Exploring Monacha cantiana (Montagu, 1803) phylogeography: cryptic lineages and new insights into the origin of the English populations (Eupulmonata, Stylommatophora, Hygromiidae)

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Academic editor: E. Neubert | Received 10 February 2018 | Accepted 23 April 2018 | Published 6 June 2018

http://zoobank.org/E5CAE122-33E5-436A-AA9B-B321D56A4D58

Citation: Pieńkowska JR, Manganelli G, Giusti F, Hallgass A, Lesicki A (2018) Exploring Monacha cantiana (Montagu, 1803) phylogeography: cryptic lineages and new insights into the origin of the English populations (Eupulmonata, Stylommatophora, Hygromiidae). ZooKeys 765: 1–41. https://doi.org/10.3897/zookeys.765.24386

Abstract

Molecular analysis of nucleotide sequences of mitochondrial cytochrome oxidase subunit 1 (COI) and 16S ribosomal DNA (16SrDNA) as well as nuclear histone 3 (H3) and internal transcribed spacer 2 of rDNA (ITS2) gene fragments together with morphological analysis of shell and genitalia features showed that English, French and Italian populations usually assigned to Monacha cantiana consist of four distinct lineages (CAN-1, CAN-2, CAN-3, CAN-4). One of these lineages (CAN-1) included most of the UK (five sites) and Italian (five sites) populations examined. Three other lineages represented populations from two sites in northern Italy (CAN-2), three sites in northern Italy and Austria (CAN-3), and two sites in south-eastern France (CAN-4). The taxonomic and nomenclatural setting is only currently available for lineages CAN-1 and CAN-4; a definitive frame for the other two requires much more research. The lineage CAN-1 corresponds to the true Monacha cantiana (Montagu, 1803) because it is the only one that includes topotypical English populations. The relationships and genetic distances support the hypothesis of the Italian origin of this lineage which was probably introduced to England by the Romans. The lineage CAN-4 is attributed to Monacha cemenelea (Risso, 1826), for which a neotype has been designated and deposited. Its diagnostic sequences of COI, 16SrDNA, H3 and ITS2 genes have also been deposited.

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in GenBank. Molecular and morphological (shell and genitalia) features showed that *M. parumcincta* (Rossmässler, 1834) is a distinct taxon from the *M. cantiana* lineages.

**Keywords**
16SrDNA, COI, H3, ITS2, molecular features, reproductive system, Roman origin, shell, structure, species distribution

**Introduction**

*Monacha* is a diverse genus of the trochuline hygromiids widespread in the western Palaearctic from western Europe to north Africa, Iran, and Arabia. It includes a large number of nominal species and shows its highest diversity in the eastern sector of southern Europe and in Turkey (Hausdorf 2000a, 2000b, Welter-Schultes 2012, Neiber and Hausdorf 2017).

*Monacha cantiana* (Montagu, 1803) is one of the westernmost species. It is a medium-sized land snail living among grass in open habitats such as grasslands, pastures, cultivated and uncultivated fields or forest edges and clearings. Its geographical distribution, probably southern European in origin, was partly shaped by anthropochorous dispersal which helped the species to reach north-western Europe. For example, in the British Isles it is considered to have been introduced and this hypothesis is supported by the absence of a Holocene fossil record in England older than the third century AD (Kerney et al. 1964, Kerney 1970, 1999, Evans 1972).

The aim of the present research was: (1) to study molecular and morphological (shell and genitalia) variation of the species in order to explore its phylogeography and detect any geographical patterns; (2) to investigate relationships between molecular and morphological variability in order to characterise clades recovered by molecular study; (3) to test the hypothesis that the English populations originated from introduced propagules.

**Material and methods**

**Taxonomic sample**

Our analysis considered a number of populations of *Monacha cantiana*, mainly from Italy and England, that represent its gross morphological, geographical, and ecological variability. Some sequences deposited in GenBank were also considered for the molecular analysis. One population from the type locality of *Theba cemenelea* Risso, 1826 a taxon regarded as a junior synonym, subspecies or species, slightly distinct from *M. cantiana*, was also included. For comparison, two other *Monacha* species were used in the molecular analysis: *Monacha cartusiana* (Müller, 1774) and *M. parumcincta* (Rossmässler, 1834). The latter was also used in the morphological analysis. While
M. cartusiana is a well-established taxon, the taxonomic and nomenclatural status of M. parumcincta is still disputed, e.g. conspecificity of Italian and Balkan populations, authorship to Rossmässler, 1834 or Menke, 1828 (see Forcart 1965, Manganelli et al. 1995, Welter-Schultes 2012).

**Material examined**

Material examined is listed as follows, when possible: geographic coordinates of locality, locality (country, region, site, municipality and province), collector(s), date, number of specimens and collection in which material is kept in parenthesis (Table 1). Collection acronyms: FGC (F. Giusti collection, Dipartimento di Scienze Fisiche, della Terra e dell’Ambiente, Università di Siena, Italy); DCBC (Department of Cell Biology Collection, Adam Mickiewicz University, Poznań, Poland).

**DNA extraction, amplification, and sequencing**

Small foot tissue fragments of alcohol-preserved snails were used for total DNA extraction with Tissue Genomic DNA extraction Mini Kits (Genoplast) according to the manufacturer’s instructions. The purified total DNA was used as template for amplification by polymerase chain reaction (PCR) of partial sequences of the following genes: mitochondrial cytochrome c oxidase subunit I (COI), 16S ribosomal DNA (16SrDNA), nuclear histone H3 (H3) and fragment enclosing partial sequence of 5.8SrDNA and complete sequence of internal transcribed spacer 2 of ribosomal DNA (ITS2). A 5’-end fragment of COI (often called “barcode sequence”) was amplified and sequenced using two degenerate primers F01-R04 (F01 5’-CATTTTTCATAAY-CATAARGATATTGG-3’ and R04 5’-TATAAACYTCDDGATGNCCAAAAA-3’; Dabert et al. 2010). The 16SrRNA gene was amplified and sequenced using primer pair 5’-CGATTTTGAACTCAGATCA-3’ (LR-J-12887, Simon et al. 1994) and 5’-GTGCAAAGGTAGCATAATCA-3’ (Gantenbein et al. 1999). The DNA fragment coding H3 was amplified and sequenced using primer pair H3F-H3R (H3F 5’-ATGGCTCGTACCAAGCAGACVGC-3’ and H3R 5’-ATATCCCTTRGGCATRATRGTGAC-3’; Colgan et al. 1998). The fragment encoding partial sequence of 5.8SrDNA and complete sequence of ITS2 was obtained for analyses using primers NEWS2 (5’-TGTGGTCGATGAAGAACGCAGC-3’) and ITS2-RIXO (5’-TTCTATGCT-TAAATTCCAGGGG-3’) (Almeyda-Artigas et al. 2000).

The amplified COI fragments consisted of 650 base pairs (bp). Polymerase chain reactions were performed in a volume of 10 µl according to the modified protocol prepared by the Biodiversity Institute of Ontario for the Consortium for the Barcode of Life (http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf). Reactions were carried out under the following thermal profile: 1 min at 94 °C followed by 42 cycles of 40 s at 94 °C, 40 s at 53 °C, 1 min at 72 °C,
Table 1. List of localities of the specimens of *Monacha cantiana* (CAN-1 to CAN-4), *M. parumcincta* and *M. cartusiana* used for molecular and morphological (SH shell, AN genitalia) research.

