Redescription of *Monacha pantanellii* (De Stefani, 1879), a species endemic to the central Apennines, Italy (Gastropoda, Eupulmonata, Hygromiidae) by an integrative molecular and morphological approach

Joanna R. Pieńkowska¹, Giuseppe Manganelli², Folco Giusti², Debora Barbato², Ewa Kosicka¹, Alessandro Hallgass², Andrzej Lesicki¹

¹ Department of Cell Biology, Institute of Experimental Biology, Faculty of Biology, Adam Mickiewicz University in Poznan, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland ² Dipartimento di Scienze Fisiche, della Terra e dell’Ambiente, Università di Siena, Via Mattioli 4, 53100 Siena, Italy

Corresponding author: Andrzej Lesicki (alesicki@amu.edu.pl)

Abstract

Specimens obtained from ten populations of a *Monacha* species from the central Apennines were compared with six molecular lineages of *Monacha cantiana* s. l. (CAN-1, CAN-2, CAN-3, CAN-4, CAN-5, CAN-6) and two other *Monacha* species (*M. cartusiana* and *M. parumcincta*), treated as outgroup, by molecular (nucleotide sequences of two mitochondrial COI and 16S rDNA as well as two nuclear ITS2 and H3 gene fragments) and morphological (shell and genital anatomy) analysis. The results strongly suggest that these populations represent a separate species for which two names are available: the older *Helix pantanellii* De Stefani, 1879 and the junior *M. ruffoi* Giusti, 1973. The nucleotide sequences created well separated clades on each phylogenetic tree. Genital anatomy included several distinctive features concerning vaginal appendix, penis, penial papilla and flagellum; instead, shell characters only enabled them to be distinguished from *M. cartusiana* and *M. parumcincta*. Remarkably, populations of *M. pantanellii* show high morphological variability. Shell variability mainly concerns size, some populations having very small
dimensions. Genital variability shows a more intricate pattern of all anatomical parts, being higher as regards the vagina and vaginal appendix. Despite this morphological variability, the K2P distance range of COI sequences between populations is narrow (0.2–4.5%), if we consider all but three of the 53 sequences obtained. This research confirmed that the species of Monacha and their molecularly distinguished lineages can only occasionally be recognised morphologically and that they have significant inter- and intrapopulation variability. The possibility of using an overall approach, including shell, genital and molecular evidence, was taken in order to establish a reliable taxonomic setting.

**Keywords**
16S rDNA, COI, H3, ITS2, molecular features, shell and genital structure, species distribution

**Introduction**

Land snail fauna of the central and southern Apennines of Italy includes many common, widespread and diversified helicoideans, such as the geomitrids Candidula Kobelt, 1871 and Xerogyra Monterosato, 1892, the hygromiid Monacha Fitzinger, 1833, the helicids Marmorana Hartmann, 1844 and Helix Linnaeus, 1758. Despite this, their taxonomy, systematics and phylogenetics have been challenging since the early studies exclusively based on shell features. Taxonomic revisions of the second half of the 20th century (e.g., Forcart 1965; Giusti 1973) lumped many of the earliest described taxa on the basis of a similar gross genital morphology. However, more recent investigations using protein electrophoresis/allozymes (Marmorana: Oliverio et al. 1993) and mitochondrial and nuclear gene sequences (Marmorana: Fiorentino et al. 2010; Helix: Fiorentino et al. 2016) shed new light on these variable species and radiation may explain the relationship between the lineages or clades distinguished in the Apennines.

Continuing work on the hygromiid Monacha (Pieńkowska et al. 2015, 2016, 2018a, 2018b, 2019a, 2019b), we studied species living in the mountain grasslands of the central Apennines, whence came reports of three species, the widespread M. cantiana (Montagu, 1803) and the endemic M. orsini (Villa and Villa, 1841) and M. ruffoi Giusti, 1973, and a number of taxa with uncertain taxonomic status (Alzona 1971; Manganelli et al. 1995). We conducted a joint molecular and morphological study of many populations, finding many different species or their molecular lineages. However, it was difficult to draw reliable nomenclatural and taxonomic conclusions because the identity of the earliest taxa, established in the past, were often based on non-diagnostic shell characters of specimens without any precise collecting record.

A first result of our research corroborated the specific distinctness of Monacha ruffoi Giusti, 1973, of which we discovered an overlooked senior synonym: Helix pantanellii De Stefani, 1879.

The aim of the present research was: 1) to investigate phylogenetic relationships of Monacha pantanellii with other Monacha species or their molecular lineages; 2) to evaluate its morphological variability; 3) to redescribe the species.
Materials and methods

Taxonomic sampling

Ten populations of *Monacha pantanellii* (Table 1, Fig. 1) were considered in our analysis of their molecular and morphological (shell and genitalia structure) variability, and compared with the *M. cantiana* s. l. lineages (Pieńkowska et al. 2018a, 2019b). The sequences deposited in GenBank were also considered for the molecular analysis (Table 2). Two other *Monacha* species were used for morphological and molecular comparison: *M. cartusiana* (Müller, 1774) and *M. parumcincta* (Rossmässler, 1834). Another 23 populations of *M. pantanellii* were studied on a qualitative morphological basis (they were not included in the statistical analysis) (Table 3).

Material examined

New material examined is listed as follows, when possible: geographic coordinates (Lat & Long and UTM references) of locality, locality (country, region, site, municipality and province), collector(s), date, number of specimens (sh/sh shells/shells; spcm/spcms specimen/specimens), and collection where material is kept in parenthesis (Tables 1, 3). The material is kept in the F. 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. 2018a: table 1, 2019b: table 1).

Molecular study

Fifty-eight specimens representing ten population of *Monacha pantanellii* were used for molecular analysis (Table 1). DNA extraction, amplification and sequencing methods are described in detail in our previous paper (Pieńkowska et al. 2018a).

Two mitochondrial and two nuclear gene fragments were analysed, namely cytochrome c oxidase subunit 1 (COI), 16S ribosomal DNA (16S rDNA), an internal transcribed spacer of rDNA (ITS2) and histone 3 (H3), respectively. All new sequences were deposited in GenBank (Table 1). The COI, 16S rDNA, ITS2 and H3 sequences obtained from GenBank for comparison are listed in Table 2.

The sequences were edited by eye using the programme BioEdit, version 7.2.6 (Hall 1999, BioEdit 2017). Alignments were performed using CLUSTALW (Thompson et al. 1994) implemented in MEGA7 (Kumar et al. 2016). The COI and H3 sequences were aligned according to the translated amino acid sequences. The ends of all sequences were trimmed. The lengths of the sequences after trimming were 592 bp for COI, 286 positions for 16S rDNA, 501 positions for ITS2 and 279 bp for H3. The sequences were collapsed to haplotypes (COI and 16S rDNA) and to common sequences (ITS2 and H3) using the programme ALTER (Alignment Transformation EnviRonment) (Glez-Peña et al. 2010). Gaps and ambiguous positions were removed from alignments prior to phylogenetic analysis. Mitochondrial (COI and 16S rDNA) and nuclear (ITS2
Table 1. List of localities of populations of *Monacha pantanellii* used for molecular and morphological research. A question mark before the geographical coordinates of the locality no. 3 denotes that the georeferencing was done a posteriori on the basis of the available information.

| No. | Coordinates / UTM references | Country and site | Collector / date / no. of specimens / (collection) | Clade | Popu- | COI | GenBank | 16S rDNA | GenBank | ITS2 | GenBank | H3 | GenBank | Figs |
|-----|-----------------------------|----------------|--------------------------------------------------|-------|-------|-----|-------|--------|-------|-----|--------|----|--------|------|
| 1   | 42°40.35’N, 12°46.29’E     | Italy, Umbria, Monte Fionchi, summit (Spoleto, Perugia), 1 340 m a.s.l. | G. Manganelli & L. Manganelli / 12.09.1999 / 5 (FGC 8140) | PAN   | Fio1  | COI 1 | MT380011 | 16S 1 | MT376031 | MT376032 | ITS2 1 | MT376088 | H3 1 | MT385776 | 5, 6, 37–40 |
| 2   | 42°40.05’N, 12°44.53’E     | Italy, Umbria, Monte Fionchi, 900 N.E. di Torrecola (Spoleto, Perugia), 680 m a.s.l. | A. Hallgass / 2010 / 5 (FGC 38944) | PAN   | Fio2  | COI 3 | MT380014 | 16S 3 | MT376035 | MT376036 | ITS2 2 | MT376090 | H3 2 | MT385781 | 7, 41–44 |
| 3   | ?42°31.13’N, 12°58.63’E   | Italy, Vallonina (Monti Reatini, Lazio) | F. Giusti / 03.08.1966 / 5 (FGC 10883, 25345) | PAN   | Val   | COI 7 | MT380019 | 16S 6 | MT376040 | MT376041 | MT376042 | MT376043 | H3 6 | MT385786 | 21–22, 45–48 |
| 4   | 42°16.74’N, 12°50.28’E    | Italy, Latium, road to Montenero Sabino, 800 m W of Ornaro Alto (Montenero Sabino, Rieti), 670 m a.s.l. | A. Hallgass / 10.2013 / 5 (FGC 41552) | PAN   | Sab   | COI 9 | MT380021 | 16S 7 | MT376045 | MT376046 | ITS2 4 | MT376095 | H3 8 | MT385789 | 8–10, 63 |
| 5   | 42°16.51’N, 12°50.70’E    | Italy, Latium, Via Salaria, 500 m WSW of Ornaro Alto (Torricella in Sabina, Rieti), 520 m a.s.l. | A. Hallgass / 10.2013 / 6 (FGC 41553) | PAN   | Alt   | COI 14 | MT380026 | 16S 8 | MT376050 | MT376051 | ITS2 7 | MT376099 | H3 2 | MT385796 | 18–20, 61 |


