Monacha samsunensis (Pfeiffer, 1868): another Anatolian species introduced to Western Europe, where it is known as Monacha atacis
Gittenberger & de Winter, 1985 (Gastropoda: Eupulmonata: Hygromiidae)

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Abstract
Populations of Monacha atacis from southern Occitania in France and of M. samsunensis from northern Anatolia in Turkey (Atakum/Samsun and Kastamonu) were investigated by an integrative approach based on morphological (shell and genitalia) and molecular (mitochondrial and nuclear gene sequences) features. Morphological examination revealed a complex pattern of variation within and between geographically separated populations, while molecular analysis showed strong similarity between the two species, confirming earlier suggestions that the species are conspecific. Pfeiffer’s name Helix samsunensis introduced in 1868 has priority over the name M. atacis given by Gittenberger & de Winter in 1985.

Keywords: Genital anatomy, molecular features, shell, species delimitation, synonymy

Introduction
Monacha Fitzinger, 1833 is a speciose hygromiid genus with species occurring from Britain and north-western France to the Caucasus, Middle East and north African coast (Hausdorf 2000a, 2000b; Welter-Schultes 2012; Neiber & Hausdorf 2017 and other references therein). After Hesse (1914), Hausdorf (2000a) recognised three subgenera within Monacha on the basis of presence/absence of penial retractor muscle and vaginal appendix: Monacha s.s. Fitzinger, 1833 (type species: Helix cartusiana Müller, 1774) for species without penis retractor but with appendix, Metatheba Hesse, 1914 (type species: Helix samsunensis Pfeiffer, 1868) for species with penial retractor but without appendix, and Paratheba Hesse, 1914 (type species: Helix fruticola Krynicki, 1833) for species with both penial retractor and appendix. In an excellent integrative phylogenetic and biogeographic analysis of Monacha based on anatomical features of the reproductive system and molecular data (mitochondrial and nuclear gene sequences), Neiber and Hausdorf (2017) established four new subgenera: Pontotheba (type species: Monacha (Paratheba) bithynica Hausdorf, 2000a), Aegaeotheba (type species: Monacha (Paratheba) cretica Hausdorf, 2003), Trichotheba (type species: Monacha (Monacha) comata Hausdorf, 2000a), Rhytidotheba (type species: Helix (Trichia) densecostulata Retowski, 1887). They also resurrected the subgenus Platytheba Pilsky, 1895 (type species: Caracolla nummus Ehrenberg, 1831) but left the status of Euthela Nordsieck, 1993 unresolved.

Most Monacha species have limited ranges of distribution, restricted to their type localities, or if...
wider, to the southern Balkans and Anatolia (especially the Pontic region along the Black Sea coast). The exceptions include three species from the subgenus *Monacha* s.s., namely *M. cartusiana* (Müller, 1774), *M. clausalis* (Rossmässler, 1834) and *M. cantiana* (Montagu, 1803). *M. cartusiana* is widespread throughout Europe except in the north-east (Scandinavia, Russia, Baltic states, Belarus, northern Ukraine) (Welter-Schultes 2012). *M. clausalis* is now spreading quickly northward (Pinter 1968; Hlaváč & Peltanová 2010; Pieńkowska et al. 2015, 2016, 2018a; Hutchinson et al. 2019; Čejka et al. 2020; Gural-Sverlova & Gural 2022) from its native range in European and Anatolian Turkey (Hausdorf 2000a) and Greece (with Corfu/Kerkyra as type locality, Welter-Schultes 2012). *M. cantiana* is found in Great Britain, northern France and Germany, in the Benelux countries as well as in Spain, where it was probably introduced in Roman times from its native area in central Italy (Kerney et al. 1964; Kerney 1970; Evans 1972; Pieńkowska et al. 2018b). *Monacha* (*Platytheba*) *ocellata* (Roth 1839), known from the vicinity of Istanbul in Turkey, was recently discovered in a single locality in Britain, probably resulting from passive introduction in unknown circumstances (Anderson et al. 2018).

All but one species of subgenus *Metatheba* occur in northern Anatolia, mainly along the Black Sea coast. The only exception is *M. (Metatheba) atacis* Gittenberger & de Winter, 1985, known from southern Occitania, France (Hausdorf 2000a; Falkner et al. 2002; Gargominy et al. 2011; Neiber & Hausdorf 2017) and a site in Catalonia, north-eastern Spain (Bertrand 2003). However, when describing the new species, Gittenberger and de Winter (1985) already drew attention to its close relationship with *M. samsunensis* (Pfeiffer, 1868). Considering the great similarity between *M. atacis* and *M. samsunensis*, Hausdorf (2000a) and Neiber and Hausdorf (2017) suggested that despite their disjunct ranges these two taxa could be conspecific and that the French populations of *M. atacis* might be the result of introduction of *M. samsunensis* in historical times. One of us (MP) collected *M. atacis* in several sites in the foothills of the Pyrenees, southern France, while another member of our team (GG) found *M. samsunensis* in two localities in northern Anatolia, Turkey, one in the vicinity of her university in Kastamonu and the other near Atakum/Samsun, i.e. in the type locality of the species. This enabled us to undertake the task of verifying the hypothesis of Hausdorf (2000a) and Neiber and Hausdorf (2017, also see Cadevall et al. 2020: p. 155). The results of our study are reported in this paper.

**Material and methods**

**Taxonomic sampling**

Specimens for research were collected on the basis of the literature data on the occurrence of *M. atacis* in France (Gittenberger & de Winter 1985) and *M. samsunensis* in Turkey (Hausdorf 2000a; Welter-Schultes 2012). Species identification was based on morphological and molecular research. Thus, the specimens were obtained from 18 populations of *Monacha atacis* from southern France and three populations of *M. samsunensis* from northern Anatolia in Turkey. They were considered in our analysis of molecular and genital structure (Table I and Figure 1). A new French population of *M. cartusiana* (Table I) as well as literature data on several *Monacha* species and lineages were used in the analysis. Several sequences deposited in GenBank (Table II) were also used for molecular analysis. Sequences of *Trochulus hispidus* (Linnaeus, 1758) from GenBank were used as an outgroup to construct phylogenetic trees.

**Material examined**

New material examined is listed as follows, when possible: geographic coordinates of locality, locality (country, region, municipality and province, site), collector(s), date, number of specimens, with the collection where the material is kept in parenthesis (Table I). The material is kept in the collection of the Department of Cell Biology, Adam Mickiewicz University, Poland (DCBC), the Małgorzata Prochów collection (MNHW; Museum of Natural History, University of Wrocław, Poland) and the Folco Giusti collection (FGC; Dipartimento di Scienze Fisiche, della Terra e dell’Ambiente, Università di Siena, Italy). The material used for comparison has already been described (see Pieńkowska et al. 2018b: table 1, 2019a: table 1, 2020: table 1).

