MONACHA OCELLATA (ROTH, 1839) (GASTROPODA: HYGROMIIDAE) ESTABLISHED IN ESSEX, AN ADDITION TO THE FAUNA OF BRITAIN AND IRELAND

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Abstract *A single adult of an unidentified hygromiid was discovered at a brownfield site on the margins of a carpark near Tilbury, south Essex, on 3rd May 2017. This was tentatively identified from photographs as a species of* Monacha, *possibly* M. ocellata *(Roth, 1839). Later in 2017, with the more widespread appearance of adults at the locality, a more detailed study was undertaken. This established, from the dissection of genitalia in sexually mature adults, that the species was indeed* Monacha ocellata, *native to the south-east Mediterranean and unknown in northern Europe. Molecular analysis (nucleotide sequencing of mitochondrial* COI *and* 16SrDNA *as well as nuclear* ITS2 *gene fragments) showed a close relationship to GenBank sequences for* Monacha ocellata *originating from the Citadel of Constantinople, Istanbul.*

Key words *Alien, introduction, ports, Mollusca, Hygromiidae,* Monacha, ITS2, COI, 16SrDNA

INTRODUCTION

The genus *Monacha* Fitzinger, 1833 (Hygromiidae) is represented in Britain and Ireland by two species, *M. cantiana* (Montagu, 1803) and *M. cartusiana* (Müller, 1774). Both are clearly climatically restricted with *M. cantiana* common only south and east of a line from Newcastle upon Tyne in the north-east to Plymouth in the south-west, and *M. cartusiana* rare and restricted to coasts in Kent, West Sussex and Suffolk in the extreme south-east (Kerney, 1999). In recent years *M. cantiana* has established isolated colonies across central and western England and Wales and in eastern Scotland. Attempts to introduce it to Ireland have failed (Kerney, 1999). The aboriginal range is probably Italy and south France (Welter-Schultes, 2012, 2017; Pieńkowska *et al*., 2018) from which it has been spread north-westwards across Europe eventually reaching England, by farmers in Roman times. It remained rare until the mediaeval period (Kerney, 1970). In its area of occupancy it typically lives on roadsides, field margins, waste ground and railway embankments. In an era of global warming it will likely continue to expand its range. *Monacha cartusiana* on the other hand, is a declining species having relinquished former strongholds in Norfolk, West Suffolk, Hampshire and Surrey in historical times (Kerney, 1999). Like *M. cantiana* it is likely to have been spread by farmers to its present sites in north-west Europe but with an earlier chronology (?Neolithic; Kerney, 1970). It originates from south-east Europe and is evidently more xeric-thermic in habitat preference compared to *M. cantiana*, preferring short-turf chalk grassland or coastal sand dunes. It was probably originally native to the Black Sea region including European Turkey (Welter-Schultes, 2012, 2017).

Into this mix we now introduce a third *Monacha* species, apparently recently arrived from some distant point within the south-east Mediterranean. The following account describes the discovery, process of identification from internal and external morphology, and confirmation of initial findings through molecular analysis.

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MATERIALS AND METHODS

Habitat A single adult snail was discovered on 3rd May 2017, during invertebrate survey work at Tilbury, south Essex (Fig. 1). The site is on the south-facing slope of a bank, about 300m in length, centred on British grid reference TQ644757 (51°27'23.1"N, 00°21'53.6"E). Snails were found in unmanaged, ruderal vegetation with extensive cover of tall herbs (Figs 2, 3) along the length

Figure 1 *Monacha ocellata*, first adult seen, 3rd May 2017. Near hard standing for imported vehicles, Port of Tilbury (M.G. Telfer).

of the bank. No investigations were undertaken beyond this area. The area has been planted with broad-leaved trees and shrubs but open habitat remains in patches where the saplings have died. Bramble and scrub are encroaching but the south-facing bank is quite steep, susceptible to drought, and the friable soil is eroding in places which may mean that succession will be slow.

Visits to the site on 16th and 30th May 2017 with the aim of collecting adult specimens succeeded only in finding immatures (two on 16th, several on 30th). Like the first adult, the immature snails were found in exposed, elevated resting positions (Fig. 1) on the dead stems of tall herbs. Ground-searching on these dates revealed that empty adult shells were abundant, making it clear that this was a well-established population.