| No. | Coordinates | Localities | Clade | Collector / date / no. of specimens | Coordinates | Country and site | Collector | Population | New haplotype (no. spcms) | GenBank ## | GenBank ## | GenBank ## | GenBank ## | GenBank ## | GenBank ## | Figs |
|-----|-------------|------------|-------|-----------------------------------|-------------|-----------------|------------|------------|------------------------|------------|------------|------------|------------|------------|------------|------|
| 6   | 42°15.53’N, 12°50.32’E | Italy, Latium, Via Salaria, 650 m NW of Poggio San Lorenzo (Poggio San Lorenzo, Rieti), 400 m a.s.l. | PAN | A. Hallgass / 10.2013 / 6 (FGC 41551) | 42°15.38’N, 12°50.32’E | Italy, Latium, Via Salaria, 650 m NW of Poggio San Lorenzo (Poggio San Lorenzo, Rieti), 400 m a.s.l. | A. Hallgass / 10.2013 / 6 (FGC 41551) | COI 19 (1) | MT380032 | MT376060 | ITS2 9 (1) | MT376104 | H3 6 (1) | MT385802 | 23–25 |
|     |             |            |       |                                   | 42°12.81’N, 12°57.80’E | Italy, Latium, near the bridge on Lago del Turano (Castel di Tora, Rieti), 260 m a.s.l. | A. Hallgass / 04.11.2013 / 7 (FGC 41654) | COI 22 (1) | MT380038 | MT376062 | ITS2 10 (1) | MT376110 | H3 13 (1) | MT385808 | 15–17, 57–59 |
| 7   | 42°07.88’N, 13°01.67’E | Italy, Latium, Valle del Turano, 1,6 km ESE di Turania (Turania, Rieti), 570 m a.s.l. | PAN | A. Hallgass / 04.11.2013 / 7 (FGC 42971) | 42°12.81’N, 12°57.80’E | Italy, Latium, near the bridge on Lago del Turano (Castel di Tora, Rieti), 260 m a.s.l. | A. Hallgass / 04.11.2013 / 7 (FGC 41654) | COI 27 (3) | MT380045 | MT376069 | ITS2 12 (1) | MT376116 | H3 10 (1) | MT385815 | 11–14, 62 |
|     |             |            |       |                                   | 42°05.74’N, 13°03.56’E | Italy, Abruzzi, Carsoli, industrial area (Carsoli, L’Aquila), 600 m a.s.l. | A. Hallgass / 04.11.2013 / 5 (FGC 41651) | COI 19 (1) | MT380052 | MT376068 | ITS2 11 (1) | MT376122 | H3 9 (1) | MT385822 | 30–31, 53–56 |
| 9   | 42°02.85’N, 12°54.33’E | Italy, Latium, Valle dell’Aniene, 600 m a.s.l. | PAN | A. Hallgass / 10.2013 / 7 (FGC 42974) | 42°05.74’N, 13°03.56’E | Italy, Abruzzi, Carsoli, industrial area (Carsoli, L’Aquila), 600 m a.s.l. | A. Hallgass / 04.11.2013 / 5 (FGC 41651) | COI 33 (2) | MT380057 | MT376081 | ITS2 5 (2) | MT376125 | H3 6 (1) | MT385826 | 26–29, 49–52, 60 |
| 10  | 42°12.81’N, 12°57.80’E | Italy, Latium, Via Salaria, 650 m NW of Poggio San Lorenzo (Poggio San Lorenzo, Rieti), 400 m a.s.l. | PAN | A. Hallgass / 10.2013 / 6 (FGC 41551) | 42°12.81’N, 12°57.80’E | Italy, Latium, near the bridge on Lago del Turano (Castel di Tora, Rieti), 260 m a.s.l. | A. Hallgass / 04.11.2013 / 7 (FGC 41654) | COI 34 (1) | MT380058 | MT376082 | ITS2 5 (2) | MT376125 | H3 9 (1) | MT385827 | 26–29, 49–52, 60 |
Figure 1. Localities of *Monacha pantanellii* and *M. cartusiana* populations listed in Tables 1, 3 (*M. pantanellii* black circles Table 1, grey circles Table 3). Details of localities of other *Monacha* species and their molecular lineages were provided in previous papers (Pieńkowska et al. 2015, 2018a, 2018b, 2019b).
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Table 2. GenBank sequences used for molecular analysis comparisons.

| Species                  | COI       | 16S rDNA  | ITS2      | H3        | References                     |
|--------------------------|-----------|-----------|-----------|-----------|--------------------------------|
| CAN-1 (Monacha cantiana s. s.) | MG208884–  | MG208960– | MH137963– | MG209031– | Pierkowska et al. (2018a)     |
|                          | MG208924  | MG208995  | MH137978  | MG209039  |                                |
|                          | –         | –         | –         | MG209041– |                                |
|                          | –         | –         | –         | MG209048  |                                |
| CAN-2 (Monacha cantiana s. s.) | MG208925–  | MG208996– | MH137979– | MG209049– | Pierkowska et al. (2018a)     |
|                          | MG208932  | MG209084  | MH137981  | MG209052  |                                |
|                          | –         | –         | –         | –         |                                |
| CAN-3 (Monacha sp.)      | MG208933– | MG209005– | MH137982– | MG209040  | Pierkowska et al. (2018a)     |
|                          | MG208938  | MG209010  | MH137983  | –         |                                |
|                          | –         | –         | –         | MG209053– |                                |
|                          | –         | –         | –         | MG209057  |                                |
| CAN-4 (Monacha cemenella) | MG208939– | MG209011– | MH137984  | MG209058– | Pierkowska et al. (2018a)     |
|                          | MG208943  | MG209015  | –         | MG209060  |                                |
|                          | –         | –         | –         | –         |                                |
| CAN-5 (Monacha sp.)      | MK066929– | MK066947– | –         | –         | Pierkowska et al. (2019b)     |
|                          | MK066941  | MK066959  | –         | –         |                                |
|                          | –         | –         | –         | MK066981– |                                |
|                          | –         | –         | –         | MK066977  |                                |
| CAN-6 (Monacha sp.)      | MK066942– | MK066960– | –         | –         | Pierkowska et al. (2019b)     |
|                          | MK066946  | MK066964  | –         | –         |                                |
|                          | –         | –         | –         | MK066999– |                                |
|                          | –         | –         | –         | MK066980  |                                |
| PAR (Monacha parumcincta) | MG208944– | MG209016– | MH137985– | MG209061– | Pierkowska et al. (2018a)     |
|                          | MG208959  | MG209030  | MH137992  | MG209071  |                                |
|                          | –         | –         | –         | –         |                                |
| CAR (Monacha cartusiana) | KM247376  | KM247391  | MH137993– | MG209072  | Pierkowska et al. (2018a)     |
|                          | –         | –         | –         | –         |                                |
|                          | MH203998  | MH204081  | –         | –         |                                |

and H3) sequences were concatenated (Table 4) before phylogenetic analysis. Finally, the sequences of COI, 16S rDNA, ITS2 and H3 were concatenated (Table 4) for Maximum Likelihood (ML) and Bayesian Inference (BI).

Estimates of evolutionary divergence between the sequences of COI obtained in this study and other sequences from GenBank were conducted with MEGA7 using the Kimura two-parameter model (K2P) (Kimura 1980). The analysis involved 83 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 615 positions in the final dataset.

Maximum Likelihood (ML) analyses were then performed with MEGA7. *Monacha cartusiana* and *Monacha parumcincta* were added as outgroup species in each analysis. For ML analysis of concatenated sequences, the following best nucleotide substitution models were specified according to the Bayesian Information Criterion (BIC): HKY+G+I (Hasegawa et al. 1985, Kumar et al. 2016) for COI and 16S rDNA concatenated sequences of 878 positions (592 COI + 286 16S rDNA), T92+G+I (Tamura 1992, Kumar et al. 2016) for ITS2+H3 concatenated sequences of 780 positions (501 ITS2 + 279 H3), and T92+G+I for COI+16S rDNA+ITS2+H3 concatenated sequences with a total length of 1658 positions (592 COI + 286 16S rDNA + 501 ITS2 + 279 H3). Bayesian analysis was conducted with MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) using the evolution model already used for ML calculation. Four Monte Carlo Markov chains were run for one million generations, sampling every 100 generations (the first 250,000 trees were discarded as 'burn-in'). This gave us
Table 3. Populations and materials of *Monacha cartusiana* (CAR) and *Monacha pantanellii* (PAN) not listed in Table 1 because they were not included in the molecular and statistical morphological analysis (apart from additional morphological analysis of *M. cartusiana*). A question mark before the geographical coordinates of some localities denotes that the georeferencing was done a posteriori on the basis of the available information.