**Morphological study**

Twenty-four specimens from 15 sites in France and ten specimens from two sites in Turkey were analysed for shell and anatomy (see Table I). Snail bodies were dissected under a light microscope (Wild M5A, or Zeiss SteREO Lumar. V12). Anatomical details were drawn using a Wild camera lucida. Adult specimens from Turkey were obtained in sufficient number to describe their genital structure, however the scarcity of specimens per population (where several were juveniles or subadults) meant that no quantitative analysis was done on the morphological characters.
| No. | coordinates | country and site | collector / date | Designation (no. specimen) | COI | 16SrDNA | H3 | dsTS2 (5.8S rDNA + ITS2) | lgiTS2 (0.58S rDNA + ITS2 + 28S rDNA) |
|-----|-------------|-----------------|-----------------|-----------------------------|-----|---------|----|----------------|-------------------------------|
| 1   | 42°44'27.8"N | France, Occitania, Altoige, Auzat near Aurzet, edge of spruce forest, 984 m a.s.l. | M. Prochôve / 30.07.2018 / 2 / (DCRC & MNHW- F18.38) | M. atacis 1 | COI1 (1) | ON325379 | 16S+1 (1) | ON350884 H3 1 (3) | ON353131                        |
|     | 01°28'34.7"E |                 |                 | M. atacis 2 | COI2 (1) | ON325480 | 16S+2 (1) | ON350885 H3 2 (3) | ON353132                        |
| 2   | 42°52'6.5"N  | France, Occitania, Aude, Le Chandelier, clearing, 826 m a.s.l. | M. Prochôve / 27.06.2018 / 1 / (DCRC & MNHW- F18.38) | M. atacis 1 | COI1 (1) | ON325891 | 16S+3 (1) | ON350886 H3 1 (3) | ON353133                        |
|     | 02°01'51.9"E |                 |                 | M. atacis 2 | COI2 (1) | ON325891 | 16S+3 (1) | ON350886 H3 1 (3) | ON353133                        |
| 3   | 42°48'35.5"N | France, Occitania, Aude, near Aurzet, vegetation along road, 466 m a.s.l. | M. Prochôve / 26.06.2018 / 5 / (DCRC & MNHW- F18.28) | M. atacis 1 | COI1 (2) | ON325928 | 16S+4 (1) | ON350887 H3 2 (3) | ON353147                        |
|     | 02°15'04.0"E |                 |                 | M. atacis 2 | COI2 (1) | ON325984 | 16S+5 (1) | ON350889 H3 2 (3) | ON353147                        |
|     |                 |                 |                 | M. atacis 3 | COI3 (1) | ON325984 | 16S+5 (1) | ON350889 H3 2 (3) | ON353147                        |
| 4   | 42°48'40.2"N  | France, Occitania, Aude, near Lapandelle, roadside, 497 m a.s.l. | M. Prochôve / 26.06.2018 / 5 / (DCRC & MNHW- F18.28) | M. atacis 1 | COI1 (2) | ON325928 | 16S+6 (1) | ON350889 H3 2 (3) | ON353147                        |
|     | 02°17'12.2"E |                 |                 | M. atacis 2 | COI2 (2) | ON325928 | 16S+6 (1) | ON350889 H3 2 (3) | ON353147                        |
|     |                 |                 |                 | M. atacis 3 | COI3 (1) | ON325928 | 16S+6 (1) | ON350889 H3 2 (3) | ON353147                        |
| 5   | 42°43'39.2"N  | France, Occitania, Ariège, Massif des Deux Alpes, vegetation between road, 1518 m a.s.l. | M. Prochôve / 26.06.2018 / 5 / (DCRC & MNHW- F18.50) | M. atacis 1 | COI1 (1) | ON325928 | 16S+7 (1) | ON350891 H3 1 (3) | ON353129                        |
|     | 02°01'22.0"E |                 |                 | M. atacis 2 | COI2 (1) | ON325928 | 16S+7 (1) | ON350891 H3 1 (3) | ON353129                        |
|     |                 |                 |                 | M. atacis 3 | COI3 (1) | ON325928 | 16S+7 (1) | ON350891 H3 1 (3) | ON353129                        |
| 6   | 42°44'19.7"N  | France, Occitania, Aude, near Girro de de Majoer, vegetation between road, 729 m a.s.l. | M. Prochôve / 26.06.2018 / 5 / (DCRC & MNHW- F18.41) | M. atacis 1 | COI1 (1) | ON325928 | 16S+8 (1) | ON350891 H3 1 (3) | ON353129                        |
|     | 02°11'35.8"E |                 |                 | M. atacis 2 | COI2 (1) | ON325928 | 16S+8 (1) | ON350891 H3 1 (3) | ON353129                        |
|     |                 |                 |                 | M. atacis 3 | COI3 (1) | ON325928 | 16S+8 (1) | ON350891 H3 1 (3) | ON353129                        |
|     |                 |                 |                 | M. atacis 4 | COI4 (1) | ON325928 | 16S+8 (1) | ON350891 H3 1 (3) | ON353129                        |
| 7   | 42°45'25.1"N, | France, Occitania, Aude, Campagna-de-Sault, vegetation along the road, 1024 m a.s.l. | M. Prochôve / 40.07.2018 / 5 / (DCRC & MNHW- F18.53) | M. atacis 1 | COI1 (1) | ON325928 | 16S+9 (1) | ON350901 H3 1 (3) | ON353131                        |
|     | 02°03'27.6"E |                 |                 | M. atacis 2 | COI2 (1) | ON325928 | 16S+9 (1) | ON350901 H3 1 (3) | ON353131                        |
|     |                 |                 |                 | M. atacis 3 | COI3 (1) | ON325928 | 16S+9 (1) | ON350901 H3 1 (3) | ON353131                        |

