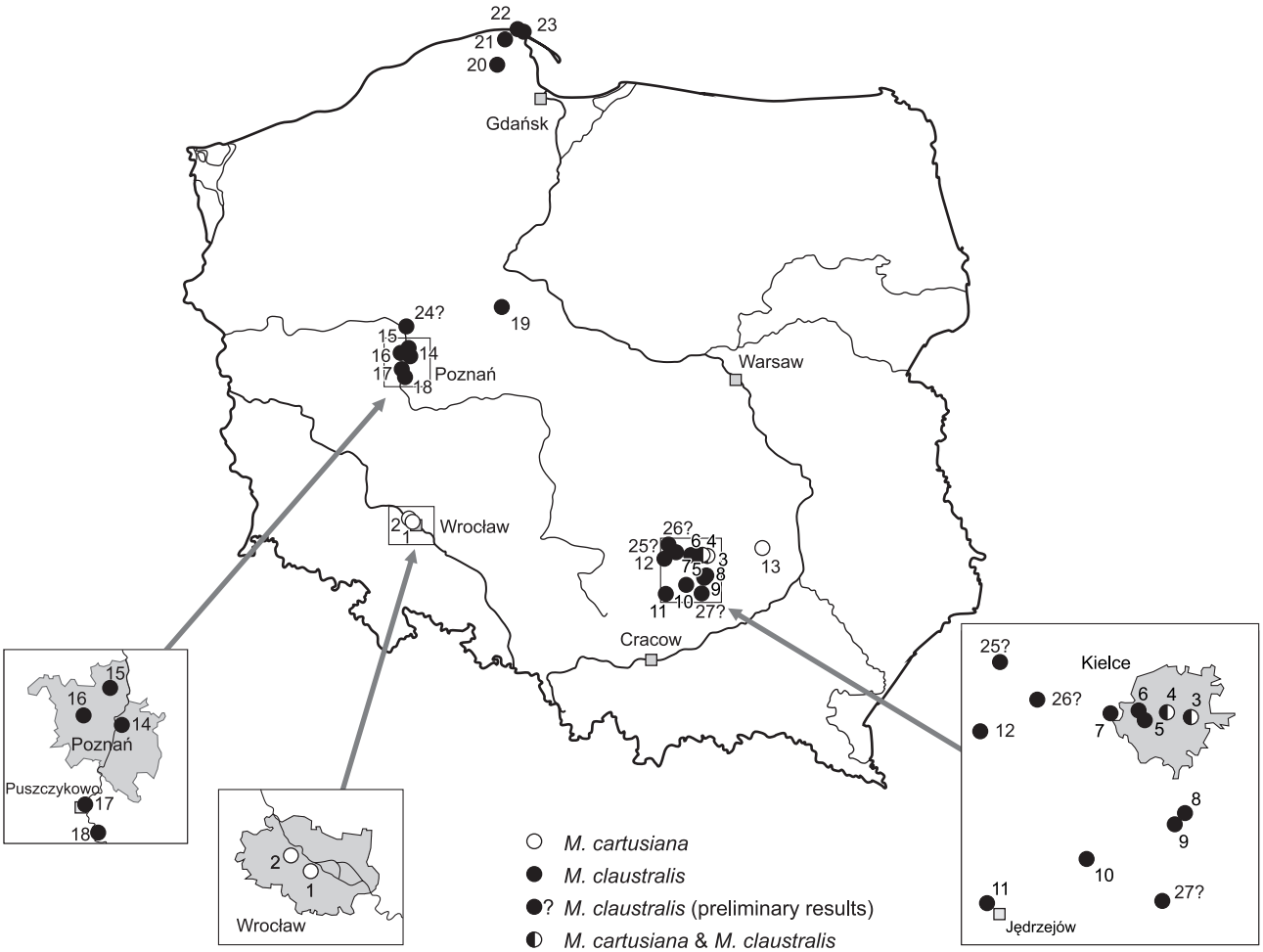

Fig. 4. Distribution of *M. cartusiana* and *M. claustralis* in Poland: 1 – Wrocław-Pilczyce; 2 – Wrocław-Legnicka; 3 – Kielce-Wietrznia; 4 – Kielce-Grzybowa; 5 – Kielce-Na Ługach; 6 – Kielce-Białogon; 7 – Jaworznia near Kielce; 8 – Morawka near Kielce; 9 – Morawica near Kielce; 10 – Sobków near Jędrzejów; 11 – Jędrzejów; 12 – Małogoszcz; 13 – Ostrowiec Świętokrzyski; 14 – Poznań-Cybina; 15 – Poznań-Morasko; 16 – Poznań-Wola; 17 – Puszczykowo near Poznań; 18 – Rogalinek near Poznań; 19 – Janikowo; 20 – Nadole; 21 – Krokowa, 22 – Chłapowo; 23 – Jastrzębia Góra; 24 – Murowana Goślina; 25 – Snochowice; 26 – Jeżynów near Bławatków; 27 – "Ślichowice" Nature Reserve

were characteristic of the Belgrade (Serbia) population. They clustered with sequences for *M. cartusiana* in both NJ and Bayesian trees (Figs 1–3). K2P distances between these sequences and those deposited in GenBank (Appendices 1 & 2) confirm that the populations from Bosnia and Serbia are conspecific. It is the first molecular confirmation of the occurrence of the true *M. cartusiana* in these countries, earlier reported only based on conchological and anatomical identification (WELTER-SCHULTES 2012). It is noteworthy that sequences COI 11 and 16S 7 were also found in some Polish populations (COI 11 from Wrocław-Pilczyce and 16S 7 from Kielce-Grzybowa and Kielce-Wietrznia).

The results presented in this paper show that *M. cartusiana* has a very limited distribution in Poland. It occurs within the city boundaries of Wrocław (Dolnośląskie Province) (Fig. 4) where it was first discovered by KOSIŃSKA (1973, 1979). Three new localities are reported in this paper (two in Kielce and one in Ostrowiec Świętokrzyski: Świętokrzyskie Province). It is noteworthy that co-occurrence of *M. cartusiana* and *M. claustralis*, previously mentioned from Prague (Czech Republic) (PIEŃKOWSKA et al. 2015), was also observed in some of the Polish localities (Kielce-Wietrznia and Kielce-Grzybowa).