By the time of the next visit on 4th August, a new generation had become fully grown and adult snails were common, again mostly in elevated resting positions. It appears that these snails climb up to a resting spot on a daily/regular basis after each foraging trip and strongly avoid resting even for one day at ground level. These observations were repeated on a final visit on 18th August, indicating that the Tilbury population is primarily annual, maturing in June or July with few adults surviving into the following year.

Amongst the herb species present were Creeping Thistle *Cirsium arvense*, Hound's-tongue *Cynoglossum officinale*, Hoary Cress *Lepidium draba*, Perennial Wall-rocket *Diplotaxis tenuifolia*, Black Horehound *Ballota nigra*, Hemlock *Conium maculatum*, Teasel *Dipsacus fullonum* and Common Mallow *Malva sylvestris*.

The bank lies at the northern edge of an area of former coastal grazing marsh known as the Ferry Fields. The bank was probably constructed using imported substrate.

Figure 2 Brownfield habitat near Tilbury, May 2017, with unmanaged ruderal vegetation (M.G. Telfer).

Shell morphology
Live animals and shells were compared with the two known British species (Figs 4, 5) and with those of other European and Mediterranean *Monacha* species. The latter comparisons were initially facilitated by the use of online media (Welter-Schultes, 2017).

Genital morphology
Live snails were killed by partially drowning in boiled tap water to cause the forebody to relax and extend from the shell aperture, followed by freezing at -10ºC for six hours. The frozen snails were thawed into 100% ethanol and allowed to equilibrate before transferring to fresh 100% ethanol. The animals were then removed from the shells and held separately in ethanol. Dissection was performed under the light microscope (Wild M5A). Anatomical details were drawn using a Wild camera lucida (Fig. 6).

DNA extraction, PCR amplification and sequencing
A small foot tissue fragment of alcohol preserved snail was used for total DNA extraction, using Tissue Genomic DNA extraction Mini Kit (*Genoplast*) according to the protocol provided by the manufacturer.

The purified total DNA was used as template for amplification of partial sequences of the following genes: mitochondrial cytochrome *c* oxidase subunit I (*COI*) and 16S ribosomal DNA (*16SrDNA*) as well as nuclear internal transcribed spacer 2 (*ITS*2) of rDNA, flanked by fragments of 5.8S ribosomal DNA (*5.8SrDNA*) and 28S ribosomal DNA (*28SrDNA*), using the polymerase chain reaction (PCR).

The amplified and sequenced 5′-end fragment of *COI* (often called "barcode sequence") was of 650 base pairs in length. Polymerase chain reactions were performed in a volume of 10 µl according to the modified protocol prepared by Biodiversity

Figure 3 View of the Tilbury site, August 2017. Snails may be seen at the base of a pole on the right (M.G. Telfer).

Institute of Ontario for the Consortium for the Barcode of Life (http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf) using two degenerate primers bcsmF1 (5′-AAYCATAAAGAYATTGGDACWT TDTA-3′) and bcsmR1 (5′-TAWACYTCWGGRT GACCAAAAAAYCA-3′) (Proćków *et al.*, 2013; the nucleotides and ambiguity codes were determined according to IUPAC). The reaction was carried out under the following thermal profile: 15 min at 94°C followed by 42 cycles of 40 s at 94°C, 40 s at 53°C, 1 min at 72°C, and finally 5 min at 72°C. The amplified and sequenced *16SrDNA* fragment was of 350 bp-long. The amplification reaction was conducted in a volume of 10 µl according to a previously described procedure (Manganelli *et al.*, 2005) with the use of primer pair 5′–CGATTTGAACTCAGATCA–3′ (LR-J-12887, Simon *et al.*, 1994) and 5′–GTGCAAAGGTAGCATAATCA–3′ (Gantenbein *et al.*, 1999). Two primers, NEWS2 5′-TGTGTCGATGAAGAACGCAG-3′ and ITS2-RIXO 5′-TTCTATGCTTAAATTCAGGGG-3′, were used to amplify a 633 bp fragment enclosing the 3′ part of *5.8SrDNA*, the *ITS2* and 5′ part of *28SrDNA* in 12.5 µl volume according to the