| No. | Species | Coordinates (Lat & Long / UTM references) | Country and site (municipality and province in parenthesis) | Collector / Date / No. of specimens (collection) | Remarks |
|-----|---------|------------------------------------------|----------------------------------------------------------|-------------------------------------------------|---------|
| 11  | CAR     | 43°18.45'N, 11°28.88'E 32TQN09           | Italy, Tuscany, Stazione di Castelnuovo Berardenga (Asciano, Siena) | G. Manganelli / 01.11.1981 / spcm (FGC 3430) |         |
| 12  | CAR     | ? 42°28.85'N, 12°50.84'E 33TUH20         | Italy, Latium, Lago Lungo (Rieti, Rieti)                | F. Giusti / 14.08.1966 / spcm (FGC 23875) |         |
| 13  | PAN     | ? 43°15.67'N, 12°48.83'E 33TUH29         | Italy, Umbria, Val Sorda (Gualdo Tadino, Perugia), 1,050 m a.s.l. | A. Minelli / 03.08.1969 / 6 spcms (FGC 25350) |         |
| 14  | PAN     | ? 43°13.72'N, 12°48.02'E 33TUH28         | Italy, Umbria, Gualdo Tadino (Gualdo Tadino, Perugia)   | E. Giusti & G. Manganelli / 13.12.1984 / 1 spcm (FGC 6371) / L. Favilli & G. Manganelli / 01.10.1992 / 4 spcms (FGC 6370) |         |
| 15  | PAN     | 43°13.72'N, 12°48.02'E 33TUH28          | Italy, Umbria, La Rocchetta (Gualdo Tadino, Perugia)   | F. Giusti & G. Manganelli / 26.10.1967 / 4 shs (FGC 636); 2 spcms (FGC 25352) |         |
| 16  | PAN     | 42°55.83'N, 12°45.83'E 33TUH15           | Italy, Umbria, 600 m a.E di Rovigletto (Foligno, Perugia), 510 m a.s.l. | A. Hallgass / 25.09.2010 |         |
| 17  | PAN     | 42°49.92'N, 13°10.87'E 33TUH54          | Italy, Umbria, Monti Sibillini, Valle Canatra (Nocera, Perugia) | F. Giusti & G. Manganelli / 13.09.1988 / 6 shs and 3 spcms (FGC 25360) |         |
| 18  | PAN     | 42°47.33'N, 12°58.55'E 33TUH33          | Italy, Umbria, Gole di Biselli (Nocera, Perugia)        | A. Hallgass / 07.10.2011 |         |
| 19  | PAN     | 42°46.00'N, 13°10.90'E 33TUH53          | Italy, Umbria, Monti Sibillini, Costa Precino (Nocera, Perugia), 1,500 m a.s.l. | A. Benocci, M. Bianchi & G. Manganelli / 29.06.2014 |         |
| 20  | PAN     | 42°42.52'N, 12°51.98'E 33TUH23          | Italy, Umbria, 750 m E of Caso (Sant’Anatolia di Narco, Perugia), 800 m a.s.l. | A. Hallgass / 18.09.2010 |         |
| 21  | PAN     | 42°38.14'N, 12°57.04'E 33TUH32          | Italy, Umbria, 1 km SSW of Ruscio (Monteleone di Spoleto, Perugia) | A. Vannozzi / 22.08.2010 |         |
| 22  | PAN     | 42°33.85'N, 12°54.17'E 3TUH21           | Italy, Latium, Monti Reatini, Strada regionale 521 di Morro (Leonezza, Rieti), 1,050 m a.s.l. | A. Hallgass / 13.09.2009 | Paratypes of *Monacha ruffoi* Giusti, 1973 |
| 23  | PAN     | ? 42°33.75'N, 12°57.63'E 33TUH31         | Italy, Latium, Monti Reatini, Leonezza (Leonezza, Rieti), 1,000 m a.s.l. | F. Giusti / 04.08.1966 / 1 sh and 1 spcm (FGC 25348) | Material collected by F. Giusti in 1966 in part published (3 spcms) and in part not published (11 shs). Unfortunately the 3 spcms, constituting paratypes of *Monacha ruffoi* Giusti, 1973, have been lost. |
| 24  | PAN     | ? 42°33.72'N, 12°56.27'E 33TUH31         | Italy, Latium, Monti Reatini, Monte Tilia (Leonezza, Rieti), 1,600 m a.s.l. | F. Giusti / 06.08.1966 / 11 shs and 3 spcms (FGC 25337) | Material collected by F. Giusti in 1966 but not published. |
| 25  | PAN     | ? 42°33.58'N, 12°56.25'E 33TUH31         | Italy, Latium, Monti Reatini, Monte Corno (Leonezza, Rieti), 1,600-1,700 m a.s.l. | F. Giusti / 12.08.1966 / 3 shs (FGC 25338) | Paratypes of *Monacha cartusiana* Giusti, 1973 |
| 26  | PAN     | ? 42°32.58'N, 12°55.05'E 33TUH21         | Italy, Latium, Monti Reatini, Monti Corno (Leonezza, Rieti), 1,600 m a.s.l. | F. Giusti / 12.08.1966 / 6 spcms, 2 of which dissected (FGC 25342) / 16 shs (FGC 25340) / 5 shs (FGC 25341) | Paratypes of *Monacha cartusiana* Giusti, 1973 |
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| No. | Species | Coordinates (Lat & Long / UTM references) | Country and site (municipality and province in parenthesis) | Collector / Date / No. of specimens (collection) | Remarks |
|-----|---------|-------------------------------------------|----------------------------------------------------------|-------------------------------------------------|---------|
| 27  | PAN     | 42°31.35'N, 12°56.45'E 33TUH31            | Italy, Latium, Monti Reatini, Rio Fuggio (Leonessa, Rieti), 1,300 m a.s.l. | E. Giusti / 05.08.1966 / 5 spcms (FGC 25351) | Paratypes of *Monacha ruffoi* Giusti, 1973 |
| 28  | PAN     | 42°31.13'N, 12°58.63'E 33TUH30            | Italy, Latium, Monti Reatini, Vallonina (Leonessa, Rieti), 1,100 m a.s.l. | E. Giusti / 03.08.1966 / 1 spcm (FGC 25343) / 12 shs and 21 spcms (FGC 25344) / 21 spcms, 3 of which dissected (FGC 25345) | Holotype (FGC 25343) and paratypes (FGC 25344 and 25345) of *Monacha ruffoi* Giusti, 1973; Other 5 paratypes from this site have been subject to molecular and morphological study (see Table 1, no. 3) |
| 29  | PAN     | 42°30.63'N, 12°58.82'E 33TUH40            | Italy, Latium, Monti Reatini, Monte Cavalli (Posta, Rieti) 1,500 m a.s.l. | E. Giusti / 15.08.1966 / 1 spcm dissected and drawn (FGC) | Material collected by F. Giusti in 1966 but not published. |
| 30  | PAN     | 42°30.30'N, 12°58.82'E 33TUH30            | Italy, Latium, Monti Reatini, pathway to Monte Sassetelli (Cantalice, Rieti) | E. Giusti / 13.08.1966 / 3 spcms (FGC 25354) | Paratypes of *Monacha ruffoi* Giusti, 1973 |
| 31  | PAN     | 42°30.12'N, 12°58.77'E 33TUH30            | Italy, Latium, Monti Reatini, pathway to Monte Sassetelli (Cantalice, Rieti) 1,500 m a.s.l. | E. Giusti / 13.08.1966 / 3 spcms (FGC 25354) | Material collected by F. Giusti in 1966 but not published. |
| 32  | PAN     | 42°29.63'N, 12°58.67'E 33TUH30            | Italy, Latium, Monti Reatini, pathway to Monte Sassetelli (Cantalice, Rieti) 1,500-1,750 m a.s.l. | E. Giusti / 13.08.1966 / 3 shs (FGC 25349) | Material collected by F. Giusti in 1966 but not published. |
| 33  | PAN     | 42°26.70'N, 12°55.77'E 33TUH20            | Italy, Latium, Monti Reatini, above Lisciano (Rieti, Rieti) 800 m a.s.l. | E. Giusti / 06.08.1966 / 14 shs and 2 spcms, 1 of which dissected and drawn (FGC 10890) | Paratypes of *Monacha ruffoi* Giusti, 1973; dissected specimen lost |
| 34  | PAN     | 42°26.09'N, 12°54.86'E 33TUH20            | Italy, Latium, Monti Reatini, Vaia (Rieti, Rieti) 400 m a.s.l. | E. Giusti / 11.08.1966 / 1 spcm dissected and drawn (FGC) | Material collected by F. Giusti in 1966 but not published and lost. |
| 35  | PAN     | 42°25.90'N, 12°58.45'E 33TUG39            | Italy, Latium, Monti Reatini, Pian di Stura (Citraducale, Rieti) | E. Giusti / 07.08.1966 / 1 spcm dissected and drawn (FGC) | Material collected by F. Giusti in 1966 but not published and lost. |

A 50% majority rule consensus tree. In parallel, Maximum Likelihood (ML) analysis was performed with MEGA7 (Kumar et al. 2016) and calculated bootstrap values were mapped on the 50% majority rule consensus Bayesian tree.

### Morphological study

One hundred and thirty-four specimens representing *M. pantanellii, M. cantiana s. l.*, *M. parumcincta* and *M. cartusiana* were considered to investigate shell variability between these four species (including six molecular lineages of *M. cantiana s. l.*) (see Table 1 and Pieńkowska et al. 2018a, 2019b); the 43 specimens of nine populations of *M. pantanellii* (Fio1, Val, Sab, Alt, Lor, Tur2, Tur1, Car and Ani, see Table 1) were also considered to investigate shell variability between specimens of these populations. Shell variability was analysed randomly choosing five adult specimens from each population, when possible. Twelve shell variables were measured to the nearest
Table 4. Concatenated sequences of COI+16S rDNA and ITS2+H3 for ML analysis (Figs 2, 3) and COI+16S rDNA+ITS2+H3 for Bayesian analysis (Fig. 4).

| Concatenated sequence | COI haplotype | 16S rDNA haplotype | Concatenated sequence | ITS2 common sequence | H3 common sequence | Concatenated sequence | COI haplotype | 16S rDNA haplotype | ITS2 common sequence | H3 common sequence | Locality / population |
|-----------------------|--------------|------------------|-----------------------|---------------------|------------------|-----------------------|--------------|------------------|-----------------------|------------------|---------------------|
| COI16S 1              | COI 33       | 16S 23           | ITS2H3 1              | ITS2 25             | H3 6             | CS 1                  | COI 33       | 16S 23           | ITS2 25             | H3 6             | Latium, Valle dell’Aniene [Ana] |
| COI16S 2              | COI 34       | 16S 16           | ITS2H3 2              | ITS2 5              | H3 1             | CS 2                  | COI 34       | 16S 16           | ITS2 5              | H3 1             | Latium, Valle dell’Aniene |
| COI16S 3              | COI 36       | 16S 25           | ITS2H3 3              | ITS2 14             | H3 9             | CS 3                  | COI 36       | 16S 25           | ITS2 14             | H3 9             | Latium, Valle dell’Aniene |
| COI16S 4              | COI 35       | 16S 24           | ITS2H3 6              | ITS2 13             | H3 15            | CS 6                  | COI 35       | 16S 24           | ITS2 13             | H3 15            | Latium, Valle dell’Aniene |
| COI16S 5              | COI 9        | 16S 7            | ITS2H3 7              | ITS2 6              | H3 10            | CS 7                  | COI 11       | 16S 8            | ITS2 5              | H3 1             | Latium, Ornaro Alto, Montereale Sabino [Sab] |
| COI16S 6              | COI 11       | 16S 8            | ITS2H3 8              | ITS2 6              | H3 10            | CS 8                  | COI 12       | 16S 8            | ITS2 6              | H3 10            | Latium, Ornaro Alto, Montereale Sabino |
| COI16S 7              | COI 13       | 16S 9            | ITS2H3 9              | ITS2 4              | H3 9             | CS 9                  | COI 13       | 16S 9            | ITS2 4              | H3 9             | Latium, Ornaro Alto, Montereale Sabino |
| COI16S 8              | COI 10       | 16S 8            | ITS2H3 10             | ITS2 8              | H3 9             | CS 10                 | COI 10       | 16S 8            | ITS2 8              | H3 9             | Latium, Ornaro Alto, Montereale Sabino |
| COI16S 9              | COI 19       | 16S 20           | ITS2H3 11             | ITS2 5              | H3 9             | CS 12                 | COI 18       | 16S 19           | ITS2 5              | H3 9             | Latium, Lago di Turano (Castel di Tora, Rieti) [Tur1] |
| COI16S 10             | COI 22       | 16S 22           | ITS2H3 12             | ITS2 9              | H3 6             | CS 19                 | COI 21       | 16S 10           | ITS2 9              | H3 6             | Latium, Lago di Turano (Castel di Tora, Rieti) [Tur1] |
| COI16S 11             | COI 24       | 16S 13           | ITS2H3 13             | ITS2 9              | H3 11            | CS 20                 | COI 21       | 16S 10           | ITS2 9              | H3 11            | Latium, Lago di Turano (Castel di Tora, Rieti) [Tur1] |
| COI16S 12             | COI 26       | 16S 15           | ITS2H3 14             | ITS2 3              | H3 1             | CS 21                 | COI 21       | 16S 10           | ITS2 3              | H3 1             | Latium, Poggio San Lorenzo [Lor] |
| COI16S 13             | COI 8        | 16S 22           | ITS2H3 15             | ITS2 7              | H3 2             | CS 22                 | COI 14       | 16S 8            | ITS2 7              | H3 2             | Latium, Orano Alto, Torricella in Sabina [Alt] |
| COI16S 14             | COI 14       | 16S 8            | ITS2H3 16             | ITS2 7              | H3 11            | CS 24                 | COI 15       | 16S 10           | ITS2 7              | H3 11            | Latium, Orano Alto, Torricella in Sabina |
| COI16S 15             | COI 25       | 16S 14           | ITS2H3 17             | ITS2 8              | H3 9             | CS 25                 | COI 16       | 16S 10           | ITS2 8              | H3 9             | Latium, Orano Alto, Torricella in Sabina |
| COI16S 16             | COI 24       | 16S 13           | ITS2H3 18             | ITS2 5              | H3 12            | CS 26                 | COI 18       | 16S 11           | ITS2 5              | H3 12            | Latium, Orano Alto, Torricella in Sabina |
| COI16S 17             | COI 17       | 16S 10           | ITS2H3 19             | ITS2 2              | H3 1             | CS 27                 | COI 30       | 16S 19           | ITS2 2              | H3 1             | Latium, Valle di Turano (Taurina, Rieti) [Tur1] |
| COI16S 18             | COI 18       | 16S 11           | ITS2H3 20             | ITS2 12             | H3 10            | CS 28                 | COI 27       | 16S 16           | ITS2 12             | H3 10            | Latium, Valle di Turano (Taurina, Rieti) |
| COI16S 19             | COI 27       | 16S 16           | ITS2H3 21             | ITS2 2              | H3 1             | CS 30                 | COI 28       | 16S 17           | ITS2 2              | H3 1             | Latium, Valle di Turano (Taurina, Rieti) |
| COI16S 20             | COI 28       | 16S 17           | ITS2H3 22             | ITS2 11             | H3 1             | CS 31                 | COI 22       | 16S 12           | ITS2 11             | H3 1             | Latium, Valle di Turano (Taurina, Rieti) |
Redescription of *Monacha pantanellii* (De Stefani, 1879)