M. claustralis is much more widespread in Poland (Fig. 4). As shown in the present study, its new populations were found in several places near Kielce (Świętokrzyskie Province, S. Poland) and near Poznań (Wielkopolskie Province, W. Poland). Four populations were found in Pomorskie Province (N. Poland). Earlier, a large population was discovered at Janikowo (Kujawsko-Pomorskie Province, central Poland, PIEŃKOWSKA et al. 2015). It can be expected that other populations will be located soon, as the species quickly expands its distribution range. There are some preliminary observations of new populations of *M. claustralis* in Murowana Goślina near Poznań (B. GOŁDYN, unpublished). We have already

found COI 1 haplotype in some specimens collected by one of us (M. GÓRKA) in Jeżynów, Snochowice and Ślichowice nature reserve, all near Kielce (Fig. 4), however these preliminary results should be confirmed based on a more extensive material, because of the possible co-occurrence of *M. cartusiana* in Świętokrzyskie Province.

M. claustralis and *M. cartusiana* are another pair of morphologically similar species which invaded Poland very recently. Similar pairs were recognised among both land and freshwater molluscs, for example *Arion vulgaris* (Moquin-Tandon, 1855) and *A. rufus* (Linnaeus, 1758) (SOROKA et al. 2009), *Corbicula fluminea* (O. F. Müller, 1774) and *C. fluminalis* (O. F. Müller, 1774) (DOMAGAŁA et al. 2004, ŁABĘDZKA et al. 2005), *Dreissena polymorpha* (Pallas, 1771) and *D. rostriformis bugensis* (Andrusov, 1897) (KOŁODZIEJCZYK et al. 2011, WOŹNICZKA et al. 2016). Molecular analysis could be useful in their correct identification.

CONCLUSIONS

M. cartusiana, discovered in Poland much earlier than *M. claustralis*, still has a very limited distribution in the country. Apart from the city of Wrocław (Dolnośląskie Province, SW. Poland) in which it was first discovered in the 1970s, it was found in three new localities (all in Świętokrzyskie Province, S. Poland).