| No. | coordinates | country and site | collector / date / no. of specimens (collection) | Localities | Clade | COI | 16SrDNA | H3 | ITS2 | PCA and RDA | Figs. |
|-----|-------------|------------------|------------------------------------------------|------------|-------|-----|---------|-----|-------|-------------|-------|
| 1   | 53°31'29"N 01°27'54"W | United Kingdom, Barrow near Barnley | R.A.D. Cameron / 10.2011 / 5 (FGC 40329) (Piekowska et al. 2015) | CAN-1 | M. cantiana | UK-COI 1 | 4 | KM247375 | 5 | KM247390 | UK-H3 1 | 3 | MH137963 | 20, 22-23, 25 |
| 2   | 51°30'30"N 00°15'38"W | United Kingdom, East Acton near London | M. Procków / 07.06.2010 / 3 (DCBC & FGC 42965) | CAN-1 | M. cantiana | UK-COI 1 | 3 | KM247388 | 3 | KM208961 | UK-ITS2 2 | 3 | MH137964 | SH, AN |
| 3   | Not available | United Kingdom, Cambridge (old material) | F. Giusti / 1981 / 3 (FGC 23773) | CAN-1 | M. cantiana | UK-COI 2 | 1 | KM208891 | UK-16S 1 | 2 | KM208972 | UK-H3 2 | 1 | MH137965 |
| 4   | 53°24'49.1"N 01°24'00.5"W | United Kingdom, Rotherham | R.A.D. Cameron / 07.2015 / 17 (DCBC) | CAN-1 | M. cantiana | UK-COI 1 | 1 | KM208883 | UK-16S 1 | 2 | KM208960 | UK-ITS2 2 | 1 | MH137966 |
| 5   | 53°24'49.1"N 01°24'36.6"W | United Kingdom, Sheffield | R.A.D. Cameron / 07.2015 / 6 (DCBC) | CAN-1 | M. cantiana | UK-COI 6 | 1 | KM208896 | UK-16S 2 | 1 | KM208976 | UK-H3 5 | 1 | MH137970 |

| new haplotype | no. sps | new haplotype | no. sps | new common sequence | no. sps | new common sequence | no. sps | SH, AN |
|---------------|---------|---------------|---------|---------------------|---------|---------------------|---------|--------|
| KM247375 | 4 | KM208884 | 5 | MG208961 | 3 | MG209031 | 2 | SH, AN |
| MG208885 | 4 | MG208886 | 5 | MG208967 | 3 | MG209032 | 2 | MH137964 |
| MG208887 | 4 | MG208962 | 5 | MG208968 | 3 | MG209033 | 2 | MH137965 |
| MG208890 | 4 | MG208962 | 5 | MG208969 | 3 | MG209033 | 2 | MH137966 |
| MG208891 | 4 | MG208962 | 5 | MG208973 | 3 | MG209034 | 2 | MH137967 |
| MG208892 | 4 | MG208962 | 5 | MG208974 | 3 | MG209035 | 2 | MH137976 |
| MG208883 | 4 | MG208962 | 5 | MG208975 | 3 | MG209036 | 2 | MH137977 |
| MG208884 | 4 | MG208962 | 5 | MG208976 | 3 | MG209037 | 2 | MH137978 |
| MG208885 | 4 | MG208962 | 5 | MG208977 | 3 | MG209038 | 2 | MH137979 |
| MG208886 | 4 | MG208962 | 5 | MG208978 | 3 | MG209039 | 2 | MH137980 |
| MG208887 | 4 | MG208962 | 5 | MG208979 | 3 | MG209040 | 2 | MH137981 |
| No. | Coordinates          | Locality                                      | Collector | Date        | no. of specimens | New haplotype no. | COI GenBank No. | 16S GenBank No. | ITS2 GenBank No. |
|-----|----------------------|-----------------------------------------------|-----------|-------------|------------------|------------------|----------------|----------------|-----------------|
| 5   | 52°34'49.1"N 01°24'36.6"W | United Kingdom, Sheffield | R.A.D. Cameron | 07.2015 | 6 (DCBC) | CAN-1 M. cantiana | MG208903 | MG208904 | MG209023 |
| 6   | 42°28'41.05"N 13°05'09.46"E | Italy, Latium, Gole del Velino, near Sigillo (Posta, Rieti) | A. Hallgass | 30.09.2012 | 8 (FGC 42960) | CAN-1 M. cantiana | MG208905 | MG208977 | MG209039 |
| 7   | Not available | Italy, Latium, Elba Island (Livorno) | F. Giusti | 19.02.1974 | 1 (FGC 23586) | CAN-1 M. cantiana | MG208913 | MG208914 | MG208915 |
| 8   | 42°02'51.18"N 12°54'19.64"E | Italy, Latium, Valle dell'Aniene (Roccagiovine, Rome) | A. Hallgass | 20.10.2013 | 6 (FGC 42973) | CAN-1 M. cantiana | MG208916 | MG208917 | MG208918 |
| 9   | 42°43'39.87"N 13°16'01.44"E | Italy, Latium, Valle del Tronto (Accumoli, Rieti) | A. Hallgass | 30.09.2012 | 4 (FGC 42962) | CAN-1 M. cantiana | MG208919 | MG208920 | MG208921 |
| 10  | 42°07'53.38"N 13°01'39.81"E | Italy, Latium, Valle del Tronto, near Turania (Rieti) | A. Hallgass | 04.11.2013 | 2 (FGC 42969) | CAN-1 M. cantiana | MG208922 | MG208923 | MG208924 |

**Figs.**
- PCA
- RDA
- ITS2
| No. | coordinates | country and site | collector / date / no. of specimens (collection) | Clade | Revised taxonomy | COI | 16SrDNA | H3 | ITS2 | PCA and RDA | Figs. |
|-----|-------------|-----------------|--------------------------------------------------|-------|----------------|-----|---------|----|-------|-------------|------|
| 11  | 43°22'59.9"N 02°59'00.0"W | Spain, Sopelana, Pais Vasco | unknown / (SP164) (Razkin et al. 2015; Neiber and Hausdorf 2015) | CAN-1 | M. cantiana | KX507234 | KJ458539 KX495428 | | | | |
| 12  | 45°11'59.85"N 10°58'49.30"E | Italy, Venetum, Sorgà (Verona) | A. Hallgass / 09.2012 / 6 (F GC 42964) | CAN-2 | M. cantiana | IT-COI 9 3 MG208925 MG208926 MG208927 MG208928 MG208929 MG208930 | GT-16S 3 2 MG208996 MG208997 | IT-H3 9 1 | MG209050 | IT-ITS2.7 1 | MH137979 SH, AN 12, 36-39 |
| 13  | 45°31'28.95"N 10°21'35.75"E | Italy, Lombardy, Rezzato (Bre- scia) | A. Hallgass / 07.2012 / 3 (F GC 42976) | CAN-2 | M. cantiana | IT-COI 10 2 MG208931 MG208932 | GT-16S 4 3 MG209000 MG209002 MG209003 MG209004 | IT-H3 9 1 | MG209051 | IT-ITS2.8 1 | MH137981 AN 31-35 |
| 14  | 43°15'58.76"N 11°28'26.20"E | Italy, Tuscany, Podere Grania (Asciano, Siena) | G. Manganeli et al. 2005 | M. sp. | | | | | | | |
| 15  | 44°22'09.98"N 11°15'11.28"E | Italy, Emilia Romagna, along Fiume Setta, upstream its confluence with Fiume Reno (Sasso Marconi, Bologna) | A. Hallgass / 09.2012 / 3 (F GC 42977) | CAN-3 | M. sp. | IT-COI 11 1 MG208933 IT-16S 6 1 MG209007 IT-H3 2 1 MG209054 IT-ITS2.9 1 | IT-COI 12 1 MG208934 IT-16S 5 2 MG209005 IT-H3 1 1 MG209053 | IT-COI 13 1 MG208935 | IT-H3 11 1 MG209040 | IT-ITS2.9 1 | MH137982 SH, AN 13, 40-42 |
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| No. | Localities | Country and site | Collector / date / no. of specimens (collection) | Clade | New haplotype no. | GenBank sps | GenBank sps | GenBank sps |
|-----|------------|------------------|--------------------------------------------------|-------|------------------|-------------|-------------|-------------|
| 16  | Italy, Friuli-Venezia Giulia, Passo di Monte, Croce Carmo | 46°36'00.9"N 12°57'59.7"E | unknown (Duda et al. 2011; Kruckenhauser et al. 2014; Cadahia et al. 2014) | CAN-3 | M. sp. | HQ204502 | HQ204543 | KF596863 |
| 17  | Austria, Breitenlee, abandoned railway station | 48°15'25.50"N 16°30'46.38"E | M. Duda / 09.2015 / 3 (FGC 44020) | ATCOI1 | ATCOI1 | MG208936 | MG208957 | AT16S2 |
| 18  | France, Alpes-Maritimes, Vallee de Peillon, Sainte Thecle | 43°46'11.79"N 07°22'21.50"E | A. Hallere / 26.10.2011 / 3 (FGC40320) | CAN-3 | M. sp. | CAN-3 | CAN-3 | CAN-3 |
| 19  | France, Arcache, Jaujac | 44°38'09"N 01°15'34"E | (Dahirel et al. 2015) | PAR | PAR | MG208954 | MG208954 | MG208954 |
| 20  | Italy, Tuscany, along the road to Medane (Asciano, Siena) | 43°18'59.40"N 11°30'04.20"E | G. Manganelli / 08.10.2000 / (FGC 12956) | IT-COI1 | IT-COI1 | MG208939 | MG208941 | MG208943 |
| 21  | Italy, Tuscany, along the road to Medane (Asciano, Siena) | 43°17'15.33"N 11°25'19.35"E | G. Manganelli / 06.10.2000 / (FGC 40407) | PAR | PAR | MG208953 | MG208953 | MG208953 |
| No. | Coordinates          | Collector / date / no. of specimens (collection) | Localities                                                                 | CoI     | Revised taxonomic name | New haplotype no. | GenBank sps. GenBank # | New common sequence no. | GenBank sps. GenBank # |
|-----|---------------------|-----------------------------------------------|----------------------------------------------------------------------------|---------|------------------------|------------------|------------------------|------------------------|------------------------|
| 22  | 43°54'18.00"N 00°49'13.63"E | A. Hallgass / 20.10.2013 / 2 (FGC 41562)      | Italy, Tuscany, Nievole (Mon-tecatini Terme, Pistoia)                      | PAR     | M. parum-cincta        | IT-COI 19        | MG208949 IT-16S 10 | 1                      | MG208947                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 20         | MG208953 IT-16S 12 | 2                      | MG208948                |
|     |                     |                                               |                                                                            |         |                        | IT-H3 12          | MG209069            | 3                      | MG209068                |
| 23  | 43°30'19.55"N 11°38'54.92"E | A. Hallgass / 10.2013 / 6 (FGC 41561)          | Italy, Tuscany, Autostrada A1: rest area near Ponte Romita (Firenze, Toscana) | PAR     | M. parum-cincta        | IT-COI 19        | MG208950 IT-16S 12 | 2                      | MG209063                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 20         | MG208956 IT-16S 14 | 3                      | MG209065                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 21         | MG208957 IT-16S 16 | 1                      | MG209067                |
| 24  | 40°13'25.49"N 15°52'17.07"E | A. Hallgass / 2012 / 5                         | Italy, Basilicata, along the road from Moliterno to Fontana di Tre Casali (Potenza) | PAR     | M. parum-cincta        | IT-COI 14        | MG208944 IT-16S 8  | 2                      | MG209061                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 15         | MG208945 IT-16S 9  | 1                      | MG209018                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 16         | MG208946 IT-16S 10 | 1                      | MG209019                |
|     |                     |                                               |                                                                            |         |                        | IT-COI 17         | MG208948            | 1                      | MG209016                |
| 25  | 46°42'10"N 17°14'38"E | J.R. Pieńkowska / 31.07.2011 / 8 (DCBC)     | Hungary, Kis-Balaton, about 50 m from Zalinda on Zalinda Karai Zala (Zala Balaton) | SH, AN  | M. car-tusiana         | KM247391 KM247396 | KX507189               | KM247390               | KX507186               |
| 26  | 45°46'38"N 10°30'12"E | B. Hausdorf / 19.08.2009                      | Italy: Brescia, Anfo towards Ponte Caffaro, calcareous rocks at branch of Ticino towards Tre Cesoli (Lombardia) | SH, AN  | M. car-tusiana         | KM247397          | KX597378               | KM247389               | KX597381               |
and finally 5 min at 72 °C. The amplified 16SrDNA fragments were of about 385 positions. The amplification reactions were conducted in a volume of 10 μl according to a previously described procedure (Manganelli et al. 2005). The amplified H3 sequences consisted of 429 bp. PCR reactions (10 μl) were performed according to the procedure described by Colgan et al. (1998). The 585 position-long sequences of regions enclosing 89 positions of 3’-end of 5.8SrDNA and 496 positions of complete sequence of ITS2 were amplified according to procedure described by Almeyda-Artigas et al. (2000).