| Concatenated sequence | COI haplotype | 16S rDNA haplotype | Concatenated sequence | ITS2 common sequence | H3 common sequence | Concatenated sequence | COI haplotype | 16S rDNA haplotype | ITS2 common sequence | H3 common sequence | Locality / population |
|-----------------------|---------------|---------------------|-----------------------|----------------------|-------------------|-----------------------|---------------|---------------------|----------------------|-------------------|----------------------|
| COI16S 28             | COI 29        | 16S 18              |                       |                      |                   |                       | COI 29        | 16S 18              |                       | H3 1              | IT, Latiurn, Valle del Taurano (Turania, Rieti) |
| COI16S 29             | COI 22        | 16S 12              | ITS2H3 23             | ITS2 10              | H3 13             | CS 34                 | COI 22        | 16S 12              | ITS2 10              | H3 13             | IT, Latiurn, Lago del Taurano (Castel di Tora, Rieti) |
| COI16S 30             | COI 23        | 16S 12              |                       |                      |                   |                       | COI 23        | 16S 12              |                       | H3 1              | IT, Latiurn, Lago del Taurano (Castel di Tora, Rieti) |
| COI16S 31             | COI 8         | 16S 6               |                       |                      |                   |                       | COI 8         | 16S 6               |                       | H3 1              | IT, Latiurn, Lago del Taurano (Castel di Tora, Rieti) |
| COI16S 32             | COI 7         | 16S 6               |                       |                      |                   |                       | COI 7         | 16S 6               |                       | H3 1              | IT, Latiurn, Lago del Taurano (Castel di Tora, Rieti) |
| COI16S 33             | COI 4         | 16S 4               | ITS2H3 24             | ITS2 2               | H3 2              | CS 35                 | COI 4         | 16S 4               | ITS2 2               | H3 2              | IT, Umbria, Monteponchi (680 m) [Fio2] |
| COI16S 34             | COI 5         | 16S 5               | ITS2H3 25             | ITS2 3               | H3 4              | CS 36                 | COI 6         | 16S 5               | ITS2 3               | H3 4              | IT, Umbria, Monteponchi (680 m) |
| COI16S 35             | COI 6         | 16S 2               | ITS2H3 26             | ITS2 2               | H3 5              | CS 38                 | COI 6         | 16S 2               | ITS2 2               | H3 5              | IT, Umbria, Monteponchi (680 m) |
| COI16S 36             | COI 3         | 16S 3               |                       |                      |                   |                       | COI 3         | 16S 3               |                       | H3 2              | IT, Umbria, Monteponchi (680 m) |
| COI16S 37             | COI 2         | 16S 1               | ITS2H3 27             | ITS2 1               | H3 2              | CS 40                 | COI 2         | 16S 1               | ITS2 1               | H3 2              | IT, Umbria, Monteponchi (summit) [Fio1] |
| COI16S 38             | COI 1         | 16S 1               |                       |                      |                   |                       | COI 1         | 16S 1               |                       | H3 1              | IT, Umbria, Monteponchi (summit) |

*Monacha cantiana* CAN-1

| CAN-1                  | MG208916      | MG208987      | CAN-1                  | MH137974    | MG209046    | CS 41                 | MG208916    | MG208987    | MH137974    | MG209046    | IT, Latiurn, Valle dell’Aniene, Rome |
|------------------------|---------------|---------------|------------------------|-------------|-------------|-----------------------|-------------|-------------|-------------|-------------|------------------------------------|
| CAN-2                  | MG208915      | MG208985      | CAN-2                  | MH137973    | MG209045    | CS 42                 | MG208915    | MG208985    | MH137973    | MG209045    | IT, Latiurn, Valle dell’Aniene, Rome |
| CAN-3                  | MG208917      | MG208989      | CAN-3                  | MH137975    | MG209047    | CS 43                 | MG208917    | MG208989    | MH137975    | MG209047    | IT, Latiurn, Valle dell’Aniene, Rome |
| CAN-4                  | MG208905      | MG208977      | CAN-4                  | MG208905    | MG208977    | CS 44                 | MG208905    | MG208977    | MH137972    | MG209039    | IT, Latiurn, Gole del Velino |
| CAN-5                  | MG208906      | MG208979      | CAN-5                  | MG208906    | MG208979    | CS 45                 | MG208906    | MG208979    | MH137976    | MG209043    | IT, Latiurn, Gole del Velino |
| CAN-6                  | MG208910      | MG208978      | CAN-6                  | MG208910    | MG208978    | CS 46                 | MG208910    | MG208978    | MH137978    | MG209048    | IT, Latiurn, Valle del Tronto |
| CAN-7                  | MG208921      | MG208990      | CAN-7                  | MG208921    | MG208990    | CS 47                 | MG208921    | MG208990    | MH137976    | MG209048    | IT, Latiurn, Valle del Tronto |
| CAN-8                  | MG208923      | MG208994      | CAN-8                  | MG208923    | MG208994    | CS 48                 | MG208923    | MG208994    | MH137978    | MG209031    | UK, Barrow near Barnsley |
| CAN-9                  | MG208984      | MG208966      | CAN-9                  | MG208984    | MG208966    | CS 49                 | MG208984    | MG208966    | MH137965    | MG209038    | UK, Rotherham |
| CAN-10                 | MG208999      | MG208976      | CAN-10                 | MG208999    | MG208976    | CS 50                 | MG208999    | MG208976    | MH137971    | MG209038    | UK, Rotherham |
| CAN-11                 | MG208993      | MG208960      | CAN-11                 | MG208993    | MG208960    | CS 51                 | MG208993    | MG208960    | MH137981    | MG209052    | IT, Venetum, Sorga |
| CAN-12                 | MG208938      | MG208908      | CAN-12                 | MG208938    | MG208908    | CS 52                 | MG208938    | MG208908    | MH137982    | MG209054    | IT, Venetum, Sorga |

*Monacha cantiana s. l. CAN-3 (Monacha sp.)

| CAN-3                  | MG208936      | MG208909      | CAN-3                  | MH137983    | MG209055    | CS 51                 | MG208936    | MG208909    | MH137983    | MG209055    | AU, Breitenlee |
|------------------------|---------------|---------------|------------------------|-------------|-------------|-----------------------|-------------|-------------|-------------|-------------|------------------------------------|
| CAN-4                  | MG208938      | MG209008      | CAN-4                  | MH137982    | MG209054    | CS 52                 | MG208938    | MG209008    | MH137982    | MG209054    | AU, Breitenlee |
| CAN-5                  | MG208933      | MG209007      | CAN-5                  | MG208933    | MG209007    | CS 53                 | MG208933    | MG209007    | MH137983    | MG209054    | IT, Emilia Romagna, Fiume Setta |
| CAN-6                  | MG208934      | MG209005      | CAN-6                  | MG208934    | MG209005    | CS 54                 | MG208934    | MG209005    | MH137984    | MG209054    | IT, Emilia Romagna, Fiume Setta |
| CAN-7                  | MG208935      | MG209006      | CAN-7                  | MG208935    | MG209006    | CS 55                 | MG208935    | MG209006    | MH137985    | MG209054    | IT, Emilia Romagna, Fiume Setta |

Redescription of *Monacha pantanellii* (De Stefani, 1879)

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| Concatenated sequence | COI haplotype | 16S rDNA haplotype | Concatenated sequence | ITS2 common sequence | H3 common sequence | Concatenated sequence | COI haplotype | 16S rDNA haplotype | ITS2 common sequence | H3 common sequence | Locality / population |
|-----------------------|---------------|---------------------|-----------------------|----------------------|------------------|-----------------------|---------------|---------------------|----------------------|------------------|------------------------|
| CAN-4                 | MG208939      | MG209011            | CAN-4                 | MH137984             | CS 53            | MG208939             | MG209011      | MH137984            | MG209058            | FR, Alpes-Maritimes, Sainte Thecle |
|                       | MG208940      | MG209012            |                       |                      |                  |                       |               |                     |                      | FR, Alpes-Maritimes, Sainte Thecle |
|                       | MG208941      | MG209013            |                       | MK067003             | MG209059        |                       |               |                     |                      | FR, Alpes-Maritimes, Sainte Thecle |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| FR, Alpes-Maritimes, Sainte Thecle |
| FR, Alpes-Maritimes, Sainte Thecle |
| FR, Alpes-Maritimes, Sainte Thecle |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| FR, Alpes-Maritimes, Sainte Thecle |
| FR, Alpes-Maritimes, Sainte Thecle |
| FR, Alpes-Maritimes, Sainte Thecle |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| IT, Tuscany, Foce di Piana |
| IT, Tuscany, Foce di Piana |
| IT, Tuscany, Foce di Piana |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| IT, Tuscany, Foce di Piana |
| IT, Tuscany, Foce di Piana |
| IT, Tuscany, Foce di Piana |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| IT, Tuscany, Campo Cecina |
| IT, Tuscany, Campo Cecina |
| IT, Tuscany, Campo Cecina |