M. claustralis, although discovered in Poland ca. 30 years later than *M. cartusiana*, turned out to be much more widespread in the country, being present in its southern, western, central and northern parts. As shown here, new populations are continuously discovered and this confirms that the species is quickly expanding its distribution range.

ACKNOWLEDGEMENTS

We thank ANDRZEJ WIKTOR and MAŁGORZATA PROĆKÓW (University of Wrocław, Poland) for providing specimens. We also thank FOLCO GIUSTI, GIUSEPPE MANGANELLI and VIVIANA FIORENTINO (Universitá di Siena, Italy) for their useful comments on the manuscript.

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Received: May 24th, 2016
Revised: August 4th, 2016
Accepted: September 2nd, 2016
Published on-line: December 5th, 2016

Appendix 1

K2P pair-wise distances of the analysed *COI* sequences from Polish, Bosnian and Serbian specimens of *M. claustralis* and *M. cartusiana*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	COI 1																																
2	COI 2	0.010																															
3	COI 3	0.035	0.035																														
4	COI 4	0.025	0.025	0.016																													
5	COI 5	0.026	0.026	0.017	0.002																												
6	COI 6	0.037	0.037	0.002	0.017	0.019																											
7	COI 7	0.026	0.026	0.017	0.002	0.003	0.019																										
8	COI 8	0.032	0.032	0.003	0.012	0.014	0.005	0.014																									
9	KM247383	0.035	0.035	0.000	0.016	0.017	0.002	0.017	0.003																								
10	KM247384	0.037	0.037	0.002	0.014	0.016	0.003	0.016	0.005	0.002																							
11	KM247385	0.000	0.010	0.035	0.025	0.026	0.037	0.026	0.032	0.035	0.037																						
12	KM247386	0.026	0.026	0.017	0.002	0.003	0.019	0.003	0.014	0.017	0.016	0.026																					
13	KM247387	0.033	0.033	0.039	0.032	0.033	0.041	0.033	0.035	0.039	0.041	0.033	0.033																				
14	KM247388	0.033	0.030	0.041	0.033	0.035	0.043	0.035	0.037	0.041	0.043	0.033	0.035	0.010																			
15	COI 9	0.136	0.136	0.136	0.129	0.131	0.138	0.132	0.131	0.136	0.133	0.136	0.131	0.125	0.127																		
16	COI 10	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.028																	
17	COI 11	0.140	0.138	0.144	0.138	0.140	0.146	0.140	0.140	0.144	0.142	0.140	0.140	0.127	0.129	0.026	0.002																
18	COI 12	0.142	0.140	0.146	0.140	0.142	0.148	0.142	0.142	0.146	0.144	0.142	0.142	0.129	0.131	0.028	0.003	0.002															
19	COI 13	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.024	0.007	0.005	0.007														
20	COI 14	0.136	0.133	0.140	0.134	0.136	0.142	0.136	0.136	0.140	0.138	0.136	0.136	0.123	0.125	0.026	0.005	0.003	0.005	0.002													
21	COI 15	0.134	0.134	0.133	0.127	0.129	0.136	0.129	0.129	0.133	0.131	0.134	0.129	0.123	0.125	0.002	0.026	0.024	0.026	0.026	0.024												
22	COI 16	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.026	0.005	0.003	0.005	0.005	0.003	0.024											
23	COI 17	0.140	0.138	0.144	0.138	0.140	0.146	0.140	0.140	0.144	0.142	0.140	0.140	0.127	0.129	0.028	0.003	0.002	0.003	0.007	0.005	0.026	0.002										
24	KM247376	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.026	0.005	0.003	0.005	0.009	0.007	0.024	0.003	0.002									
25	KM247377	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.026	0.005	0.003	0.005	0.005	0.003	0.024	0.003	0.005	0.007								
26	KM247378	0.138	0.136	0.142	0.136	0.138	0.144	0.138	0.138	0.142	0.140	0.138	0.138	0.125	0.127	0.024	0.003	0.002	0.003	0.003	0.002	0.023	0.002	0.003	0.005	0.002							
27	KM247379	0.140	0.138	0.144	0.138	0.140	0.146	0.140	0.140	0.144	0.142	0.140	0.140	0.127	0.129	0.026	0.002	0.000	0.002	0.005	0.003	0.024	0.003	0.002	0.003	0.003	0.002						
28	KM247380	0.142	0.140	0.146	0.140	0.142	0.148	0.142	0.142	0.146	0.144	0.142	0.142	0.129	0.131	0.028	0.003	0.002	0.000	0.007	0.005	0.026	0.005	0.003	0.005	0.005	0.003	0.002					
29	KM247381	0.144	0.142	0.148	0.142	0.144	0.151	0.144	0.144	0.148	0.146	0.144	0.144	0.131	0.134	0.030	0.005	0.003	0.002	0.009	0.007	0.028	0.007	0.005	0.007	0.007	0.005	0.003	0.002				
30	KM247382	0.142	0.140	0.146	0.140	0.142	0.148	0.142	0.142	0.146	0.144	0.142	0.142	0.129	0.127	0.028	0.003	0.002	0.003	0.007	0.005	0.026	0.005	0.003	0.005	0.005	0.003	0.002	0.003	0.005			
31	KM247389	0.142	0.140	0.146	0.140	0.142	0.148	0.142	0.142	0.146	0.144	0.142	0.142	0.129	0.132	0.030	0.005	0.003	0.005	0.009	0.007	0.028	0.003	0.002	0.003	0.007	0.005	0.003	0.005	0.007	0.005		
32	KM247375	0.215	0.215	0.210	0.212	0.214	0.210	0.212	0.212	0.210	0.207	0.215	0.214	0.192	0.206	0.200	0.200	0.203	0.205	0.196	0.198	0.203	0.200	0.202	0.205	0.203	0.200	0.203	0.205	0.207	0.205	0.200	