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

**Phylogenetic inference**

All individual sequences were deposited in GenBank (Table 1). The following COI sequences from GenBank were used: HQ204502 (Duda et al. 2011), KF596907 (Cadahia et al. 2014), KF986833 (Dahirel et al. 2015), KM247375 (Pieńkowska et al. 2015) and KX507234 (Neiber and Hausdorf 2015) of *M. cantiana*, as well as KM247376, KM247389 (Pieńkowska et al. 2015) and KX507189 (Neiber and Hausdorf 2015) of *M. cartusiana* (as an outgroup). Regarding 16SrDNA, the following sequences from GenBank were used: AY741419 (Manganelli et al. 2005), HQ204543 (Duda et al. 2011), KF596863 (Cadahia et al. 2014), KJ458539 (Razkin et al. 2015), KM247390 (Pieńkowska et al. 2015) and KX495428 (Neiber and Hausdorf 2015) of *M. cantiana*, AY741418 (Manganelli et al. 2005) of *M. parumcincta* and KM247391, KM247397 (Pieńkowska et al. 2015) and KX49537 (Neiber and Hausdorf 2015) of *M. cartusiana* (as an outgroup). In analysis of H3 relationships the sequence KF596955 deposited in GenBank by Cadahia et al. (2014) was used.

Sequences were edited by eye using the program BIOEDIT, version 7.0.6 (Hall 1999). The alignments were performed using the CLUSTAL W programme (Thompson et al. 1994) implemented in MEGA 7 (Kumar et al. 2016). The COI sequences 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 cutting were 592 bp for COI, 287 positions for 16SrDNA, 315 bp for H3 and 496 positions for ITS2. The sequences were collapsed to haplotypes (COI and 16SrDNA) and to common sequences (H3 and ITS2) using the programme ALTER (Alignment Transformation EnviRonment) (Glez-Peña et al. 2010). Gaps and ambiguous positions were removed from alignments prior to phylogenetic analysis.

Maximum Likelihood (ML) analyses were performed with MEGA 7. For each alignment file best nucleotide substitution models were specified according to the
Bayesian Information Criterion (BIC): HKY+I for COI sequences (Hasegawa et al. 1985, Kumar et al. 2016), T92+I for 16SrDNA (Tamura 1992, Kumar et al. 2016), TN93+G+I for H3 (Tamura and Nei 1993, Kumar et al. 2016) and JC+G for ITS2 (Jukes and Cantor 1969, Kumar et al. 2016). In parallel, the sequences of COI and 16SrDNA obtained in the present work together with other sequences obtained from GenBank were analysed by the genetic distance Neighbour-Joining method (Saitou and Nei 1987) implemented in MEGA7 (Kumar et al. 2016) using the Kimura two-parameter model (K2P) for pairwise distance calculations (Kimura 1980). Next, mitochondrial sequences of COI and 16SrDNA, and nuclear sequences of H3 and ITS2 were combined and as two data sets subjected to ML analysis. The combined sequences were of length of 879 positions for COI+16SrDNA pair and of 811 positions for H3+ITS2. The specified best nucleotide substitution models for ML analysis according to the Bayesian Information Criterion (BIC) were: HKY+I (Hasegawa et al. 1985, Kumar et al. 2016) for COI+16SrDNA combined sequences and TN93+G+I (Tamura and Nei 1993, Kumar et al. 2016) for H3+ITS2. Finally, sequences of COI, 16SrDNA and H3 were combined for Bayesian inference. Before doing so, uncertain regions were removed from 16SrDNA alignment with the programme GBLOCKS 0.91b (Castresana 2000, Talavera and Castresana 2007) with parameters for relaxed selection of blocks. This procedure shortened alignment of 16SrDNA sequences from 287 to 271 positions. The combined sequences with a total length of 1178 positions (592 COI + 271 16SrDNA + 315 H3) were used to infer group phylogeny by Bayesian analysis conducted with the program MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). Monacha cartusiana was added as an outgroup species in each analysis. Using JMODELTEST2 (Darriba et al. 2012) according to the Bayesian Information Criterion (BIC), we specified a HKY substitution model for our data set (Hasegawa et al. 1985), assuming a gamma distributed rate variation among sites. Four Monte Carlo Markov chains were run for 1 million generations, sampling every 100 generations (the first 250 000 trees were discarded as ‘burn-in’). This gave us 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.

The haplotype network was inferred with NETWORK 5.0.0.1 to reflect all relationships between COI and 16SrDNA haplotypes. During the analysis, a median-joining calculation implemented in NETWORK 5.0.0.1 was used (Bandelt et al. 1999).

Morphological study

Approximately 70 specimens of five clades (four lineages of the M. cantiana group: CAN-1, CAN-2, CAN-3 and CAN-4; one lineage of M. parumcincta) were considered for shell variability (see Table 1). Shell variability was analysed randomly, choosing when possible five adult specimens from each population. Thirteen shell variables were measured to the nearest 0.1 mm using ADOBE PHOTOSHOP 7.0.1 on digital im-
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...ages 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, LWH last whorl height, 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, UD umbilicus diameter (Fig. 1).