**Monacha parumcincta PAR**

| Locality / population |
|-----------------------|------------------------|
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |

**Monacha cartusiana CAR**

| Locality / population |
|-----------------------|------------------------|
| DE, Lower Saxony, Hannover, Schudehöhe |
| HU, Kis-Balaton |

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**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| ST, Tuscany, Foce di Piana |
| ST, Tuscany, Foce di Piana |
| ST, Tuscany, Foce di Piana |

**Monacha cantiana s. l.**

| Locality / population |
|-----------------------|------------------------|
| ST, Tuscany, Campo Cecina |
| ST, Tuscany, Campo Cecina |
| ST, Tuscany, Campo Cecina |

**Monacha parumcincta PAR**

| Locality / population |
|-----------------------|------------------------|
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |

**Monacha cartusiana CAR**

| Locality / population |
|-----------------------|------------------------|
| DE, Lower Saxony, Hannover, Schudehöhe |
| HU, Kis-Balaton |

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**Monacha parumcincta PAR**

| Locality / population |
|-----------------------|------------------------|
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |

**Monacha cartusiana CAR**

| Locality / population |
|-----------------------|------------------------|
| DE, Lower Saxony, Hannover, Schudehöhe |
| HU, Kis-Balaton |

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**Monacha parumcincta PAR**

| Locality / population |
|-----------------------|------------------------|
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |
| IT, Basilicata, Moliterno to Fontana d’Eboli |

**Monacha cartusiana CAR**

| Locality / population |
|-----------------------|------------------------|
| DE, Lower Saxony, Hannover, Schudehöhe |
| HU, Kis-Balaton |
Redescription of *Monacha pantanellii* (De Stefani, 1879)

0.1 mm using ADOBE PHOTOSHOP 7.0.1 on digital images of apertural and umbilical standard views taken with a Canon EF 100 mm 1:2.8 L IS USM macro lens mounted on a Canon F6 camera: \( AH \) aperture height, \( AW \) aperture width, \( LWfW \) last whorl final width, \( LWmW \) last whorl medial width, \( LWAH \) height of adapical sector of last whorl, \( LWMH \) height of medial sector of last whorl, \( PWH \) penultimate whorl height, \( PWfW \) penultimate whorl final width, \( PWmW \) penultimate whorl medial width, \( SD \) shell diameter, \( SH \) shell height, and \( UD \) umbilicus diameter (Pieńkowska et al. 2018a: fig. 1).

One hundred and thirty-five specimens of *M. pantanellii*, *M. cantiana* s. l. (with its six molecular lineages), *M. parumcincta* and *M. cartusiana* were analysed to examine anatomical variability between species; the 50 specimens of ten populations of *M. pantanellii* were also considered to investigate genital variability between populations of this species (see Table 1 and Pieńkowska et al. 2018a, 2019b). Snail bodies were dissected under the light microscope (Wild M5A or Zeiss SteREO Lumar V12). Anatomical details were drawn using a Wild camera lucida. Acronyms: \( BC \) bursa copulatrix, \( BW \) body wall, \( DBC \) duct of bursa copulatrix, \( DG \) digitiform glands (also known as mucous glands), \( E \) epiphallus (from base of flagellum to beginning of penial sheath), \( F \) flagellum, \( FO \) free oviduct, \( GA \) genital atrium, \( GAR \) genital atrium retractor, \( OSD \) ovispermiduct, \( P \) penis, \( PP \) penial papilla (also known as glans), \( V \) vagina, \( VA \) vaginal appendix (also known as appendicula), \( VAS \) vaginal appendix basal sac, \( VS \) vaginal sac (only present in *M. cartusiana*; see Pieńkowska et al. 2015: figs 11, 12), \( VD \) vas deferens. Seven anatomical variables (DBC, E, F, P, V, VS, VA) were measured under a light microscope (0.01 mm) using callipers (see: Pieńkowska et al. 2018a: fig. 2).

Detailed methods of multivariate ordination by Principal Component Analysis (PCA) and Redundancy Analysis (RDA), performed on the original shell and genitalia matrices as well as on the shape-related Z-matrices, are described in a previous paper (Pieńkowska et al. 2018a).

Differences between species for each shell and genital character were assessed through box-plots and descriptive statistics. Overall significance of differences was obtained using the Kruskal-Wallis test; when the test proved significant, multiple comparisons between pairs of species were performed using Dunn's test. In order to control the false discovery rate (FDR), the Benjamini-Hochberg correction was used to adjust \( P \)-values for multiple comparisons. The dunn.test function with the altp = TRUE option and \( \alpha = 0.01 \) in the dunn.test R package were used for analysis (Dinno 2017).

**Results**

**Molecular study**

DNA sequencing resulted in 53 and 57 sequences of mitochondrial COI and 16S rDNA as well as 43 and 57 sequences of nuclear ITS2 and H3 gene fragments, respectively. They were all deposited in GenBank as MT380011–MT380063 (COI), MT376031–
Figure 2. Maximum Likelihood (ML) tree of concatenated COI and 16S rDNA haplotypes of *Monacha pantanellii* (see Table 4). New COI and 16S rDNA sequences of *M. pantanellii* (Table 1) were compared with COI and 16S rDNA sequences of *M. cantiana* s. l. and *M. parumcincta* obtained from GenBank (Tables 2, 4). Numbers next to branches indicate bootstrap support above 50% calculated on 1000 replicates (Felsenstein 1985). The tree was rooted with *M. cartusiana* concatenated sequences obtained from GenBank (Table 2).
Redescription of *Monacha pantanellii* (De Stefani, 1879)

Figure 3. Maximum Likelihood (ML) tree of concatenated ITS2 and H3 common sequences of *Monacha pantanellii* (see Table 4). New ITS2 and H3 sequences of *M. pantanellii* (Table 1) were compared with ITS2 and H3 sequences of *M. cantiana* s. l. and *M. parumcincta* obtained from GenBank (Tables 2, 4). Numbers next to branches indicate bootstrap support above 50% calculated on 1000 replicates (Felsenstein 1985). The tree was rooted with *M. cartusiana* concatenated sequences obtained from GenBank (Table 2).
Figure 4. Bayesian 50% majority-rule consensus tree of the concatenated data set of COI and 16S rDNA haplotypes, and ITS2 and H3 common sequences (see Table 4). Sequences of *M. pantanellii* were compared with appropriate sequences of *M. cantiana* s. l. and *M. parumcincta* obtained from GenBank (Tables 2, 4). Posterior probabilities (left) and bootstrap support above 50% from ML analysis (right) are indicated next to the branches. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with *M. cartusiana* concatenated sequences obtained from GenBank (Table 2).
Redescription of *Monacha pantanellii* (De Stefani, 1879)

MT376087 (16S rDNA), MT376088–MT376130 (ITS2) and MT385776–MT385832 (H3) (Table 1). Thirty-six COI (COI 1–COI 36) and 25 16S rDNA (16S 1–16S 25) haplotypes, as well as 15 ITS2 (ITS2 1–ITS2 15) and 15 H3 (H3 1–H3 15) common sequences were recognised among them (Table 1). They were used for phylogenetic analysis with appropriate sequences representing *M. parumcincta* (PAR) and *M. cartusiana* (CAR), as well as six molecular lineages of *M. cantiana* s. l. (CAN-1–CAN-6) obtained from GenBank (Table 2). ML trees for concatenated sequences of mitochondrial COI and 16S rDNA (Fig. 2, Table 4) and of nuclear ITS2 and H3 (Fig. 3, Table 4) gene fragments, as well as the Bayesian Inference (BI) phylogenetic tree of concatenated sequences of COI+16S rDNA+ITS2+H3 gene fragments (Fig. 4, Table 4) clustered the concatenated sequences in one clade (PAN) separated from all other clades hitherto recognised for *M. cantiana* s. l. (CAN-1, CAN-2, CAN-3, CAN-4, CAN-5, CAN-6), *M. parumcincta* (PAR) and *M. cartusiana* (CAR) populations (Pieńkowska et al. 2018a, 2018b, 2019b).

K2P genetic distances between COI haplotypes are summarised in Table 5. Differences in COI haplotypes of *M. pantanellii* are rather small (up to 4.5%). Three varied somewhat more (COI 8 from populations from Vallonina [Val] and Carsoli [Car], COI 30 from Valle del Turano [Tur1] and COI 32 from Carsoli [Car]), bringing the mean for all populations to 0.2–6.7%. It was not possible to differentiate one population from the others. It is noteworthy that haplotypes of *M. pantanellii* are very different (15.5–22.0%) from the others representing *M. cantiana* s. l. (i.e., *M. cantiana* CAN-1–CAN-3, *M. ceme-nelea* (Risso, 1826) CAN-4, and *M. sp. CAN-5–CAN-6) as well as from *M. parumcincta* (18.1–21.4%) and *M. cartusiana* (16.6–18.3%) (Pieńkowska et al. 2018a, 2019b).

| Comparison                                      | COI (%)     |
|------------------------------------------------|-------------|
| Within *M. pantanellii* PAN                     | 0.2–6.7     |
| Between *M. pantanellii* PAN and *M. cantiana*  | 17.2–21.2   |
| CAN-1                                            |             |
| Between *M. pantanellii* PAN and *M. cantiana*  | 19.1–22.0   |
| CAN-2                                            |             |
| Between *M. pantanellii* PAN and *M. cantiana*  | 16.8–18.9   |
| s. l. CAN-3 (M. sp.)                            |             |
| Between *M. pantanellii* PAN and *M. cantiana*  | 15.5–17.4   |
| s. l. CAN-4 (M. ceme-nelea)                     |             |
| Between *M. pantanellii* PAN and *M. cantiana*  | 17.1–19.9   |
| s. l. CAN-5 (M. sp.)                            |             |
| Between *M. pantanellii* PAN and *M. cantiana*  | 15.5–18.6   |
| s. l. CAN-6 (M. sp.)                            |             |
| Between *M. pantanellii* PAN and *M. parumcincta* | 18.1–21.4   |
| PAR                                              |             |
| Between *M. pantanellii* PAN and *M. cartusiana* | 16.6–18.3   |
| CAR                                              |             |

**Table 5.** Ranges of K2P genetic distances between analysed COI sequences.

**Morphological study: shell**

*Monacha pantanellii* has a globose to sub-globose shell, variable in size, colour, and presence of paler subsutural and peripheral bands, with roundish to oval slightly descending aperture, a brownish peristome and a very small to small umbilicus (Figs 5–31).