(Continued)
| No. | coordinates | collector / date / locality | Designation | COI new haplotype | GenBank no. | 16SrDNA new haplotype | GenBank no. | HB new common haplotype | GenBank no. | sITS2 new common haplotype | GenBank no. | lgITS2 (5.8S rDNA + sITS2) | GenBank no. | IT + 28S rDNA | GenBank no. | AA | Flgs |
|-----|-------------|-----------------------------|-------------|----------------|-------------|-----------------------|-------------|------------------------|-------------|--------------------------|-------------|-------------------------|-------------|--------|-------------|-------|
| 8   | 42°51'20.8"N | 02°13'32.5"E | Occitania, Aude, Saint-Ferrol 1, vegetation, 454 m a.s.l. | M. aatis | For2-1 | COI 1 (no. spec.) | ON32602 | 145 137 (1) | ON32602 | 145 137 (2) | ON32636 | sITS2 2 (1) | ON32753 | + | | |
| 9   | 42°57'14.4"N | 02°12'39.4"E | Occitania, Aude, Saint-Ferrol 2, garigue shrubs, 338 m a.s.l. | M. aatis | For2-1 | COI 6 (no. spec.) | ON32607 | 145 127 (1) | ON32607 | 145 127 (2) | ON32631 | sITS2 2 (1) | ON32756 | + | | | 3 |
| 10  | 42°49'34.2"N | 02°13'38.8"E | Occitania, Aude, Saint-Martin-Lys, vegetation on rocky wall, 335 m a.s.l. | M. aatis | Lys 1 | COI 8 (no. spec.) | ON32612 | 145 149 (1) | ON32612 | 145 149 (2) | ON32645 | sITS2 1 (1) | ON32759 | + | | | |
| 11  | 42°49'55.8"N | 02°03'23.4"E | Occitania, Aude, Belbœuf-Roubiny 1, roadside, vegetation under trees, 704 m a.s.l. | M. aatis | Reb1-1 | COI 1 (no. spec.) | ON32617 | 145 169 (1) | ON32617 | 145 169 (2) | ON32649 | sITS2 10 (1) | ON32763 | + | | | |
| 12  | 42°40'47.2"N | 02°18'32.6"E | Occitania, Aude, Sauvignes, roadside, 547 m a.s.l. | M. aatis | Sá 1 | COI 2 (no. spec.) | ON32621 | 145 221 (1) | ON32621 | 145 221 (2) | ON32653 | sITS2 12 (1) | ON32766 | + | | | |
| 13  | 42°40'41.0"N | 02°13'06.7"E | Occitania, Aude, Georges de Saint-Georges, vegetation along road, 440 m a.s.l. | M. aatis | Gor 1 | COI 5 (no. spec.) | ON32626 | 145 184 (1) | ON32626 | 145 184 (2) | ON32658 | sITS2 7 (1) | ON32769 | + | | | |
| 14  | 42°38'32.2"N | 02°02'04.3"E | Occitania, Aude, Belbœuf-Roubiny 2, woodland near eratic stones, 750 m a.s.l. | M. aatis | Reb2-1 | COI 8 (no. spec.) | ON32631 | 145 149 (1) | ON32631 | 145 149 (2) | ON32662 | sITS2 3 (2) | ON32771 | + | | | |
| No. | collector / date | coordinates | country and site | COI (5.8S rDNA) | 16S rDNA | H3 | sITS2 (5.8S rDNA + ITS2) | lgITS2 (5.8S rDNA + ITS2 + 28S rDNA) |
|-----|-----------------|-------------|-----------------|----------------|-----------|----|----------------|------------------------------------------|
| 18  | M. Paschoire / 29.06.2018 / 5 / (DCBC & MINHFW, F 18.42) | 42°36'37"N, 5°43'00"E | France, Occitania, Aude, vegetation beneath rooks, 838 m a.s.l. | M. scissifrons | COR 4 (1) | ON32365 | 16S 7' (1) | ON30941 | H3 1 (1) | ON325364 |
| 19  | G. Mangendie / 15.07.2004 / 1 / (FGC35773) | 41°23'00"N, 0°22'30"E | France, Occitania, Aude, Carcassonne | M. scissifrons | FGC 35773 | = M. scissifrons | KX97213 | KX495402 |
| 20  | unknown (Neber & Hauffen 2017: ZMH 119337 / 2637) | 42°22'30"N, 0°22'30"E | France, Occitania, Aude, Carcassonne | M. scissifrons | | | | |
| 21  | G. Gürelli / 20.09.2017 / 1 | 41°24'30"N, 0°22'30"E | Turkey, Kásefent naighbourhood, Kartonosu, near University Central Research Laboratory | M. samsunensis | Sam2 | 16S 19' (3) | ON30946 | H3 1 (7) | ON325355 | sITS2 13 (3) | ON319277 |
| 22  | Sam3 | 16S 20' (1) | ON30948 | ON32571 | ON31728 |
| 23  | G. Gürelli / 30.09.2020 / 6 | 41°24'30"N, 0°22'30"E | Turkey, Kásefent naighbourhood, Kartonosu, near University Central Research Laboratory | M. samsunensis | Kut1 | 16S 19' (3) | ON30949 | ON32572 | sITS2 10 (4) | ON319277 |
| 24  | Kut2 | 16S 20' (1) | ON30950 | ON32573 | ON319278 |
| 25  | Kut3 | 16S 21' (1) | ON30951 | ON32574 | ON319281 |
| 26  | Kut4 | 16S 22' (1) | ON30952 | ON32575 | ON319282 |
| 27  | Kut5 | 16S 23' (1) | ON30953 | ON32576 | ON319283 |
| 28  | Kut6 | 16S 24' (1) | ON30954 | ON32577 | ON319284 |
| 29  | Kut7 | 16S 25' (1) | ON30955 | ON32578 | ON319285 |
| 30  | G. Gürelli / 8.09.2021 / 4 | 41°18'34"N, 0°22'30"E | Turkey, Balq, neighbourhood, Atabak, Samsun | M. samsunensis | Rey1 | 16S 17' (1) | ON30956 | H3 1 (2) | ON325379 | sITS2 13 (1) | ON319286 |
| 31  | Rey2 | 16S 21' (1) | ON30957 | ON325380 | sITS2 14 (3) | ON319287 |
| 32  | Rey3 | 16S 22' (1) | ON30958 | ON325381 | ON319288 |
| 33  | Rey4 | 16S 23' (1) | ON30959 | ON325382 | ON319289 |
| No. | coordinates | country and site | collector / date / no. of specimens (collection) | Designation of voucher sps | COI GenBank no. | 16SrDNA GenBank no. | H3 GenBank no. | sITS2 (5.8S rDNA + ITS2) GenBank no. | lgITS2 (5.8S rDNA + ITS2 + 28S rDNA) GenBank no. | AA | Figs |
|-----|-------------|-----------------|-----------------------------------------------|---------------------------|----------------|-------------------|---------------|-------------------------------------|-----------------------------------------------|-----|------|
| 21  | 40°42'23"N 39°04'09"E | Turkey, Gümüşhane, Kızıltepe towards Timbula, 0.1-0.3 km along the road from junction towards Taslica kyü (Harsit river valley) | unknown (Neber & Haasdorf 2017) | M. samsunensis | ZMH 96199/2241 | KX507202 | KX495391 |                                  |                                  |     |
|     |              |                 |                                               |                           |                |       |               |                                     |                                     |     |
| 22  | 42°02'09.7"N 02°29'06.0"E | France, Occitania, Aude, Carcassonne-Cambes, roadside, 419 m a.s.t. | M. Prochowny / M. antiquata |                                  |                   |               |               |                                     |                                     |     |

Particular gene sequences were trimmed and then deposited in GenBank with the following lengths:
- Sequences COI were 684 bp long, except those for Kas1 - Kas5 which were 678 bp;
- Sequences 16SrDNA were 308-317 bp long, except those marked by asterisk which were 814-821 bp;
- Sequences H3 were 303 bp long;
- Sequences sITS2 (ITS2 flanked by 5.8S rDNA fragment) were of 558-581 bp (71 bp 5.8S + 487-510 bp ITS2);
- Sequences lgITS2 (complete ITS2 flanked by 5.8S and 28S rDNA fragments) were of 835-856 bp long (89 bp 5.8S + 489-510 bp ITS2 + 257 bp 28S).