Approximately 60 specimens of five clades (all lineages of the *M. cantiana* group plus one lineage of *M. parumcincta*) were analysed for anatomical variability (see Table 1). Snail bodies were dissected under the light microscope (Wild M5A or Zeiss SteREO Lumar V12). Anatomical structures were drawn using a Wild camera lucida. Acronyms: BC bursa copulatrix, BW body wall, DBC duct of bursa copulatrix, DG digitiform glands, E epiphallus (from base of flagellum to beginning of penial sheath), F flagellum, FO free oviduct, GA genital atrium, GAR genital atrium retractor, OSD ovispermiduct, P penis, V vagina, VA vaginal appendix (also known as appendicula), VAS vaginal appendix basal sac, VD vas deferens. Six anatomical variables (DBC, E, F, P, V, VA) were measured using a calliper under a light microscope (0.01 mm) (Fig. 2).

Multivariate ordination by Principal Component Analysis (PCA) was performed on shell and genitalia matrices separately in order to determine the degree of correlation between variables and their role in explaining variability. Before PCA, variables were log-transformed to obtain a linear relationship. Since variation in size is the first determinant of biometric variation (e.g., Cadima and Jolliffe 1996, Klingenberg 2016), multivariate morphometrics to distinguish size and shape components by removing isometric effects are nowadays routinely applied in shell biometry studies (Madec et al. 2003, Paquette and Lapointe 2007, Fiorentino et al. 2008, Caruso and Chemello 2009). We therefore performed two PCAs for each data set (shell, genitalia), one on the original matrices and one on the Z-matrices, the latter only consider shape components according to the methods proposed by Cadima and Jolliffe (1996).

Redundancy analysis (RDA; ter Braak 1986) was then applied to the original matrices and Z-matrices in order to detect any multivariate relationships between shell/genitalia variables and the taxonomic assignment. The factors “clade/lineage” were used as constraint factor. An ANOVA-like permutation test for constrained ordination was used to assess the significance (P-value < 0.05) of the constraint for the first two RDA axes. Vegan package (Oksanen et al. 2016) in RStudio 1.0.136 (RStudio Team 2016) was used for processing.

Differences between species for each shell and genitalia characters were assessed through box-plots and descriptive statistics. The significance of differences (P < 0.01) was obtained using analysis of variance (ANOVA); where the test proved significant, an adjusted a posteriori pair-wise comparison between pairs of species was performed using Tukey’s honestly significant difference (HSD) test. All variables were log transformed before analysis.
Results

Molecular study

Thirty-nine and 18 haplotypes of COI and 16SrDNA mitochondrial gene fragments, respectively, as well as 23 and 18 common nucleotide sequences of histone H3 and ITS2 nuclear gene fragments, respectively, were established (Table 1). As a result, 77 sequences of COI as MG208883–MG208959, 71 sequences of 16SrDNA as MG208960–MG209030, 42 sequences of H3 as MG209031–MG209072 and 31 sequences of ITS2 as MH137963–MH137993 were deposited in GenBank (see also Table 1). ML tree for combined sequences of COI and 16SrDNA (Fig. 3, Table 2) as well as Bayesian phylogenetic tree for combined sequences of COI+16SrDNA+H3 gene fragments (Fig. 4, Table 2) clustered the received combined sequences in five
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Figure 3. Maximum Likelihood (ML) tree of combined COI and 16SrDNA haplotypes of Monacha cantiana group (see: Table 2). Bootstrap support above 50% from maximum likelihood analysis is marked at the nodes. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with M. cartusiana combined sequences obtained from GenBank: KM247376 and KM247391.

clearly separate clades. ML tree of combined sequences of nuclear H3 and ITS2 gene fragments (Fig. 5, Table 2) clustered the combined sequences in three clades.

First clade CAN-1 includes 14 combined sequences in particular trees (Figs 3–4). The clade includes haplotypes and common sequences (Table 1) which have been found in specimens from the following UK populations: Barrow near Barnsley, East Acton, Cambridge, Rotherham and Sheffield, together with those found in specimens from Italian populations from Latium (Gole del Velino, Valle dell’Aniene, Valle del Tronto and Valle del Turano), as well as from Elba island (Tuscan Archipelago). It is noteworthy that sequences of haplotypes UK-COI 1 and UK-16S 1 are identical to sequences KM247375 and KM247390 deposited in GenBank for COI and 16SrDNA of M. cantiana, respectively (Pieńkowska et al. 2015). It is also important that UK haplotypes UK-COI 2, UK-16S 2 and UK-ITS 2 are identical to Italian IT-COI 2,
Figure 4. Bayesian 50% majority-rule consensus tree obtained from analysis of the combined data set of COI, 16SrDNA, and H3 sequences (see: Table 2). Posterior probabilities (left) and bootstrap support above 50% from Maximum Likelihood analysis (right) are marked at the nodes. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with \textit{M. cartusiana} combined sequences KM247376, KM247391 and MG209072. It-16S 1 and IT-ITS2 1, respectively. Moreover, sequences KX507234, KJ458539 and KX495428 deposited in GenBank for \textit{M. cantiana} from País Vasco, Sopelana (Neiber and Hausdorf 2015, Razkin et al. 2015), suggest that this Spanish population also belongs to the clade CAN-1. K2P genetic distances between COI and 16SrDNA haplotypes are rather small within the clade CAN-1 (Table 3).

Clade CAN-2 (Figs 3–4) includes four COI+16SrDNA combined haplotypes and four COI+16SrDNA+H3 combined sequences. All came from two north Italian populations: Sorgà in Venetum and Rezzato in Lombardy (Table 1). K2P distances between COI and 16SrDNA haplotypes of the clade CAN-2 are very small (Table 3).
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Figure 5. Maximum Likelihood (ML) tree of combined H3 and ITS2 sequences of *Monacha cantiana* group (see: Table 2). Bootstrap support above 50% from maximum likelihood analysis is marked at the nodes. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). The tree was rooted with *M. cartusiana* combined sequences MG209072 and MH137993.

This CAN-2 clade is not separated from CAN-1 and CAN-3 on the tree of combined nuclear gene sequences (Fig. 5).

Clade CAN-3 is composed of five combined sequences both in COI+16SrDNA (Fig. 3) and COI+16SrDNA+H3 (Fig. 4) trees. It is also not separated in the tree of combined sequences of nuclear H3+ITS2 gene fragments (Fig. 5). The sequences, i.e., COI, 16SrDNA, H3 and ITS2 were from specimens either from Breitenlee in Austria (in Figs 3–5, and Table 1 marked as AT-) or from northern Italy (near Bologna, marked IT-). Sequences deposited in GenBank by Duda et al. (2011), Kruckenhauser et al. (2014) (COI HQ204502, 16SrDNA HQ204543) and by Cadahia et al. (2014) (COI KF596907, 16SrDNA KF596863, H3 KF596955) for *M. cantiana* from the Carnic Alps, Friuli Venezia Giulia, also belong to the CAN-3 lineage. K2P genetic distances of haplotypes within clade CAN-3 varied in a small range (Table 3).

Clade CAN-4 (Figs 3–5) includes three COI+16SrDNA, one H3+ITS2 and three COI+16SrDNA+H3 combined sequences. All were from specimens of a French population in the Maritime Alps near Nice (Sainte Thecle, Table 1). Again K2P genetic distances in this population were small (Table 3). COI sequence KF986833 deposited in GenBank by Dahirel et al. (2015) for *M. cantiana* from Monts d’Ardèche Natural Regional Park near Jaujac (S France) seems to belong to the same clade.