Figure 4 Habitus of *Monacha ocellata* from Tilbury; specimen received 18th August 2017 (R. Anderson)

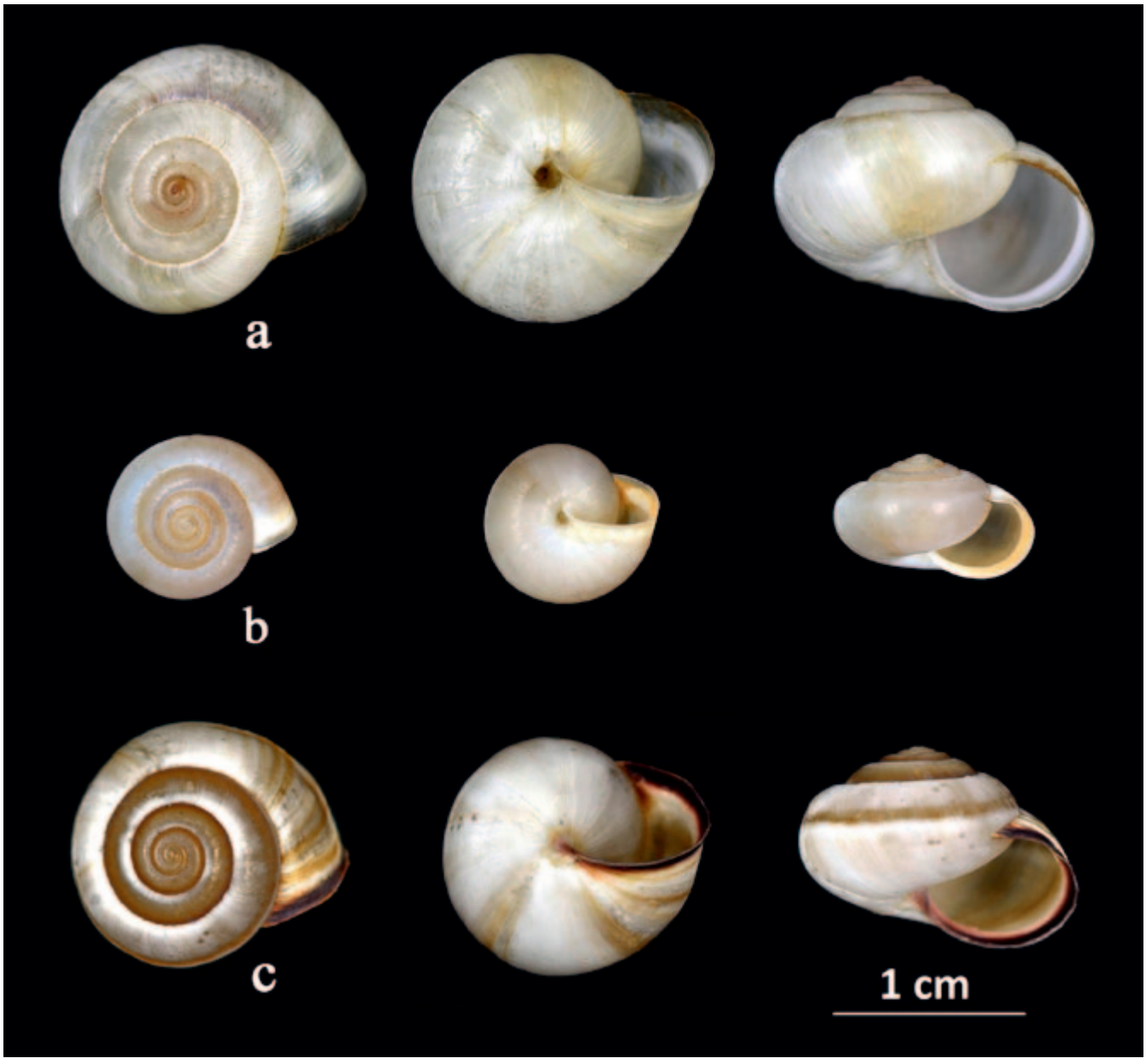

Figure 5 British *Monacha* species: **a** *M. cantiana,* Luton Airport, 6th July 2003, coll. R. Anderson; **b** *M. cartusiana,* Dover, 1899, coll. A.G. Stubbs (Ulster Museum accession Mn3201); **c** *M. ocellata,* near Tilbury, 4th August 2017, coll. M. G. Telfer.

procedure described by Almeyda-Artigas *et al.* (2000).