AA – specimens used in anatomical studies marked with +.
Abbreviations: BC bursa copulatrix (also known as gametolytic gland), BW body wall, DBC duct of bursa copulatrix (also known as pedunculus), DG digitiform glands (also known as mucous glands or glandulae mucosae), DV distal vagina (from digitiform glands to genital atrium), E epiphallus (from base of flagellum to beginning of penial sheath), F flagellum, FO free oviduct, GA genital atrium, GAR genital atrium retractor, OSD ovispermiduct (also known as spermoviduct), P penis (from beginning of penial sheath to genital atrium), PP penial papilla (also known as glans), PR penial retractor, PV proximal vagina (from confluence of free oviduct and duct of bursa copulatrix to digitiform glands), VD vas deferens.

**Molecular study**

Sixty-three specimens of *M. atacis* and 14 of *M. samsunensis* were used in the molecular analysis (Table I). Total genomic DNA was extracted from 20 mg of foot tissue using Tissue Genomic DNA extraction MiniKit (Genoplast) following the manufacturer's instructions. Purified total DNA was used as template for amplification by polymerase chain reaction (PCR) of partial sequences of the following gene fragments: mitochondrial 5'-end of cytochrome c oxidase subunit I (COI) and large subunit ribosomal DNA gene (16S rDNA), as well as nuclear internal transcribed spacer 2 (ITS2) in ribosomal DNA flanked with 5.8S and 28S ribosomal DNA fragments (5.8S rDNA and 28S rDNA, respectively) and histone 3 (H3). Partial sequences of these gene fragments were obtained by PCR with the primer sets listed in Table III.

All PCRs were carried out with total volumes of 10 µl. The following thermal profile was used for COI amplification: 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 1 min at 50°C, 1 min at 72°C, and finally 5 min at 72°C using Type-it Microsatellite PCR kit (Qiagen) or 5 min at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C, and finally 7 min at 72°C using tTaq Polymerase (EUR). Amplitifications of fragments of 16S rDNA (short fragment, see Table III), H3 and ITS2 (flanked with 5.8S rDNA and short fragment of 28S rDNA, see Table III) were performed according to procedures previously described by Manganelli et al. (2005), Colgan et al. (1998) and Almeyda-Artigas et al. (2000), respectively. Amplitifications of 16S rDNA (longer fragment, see Table III) and ITS2 (flanked with 5.8S rDNA and longer fragment of 28S rDNA, see Table III) were performed with the same thermal profile as for COI amplification with tTaq Polymerase (EUR), however for this ITS2 sequence, two rounds of amplifications were performed: the first with the purified total DNA as template and the second with 1 µl of the 10x diluted product from the first round as template. Lengths of amplification products were as follows: COI – 710 bp; 16S rDNA – 371–382 (short fragments) or 873–882 bp (long fragments); ITS2 (flanked with 5.8S rDNA and short fragment of 28S rDNA) – 655–681 bp; ITS2 (flanked with 5.8S rDNA and longer fragment of 28S rDNA) – 911–932 bp; H3 – 375 bp.

The PCR products were verified by agarose gel electrophoresis (1% agarose) and purified for sequencing with thermostable Exonuclease I and FastAP alkaline phosphatase (Fermentas, Thermo Scientific). Finally, the amplified products were sequenced in both directions using the BigDye Terminator v3.1 sequencing kit on an ABI Prism 3130XL Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

Sequences were edited with BioEdit version 7.0.6 (Hall 1999; BioEdit 2017). Alignments were performed with ClustalW, implemented in BioEdit (Thompson et al. 1994). The COI and H3 sequences were aligned according to the translated amino acid sequences to correct errors that could arise from the presence of ambiguous nucleotides after sequencing. The ends of all sequences were trimmed and deposited in GenBank (Table I). Sequences obtained by PCR with NEWS2 and ITS2-RIXO, and LSU1 and LSU3 primer pairs (Table III) were joined in longer sequences marked as lgITS2 (see Table I). After trimming, the lengths of sequences were 684 and 678 bp for COI, 309–317 bp for 16S rDNA short fragment, 814–821 for 16S rDNA long fragment, 558–581 for short ITS2 (marked sITS2, including 71 bp 5.8S rDNA + 487–510 bp ITS2), 835–856 bp for long ITS2 (marked lgITS2, including 89 bp 5.8S rDNA + 489–510 bp ITS2 + 257 bp 28S rDNA) and 303 bp for H3 (see also Table I). For phylogenetic analysis, the following alignments were made: 600 positions long for COI, 332 or 869 positions long for 16S rDNA, 279 position long for H3. Sequences of ITS2 alone and sequences of ITS2 flanked by fragments of 5.8S rDNA at the 5′-end and 28S rDNA at the 3′-end, for comparison with sequences obtained from GenBank, were 546 (or 564) and 856 positions of alignment length, respectively. The sequences were collapsed to haplotypes (COI, 16S rDNA) and to common sequences (H3, ITS2 flanked with 5.8S rDNA, ITS2 flanked with 5.8S rDNA and 28S rDNA) using the programme ALTER (Alignment Transformation EnviRonment)
Finally COI and 16S rDNA haplotypes were joined into concatenated sequences COI+16S rDNA, 932 or 1469 positions (600 COI + 332 16S rDNA or 600 COI + 869 16S rDNA) in length, ITS2 and H3 common sequences were joined into concatenated sequences ITS2 + H3, 825 positions (546 ITS2 + 279 H3) in length and COI and 16S rDNA haplotypes were joined with ITS2 (flanked with 5.8S and 28S rDNA fragments) common sequences into concatenated sequences of COI + 16S rDNA + ITS2, 2325 positions in length (600 COI + 869 16S rDNA + 42 5.8S rDNA + 557 ITS2 + 257 28S rDNA).

For each alignment file, best nucleotide substitution models were specified according to the Bayesian Information Criterion (BIC): for COI (600 bp) – HKY+G+I, for concatenated sequences COI + short 16S rDNA (932 positions) – T92+G+I, for COI + 16S rDNA + ITS2 (flanked with 5.8S and 28S rDNA) – GTR+G+I, for COI + long 16S rDNA (1469 positions) – GTR+G, for ITS2 (564 bp) and for concatenated sequences ITS2 + H3 (825 positions) – K2 + G (Kimura 1980; Hasegawa et al. 1985; Tamura 1992; Nei & Kumar 2000; Kumar et al. 2016). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Neighbour Joining (NJ) analysis (Saitou & Nei 1987) and Maximum Likelihood (ML) analysis were performed with MEGA7 (Kumar et al. 2016). Calculated bootstrap values obtained by ML and NJ analysis were mapped on the ML trees. In addition, Bayesian Inference (BI) was conducted for concatenated COI + 16S rDNA + ITS2 (flanked with 5.8S and 28S rDNA) sequences with the use of the programme MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003; Ronquist et al. 2012). Four Monte Carlo Markov chains were run for 1 million generations, sampling every 100 generations (the first 25% of trees were discarded as “burn-in”). Posterior probability (PP) values obtained on the 50% majority rule consensus Bayesian tree of concatenated sequences were mapped together with bootstrap values obtained by ML and NJ analysis on the ML tree.