The fifth clade PAR was composed of sequences from specimens identified as *M. parumcineta*. Eight COI and six 16SrDNA haplotypes, as well as two H3 and four ITS2 common sequences were recognised among specimens from four populations from central and southern Italy (Table 1). K2P genetic distances within this clade were larger than
Table 2. Combined Sequences of the following gene sequences: COI+16SrDNA and H3+ITS2 for ML analysis and of COI+16SrDNA+H3 for Bayesian analysis.

| Combined Sequence | COI haplotype | 16S haplotype | Combined Sequence | H3 sequence | ITS2 sequence | Combined Sequence | COI haplotype | 16S haplotype | H3 sequence | Locality (number of specimens) |
|-------------------|---------------|---------------|-------------------|-------------|---------------|-------------------|---------------|---------------|-------------|---------------------------------|
| UK-COI16S-1       | UK-COI 12     | UK-16S 1      | UK-COI16S-2       | UK-COI 1    | UK-16S 1      | UK-H3ITS2-1       | UK-H3 1       | UK-ITS2 2     | UK-CS_2     | UK, Sheffield (1)               |
| IT-COI16S-1       | IT-COI 3      | IT-16S 1      | IT-H3ITS2-3       | IT-H3 7     | IT-ITS2 3     | IT-CS_1           | IT-CS_1       | IT-ITS2 3     | IT-H3 7     | Italy, Latium, Valle dell’Aniene (1) |
| IT-COI16S-2       | IT-COI 3      | IT-16S 1      | IT-H3ITS2-2       | IT-H3 6     | IT-ITS2 2     | IT-CS_2           | IT-CS_2       | IT-ITS2 2     | IT-H3 6     | Italy, Latium, Valle dell’Aniene (1) |
| IT-COI16S-3       | IT-COI 3      | IT-16S 1      | IT-H3ITS2-4       | IT-H3 8     | IT-ITS2 3     | IT-CS_3           | IT-CS_3       | IT-ITS2 3     | IT-H3 8     | Italy, Latium, Valle dell’Aniene (1) |
| IT-COI16S-4       | IT-COI 1      | IT-16S 1      | IT-H3ITS2-5       | IT-H3 1     | IT-ITS2 4     | IT-CS_4           | IT-CS_4       | IT-ITS2 4     | IT-H3 1     | Italy, Latium, Gole del Velino (1) |
| IT-COI16S-5       | IT-COI 1      | IT-16S 1      | IT-H3ITS2-6       | IT-H3 4     | IT-ITS2 5     | IT-CS_5           | IT-CS_5       | IT-ITS2 5     | IT-H3 4     | Italy, Latium, Gole del Velino (1) |
| IT-COI16S-6       | IT-COI 1      | IT-16S 1      | IT-H3ITS2-1       | IT-H3 3     | IT-ITS2 1     | IT-CS_6           | IT-CS_6       | IT-ITS2 1     | IT-H3 3     | Italy, Latium, Valle del Tronto (2) |
| IT-COI16S-7       | IT-COI 1      | IT-16S 1      | IT-H3ITS2-7       | IT-H3 10    | IT-ITS2 8     | IT-CS_12          | IT-CS_12      | IT-ITS2 8     | IT-H3 10    | Italy, Lombardia, Rezzato (1) |
| IT-COI16S-8       | IT-COI 2      | IT-16S 1      | IT-H3ITS2-2       | IT-H3 10    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 12    | Italy, Emilia Romagna (1) |
| IT-COI16S-9       | IT-COI 9      | IT-16S 3      | IT-CS_9           | IT-CS_9     | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 2     | Italy, Emilia Romagna (1) |
| IT-COI16S-10      | IT-COI 9      | IT-16S 4      | IT-CS_10          | IT-CS_10    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 2     | Italy, Emilia Romagna (1) |
| IT-COI16S-11      | IT-COI 10     | IT-16S 4      | IT-CS_11          | IT-CS_11    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 1     | Italy, Emilia Romagna (1) |
| IT-COI16S-12      | IT-COI 10     | IT-16S 4      | IT-H3ITS2-7       | IT-H3 10    | IT-ITS2 8     | IT-CS_12          | IT-CS_12      | IT-ITS2 8     | IT-H3 10    | Italy, Lombardia, Rezzato (1) |
| IT-COI16S-13      | IT-COI 12     | IT-16S 5      | IT-CS_13          | IT-CS_13    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 11    | Italy, Emilia Romagna (1) |
| IT-COI16S-14      | IT-COI 11     | IT-16S 6      | IT-H3ITS2-8       | IT-H3 2     | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 2     | Italy, Emilia Romagna (1) |
| IT-COI16S-15      | IT-COI 13     | IT-16S 5      | IT-CS_15          | IT-CS_15    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 1     | Italy, Emilia Romagna (1) |
| IT-COI16S-16      | IT-COI 14     | IT-16S 8      | IT-CS_16          | IT-CS_16    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 12    | Italy, Basilicata (1) |
| IT-COI16S-17      | IT-COI 15     | IT-16S 9      | IT-CS_17          | IT-CS_17    | IT-ITS2 9     | IT-CS_14          | IT-CS_14      | IT-ITS2 9     | IT-H3 12    | Italy, Basilicata (1) |
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| Combined Sequence | COI haplotype | 16S haplotype | Combined Sequence | H3 haplotype | ITS2 sequence | Combined Sequence | COI haplotype | 16S haplotype | H3 haplotype | ITS2 sequence | Localities (number of specimens) |
|-------------------|---------------|---------------|------------------|--------------|---------------|------------------|---------------|---------------|--------------|---------------|-------------------------------|
| IT-COI16S-18      | IT-COI 18     | IT-16S 10     | IT-H3ITS2-9      | IT-H3 12     | IT-ITS2 11    | IT-CS_18         | IT-COI 18     | IT-16S 10     | IT-H3 12     | IT-ITS2 11    | Italy, Tuscany, Nievole (1) |
| IT-COI16S-19      | IT-COI 19     | IT-16S 12     | IT-H3ITS2-10     | IT-H3 12     | IT-ITS2 11    | IT-CS_19         | IT-COI 19     | IT-16S 12     | IT-H3 12     | IT-ITS2 11    | Italy, Tuscany, Arezzo (1)  |
| IT-COI16S-20      | IT-COI 20     | IT-16S 11     | IT-H3ITS2-10     | IT-H3 12     | IT-ITS2 11    | IT-CS_20         | IT-COI 20     | IT-16S 11     | IT-H3 12     | IT-ITS2 11    | Italy, Tuscany, Arezzo and Nievole (3) |
| IT-COI16S-21      | IT-COI 21     | IT-16S 12     | IT-H3ITS2-11     | IT-H3 13     | IT-ITS2 11    | IT-CS_21         | IT-COI 21     | IT-16S 12     | IT-H3 13     | IT-ITS2 11    | Italy, Tuscany, Arezzo (1)  |
| IT-COI16S-22      | IT-COI 21     | IT-16S 12     | IT-H3ITS2-12     | IT-H3 12     | IT-ITS2 12    | IT-CS_22         | IT-COI 21     | IT-16S 12     | IT-H3 12     | IT-ITS2 11    | Italy, Tuscany, Arezzo and La Casella (2) |
| FR-COI16S-1       | FR-COI 1      | FR-16S 1      | FR-H3ITS2-1      | FR-H3 1      | FR-ITS2 1     | FR-CS_1          | FR-COI 1      | FR-16S 1      | FR-H3 1      | FR-ITS2 1     | France, Alpes-Maritimes, Sainte Thecle (1) |
| FR-COI16S-2       | FR-COI 2      | FR-16S 1      | FR-H3ITS2-1      | FR-H3 1      | FR-ITS2 1     | FR-CS_2          | FR-COI 2      | FR-16S 1      | FR-H3 2      | FR-ITS2 1     | France, Alpes-Maritimes, Sainte Thecle (1) |
| FR-COI16S-3       | FR-COI 2      | FR-16S 1      | FR-H3ITS2-1      | FR-H3 1      | FR-ITS2 1     | FR-CS_3          | FR-COI 2      | FR-16S 1      | FR-H3 3      | FR-ITS2 1     | France, Alpes-Maritimes, Sainte Thecle (1) |
| AT-COI16S-1       | AT-COI 1      | AT-16S 2      | AT-H3ITS2-1      | AT-H3 1      | AT-ITS2 1     | AT-CS_1          | AT-COI 1      | AT-16S 2      | AT-H3 1      | AT-ITS2 1     | Austria, Breitenlee (1)  |
| AT-COI16S-2       | AT-COI 2      | AT-16S 1      | AT-H3ITS2-1      | AT-H3 1      | AT-ITS2 1     | AT-CS_2          | AT-COI 2      | AT-16S 1      | AT-H3 1      | AT-ITS2 1     | Austria, Breitenlee (2)  |
| HU-COI16S-1       | KM247376      | KM247391      | HU-H3ITS2-1      | HU-H3 1      | HU-ITS2 1     | HU-CS_1          | KM247376      | KM247391      | HU-H3 1      | HU-ITS2 1     | Hungary, Kis-Balaton (1)   |
Table 3. Ranges of K2P genetic distances for COI and 16SrDNA sequences analysed (mean values in parentheses).