RDA with species or molecular lineage constraint on the shape and size matrix (Fig. 32) showed that RDA 1 (33%, P < 0.001) separated all the species or molecular lineages from PAR. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (70%) was a positive combination of all variables. On
Figures 5–14. Shell variability in *Monacha pantanellii* from Monte Fionchi, summit [Fio1] (FGC 8140) (5, 6), Monte Fionchi, Torrecola [Fio2] (FGC 38944) (7), road to Montenero Sabino [Sab] (FGC 41552) (8–10) and Turania [Tur1] (FGC 42971) (11–14).
Figures 15–22. Shell variability in *Monacha pantanellii* from Lago del Turano [Tur2] (FGC 41654) (15–17), Via Salaria, Ornaro Alto [Alt] (FGC 41553) (18–20) and Vallonina [Val] (FGC 25345) (21, 22).
Figures 23–31. Shell variability in *Monacha pantanellii* from Via Salaria, Poggio San Lorenzo [Lor] (FGC 41551) (23–25), Valle dell’Aniene, Roccagiovine [Ani] (FGC 42974) (26–29) and Carsoli [Car] (FGC 41651)(30, 31).
the contrary, RDA 2 (7.3%, \( P < 0.001 \)) slightly separated CAN-1, CAN-2 and CAN-3 from CAN-4, CAN-5, CAN-6 and PAN with PAR in intermediate position. In this regard, PC2 (13%) mostly accounted for contrast between LWmH vs. LWaH and PWH.

RDA on the shape (Z) matrix (Fig. 33) showed a hazier separation of species or molecular lineages, confirming that size is a major source of morphological variation, although both RDA axes proved to be significant. In particular, RDA 1 separated CAR, CAN-5, CAN-6 from PAR, CAN-1 and CAN-3, with the other groups in a more or less intermediate position. Conversely, RDA 2 separated PAR and CAR from all the other species or molecular lineages. Shape-related PCA indicated that SH, LWaH and PWH vs. LWfW were the principal shape determinants on PC1 and PWmW, AH and AD vs. UD on PC2.

Box plots (Fig. 34) proved that the shell characters only have discriminating value in distinguishing Monacha pantanellii from other species or molecular lineages in a few cases. In fact, according to Dunn’s test with Benjamini-Hochberg adjustment \( (\alpha = 0.01) \), no character significantly distinguished PAN from CAN-1, CAN-2 and CAN-4, only one distinguished it from CAN-5 (UD), only two from CAR (LWaH, PWH), four from CAN-6 (SD, LWmW, LWfW, UD), six from CAN-3 (SH, AH, SD, AD,
Figure 34. Box plots for shell characters of the nine *Monacha* species or molecular lineages investigated. 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).
Table 6. Results of Dunn’s test with Benjamini-Hochberg correction ($\alpha = 0.01$) for shell and genital characters (in bold $P \leq 0.01$).

| Pairs       | SH   | AH   | LWmH | LWaH | PWH  | SD  |
|-------------|------|------|------|------|------|-----|
| PAN vs. CAN-1 | 0.0956 | 0.1431 | 0.3784 | 0.0134 | 0.1993 | 0.2703 |
| PAN vs. CAN-2 | 0.2257 | 0.0763 | 0.9541 | 0.8128 | 0.9275 | 0.0517 |
| PAN vs. CAN-3 | **0.0075** | **0.0039** | **0.7552** | **0.1309** | **0.6223** | **0.0063** |
| PAN vs. CAN-4 | 0.1428 | 0.4689 | 0.3232 | 0.0750 | 0.0467 | 0.1496 |
| PAN vs. CAN-5 | 0.8439 | 0.4087 | 0.8724 | 0.1396 | 0.8163 | 0.3364 |
| PAN vs. CAN-6 | 0.0514 | 0.0895 | 0.1007 | 0.8442 | 0.3559 | 0.0039 |
| PAN vs. CAR  | 0.0046 | 0.0330 | 0.1163 | **0.0009** | **0.0026** | **0.0044** |

| Pairs       | AW   | LWmW | PWmW | PWfW | LWfW | UD  |
|-------------|------|------|------|------|------|-----|
| PAN vs. CAN-1 | 0.1792 | 0.5046 | 0.0468 | 0.4863 | 0.8655 | 0.9405 |
| PAN vs. CAN-2 | 0.0488 | 0.0189 | 0.0434 | 0.1789 | 0.0826 | 0.5901 |
| PAN vs. CAN-3 | **0.0054** | **0.0046** | **0.0085** | **0.0265** | **0.0711** | 0.5962 |
| PAN vs. CAN-4 | 0.3094 | 0.1947 | 0.1515 | 0.1979 | 0.3344 | 0.1765 |
| PAN vs. CAN-5 | 0.8931 | 0.2051 | 0.7961 | 0.8167 | 0.3478 | 0.0015 |
| PAN vs. CAN-6 | 0.0330 | 0.0043 | 0.0434 | 0.0249 | **0.0030** | **0.0029** |
| PAN vs. CAR  | 1.0000 | 0.9480 | 0.4609 | 0.4984 | 0.1652 | 0.1370 |
| PAN vs. PAR  | **0.0046** | **0.0028** | 0.0365 | **0.0054** | **0.0008** | **0.0000** |

| Pairs       | DBC  | V    | F    | E    | P    | VA  | VS  |
|-------------|------|------|------|------|------|-----|-----|
| PAN vs. CAN-1 | 0.3802 | 0.0992 | 0.0000 | 0.0072 | 0.0001 | 0.0000 | 1.0000 |
| PAN vs. CAN-2 | 0.0808 | 0.1870 | 0.0001 | 0.0003 | 0.5535 | 0.0000 | 0.0000 | 1.0000 |
| PAN vs. CAN-3 | 0.9561 | 0.4778 | 0.0000 | 0.0057 | 0.5350 | 0.0000 | 0.0000 | 1.0000 |
| PAN vs. CAN-4 | 0.3528 | 0.9287 | 0.0708 | 0.9913 | 0.0001 | 0.0013 | 0.0000 | 1.0000 |
| PAN vs. CAN-5 | 0.0813 | 0.1862 | 0.6815 | **0.0002** | **0.0006** | **0.0001** | **0.0000** | **1.0000** |
| PAN vs. CAN-6 | 0.1163 | 0.3350 | 0.7574 | 0.0328 | 0.0101 | **0.0001** | **0.0001** | 1.0000 |
| PAN vs. CAR  | **0.0009** | **0.2609** | 0.0000 | 0.0122 | 0.0000 | 0.6581 | **0.0000** |
| PAN vs. PAR  | 0.0430 | **0.0000** | 0.0000 | 0.1266 | **0.0000** | 0.5918 | 1.0000 |

RDA with population constraint on the shape and size matrix (Fig. 35) showed that RDA 1 (53.6%, $P < 0.001$) separated them into two groups, the first, including populations from Via Salaria, Ornano Alto [Alt], Valle dell’Aniene, Roccagiovine [Ani], Monte Fionchi, summit [Fio1], Via Salaria, Poggio San Lorenzo [Lor], Montero Sabino [Sab] and Lago del Turano [Tur2] was separate from the second consisting of populations from Carsoli [Car], Turania [Tur1] and Vallonina [Val]. On the contrary, RDA 2 (4.0%, $P > 0.05$) showed no significant separation of populations. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (67.0%) was a negative combination of all variables.

RDA on the shape (Z) matrix (Fig. 36) showed no significant separation between populations, again confirming that size is a major source of morphological variation. Shape-related PCA indicated that LWaH and PWH vs. LWfW were the two principal shape determinants on PC1 and AH vs. LWmH on PC2.

Morphological study: anatomy

*Mona*cha pantanellii has distal genitalia very similar to those of the *Mona*cha cantiana group. The most remarkable features are the usually short vaginal appendix with mid or proximal vaginal insertion, the long flagellum and the penial papilla with thick LWmW, PWmW) and eight from PAR (SH, AH, SD, AD, LWmW, PWfW, LWfW, UD) (Table 6).
external wall bordering a central duct without strips joining it to the external wall and with a lumen filled by many variably sized pleats (Figs 37–63).

RDA with species or molecular lineage constraint on the shape and size matrix (Fig. 64) showed that RDA 1 (27%, $P < 0.001$) separated $M.\ cantiana$ s. l. (CAN-1, CAN-2, CAN-3, CAN-4, CAN-5 and CAN-6) from PAN, PAR and CAR. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (43%) accounted for VS vs. all the other variables. On the contrary, RDA 2 (22%, $P < 0.001$) separated CAN-5, CAN-6 and PAN from CAR and PAR. The group CAN-1, CAN-2, CAN-3 and CAN-4 was in intermediate position. In that regard, PC2 (20%) accounted for a contrast between E and VA vs. F, P, V and VS.

RDA with species or molecular lineage constraint on the shape (Z) matrix (Fig. 65) showed that RDA 1 (43%, $P < 0.001$) separated PAN from the group CAN-1, CAN-2, CAN-3, CAN-4 and CAN-6, with CAN-5, PAR and CAR in intermediate position, and that RDA 2 (20%, $P < 0.001$) separated CAR from all the others. Shape-related PCA indicated that VA and E vs. V and P were the principal shape determinants on PC1 and VS and V vs. DBC and F on PC2. In the latter case, removing the size effect altered the overall relationship patterns.
Figures 37–40. Genitalia (proximal parts excluded) (37), internal structure of distal genitalia (38), transverse sections of medial epiphallus (39) and apical penial papilla (40) of *Monacha pantanellii* from Monte Fionchi summit [Fio1] (FGC 8140).
Figures 41–44. Genitalia (proximal parts excluded) (41), internal structure of distal genitalia (42), transverse sections of medial epiphallus (43) and apical penial papilla (44) of *Monacha pantanellii* from Monte Fionchi, Torrecola [Fio2] (FGC 38944).