Appendix 2

K2P pair-wise distances of the analysed *16SrDNA* sequences from Polish, Bosnian and Serbian specimens of *M. claustralis* and *M. cartusiana*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 16S 1																				
2 16S 2	0.004																			
3 16S 3	0.011	0.008																		
4 16S 4	0.015	0.011	0.011																	
5 16S 5	0.011	0.008	0.000	0.011																
6 16S 6	0.011	0.008	0.000	0.011	0.000															
7 KM247392	0.019	0.015	0.008	0.004	0.008	0.008														
8 KM247393	0.015	0.011	0.011	0.000	0.011	0.011	0.004													
9 KM247394	0.008	0.004	0.004	0.015	0.004	0.004	0.011	0.015												
10 KM247395	0.019	0.015	0.023	0.027	0.023	0.023	0.031	0.027	0.019											
11 KM247396	0.000	0.004	0.011	0.015	0.011	0.011	0.019	0.015	0.008	0.019										
12 16S 7	0.101	0.097	0.105	0.101	0.105	0.105	0.106	0.101	0.105	0.119	0.101									
13 16S 8	0.101	0.097	0.105	0.101	0.105	0.105	0.106	0.101	0.105	0.110	0.101	0.019								
14 16S 9	0.106	0.101	0.110	0.106	0.110	0.110	0.110	0.106	0.110	0.114	0.106	0.023	0.004							
15 16S 10	0.097	0.093	0.101	0.097	0.101	0.101	0.101	0.097	0.101	0.106	0.097	0.023	0.004	0.008						
16 16S 11	0.106	0.101	0.110	0.106	0.110	0.110	0.110	0.106	0.110	0.110	0.106	0.023	0.004	0.008	0.008					
17 16S 12	0.097	0.093	0.101	0.097	0.101	0.101	0.101	0.097	0.101	0.106	0.097	0.023	0.004	0.008	0.008	0.008				
18 KM247391	0.101	0.097	0.105	0.101	0.105	0.105	0.106	0.101	0.105	0.110	0.101	0.019	0.000	0.004	0.004	0.004	0.004			
19 KM247397	0.106	0.101	0.110	0.106	0.110	0.110	0.110	0.106	0.110	0.110	0.106	0.023	0.004	0.008	0.008	0.000	0.008	0.004		
20 KM247390	0.219	0.224	0.213	0.219	0.213	0.213	0.214	0.219	0.218	0.238	0.219	0.213	0.208	0.213	0.203	0.213	0.213	0.208	0.213	