| Comparison                                      | COI (%)     | 16SrDNA (%)  |
|------------------------------------------------|-------------|--------------|
| Within *M. cantiana* CAN-1                    | 0.2–2.2 (0.9) | 0.7–1.4 (0.7) |
| Within *M. cantiana* CAN-2                    | 0.3 (0.3)   | 0.7 (0.7)   |
| Within *M. sp.* CAN-3                         | 0.2–1.9 (1.2) | 0.4–2.6 (1.5) |
| Within *M. cemenelea* CAN-4                   | 0.2–0.5 (0.3) | 0.7 (0.7)   |
| Within *M. parumcincta*                       | 0.2–4.6 (2.8) | 0.8–4.7 (2.5) |
| Between *M. cantiana* CAN-1 and *M. cantiana* CAN-2 | 3.3–5.3 (3.9) | 1.8–2.9 (2.5) |
| Between *M. cantiana* CAN-1 and *M. sp.* CAN-3 | 17.6–19.3 (18.6) | 17.5–18.9 (18.1) |
| Between *M. cantiana* CAN-1 and *M. cemenelea* CAN-4 | 17.1–18.9 (18) | 20.4–21.9 (21.4) |
| Between *M. cantiana* CAN-1 and *M. parumcincta* | 19.9–22.1 (20.9) | 24.7–26.4 (25.5) |
| Between *M. cantiana* CAN-2 and *M. sp.* CAN-3 | 17.8–18.2 (18.1) | 15.7–17.1 (16.4) |
| Between *M. cantiana* CAN-2 and *M. cemenelea* CAN-4 | 18.2–18.7 (18.4) | 19.6–20.6 (20.1) |
| Between *M. cantiana* CAN-2 and *M. parumcincta* | 19.7–20.9 (20.3) | 23.0–26.5 (24.3) |
| Between *M. sp.* CAN-3 and *M. cemenelea* CAN-4 | 5.1–6.2 (5.3) | 4.1–5.3 (4.8) |
| Between *M. sp.* CAN-3 and *M. parumcincta*     | 17.9–22.0 (19.7) | 19.3–21.8 (20.3) |
| Between *M. cemenelea* CAN-4 and *M. parumcincta* | 19.5–21.1 (20.1) | 20.4–22.4 (20.8) |

for other clades (up to 4.6% in COI haplotypes, Table 3). The clade PAR was clearly separated from other clades in each tree (Figs 3–5). Combined haplotypes IT-COI16S-16 – IT-COI16S-17 from Basilicata (S Italy) seem to form a separate subclade against haplotypes IT-COI16S-18 – IT-COI16S-21 from three other populations in Tuscany (Fig. 3).

K2P genetic distances between COI and 16SrDNA haplotypes are summarised in Table 3. The smallest distances are between haplotypes of CAN-1 and CAN-2 clades; however they are larger than distances within these clades. The largest K2P distances between COI sequences separate clade of haplotypes found in *M. parumcincta* from all other clades (by ca. 20–25%). Very large distances also separate clade CAN-1 from clades CAN-3 and CAN-4 (COI 18.0% and 18.6%, respectively). Distances between clade CAN-2 and CAN-3 on one hand, and CAN-4 on the other, are also large. Only distances between clades CAN-3 and CAN-4 are smaller (COI 5.3%) although they are much larger than within each of these clades.

Networks of COI (Fig. 6) and 16SrDNA (Fig. 7) confirm separateness of five clades. Clades CAN-1 and CAN-2 are much closer than the others; French haplotypes of clade CAN-4 are separate from the Austrian-Italian CAN-3; clade PAR of *M. parumcincta* haplotypes is differentiated into two subgroups.

Morphological study: shell

The *M. cantiana* group (clades CAN-1, CAN-2, CAN-3, CAN-4; Figs 8–15) and that of *M. parumcincta* (clade PAR; Fig. 16) have a globose-subglobose shell, variable in colour and size, with roundish aperture and very small or closed umbilicus. The main
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**Figure 6.** The median-joining haplotype network for COI haplotypes of Monacha cantiana group. The colours of the circles indicate Monacha species, and their size is proportional to haplotype frequencies. Small black circles are hypothetical missing intermediates.

**Figure 7.** Haplotype network for 16SrDNA of Monacha cantiana group. Other explanations as in Figure 6.

difference between the two groups consists in the umbilicus (very small, but always open in M. cantiana s.l.; closed in M. parumcincta). Some populations of M. parumcincta have variably evident whitish peripheral and subsutural bands (evident if the last whorl is reddish) and/or a less glossy (more opaque) shell surface.
Figures 8–16. Shell variability in *Monacha cantiana* s.l. group (8–15) and *Monacha parumcincta* (16). CAN-1 from Valle dell’Aniene (FGC 42973) (8), Gole del Velino, near Sigillo (FGC 42960) (9), Elba Island, Sant’Ilario in Campo (FGC 23586) (10) and Valle del Turano, near Turania (FGC 42969) (11); CAN-2 from Sorgà (FGC 42964) (12); CAN-3 from Fiume Setta (FGC 42977) (13) and Breitenlee (FGC 44020) (14); CAN-4 from Vallée de Peillon, Sainte Thecle (FGC 40320) (15); PAR from La Casella (FGC 44077) (16).
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RDA with “clade/lineage” constraint on the shape and size matrix (Fig. 17) showed that RDA 1 (47%, P < 0.001) separated the groups CAN-1, CAN-2 and CAN-3 from PAR with CAN-4 in intermediate position. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (78%) was a positive combination of all variables. On the contrary, RDA 2 (3%, P < 0.05) showed a statistically significant separation between CAN-4 and the others; no difference was found between the CAN-1, CAN-2 and CAN-3 groups. In this regard, PC2 (9%) accounted for a contrast between LWmH and LWaH / PWH variables. RDA on the shape (Z) matrix (Fig. 18) confirmed a statistically significant separation between PAR and CAN-4 with the large group CAN-1-CAN-2-CAN-3 in intermediate position. Shape-related PCA indicated that LWfW / LWmW / LWmH / SD / AD vs LWaH / PWH were the two principal shape determinants on PC1 and PWmW vs UD on PC2.

Box plots (Fig. 19) prove the poor discriminating value of shell characters in distinguishing species pairs (no character distinguishes more than four clade pairs according to Tukey’s honestly significant difference test). The most recognisable pairs are CAN-1 vs. PAR, CAN-2 vs. PAR, and CAN-3 vs. PAR (11, 9, and 10 significant
Figure 19. Box plots for shell characters of the five *Monacha* clades 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).
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Characters, respectively). Only two significant characters distinguish CAN-1 vs. CAN-4 and only one CAN-3 vs CAN-4 or CAN-4 vs. PAR. No significant character distinguishes CAN-1 vs. CAN-2, CAN-1 vs. CAN-3 or CAN-2 vs. CAN-3 (Table 4).

**Morphological study: anatomy**

The bodies (generally pinkish or yellowish white) and mantle (with sparse, variably numerous brown or blackish spots near mantle border or on the lung surface, one larger close to the pneumostomal opening) are very similar in the two species group, whereas the distal genitalia show some diagnostic features (Figs 20–50 vs. Figs 51–59): vagi-

**Table 4. Results of Tukey's honestly significant difference (HSD) test for shell and genitalia characters (in bold Tukey's post-hoc P < 0.01).**

| pairs          | SH   | AH   | LWmH | LWaH  | PWH  | SD  |
|----------------|------|------|------|-------|------|-----|
| CAN-1 vs CAN-2 | 0.97573 | 0.64561 | 0.99140 | 0.46817 | 0.95652 | 0.47286 |
| CAN-1 vs CAN-3 | 0.39185 | 0.18401 | 0.57940 | 1.00000 | 0.99945 | 0.15274 |
| CAN-1 vs CAN-4 | 0.05983 | 0.42921 | 0.92651 | 0.006065 | 0.00567 | 0.23583 |
| CAN-1 vs PAR   | 0.00001 | 0.00000 | 0.97255 | 0.00001 | 0.00144 | 0.00030 |
| CAN-2 vs CAN-3 | 0.97242 | 0.99963 | 0.98207 | 0.59785 | 0.98906 | 1.00000 |
| CAN-2 vs CAN-4 | 0.11515 | 0.14765 | 0.87857 | 0.38505 | 0.24954 | 0.04877 |
| CAN-2 vs PAR   | 0.00340 | 0.00008 | 1.00000 | 0.35237 | 0.39229 | 0.00082 |
| CAN-3 vs CAN-4 | 0.00569 | 0.02947 | 0.42967 | 0.00414 | 0.03203 | 0.01007 |
| CAN-3 vs PAR   | 0.00000 | 0.00000 | 0.92716 | 0.00047 | 0.03296 | 0.00001 |
| CAN-4 vs PAR   | 0.84947 | 0.12731 | 0.78714 | 0.99908 | 0.96245 | 0.84026 |