Figure 1. Map of localities of the populations of Monacha ataxis, M. samsunensis and M. cartusiana analysed (see Table I for details).
Table II. GenBank sequences used for molecular analysis comparisons.

| Species              | COI       | 16S rDNA | H3                  | (5.8S rDNA) + ITS2 + (5.8S rDNA) + ITS2 + (28S rDNA) | References                                      |
|----------------------|-----------|----------|---------------------|---------------------------------|-----------------------------------------------|
| Monacha atacis       | KX507213, KX507236 | KX495402, KX495430 | KX495455, KX495480 | Neiber and Hausdorf (2017) |
| Monacha cantiana     | KX507234 | KX495428 | KX496547 | Neiber and Hausdorf (2017) |
| Monacha cantiana CAN-1 | MG208884, MG208905 | MG208966, MG209038, MG209048 | MH137963*, MH137971*, MH137972*, MH137978* | Pieńkowska et al. (2018b) |
| Monacha cantiana CAN-2 | MG208925, MG208931 | MG208996, MG209050, MG209052 | MH137981* | Pieńkowska et al. (2018b) |
| Monacha cantiana CAN-3 | MG208933, MG208936 | MG209005, MG209040 | MK067000*, MK067001* | Pieńkowska et al. (2019a) |
| Monacha cantiana CAN-4 | MG208939, MG208940 | MG209011, MG209055, MG209059 | MH137984* | Pieńkowska et al. (2018b) |
| Monacha cantiana CAN-5 | MT947641 | MT952445 | MK066934, MK066938 | Pieńkowska et al. (2019a) |
| Monacha cantiana CAN-6 | MK066942, MK066943 | MK066960, MK066980 | MK066995*, MK066999* | Pieńkowska et al. (2019a) |
| Monacha cartusiana   | KX507189, KX507235 | KX495378, KX495429 | KX495431, KX495470, MG209072 | MH137993* | Pieńkowska et al. (2018b) |
| Monacha claustralis  | KX507199 | KX495388 | KX495411 | Neiber and Hausdorf (2017) |
| Monacha cretica      | KX507190 | KX495379 | KX495470 | Neiber and Hausdorf (2017) |
| Monacha devrekensis  | KX507200, KX507201 | KX495389, KX495390 | KX495441, KX495443, KX495443 | Neiber and Hausdorf (2017) |
| Monacha laxa         | KX507220 | KX495409 | KX495442, KX495443 | Neiber and Hausdorf (2017) |
| Monacha ocellata     | MG918127 | MG918128 | MG918129 | Anderson et al. (2018) |
| Monacha pantanellii  | MT380013, MT380014, MT380038, MT380063 | MT376033, MT385778, MT385781, MT385808 | MT376088*, MT376090*, MT376110* | Pieńkowska et al. (2020) |
| Monacha samuensis    | KX507202 | KX495391 | KX494444 | Neiber and Hausdorf (2017) |
| Monacha parumcinta   | MG208949, MG208955 | MG209023, MG209066, MG209067 | MH137985*, MH137987* | Pieńkowska et al. (2018b) |
| Monacha perfrequens  | KX507191 | KX495380 | KX495433 | Neiber and Hausdorf (2017) |
| Monacha tibarenica   | KX507227 | KX495421 | KX495472 | Neiber and Hausdorf (2017) |
Results

Morphological study

Monacha atacis and M. samsunensis have globose to subglobose shell (Figures 2–7), sometimes variably depressed, variable in size (in M. atacis: diam. 7.5–15.2 mm; Gittenberger & de Winter 1985; in M. samsunensis: diam. 11.0–19.1 mm; Hausdorf 2000a), pale yellowish, pale ochre or whitish in colour (creamy-white, sometimes with sparse darker stripes in M. samsunensis), sometimes with one whitish peripheral band (only in some light horn-coloured specimens of M. samsunensis according to Hausdorf 2000a), surface with fine irregular growth-ridges, very fine spiral striae and evident hair scars on the first whorls. Aperture roundish to oval, slightly descending, with a variably thick white internal rib (only very evident in M. samsunensis). Peristome interrupted, its columellar margin reflexed to more or less cover the umbilicus, which may be very small to almost closed or rather open (for M. atacis, see also Gittenberger & de Winter 1985: fig. 2; Welter-Schultes 2012: fig. at p. 503; for M. samsunensis, see also Hudec & Ležava 1969: pl.

Table II. (Continued).

| Species            | COI     | 16S rDNA  | H3          | References          |
|--------------------|---------|-----------|-------------|---------------------|
| Trochulus hipidus  | KY818415| KY818541  | KY818647    | Neiber et al. (2017) |
|                    |         |           | KX495451    | Neiber and Hausdorf (2017) |
|                    |         |           | MG585474    | Caro et al. (2019)    |
|                    |         |           | MT758614    |                     |

Table III. Primers used in molecular analysis.

| Name                   | Sequence 5’ – 3’ | References                        |
|------------------------|------------------|-----------------------------------|
| COI                    |                  |                                   |
| LCO1490                | GGTCACAACATCATAAAGATATTGG | Folmer et al. (1994) |
| HC02198                | TAAACTTCAGGGTGACCAAAAAATCA | Dabert et al. (2010) |
| F01                    | CATTTCACCCCACCATGATTTGG |                                   |
| R04                    | TATACACTCTCGACTATCA |                                   |
| 16S rRNA (shorter fragment) |                  |                                   |
| LR-J-12887 (reverse)   | CGATTTTGAACTCGATCA | Simon et al. (1994) |
| 16S rRNA (longer fragment) |                  |                                   |
| 16Scs1                 | AAAACATACCTTTTGCATAATGG | Chiba (1999) |
| 16Scs2                 | AGAACCTGACCTGCCGTTACG |                                   |
| ITS2 (flanked with 5.8S rDNA and short fragment of 28S rDNA) |        |                                   |
| NEWS2                  | TGTTGCTGAGAAGACGCAG | Almeyda-Artigas et al. (2000) |
| ITS2-RIXO              | TCTATGCTTAAAAATCAGGGG |                                   |
| ITS2 (flanked with for 5.8S rDNA and longer fragment of 28S rDNA) |     |                                   |
| LSU1                   | CTTGCTGCAAGAATATGGA | Wade and Mordan (2000) |
| LSU3                   | ACTTTCCTCAGCGTACTTG |                                   |
| H3                     |                   |                                   |
| H3F                    | ATGTCGATCCAAGCACCGC | Colgan et al. (1998) |
| H3R                    | ATATCCCTTGGCATRATGCA |                                   |
Monacha atacis and M. samsunensis show distal genitalia of the *Matatheba* type, i.e. with penial retractor muscle but without vaginal appendix. The literature reports evidence of a long vagina, bursa copulatrix duct and flagellum in *M. atacis* (Gittenberger & de Winter 1985) and a variably long vagina, bursa copulatrix duct and flagellum in *M. samsunensis* (Hesse 1931; Hudec & Ležava 1969; Hudec 1973; Schileyko 1978; Hausdorf 2000a). This picture was confirmed by our anatomical study. Both species showed vagina with very short proximal section and variably long distal section. In *M. atacis*, the distal vagina is long and variably slender, sometimes widening slightly in its subterminal portion where the internal surface shows a series of small swollen pleats (Figures 8 and 12). In *M. samsunensis*, the distal vagina is variably long and wide: sometimes long and slender (Figures 15 and 16; see also Hesse 1931: pl. 6 figs 55a-b; Hudec & Ležava 1969: fig. 22; Hudec 1973: fig. 6; Hausdorf 2000a: fig. 50) and at other times rather or very short (Figures 20 and 21). When short or very short, the vagina is also wider distally (Figure 21) or very wide with an internal ring of variably raised pleats (Figure 22), a detail never mentioned before. The duct of the bursa copulatrix is very long in *M. atacis* (Figures 8 and 12; see also Gittenberger & de Winter 1985: figs. 7–8) and variably long in *M. samsunensis*, ranging from very short or short (Hesse 1931: pl. 6 fig. 55a; Hudec & Ležava 1969: fig. 22) to long or very long (Figures 15, 16, 20 and 21; see also Hudec 1973: fig. 6; Hausdorf 2000a: fig. 50). Finally the flagellum is rather long in *M. atacis* (Figures 8, 12; see also Gittenberger & de Winter 1985: figs. 7–8) and variably long in *M. samsunensis*, ranging from short (Figures 20 and 21; see also Hesse 1931: pl. 6 fig. 55a; Hudec & Ležava 1969: fig. 22; Hudec 1973: fig. 6) to medium or rather long (Figures 15 and 16; see also Schileyko 1978: fig. 377; Hausdorf 2000a: fig. 50).