All PCR products were verified by 1% agarose gel electrophoresis. 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 manufacturer's protocols.

Phylogenetic inference

The individual sequences are deposited in GenBank, *COI*: MG918127, *16SrDNA*: MG918128, and *ITS-2*: MG918129. The following *Monacha* sequences from GenBank were also used: *M. ocellata* – KX507220 (*COI*), KX495409 (*16SrDNA*), KX495462 (*ITS2*) deposited in GenBank by Neiber & Hausdorf (2015), *M. cartusiana* – KX507189, KX507235 and KM247379 (*COI*), KX495378, KX495429 and KM247391 (*16SrDNA*), KX495431, KX495479 and KM247399 (*ITS2*) deposited by Neiber & Hausdorf (2015, KX sequences) and

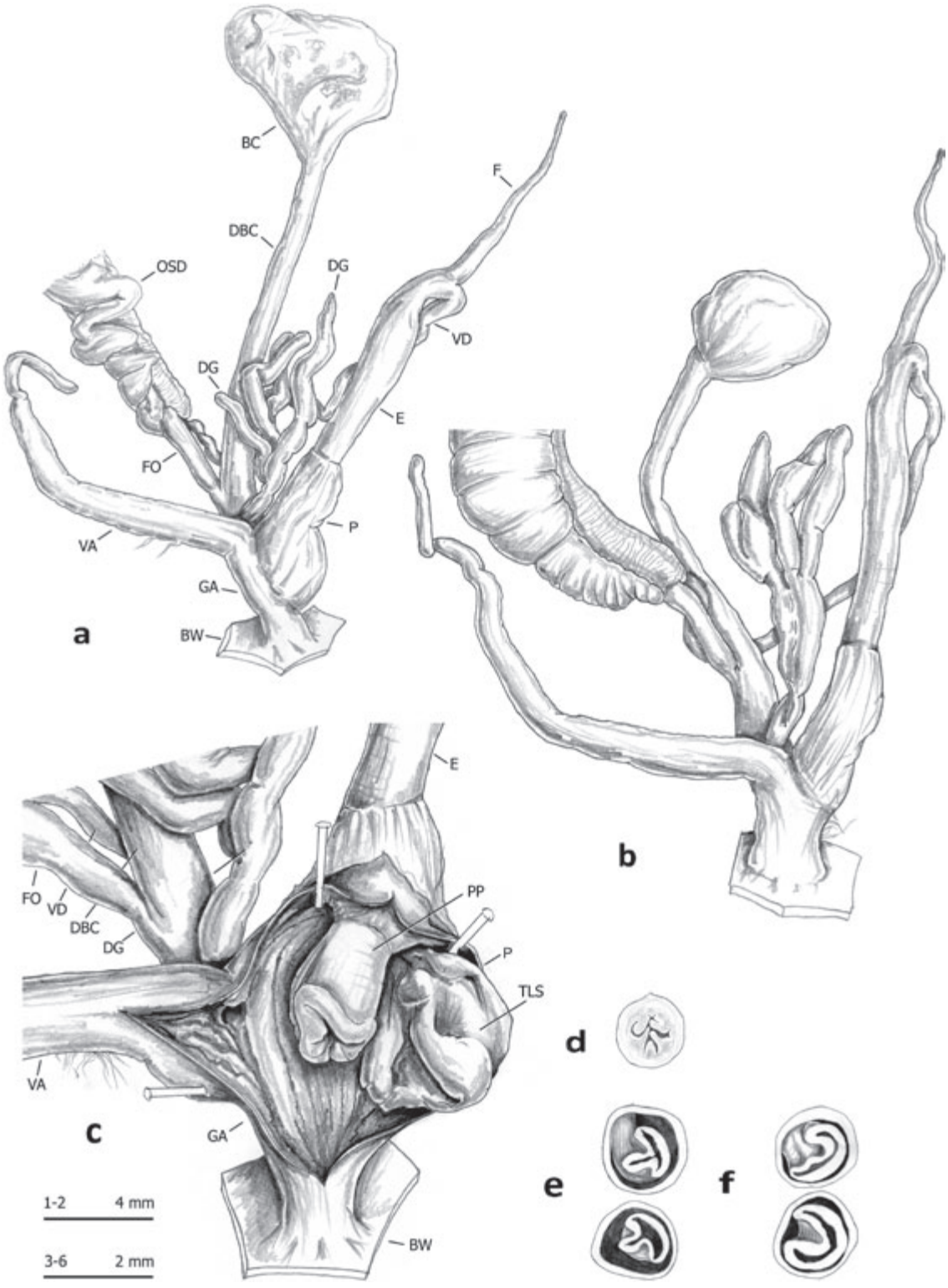

Figure 6 Dissection of genitalia in *Monacha ocellata* from Tilbury: **a** overall layout, specimen 1; **b** layout, specimen 2; **c** internal structures of penis and genital atrium, specimen 1; **d** transverse section of median epiphallus, specimen 1; **e–f** transverse sections of penial papilla; at ca. half its length: above sections towards papilla apex; below sections towards papilla base, specimens 1 and 2.

Acronyms: BC bursa copulatrix; BW body wall; DBC duct of bursa copulatrix; DG digitiform mucus glands; E epiphallus (from base of flagellum to beginning of penial sheath); F flagellum; FO free oviduct; GA genital atrium; OSD ovispermiduct; P penis; PP penial papilla; TLS tongue-like structure; VA vaginal appendix (also known as appendicula); VD vas deferens.

Pieńkowska *et al.* (2015, KM), *M. cantiana* – KX507234 and KM247375 (*COI*), KX495428 and KM247390 (*16SrDNA*), KX495478 and KM247398 (*ITS2*) deposited by Neiber & Hausdorf (2015, KX) and Pieńkowska *et al.* (2015, KM). For phylogenetic analysis, the following sequences of *Trochulus hispidus* (Linnaeus, 1758) were used as outgroups: KX507209 (*COI*), KX495398 (*16SrDNA*) and KX495451 (*ITS2*) deposited by Neiber & Hausdorf (2015).