| pairs          | AD   | LWmW | PWmW | PWfW  | LWfW | UD  |
|----------------|------|------|------|-------|------|-----|
| CAN-1 vs CAN-2 | 0.51068 | 0.08476 | 0.82369 | 0.68103 | 0.18598 | 0.87507 |
| CAN-1 vs CAN-3 | 0.19064 | 0.03926 | 0.45194 | 0.22487 | 0.12364 | 0.99947 |
| CAN-1 vs CAN-4 | 0.33899 | 0.38635 | 0.06390 | 0.44613 | 0.90473 | 0.75084 |
| CAN-1 vs PAR   | 0.00010 | 0.00008 | 0.00206 | 0.00241 | 0.00002 | 0.00000 |
| CAN-2 vs CAN-3 | 1.00000 | 0.99124 | 0.99994 | 0.99975 | 0.99254 | 0.86022 |
| CAN-2 vs CAN-4 | 0.07939 | 0.01170 | 0.05068 | 0.16856 | 0.12920 | 0.48690 |
| CAN-2 vs PAR   | 0.00052 | 0.00002 | 0.01253 | 0.00695 | 0.00003 | 0.00000 |
| CAN-3 vs CAN-4 | 0.02106 | 0.00660 | 0.00750 | 0.03792 | 0.12320 | 0.89763 |
| CAN-3 vs PAR   | 0.00000 | 0.00000 | 0.00029 | 0.00009 | 0.00000 | 0.00000 |
| CAN-4 vs PAR   | 0.60652 | 0.53369 | 0.99999 | 0.86111 | 0.07669 | 0.00000 |

| pairs          | DBC  | V    | F    | E    | P    | VA  |
|----------------|------|------|------|------|------|-----|
| CAN-1 vs CAN-2 | 0.04626 | 0.99611 | 0.59664 | 0.09790 | 0.14384 | 0.00002 |
| CAN-1 vs CAN-3 | 0.87421 | 0.99165 | 0.91278 | 0.61442 | 0.07853 | 0.03767 |
| CAN-1 vs CAN-4 | 0.99873 | 0.47088 | 0.12512 | 0.69751 | 0.65012 | 0.57764 |
| CAN-1 vs PAR   | 0.86530 | 0.00445 | 0.00938 | 0.00053 | 0.95393 | 0.00000 |
| CAN-2 vs CAN-3 | 0.43904 | 0.96413 | 0.97735 | 0.82401 | 1.00000 | 0.14098 |
| CAN-2 vs CAN-4 | 0.14954 | 0.46577 | 0.02416 | 0.03608 | 0.04286 | 0.05841 |
| CAN-2 vs PAR   | 0.01497 | 0.10864 | 0.67653 | 0.00001 | 0.07788 | 0.00000 |
| CAN-3 vs CAN-4 | 0.89019 | 0.77914 | 0.06102 | 0.21675 | 0.02722 | 0.94002 |
| CAN-3 vs PAR   | 0.48631 | 0.01053 | 0.24592 | 0.00012 | 0.04367 | 0.00000 |
| CAN-4 vs PAR   | 0.99374 | 0.00166 | 0.00030 | 0.38095 | 0.93760 | 0.00000 |
nal appendix or “appendicula” rather long, always with thin walled terminal portion and with variably evident basal sac (i.e., the “sac-like diverticulum of the appendicula vaginalis” first described by Giusti and Manganelli 1987: 135, Fig. 3A, C – in “M. cantiana” specimens from Corsica); short, only occasionally with very short terminal portion and always without basal sac in M. parumcincta; the vaginal-atrial pilaster (present and variably evident in the M. cantiana group; absent in M. parumcincta); penial papilla (glans) with central canal wide, thin walled, internally irregularly jagged and with a sort of solid pilaster on one side; central canal connected to external wall of penial papilla by many muscular/connective strings in the M. cantiana group; penial
Figures 26–30. Genitalia (proximal parts excluded) (26–27), internal structure of distal genitalia (28) and transverse sections of medial epiphallus (29) and penial papilla (30) of *Monacha cantiana*. CAN-1 from Gole del Velino, near Sigillo (FGC 42960) (26, 28–30) and Valle del Turano, near Turania (FGC 42969) (27).

papilla with central canal thin walled, internally smooth or slightly jagged, almost completely filled by large invagination; central canal not connected to external wall of penial papilla in *M. parumcincta.*
Figures 31–35. Genitalia (proximal parts excluded) (31), internal structure of distal genitalia (32–32) and transverse sections of medial epiphallus (34) and penial papilla (35) of *Monacha cantiana*. CAN-2 from Rezzato (ex. 1: 31–32, 34–35; ex. 2: 33) (FGC 42976).
**Figures 36–39.** Genitalia (proximal parts excluded) (36), transverse sections of medial epiphallus (37) and penial papilla (38) and internal structure of distal genitalia (39) of *Monacha cantiana*. CAN-2 from Sorgà (FGC 42964).
Figures 40–42. Genitalia (proximal parts excluded) (40) and transverse sections of medial epiphallus (41) and penial papilla (42) of *Monacha cantiana*. CAN-3 from Fiume Setta (FGC 42977).

RDA with “clade/lineage” constraint on the shape and size matrix (Fig. 60) showed that RDA 1 (45%, P < 0.001) tended to separate the group CAN-1, CAN-2, CAN-3 and CAN-4 from PAR. The preliminary classic PCA revealed size as the first major source of morphological variation, since PC1 (53%) was a positive combination of all variables. On the contrary, RDA 2 (6%, P < 0.002) showed statistically significant separation of CAN-1, CAN-2, CAN-3 and PAR from CAN-4. In that regard, PC2 (20%) accounted for a contrast between F and P variables. RDA with species constraint on the shape (Z) matrix (Fig. 61) showed that RDA 1 (20%, P < 0.001) confirmed a statistically significant separation between PAR and CAN-4, while the large group CAN-1-CAN-2-CAN-3 remained completely unexplained. Shape-related PCA indicated that VA and F vs E and P were the two principal shape determinants on PC1 and V vs BCD on PC2.

Box plots (Fig. 62) for anatomical characters showed that VA has the best discriminating value (it distinguishes five clade pairs according to Tukey’s honestly significant difference test), followed by E and V (three pairs). The most recognisable pairs are CAN-1 vs. PAR (four significant characters), CAN-2 vs. PAR, CAN-3 vs.
Figures 43–46. Genitalia (proximal parts excluded) (43), internal structure of distal genitalia (44) and transverse sections of medial epiphallus (45) and penial papilla (46) of Monacha cantiana. CAN-3 from Breitenlee (FGC 44020).
Figures 47–50. Genitalia (proximal parts excluded) (47), internal structure of distal genitalia (48) and transverse sections of medial epiphallus (49) and penial papilla (50) of Monacha cantiana. CAN-4 from Vallée de Peillon, Sainte Thecle (FGC 40320).

PAR, and CAN-4 vs. PAR (three significant characters). Only one significant character distinguishes CAN-1 vs. CAN-2 and none distinguish CAN-1 vs. CAN-3, CAN-1 vs. CAN-4, CAN-2 vs. CAN-3, CAN-2 vs. CAN-4, or CAN-3 vs. CAN-4 (Table 4).

Discussion

The finding that M. cantiana, as usually conceived, actually consists of four distinct lineages (CAN-1, CAN-2, CAN-3, CAN-4) is an absolute novelty. One of these lineages (CAN-1) included most of the populations examined (11 populations). It is widespread
**Figures 51–59.** Genitalia (proximal parts excluded) (51, 56), internal structure of distal genitalia (52–53, 59) and transverse sections of medial epiphallus (54, 57) and penial papilla (55, 58) of *Monacha parumcincta*. Specimens from La Casella (FGC 44077) (51–55) and along the road from Moliterno to Fontana d’Eboli (FGC 42962) (56–59).
Figures 60–61. Principal component analysis (PCA) and Redundancy analysis (RDA) with clade applied to the original genitalia matrix (60) and Z-matrix (shape-related) (61). Ellipses show the 95% confidence intervals associated with each group.

and reported from the United Kingdom, Spain and Italy. The other three lineages include only two (CAN-2 and CAN-4) or three (CAN-3) populations, respectively, and at present have a narrow distribution, being known only from two sites in northern Italy (CAN-2), three sites in northern Italy and Austria (CAN-3) and two sites in south-eastern France (CAN-4) (Fig. 63). If these lineages were treated as distinct species, a taxonomical and nomenclatural setting would only be possible for CAN-1 and CAN-4 at present (a definitive framework for the other two requires more research).