Molecular study

Two hundred and ninety-two new sequences were obtained and deposited in GenBank: 221 for *M. atacis* (61 COI, 62 16S rDNA, 58 H3, 20 sITS2 and 20 lITS2), 54 for *M. samsunensis* (12 COI, 14 16S rDNA, 14 H3, 3 sITS2 and 11 lITS2) and 17 for *M. cartusiana* (4 COI, 5 16S rDNA, 5 H3, 2 sITS2 and 1 lITS2) (for details on their lengths and GenBank accession numbers, see Table I). We identified 24 COI haplotypes (eleven COI 1 – COI 11 for *M. atacis*, nine COI 12 – COI 20 for *M. samsunensis*, four COI 17 – COI 20 for *M. cartusiana*) and 35 16S rDNA haplotypes (18 16S 1–16S 18 for *M. atacis*; 12 16S 19–16S 30 for *M. samsunensis*; 5 16S 31–16S 35 for *M. cartusiana*). Among sequences of the H3 gene, 10 common sequences were identified (6 H3 1 – H3 6 for *M. atacis* and *M. samsunensis*; 4 H3 7 – H3 10 for *M. cartusiana*). We established 15 short ITS2 (sITS2: 5.8S rDNA + ITS2) and 15 long ITS2 (lITS2: 5.8S rDNA + ITS2 + 28S rDNA) common sequences (for *M. atacis* – 12 sITS2: sITS2 1 – sITS2 12 and 9 lITS2: lITS2 1 – lITS2 9; for *M. samsunensis* – one sITS2; sITS2 13 and 4 lITS2: lITS2 10 – lITS2 14; for *M. cartusiana*: two sITS2: sITS2 14 – sITS2 15 and one lITS2: lITS2 13). Haplotypes and common sequences were used for phylogenetic analysis.

In the case of mitochondrial gene fragments (COI and 16S rDNA), sequences obtained from *M. atacis* and *M. samsunensis* were analysed separately as single locus data sets (not shown, except COI sequence analysis, see Supplementary Material Figure S1) or as concatenated COI + short 16S rDNA sequences (Figure 23 and Supplementary Material Table S1). A tree of similar topology was obtained from analysis of concatenated COI + long 16S rDNA sequences (Figure S2 and Supplementary Material Table S1). The *M. atacis* and *M. samsunensis* sequences clustered together in these trees, although two groups, one for *M. atacis* and another for *M. samsunensis* sequences, can be seen. In the case of new sequences obtained from the population of *M. cartusiana* from Cubières-sur-Cinoble, southern France, in the concatenated COI + 16S rDNA tree (Figure 23, see also Supplementary Material Table S1), they clustered quite separately from *M. atacis* and *M. samsunensis*, as well as from other representatives of *Matatheba*, but together with other COI and 16S rDNA sequences of *M. cartusiana* obtained from GenBank.

K2P distances for COI sequences characteristic of intraspecies differentiation were very small: mean 0.6% (range 0.0–1.4%) for *M. atacis* and mean 3.5% (range 0.0–7.6%) for *M. samsunensis* (Table IV). K2P distances between COI sequences of *M. atacis* and *M. samsunensis* were also small (mean 3.5%, range 1.2–6.9%, Table IV). They were an order of magnitude smaller than the distances that distinguished these two taxa from the other species of the
subgenus Metatheba i.e. *M. perfrequens*, *M. laxa* and *M. tibarenica* (3.5% vs. 13.0–18.6%) analysed here. The K2P distances separating the COI sequences of *M. atacis* and *M. samsunensis* from species of other *Monacha* subgenera were even greater (19.1–22.9%). K2P distances within
the southern French *M. cartusiana* populations, as well as between them and other *Monacha* species (0.4% intraspecies, 14.5% and 19.9% *M. cartusiana* vs. *M. claustralis* and *M. cantiana*, respectively; Table IV) were similar to those reported in our previous papers (Pieńkowska et al. 2015, 2016, 2018a).

Analysis of nuclear genes was hindered by the fact that only the 5.8S rDNA + ITS2 + 28S rDNA gene sequences (lgITS2) were deposited in great number in GenBank by Neiber and Hausdorf (2017), including *Metatheba* subgenus representatives. For the H3 gene, GenBank only contains sequences of the *M. cantiana* s.l. complex, deposited in connection with our previous papers (Pieńkowska et al. 2018b, 2019a, 2020). We therefore present two trees, based on various analyses of single or multiple locus data sets, one consisting of the ITS2 gene sequences cut off from flanking fragments (Figure 24) and the other built from concatenated ITS2 + H3 sequences (Figure 25, Supplementary Material Table S2). They confirm the results obtained with mitochondrial genes. The sequences obtained from *M. atacis* and *M. samsunensis* specimens are grouped into a common clade, and because they are mixed with each other, no separate subgroups can be distinguished (Figures 24 and 25). It is noteworthy that the sequence KX495444 deposited in GenBank by Neiber and Hausdorf (2017) for ITS2 of *M. samsunensis* is identical to the sequences lgITS2 1, found in some specimens of *M. atacis* from different French populations (Arties, Le Chandelier, Axat, Saint-Martin-Lys, Belfort-sur-Rebenty, Salvezines and Roquefort-de-Sault; Table I). Moreover, the H3 1 sequence was found in 9 out of the 10 specimens of *M. samsunensis* from Kastamonu as well as in nine specimens of *M. atacis* from eight French populations (Arties, Le Chandelier, Mijanes, Campagna-de-Sault, Saint-Ferriol 2, Belfort-sur-Rebenty 1, Salvezines and Roquefort-de-Sault; Table I) and the H3 2 sequence was found in four *M. samsunensis* specimens from Atakum/Samsun as well as in 44 specimens from 14 French populations (i.e. all but one: Le Chandelier, however only one specimen was available from this population; Table I).