Box plots (Fig. 66) for anatomical characters showed that VA, F and P have the best discriminating value in distinguishing PAN: they distinguished 6 (VA) and 5 (F and P) species or molecular lineage pairs, respectively, according to Dunn’s test with Benjamini-Hochberg adjustment ($\alpha = 0.01$), followed by E and V with four and three species or molecular lineage pairs, respectively (Table 6). The most recognisable pairs were PAN vs. CAR and PAN vs. CAN-1 (four significant characters), PAN vs. CAN-2, PAN vs. CAN-3, PAN vs. CAN-5 and PAN vs. PAR (3 significant characters). Only two characters significantly distinguished PAN vs. CAN-4 and only one PAN vs. CAN-6 (Table 6). Anatomical characters have high discriminating value as testified by very low p values after Dunn’s test: in most cases (19 of 22) $P < 0.001$ (Table 6).
Redescription of *Monacha pantanellii* (De Stefani, 1879)

Figures 45–48. Genitalia (proximal parts excluded) (45), internal structure of distal genitalia (46), transverse sections of medial epiphallus (47) and apical penial papilla (48) of *Monacha pantanellii* from Vallonina [Val] (FGC 25345).

RDA with population constraint on the shape and size matrix (Fig. 67) showed that RDA 1 (64%, $P < 0.001$) separated populations Carsoli [Car], Monte Fionchi, Torrecola [Fio2], Turania [Tur1] and Vallonina [Val] from populations Via Salaria, Ornaro Alto [Alt], Valle dell’Aniene [Ani], Via Salaria, Poggio San Lorenzo [Lor] and Lago del Turano [Tur2], with Monte Fionchi, summit [Fio1] and Montero Sabino [Sab] in intermediate position. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (65%) was a positive combination of all variables. On the contrary, RDA 2 (13%, $P < 0.001$) separated population Val
Figures 49–52. Genitalia (proximal parts excluded) (49), internal structure of distal genitalia (50), transverse sections of medial epiphallus (51) and apical penial papilla (52) of *Monacha pantanellii* from Valle dell’Aniene, Roccagiovine [Ani] (FGC 42974).
Redescription of *Monacha pantanellii* (De Stefani, 1879)

Figures 53–56. Genitalia (proximal parts excluded) (53), internal structure of distal genitalia (54), transverse sections of medial epiphallus (55) and apical penial papilla (56) of *Monacha pantanellii* from Carsoli [Car] (FGC 41651).

from populations Sab and Fio1, with all the other populations (Car, Fio2, Tur1, Alt, Ani, Lor and Tur2) in intermediate position. In that regard, PC2 (16.5%) accounted for a contrast between V and VA vs. F and DBC.

RDA on the shape (Z) matrix (Fig. 68) showed a less clear separation between populations. RDA 1 (43%, P < 0.001) separated the population Sab from the group of populations Tur2, Val, Alt and Lor, with Ani, Fio1, Fio2 and Car in intermediate
Figures 57–59. Genitalia (proximal parts excluded) (57), internal structure of distal genitalia (58) and transverse section of apical penial papilla (59) of Monacha pantanellii from Lago del Turano [Tur2] (FGC 41654).
Figures 60–63. Internal structure of distal genitalia of *Monacha pantanellii* from Valle dell’Aniene, Roccagiovine [Ani] (FGC 42974) (60), Via Salaria, Ornaro Alto [Alt] (FGC 41553) (61), Turania [Tur1] (FGC 42971) (62) and road to Montenero Sabino [Sab] (FGC 41552) (63).
Figures 64, 65. Principal component analysis (PCA) and Redundancy analysis (RDA) with species or molecular lineage constraint applied to the original genital matrix (64) and shape-related Z-matrix (65).

Discussion

Molecular analysis of nucleotide sequences obtained from specimens originating from ten populations occurring in the grasslands of the central Apennines suggests that these populations represent a different species from other Italian *M. cantiana* s. l. lineages (CAN-1, CAN-2, CAN-3, CAN-5, CAN-6) and *Monacha* species (*M. cartusiana* and *M. parumcincta*), populations of which were previously subject to molecular analysis (Pieńkowska et al. 2018a, 2019a, 2019b). In each of the phylogenetic trees, i.e., ML of concatenated sequences for mitochondrial COI+16S rDNA (Fig. 2) and nuclear ITS2+H3 (Fig. 3) gene fragments as well as the BI tree of concatenated sequences COI+16S rDNA+ITS2+H3 (Fig. 4), sequences from these ten populations created
Figure 66. Box plots for genital characters of the ten Monacha species or molecular lineages investigated. 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).

well separated monophyletic clades. Two of these populations represent species described in the past: Monte Fionchi, Summit [Fio1]: Helix pantanellii De Stefani, 1879; Vallonina [Val]: Monacha ruffoi Giusti, 1973. Molecular analysis confirmed the validity of the species described by Giusti (1973) from the Reatini Mountains, although an older discarded name, introduced by De Stefani (1879), turned out to be available for it.

The range of K2P genetic distances between COI sequences obtained from the ten populations of M. pantanellii was 0.2–6.7% (Table 5). We previously found a similar range of K2P distances within populations of M. cantiana s. l. CAN-1/ CAN-2 (0.2–5.3%; Pieńkowska et al. 2018a, 2019b), M. cartusiana (0.0–3.3%; Pieńkowska et al. 2015, 2016, 2018b), M. parumcincta (0.2–4.6%; Pieńkowska et al.
Figures 67, 68. Principal component analysis (PCA) and Redundancy analysis (RDA) with population constraint applied to the original genital matrix (67) and shape-related Z-matrix (68) of specimens of *Monacha pantanellii*.

It is worth noting that this K2P distance range was even narrower (0.2–4.5%) if we considered all but three of 53 the COI sequences obtained from *M. pantanellii* specimens. The three COI sequences excluded were found in single (one or two) specimens of populations from Carsoli [Car], Valle del Turano [Tur1] and Vallonina [Val], however COI sequences obtained from the other specimens of these populations were more similar to others found in *M. pantanellii*. This suggests higher intrapopulation variation within these three populations, which may prove speciation seen in a rapidly evolving mitochondrial genome (Thomaz et al. 1996; Remigio and Hebert 2003).

The conclusion that ten populations from the central Apennines form a different species is supported by the analysis of K2P genetic distances of COI sequences (Table 5). Although the utility of the 3% barcode threshold as a marker for species distinction, applied in the so-called “barcode method” based on COI sequences (Hebert et al. 2003a, 2003b, 2018; Pentinsaari et al. 2020), is disputable (Davison et al. 2009; Sauer and Hausdorf 2010, 2012; Köhler and Johnson 2012; Batomalaque et al. 2018a, 2019b) and *M. clausuralis* (0.0–5.7%; Pieńkowska et al. 2015, 2016, 2018b).
Redescription of *Monacha pantanellii* (De Stefani, 1879)

COI sequences have been used to analyse taxonomic problems in different gastropod families (e.g., Remigio and Hebert 2003; Elejalde et al. 2008; Duda et al. 2011; Breugelmans et al. 2013; Prockow et al. 2013, 2019; Cande and Kuntner 2015; Walther et al. 2016; Kruckenhauser et al. 2017; Galan et al. 2018; Gladstone et al. 2019; Harl et al. 2019; Kneubühler et al. 2019; Bamberger et al. 2020). They were also useful in our previous studies on *Monacha* species (Pieńkowska et al. 2015, 2018a, 2019a, 2019b). Indeed, we have always emphasised that phylogenetic analysis cannot be based on a single gene locus but should combine several mitochondrial and nuclear genes (Pieńkowska et al. 2015, 2018a, 2019a, 2019b). Note that the conclusion that ten populations are distinct from other *Monacha* species at species level is not only supported by the analysis of COI sequences, but also of 16S rDNA, ITS2, and H3.

Moreover, we have always stressed (Pieńkowska et al. 2015, 2018a, 2019a, 2019b) that molecular features alone are insufficient to define species but need to be supported by morphological (shell and anatomy) features. Inconsistency between molecular and morphological features may occur among snail populations or species (Cameron 1992; Cameron et al. 1996; Sauer and Hausdorf 2012; Falniowski et al. 2020), because according to the concept of morphostatic evolution (Gittenberger 1991; Davis 1992; Koch et al. 2020) speciation may be reflected earlier in molecular than in morphological features.

It is not possible to distinguish *M. pantanellii* from the lineages of the *M. cantiana* group on the basis of shell characters, perhaps with the exception of CAN-6 (see Figs 32–34; Table 6). However, this may be biased by the fact that only one population of this lineage was available for study (Pieńkowska et al. 2019b). With regard to the other two species examined by comparison, *M. cartusiana* and *M. parumcincta*, the analysis found that distinguishing *M. pantanellii* from the former is difficult (only two characters have discriminating value), but from the latter is easy (eight characters have discriminating value). Anyway, these species are readily distinguished by colour pattern. *M. cartusiana* has a smoother more glossy shell, usually whitish, often with sharp milky-white sub MUTURAL and peripheral bands, intensely reddish-brown peristome, externally bordered by a ring of bright milky white. *M. parumcincta* has a shell similar to that of *M. pantanellii*, but less glossy and more opaque, sometimes with paler peripheral and sub MUTURAL bands and brownish peristome, externally bordered by a pale whitish ring.

The distinction of *M. pantanellii* based on anatomical characters is clear from the lineages of the *M. cantiana* group and the other two species examined by comparison, *M. cartusiana* and *M. parumcincta*. However, contrary to the situation with shell characters, CAN-6 is the lineage least distinct from *M. pantanellii*: again, the few specimens available may have biased the result. The analysis confirmed the high discriminating value of the vaginal appendix which distinguishes *M. pantanellii* from all the lineages of the *M. cantiana* group and *M. cartusiana*. The penis and flagellum are also important because they significantly distinguish *M. pantanellii* from five other species or molecular lineages (Table 6).
Table 7. Other anatomical features distinguishing Monacha species.

| Characters             | M. pantanellii | M. cantiana group | M. cartusiana | M. parumcincta |
|------------------------|----------------|-------------------|---------------|----------------|
| insertion of VA        | vaginal        | atrial            | vaginal       | atrial         |
| shape of VA            | usually short and slender, calibre almost constant; however, in two populations it is long or very long with proximal portion (ca. half its length or more) very enlarged and distal portion slender | long or very long, not slender nor enlarged, calibre initially large then progressively tapered; sometimes with variably evident basal sac | long or very long with proximal portion (ca. half its length or less) enlarged and distal portion slender | usually short and enlarged, calibre almost constant |
| PP                     | with thick external walls and narrow space between external walls and central duct; central duct circular in transverse section, usually rather small in diameter, not joined by strips to external walls and with its lumen almost totally filled by large pleats | with thick external walls, and narrow to wide space between external walls and central duct; central duct circular in transverse section, usually rather large in diameter, joined by strips to external walls and with its lumen not filled by large pleats | with thick external walls and narrow to wide space between external walls and circular central duct; central duct circular in transverse section, usually medium-sized in diameter, not joined by strips to external walls and with its lumen almost totally filled by large pleats | with thin external walls and narrow space between external walls and central duct; central duct horseshoe-shaped in transverse section, large in diameter, not joined by strips to external walls and with its lumen apparently not filled by pleats |

References

Giusti (1973: figs 26A, B), this paper (Figs 38–64)  
Pieńkowska et al. (2018a: figs 20–50; 2019: figs 2–3; 2019b: figs 19–41)  
Giusti and Manganeli (1987: figs 1A–G), Pieńkowska et al. (2015: figs 11–12, 15–16, 18–21)  
Pieńkowska et al. (2018a: figs 51–59)

Other anatomical features that distinguish M. pantanellii from the M. cantiana group, M. cartusiana and M. parumcincta were not included in the analysis, since it is impossible to quantify them. They are the insertion of the vaginal appendix, the shape of the vaginal appendix, and the section of the penial papilla (Table 7).