Statistical analysis of a series of shell and anatomical characters shows that at least three lineages (CAN-1, CAN-2, CAN-3) cannot be distinguished from each other based on morphology and that one lineage (CAN-4) is only marginally distinct. On the contrary, these four lineages are anatomically well distinct from the *Monacha* species used for comparison (*M. parumcincta*), and three of them (CAN-1, CAN-2, CAN-3) are also conchologically distinct on the basis of many significant characters (11, 9, and 10, respectively). The major bias of morphological analysis was the small sample available for lineages CAN-2, CAN-3, and CAN-4, which prevented a realistic account of their variability.
Sequences characteristic of clade CAN-1 formed a well-separated group in ML and Bayesian trees (Figs 3–5). Although they were all from UK and Italian populations, they are mixed together in the trees without separate branches for UK and Italian populations. Interestingly, three pairs of haplotypes or common sequences are identical: UK-COI 2 / IT-COI 2, UK-16S 2 / IT-16S 1 and UK-ITS2 2 / IT-ITS2 1. This and small K2P genetic distances within this clade (0.9% in COI, 0.5% in 16SrDNA) suggest that the clade represents one taxon. CAN-1 corresponds to the true *M. cantiana* because it is the only clade that includes topotypical English populations. Close rela-
Figure 63. Localities of *Monacha cantiana*, *M. parumcincta* and *M. cartusiana* specimens where they were collected for the research (see Table 1 for locality numbers).

Comparisons between the sequences studied (clade CAN-1 in Figs 3–5) support the conclusion that the populations have a common Mediterranean origin (Neiber and Hausdorf 2017), which in view of available fossil record (Kerney et al. 1964, Kerney 1970, Evans 1972), may be postulated to date back to the Roman conquest. The same is also true for the Spanish populations from Pais Vasco (Sopelana), whose sequences (KX507234 and KJ458539 / KX495428), deposited in GenBank for COI and 16SrDNA of *M. cantiana* (Neiber and Hausdorf 2015, Razkin et al. 2015), respectively, were located between our UK and Italian (Latium sites close to Rome) populations in our ML trees (Fig. 64). Nevertheless further studies on molecular characteristics of *M. cantiana* populations from Scotland, N France, N Germany, Belgium, and The Netherlands are necessary in order to test this hypothesis.
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**Figure 64.** Maximum Likelihood trees of COI, 16SrDNA, H3, and ITS2 sequences of Monacha cantiana group. Bootstrap analysis was run with 1000 replicates (Felsenstein 1985). Numbers on branches represent bootstrap support above 50%. A the COI sequences of Monacha cartusiana KM247389, KM247376 and KX507189 were used as an outgroup, and those of M. cantiana KF986833, KX507234 and HQ204502 as reference sequences. 592-bp sequences of new COI haplotypes (Table 1) were shortened to a 556-bp fragment for alignment with the GenBank sequences used as outgroup or references. B the 16SrDNA sequences of Monacha cartusiana KM247391, KM247397 and KX495378 sequences were chosen as outgroup. M. cantiana AY741419, HQ204543, KJ458539 and KX495428 as well as M. parumcincta AY741418 sequences were used as references. The final dataset contained 287 positions. C the ITS2 tree was rooted with Monacha cartusiana sequence MH137993. D the H3 tree was rooted with Monacha cartusiana sequence MG209072. Monacha cantiana KF596955 was used as a reference.

The three percent threshold for genetic distance between COI barcode sequences was established by Hebert et al. (2003a, 2003b) as a criterion for the description of a new taxon at species level. There are many papers concerning usefulness of barcoding in taxonomy (e.g., Ebach and Holdrege 2005, Gregory 2005, Goldstein and DeSalle 2010) and showing that 3% threshold should be higher (4% or even higher) for stylommatophoran gastropods (Davison et al. 2009, Sauer and Hausdorf 2012 and references cited therein). Aware of it we think that the slightly exceeded barcode threshold in K2P distances between COI sequences of CAN-1 and CAN-2 clades together with the lack of significant differences in shell (Fig. 19) and genitalia features (Fig. 62), do
not permit to introduce a distinct taxon, even at subspecies level. Rather, the K2P distances show that some Italian populations of the *M. cantiana* group are in a process of speciation and differentiation.

The cases of the clades CAN-3 and CAN-4 are completely different, since K2P genetic distances distinguish the haplotypes of these two clades from the others (CAN-1, CAN-2, PAR) and were well above Hebert’s threshold (even enlarged according to Davison et al. 2009). However, due to the lack of differences in anatomical and conchological features between CAN-3 and clades CAN-1 and CAN-2, we treat CAN-3 as mitochondrialy distinct lineage only. Any taxonomic conclusion would be premature.

The situation of clade CAN-4 is distinct because this lineage includes a French population which can be considered toptotypical of *Theba cemenelea*. Live specimens were collected by one of us (AH) at Sainte Thecle, Vallée de Peillon, a site located 10 km NE of Risso’s original locality: Colline de Cimiez at Nice, now in the urban area of Nice. It was regarded as a junior synonym or at least a subspecies of *M. cantiana* until the early 2000s, when Falkner et al. (2002) separated it again on the basis of the presence of well evident basal sac of the vaginal appendix considered instead absent in *M. cantiana*. Since type material of *T. cemenelea* no longer exists (Chevallier 1976, Arnaud 1977), only designation of a neotype can ensure correct univocal application of Risso’s name. We therefore select a specimen collected at Sainte Thecle in Vallée de Peillon as the neotype. The neotype is deposited in the malacological collection of the Museo di Storia Naturale dell’Accademia dei Fisiocritici, Siena (MOLL/3309). Its shell is illustrated in Fig. 16 and its genital anatomy in Figs 38–41. The separation of CAN-4 (*M. cemenelea*) is strongly supported by nucleotide sequence analysis of both mitochondrial and nuclear genes (Figs 3–5, 64). Therefore haplotypes of COI and 16SrDNA as well as sequences of H3 and ITS2 gene fragments characteristic of specimens from this population have been deposited in GenBank (accession Numbers for FR-COI 1–4: MG208939–MG208943; for FR-16S 1–2: MG209011–MG209015; for FR-H3 1–3: MG209058–MG209060; for FR-ITS2 1: MH137984).

Designation of the neotype is in line with the current concept of this *Monacha* species (e.g., Falkner et al. 2002) i.e., a species distinguished by a well evident basal sac of the vaginal appendix. Contrarily to what has been stated by Falkner et al. (2002), this basal sac is present but smaller or sometimes absent in *M. cantiana*. Moreover this taxonomic setting based on genitalia features is supported by molecular features of mitochondrial and nuclear genes.

A singular sequence AY741419 from Podere Grania, Asciano, Siena deposited in GenBank by Manganelli et al. (2005) for 16SrDNA (Fig. 64B, Table 1) as well as our not yet published molecular results for certain Italian populations (from Alpi Apuane, Tuscany) suggest that Italian *M. cantiana* may include other lineages.

All our results, namely shell (Figs 17–19) and genital (Figs 60–62) structures and molecular evidence of separate clades for each tree (Figs 3–5, 64), show that *M. parumcincta* and *M. cantiana* are distinct taxa. However the definitive taxonomic and nomenclatural setting of *M. parumcincta* is still unclear (see Forcart 1965, Manganelli et al. 1995, Welter-Schultes 2012). This and its infraspecific variation will be the subject of further studies.
Acknowledgements

We are grateful to Robert A.D. Cameron (University of Sheffield, UK), Michael Duda (Natural History Museum Vienna, Austria) and Małgorzata Proćków (University of Wroclaw, Poland) for providing specimens. We also thank Bernhard Hausdorf (University of Hamburg, Germany) and Robert A.D. Cameron (University of Sheffield, UK) for their comments on the manuscript, Francisco Welter-Schultes (University of Göttingen, Germany) for a discussion on the nomenclatural items, Jarosław Bogucki (Poznań, Poland) for drawing the Fig. 63, Helen Ampt (Siena, Italy) for revising English, and Giovanni Cappelli (Siena, Italy) for taking photos of the shells.

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