Finally, we present an analysis of concatenated mitochondrial and nuclear gene sequences: COI + 16S rDNA + 5.8S rDNA + ITS2 + 28S rDNA conducted by three different methods (ML, NJ and BI) (Figure 26, Supplementary Material, Table S3). Again, sequences from *M. atacis* and *M. samsunensis* clustered in two slightly separate subgroups, but as a common clade they were clearly separate from sequences of other *Monacha* (*Metatheba*) and *Monacha* s.s. species.

**Discussion**

The shells of *M. atacis* from southern France and *M. samsunensis* from Turkey (Atakum/Samsun and Kastamonu) are very similar and do not differ from the lectotype of *M. samsunensis* deposited in the Naturhistorisches Museum Wien (Figure 27, see also Hausdorf 2000a: pl. 11, fig. 54).

The distal genitalia of *M. atacis* specimens from Carcassonne (Figures 8–11) and Lapradelle (Figures 12–14), characterised by a long vagina, a long bursa copulatrix duct and a long flagellum, exactly match those of the original description of this species (Gittenberger & de Winter 1985: figs 7–8). Specimens of *M. samsunensis* from the type locality (Figures 15–19) and the literature (Hesse 1931: pl. 6 fig. 55a; Hudec & Ležava 1969: fig. 22; Hudec 1973: fig. 6; Schileyko 1978: fig. 377; Hausdorf 2000a: fig. 50; Schileyko 2005: fig. 2534b) are usually characterised by a shorter flagellum, vagina and bursa copulatrix duct. Specimens of *M. samsunensis* from Kastamonu also seem to have a much shorter vagina than the others (Figures 20 and 21). However, vagina length is variable in *M. samsunensis* populations and possibly depends on sexual maturation (Hausdorf 2000a: tables 10 & 11, vagina total length 1.7–7.2 mm, measured in 35 specimens from different populations). In contrast to *M. samsunensis*, the features of the distal genitalia of *M. atacis* seem to vary little. In the absence of a more integrative approach to the study of the Turkish *Metatheba*, it is difficult to explain the significance of this pattern. For example, Gittenberger and de Winter (1985) wondered if the various figures of *M. samsunensis* reported in the literature really belong to a single species. However, high intra- and inter-population variability is well known among the *Monacha* species so much so that the species of this genus can only occasionally be recognised morphologically (Pieńkowska et al. 2018b, 2019a, 2020).

Some sequences of nuclear genes (H3 and lgITS2) obtained from specimens of *M. atacis* and *M. samsunensis* are exactly the same. The sequences of nuclear genes from these two species mixed and grouped together in a common clade on phylogenetic trees (Figures 24 and 25). The sequences of their mitochondrial genes also cluster together (Figure 23, also Supplementary Material Figure S1) as found also in those of the concatenated mitochondrial and nuclear genes (Figure 26). Although there are two separate subgroups for sequences of *M. atacis* and *M. samsunensis* in these analyses, they create a single strongly supported clade on both phylogenetic trees (Figures 23 and
The mean K2P distance for COI sequences between *M. atacis* and *M. samsunensis* is small, reaching 3.5% (Table IV) which is almost at the 3% threshold of the “barcode method” based on COI sequences (Hebert et al. 2003a, 2003b; Pentinsaari et al. 2020).

It is noteworthy that the mean K2P distance between the French *M. atacis* and the topotypical

Figures 8–14. Distal genitalia (8, 12), internal structure of distal genitalia (9, 13), transverse sections of medial epiphallus (10) and apical penial papilla (11, 14) of *Monacha atacis* from France: Carcassonne (FGC 35773) (8–11) and Lapradelle (DCBC & MNHW-F.18.27; FGC 51247) (12–14).
M. samsunensis from Atakum/Samsun is smallest (2.8%) when the Turkish populations are analysed separately (Table V). The mean K2P distances between M. atacis and M. samsunensis from the Kastamonu population as well as between M. atacis and M. samsunensis from Kürtün (sequence KX507202 from Neiber & Hausdorf 2017) were 3.8% and 4.4%, respectively (Table V).

Figures 15–19. Distal genitalia (15–16), internal structure of distal genitalia (17), transverse sections of medial epiphallus (18) and apical penial papilla (19) of Monacha samsunensis from Turkey: Atakum/Samsun [Bey3: 15; Bey2: 16–19] (DCBC; FGC 51175).
Nevertheless it must be stressed that Turkish populations vary in K2P distances between COI sequences (see K2P distances between three Turkish populations as well as the ranges of K2P distances, Table V), which may suggest that they are somewhat genetically differentiated. We are aware of limits of the barcode method in the analysis of taxonomic relations of stylommatophoran snails.

Figures 20–22. Distal genitalia (20–21) and internal structure of distal genitalia (22) of Monacha samsunensis from Turkey: Kastamonu [Kas3: 20; Sam3: 21–22] (DCBC; FGC 51094, 51095).
Figure 23. Maximum Likelihood (ML) tree of concatenated COI + (short) 16S rDNA haplotypes obtained from specimens of *Monacha ataxis* and *Monacha samsunensis* compared with sequences obtained from GenBank for representatives of the other *Monacha* species. Concatenated COI + 16S rDNA sequences (Table S1) were cut to 932 positions (600 bp COI + 332 bp 16S) in length. Numbers next to the branches indicate bootstrap support above 50% calculated for 1000 replicates from ML (left) and NJ (right) analysis (Felsenstein 1985). The tree was rooted with *Trochulus hispidus* concatenated sequences KY818415 + KY818541 deposited in GenBank by Neiber et al. (2017).
Table IV. Ranges of K2P genetic distances between analysed COI sequences (mean value in parenthesis).