Sequences were edited by eye using the program BioEdit, version 7.0.5 (Hall, 1999). The alignments were performed using the Clustal W programme (Thompson *et al.*, 1994) implemented in MEGA 7 (Kumar *et al.*, 2016). The borders of ITS2 sequence were identified using ITS2-Database (http://its2-old.bioapps.biozentrum.uni-wuerzburg.de) (Eddy, 1998, Koetschan *et al.*, 2010). The identified length of *ITS2* was 494 bp. The ends of all sequences were trimmed to mentioned above reference sequences downloaded from GenBank. The length of the sequences after cutting were 633 bp for *COI*, 285 positions for *16SrDNA* and 362 positions for *ITS2.* Gaps and ambiguous positions were removed from alignments prior to phylogenetic analysis.

To infer the phylogenetic relationships, the sequences obtained in the present work together with other sequences obtained from GenBank were analysed by a genetic distance Neighbour-Joining method (Saitou & Nei, 1987) implemented in MEGA7 (Kumar *et al*., 2016) using the Kimura two-parameter model (K2P) for pairwise distance calculations (Kimura, 1980). NJ trees were tested by bootstrap analysis with 1,000 replicates (Felsenstein, 1985). Finally, sequences of *COI*, *16SrDNA* and *ITS2* were combined. Before sequence combining, uncertain regions were removed from *16SrDNA* and *ITS2* alignments with Gblocks 0.91b programme (Castresana, 2000; Talavera & Castresana, 2007). This procedure shortened alignments, that of *16SrDNA* sequences from 285 to 275 positions and that of *ITS2* sequences from 362 to 334 positions. The combined sequences with the total length of 1242 positions (633 *COI* +275 *16SrDNA* +334 *ITS2*) were used to infer the phylogeny of this group by Bayesian analysis conducted with the program MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). *Trochulus hispidus* was added as an outgroup species. Using jModelTest2 (Darriba *et al*., 2012) according to the Bayesian Information Criterion (BIC), we specified a GTR 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'). A 50% majority rule consensus tree was obtained as a result. Simultaneously, 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.