Intraspecific variability in M. pantanellii is high and concerns both shell and genitalia. Inter-population shell variability mainly affects the size features: some populations are distinguished by reduced size, notably the one from Carsoli [Car] (Figs 30, 31) and the slightly larger populations from Turania [Tur1] (Figs 11–14) and Vallonina [Val] (Figs 21, 22). This pattern was confirmed by RDA on the original shell matrix (Fig. 32) and by its disappearance when the size effect was removed (Fig. 33). Anatomically, these populations agree very well with the characters typical of the species (e.g., VA, PD, F) suggesting that shell size has no phylogenetic signal and cannot be used to support taxonomic distinctions. We can hypothesize that it depends on local conditions of drought, food availability and lack of refuges.

Intra-population shell variability is smaller, but the variation of UD from Via Salaria, Ornaro Alto [Alt] is notable (0.9–2.4 mm) including almost the extremes of the range (Figs 18–20).

Inter-population genital variability is more intricate although the size effect is again evident: RDA 1 (Fig. 35) separates the populations of smaller size, i.e., those from Car-
soli [Car], Monte Fionchi, Torrecola [Fio2], Turania [Tur1] and Vallonina [Val], from those of larger size, namely Via Salaria, Ornaro Alto [Alt], Valle dell’Aniene, Roccagiovine [Ani], Via Salaria, Poggio San Lorenzo [Lor] and Lago del Turano [Tur2]. When the size effect is removed (Fig. 36) some patterns persist, albeit less clear because conflicting variables are involved. Inter-population genital variability concerns all anatomical sections but is higher in V and VA (as shown by PCA). The former (V) is very short in Montenero Sabino [Sab] (Fig. 63), Monte Fionchi, Torrecola [Fio2] (Fig. 41) and Car-soli [Car] populations (Fig. 53) and long in those from Via Salaria, Ornaro Alto [Alt] (Fig. 61), Via Salaria, Poggio San Lorenzo [Lor] (not shown), Valle dell’Aniene, Roccagiovine [Ani] (Fig. 49) and Lago del Turano [Tur2] (Fig. 57). The latter (VA) is usually short but is long in Valle dell’Aniene, Roccagiovine [Ani] (Fig. 49) and very long in Lago del Turano [Tur2] populations (Fig. 57), where however intra-population range is wide.

According to RDA on the shape (Z) matrix, some of the most divergent populations are those from Montenero Sabino [Sab] and Lago del Turano [Tur2], which fall at the extremes of the ordination figure (Fig. 68).

This revision is the first result of research on the Monacha species living in the mountain grasslands of the central Apennines. It confirms the validity of the species described by Giusti (1973) from the Reatini Mountains, though an older discarded name, introduced by De Stefani (1879), turned out to be available for it.

It is evident from the above discussion that the species of Monacha and the lineages of M. cantiana s. l. can only occasionally be recognised morphologically and are also subject to significant inter- and intra-population variability. In this situation, revision based on type material consisting of shells may be not decisive. We therefore took an overall approach that considers shell, genital and molecular evidence to establish a reliable taxonomic setting. Only a multidisciplinary investigation of populations from the type locality, matching type specimens, can clarify the identity of old established Monacha taxa. This what we tried to do, although it was made difficult by the fact that the type locality was not always reported in a detailed way. Luckily this was not the case of the species described by De Stefani (1879). Thus, the investigation of specimens from the type locality, the summit of Monte Fionchi near Spoleto in Umbria, enabled us to ascertain that they have the same anatomical features as M. ruffoi. Conspecificity of the topotypical populations of M. pantanellii and M. ruffoi is also strongly supported by molecular analysis. Consequently, the latter has to be regarded as a junior synonym of De Stefani’s species.

Since M. pantanellii is a Monacha species with distinctive anatomical features, we checked all the material accessible to us. This enabled us to find other populations of the species, some from the Reatini Mountains, where they were collected by one of us in the 1960s during field work, some from other more northern mountain ranges (Table 3).

Regarding relationships of M. pantanellii with other taxa described or reported from the central Apennines, research is underway. So far we can only reveal that they belong to lineages different from this species and the M. cantiana group.
Redescription of *Monacha pantanelli* (De Stefani, 1879)

*Monacha pantanelli* (De Stefani, 1879)  
Figures 5–31, 37–63

*Helix pantanelli* De Stefani, 1879: 40–41.  
*Monacha ruffoi* Giusti, 1973: 533–537, pl. 6.

**Diagnosis.** A species of *Monacha* (s. str.) (according to the subgeneric division proposed by Neiber and Hausdorf 2017) with vaginal appendix usually short and slender (having shape and size of a digitiform gland) inserted at mid vagina; proximal vaginal sac absent; penial flagellum long to very long; penial papilla with narrow space between external walls and central duct; central duct circular in transverse section, usually rather small in diameter, not joined by strips to external walls and with its lumen almost totally filled by large pleats.

**Redescription.** *Shell* (Figs 5–31) dextral, sub-globose to globose, small to medium in size, variable in colour, sometimes (when colour is brownish yellow) with paler sub-sutural and peripheral bands, with 5½–6 slightly convex whors separated by superficial sutures; aperture slightly prosocline, round to oval; peristome not reflected, thickened, with variably evident whitish callous rim lining the outer margin; umbilicus open, very small to small; protoconch and teleoconch smooth, with very faint scattered collabral growth lines. *Shell* dimensions: H: 10.3 ± 1.5 mm; D: 16.2 ± 2.3 mm (n = 45).

*Radula* not examined.

*Female distal genitalia* (Figs 37, 38, 41, 42, 45, 46, 49, 50, 53, 54, 57, 58, 60–63; Table 7) include free oviduct, bursa copulatrix and its duct, digitiform glands, vagina and vaginal appendix. Free oviduct short and variably wide. Bursa copulatrix bean-like or pyriform with long wide duct. Vagina short to long and wide. Digitiform glands disposed on opposite sides of vagina in two groups of 1–3 tufts, each with 1–3 units. Vaginal appendix usually short (having shape and size of a digitiform gland) and inserted approximately half-way along the vagina.

*Male distal genitalia* (Figs 37–63, Table 7) include vas deferens, flagellum, epiphallus and penis. Vas deferens very long and very slender. Flagellum long to very long and slender. Epiphallus long to very long and wide. Penis short and wide, enveloped by thin sheath, consisting of proximal portion (from start of penial sheath to base of penial papilla) and distal portion (from base of penial papilla to genital atrium). Penial papilla variable in shape (perhaps due to pre-mortem stress or spirit fixation), with apical opening, thick external walls and narrow space between external walls and central duct; central duct circular in section, usually rather small in diameter, not joined by strips to external walls of penial papilla and with its lumen almost totally filled with large pleats.

Genital atrium large, receiving vagina and penis, internally smooth or with variably developed longitudinal pleats.
Redescription of *Monacha pantanellii* (De Stefani, 1879)

**Type locality.** “Sulla cima del Monte Fionghi al sud di Spoleto a circa mille metri sul livello del mare “, i.e., on the summit of Monte Fionchi, south of Spoleto, at an altitude of ca. 1000 m (municipality of Spoleto, province of Perugia), UTM references 32T UH 1726, Lat and Long: 42°40.455’N, 12°46.340’E.

**Type material.** Probably lost.

**Topotype sequences.** Sequences obtained from individuals from the type locality of *M. pantanellii* are designated as typical for this species: COI – MT380011–MT380018, 16S rDNA – MT376031–MT376039, ITS2 – MT376088–MT376094, H3 – MT385776–MT385785.

**Etymology.** Named after Dante Pantanelli (1844–1913), Italian palaeontologist and geologist at the University of Modena. He published many papers on Miocene and Pliocene molluscs, some of which were co-authored by his friend Carlo De Stefani (1851–1924). He was also the secretary of the Italian Malacological Society and the editor of the Bullettino della Società Malacologica Italiana for many years (Manganelli et al. 2017, with references).

Giusti’s species was named after Sandro Ruffo (1915–2010), a major Italian twentieth-century zoologist and director of the Museo Civico di Storia Naturale di Verona for many years (Latella 2011).

**Distribution.** Endemic to Umbria-Marche Apennines and Latium Sub-Apennines. It occurs from the Apennines of Gualdo Tadino in the north to the Aniene and Turano valleys in the south.

**Ecology.** Mesophile species living among grass in open habitats such as grasslands, pastures, forest edges and clearings in hill and mountain areas.

**Conservation.** Apparently common and widespread species within its range, but in some sites (e.g., Vallonina) it was no longer found during a field survey in the summer of 2019. Like other mesophilic species it could be sensitive to global warming.

**Remarks.** This species was distinguished from *Monacha cantiana* on the basis of a few shell characters (“more depressed, more fragile and paler shell, with fine growth lines, less rounded opening and deeper umbilicus”) and was disregarded by its author as an “extreme variety” of the former. Subsequently it was only reported in two catalogues by Westerlund (1889: 95) and Pilsbry (1895: 266) so that when Alzona prepared the catalogue of Italian non-marine malacofauna, they included it as a doubtful species (Alzona 1971: 183).

On the contrary, our analysis showed that it matches a valid species, currently known as *Monacha ruffoi*, described from the Reatini mountains by Giusti (1973) as a *Monacha* species with a shell resembling that of *cantiana*, but with a much smaller vaginal appendix.

This is an unexpected result: indeed, De Stefani’s species is one of thousands of mollusc species established since the second half of the nineteenth century on the basis of very few shell features of no diagnostic value due to dramatic intra- and inter-population variability. In describing thousands of species and varieties, past authors hit on some that remained valid.
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