| Comparison                      | COI (%)   |
|---------------------------------|-----------|
| Within M. atacis                | 0.0–1.4 (0.6) |
| Within M. samsunensis           | 0.0–7.6 (3.5) |
| Within M. cartusiana            | 0.0–0.7 (0.4) |
| Between M. atacis and M. samsunensis | 1.2–6.9 (3.5) |
| Between M. atacis and M. perfrequens | 12.6–13.6 (13.0) |
| Between M. atacis and M. laxa   | 17.3–18.6 (17.9) |
| Between M. atacis and M. tibarenica | 17.5–18.1 (17.8) |
| Between M. atacis and M. parumcincta | 17.9–20.1 (19.1) |
| Between M. atacis and M. claustralis | 18.7–20.5 (19.5) |
| Between M. atacis and M. cartusiana | 19.6–21.7 (20.6) |
| Between M. atacis and M. cantiana | 22.2–23.4 (22.9) |
| Between M. samsunensis and M. perfrequens | 12.6–15.9 (13.4) |
| Between M. samsunensis and M. laxa | 17.7–21.9 (18.6) |
| Between M. samsunensis and M. tibarenica | 16.4–21.0 (17.4) |
| Between M. samsunensis and M. parumcincta | 18.8–22.5 (20.3) |
| Between M. samsunensis and M. claustralis | 19.6–23.0 (20.6) |
| Between M. samsunensis and M. cartusiana | 20.1–23.2 (21.4) |
| Between M. samsunensis and M. cantiana | 21.7–25.8 (22.5) |
| Between M. perfrequens and M. laxa | 16.9 |
| Between M. perfrequens and M. tibarenica | 18.1 |
| Between M. perfrequens and M. parumcincta | 19.7–20.6 (20.2) |
| Between M. perfrequens and M. claustralis | 19.2 |
| Between M. perfrequens and M. cartusiana | 20.7–21.6 (21.1) |
| Between M. perfrequens and M. cantiana | 23.1–23.3 (23.2) |
| Between M. laxa and M. tibarenica | 17.2 |
| Between M. laxa and M. parumcincta | 17.2 |
| Between M. laxa and M. claustralis | 17.5 |
| Between M. laxa and M. cartusiana | 18.2–18.7 (18.4) |
| Between M. laxa and M. cantiana | 18.5 |
| Between M. tibarenica and M. parumcincta | 18.8–19.2 (19.0) |
| Between M. tibarenica and M. claustralis | 18.0 |
| Between M. tibarenica and M. cartusiana | 20.4–20.9 (20.7) |
| Between M. tibarenica and M. cantiana | 20.7–20.9 (20.8) |
| Between M. claustralis and M. cartusiana | 14.2–14.6 (14.5) |
| Between M. cartusiana and M. cantiana | 19.4–20.3 (19.9) |

(Davison et al. 2009; Sauer & Hausdorf 2010, 2012; Köhler & Johnson 2012; see also discussions in our previous papers Pienkowska et al. 2018b, 2019a, 2020). Nevertheless in this study we used Hebert’s method to confirm conspecificity and not to support the conclusion about species distinctness.

Incidentally, we used molecular analysis to confirm the occurrence of M. cartusiana in southern France (population from Cubières-sur-Cinoble in Aude, Table 1), where it may co-occur with M. atacis. This confirms previous molecular reports of M. cartusiana in France (Dahirel et al. 2015: northwestern France 48°07’51”N, 01°41’34”W, near Rennes; Čejka et al. 2020: fig. 3 – in Provence, southern France: 43°31’34.7”N, 05°04’30.7”E, L’Etang de Berre; 43°49’13.1”N, 05°18’29.5”E, Bonnieux; 43°37’03.7”N, 05°18’37.8”E, St. Cannat; 43°37’58.4”N, 05°38’37.3”E, Jouques; 43°39’33.5”N, 05°20’43.1”E, Rognes).

In conclusion, our morphological (shell and genitalia, Figures 2–22 and 27) and molecular (mitochondrial and nuclear gene sequences, Figures 23–26) findings corroborate that M. atacis and M. samsunensis are conspecific and that the former should be named M. samsunensis because the name introduced by Pfeiffer in 1868 has priority over that established by
Figure 24. Maximum Likelihood (ML) tree of ITS2 common sequences obtained from specimens of Monacha atacis and Monacha samsunensis compared with sequences obtained from GenBank for representatives of the other Monacha species. ITS2 sequences were cut to 564 positions in length. Numbers next to the branches indicate bootstrap support above 50% calculated for 1000 replicates from ML (left) and NJ (right) analysis (Felsenstein 1985). The tree was rooted with Trochulus hispidus ITS2 sequences KX495451 and MG585474 deposited in GenBank by Neiber and Hausdorf (2017) and Caro et al. (2019), respectively.
Figure 25. Maximum Likelihood (ML) tree of concatenated ITS2 + H3 common sequences obtained from specimens of *Monacha atacis* and *Monacha samunensis* compared with sequences obtained from GenBank for representatives of the other *Monacha* species. Concatenated ITS2 + H3 sequences (Table S2) were cut to 825 positions (546 positions ITS2 and 279 positions H3) in length. Numbers next to the branches indicate bootstrap support above 50% calculated for 1000 replicates from ML (left) and NJ (right) analysis (Felsenstein 1985). The tree was rooted with *Trochulus hispidus* ITS2 + H3 concatenated sequences MT755395 and MT758614 deposited in GenBank by Proćków et al. (2021).
Gittenberger & de Winter in 1985. According to Hausdorf (2000a) the occurrence of *M. atacis* in France is the result of an introduction of *M. samsunensis* in historic times. This possibility is supported by the fact that *M. atacis* occurs in a rather small area of France. However, the diversity of rapidly evolving mitochondrial genes may indicate that the French populations differentiated since their introduction. They may represent a distinct lineage that originated in France after their introduction (according to Falkner et al. 2002, the species has been reported from France at least since the 19th century) by the founder effect or by selection. This hypothesis cannot be verified without further research on a greater number of French populations of *M. samsunensis* (possibly also those from Catalonia, Spain).

On the other hand, it seems that there is greater genetic differentiation between Turkish populations from Atakum/Samsun, Kastamonu and Kürtün. It is noteworthy that there is more genetic similarity between specimens of *M. atacis* from France and the toptypical *M. samsunensis* from Atakum/Samsun than between Atakum/Samsun and Kastamonu populations. *M. samsunensis* has a wider distribution in Turkey than in France, and has probably existed there for much longer. A reason for the lower variabiliy observed within French populations may be their smaller range and shorter evolution. The variability in populations of *M. samsunensis* occurring in northern Anatolia and along the Black Sea coast is worthy of further study.

Figure 26. Maximum Likelihood (ML) tree of concatenated COI + 16S rDNA + (5.8S rDNA + ITS2 + 28S rDNA) sequences of Monacha atacis and Monacha samsunensis compared with sequences obtained from GenBank for representatives of the other Monacha species. Concatenated sequences (Table S3) were 2325 positions in length (600 COI + 869 16S rDNA + 42 5.8S rDNA + 557 ITS2 + 257 28S rDNA). Bootstrap support above 50% from ML (left) and NJ (middle) analysis as well as posterior probabilities (right) from Bayesian inference analysis are marked at the nodes. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with Trochulus hispidus COI + 16S rDNA + (5.8S rDNA + ITS2 + 28S rDNA) concatenated sequences KY818415 + KY818541 + KY818647 deposited in GenBank by Neiber et al. (2017).
The introduction of *M. samsunensis* to Western Europe is not an isolated case among the *Monacha* hygromiids. A population of *M. ocellata* was recently found in England, where it was accidentally introduced from the Istanbul area, Turkey (Anderson et al. 2018).

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