Results

Shell morphology

Shells from Tilbury were compared with the two known British *Monacha* species, *M. cantiana* and *M. cartusiana*. These showed distinct differences. In particular, shells were more conspicuously pigmented than either of the two other *Monacha* species, with a red-brown lip, a thin mid-brown peripheral line on the body whorl which broadens and becomes more diffuse along the spire with a pale band inside it of variable width (Fig. 5). In all of the specimens examined, the umbilicus was fully closed whereas in *M. cantiana* and *M. cartusiana,* the umbilicus is partly to fully open. These characters led to an initial conclusion that we were dealing with an east Mediterranean species, either *M. syriaca* (Ehrenberg, 1831) or the closely similar *M. ocellata.* These cannot be separated reliably on shell morphology and require dissection (Hausdorf, 2000).

Genital morphology

Alcohol specimens were dissected and the genital tract compared with those of *M. syriaca* and the two known British species. Structures in the Tilbury *Monacha* corresponded closely to descriptions of *M. ocellata* (Hausdorf, 2000; Irikov, 2008; Örstan, 2014 – this author helpfully compares the genitalia of *M. ocellata* with those of *M. syriaca*).

The genital system is illustrated in Fig. 6:a–f. The Tilbury *Monacha* possesses a single mucus gland with four digitate branches (DG in Fig. 6:a) and it and the appendix are inserted at the junction of the penis and the vagina. In *M. syriaca* the mucus gland and the appendix insert on the proximal vagina where the duct of the bursa copulatrix and the free oviduct unite. In *M. syriaca* the penis is (externally) distinct from the

Table 1 K2P genetic distances for analysed *COI*, *16SrDNA* and *ITS2* sequences

Comparison	*COI* (%)	*16SrDNA* (%)	*ITS2* (%)
Within *M. ocellata*	0.2	0.7	0.0
Within *M. cartusiana*	0.2–0.3	0.4–0.7	0.6–1.8
Within *M. cantiana*	0.3	0.7	0.0
Between *M. ocellata* and *M. cartusiana*	16.8–17.3	17.9–18.3	6.3–7.0
Between *M. ocellata* and *M. cantiana*	19.7–20.1	28.3–29.4	4.9
Between *M. cartusiana* and *M. cantiana*	18.7–19.1	21.0–22.5	4.9–5.5

Table 2 Combined sequences of *COI+16S+ITS2* gene fragments for Bayesian analysis

Combined sequence	*COI* sequence	*16S* sequence	*ITS2* sequence	Locality
Mocel CS 1	MG918127	MG918128	MG918129	UK, Tilbury (51°27'23.1"N, 00°21'53.6"E)
Mocel CS 2	KX507220	KX495409	KX495462	TR, Istanbul (41°00'00"N, 28°59'00"E)
Mcart CS 1	KX507189	KX495378	KX495431	IT, Lombardia (45°46'38"N, 10°30'12"E)
Mcart CS 2	KX507235	KX495429	KX495479	ES, Castilla La Mancha (41°00'00"N, 02°38'00"E)
Mcart CS 3	KM247379	KM247391	KM247399	PL, Wrocław (51°08'30.5"N, 16°56'55.9"E)
Mcan CS 1	KX507234	KX495428	KX495478	ES, Pais Vasco, Sopelana (43°23'00"N, 02°59'00"E)
Mcan CS 2	KM247375	KM247390	KM247398	UK, East Acton (51°30'30"N, 00°15'38"W)
This CS 1	KX507209	KX495398	KX495451	DE, Hamburg (53°40'12"N, 10°06'19"E)

epiphallus whereas in *M. ocellata* the demarcation is unclear. Finally, the penis plus epiphallus of *M. ocellata* is shorter in total length than the duct of the bursa copulatrix, whereas in *M. syriaca* penis plus epiphallus is longer than the duct of the bursa copulatrix.

Layout of the distal genitalia in *M. ocellata* is broadly similar to those of *M. cantiana* and *M. cartusiana*. However, instead of the single mucus gland found in *M. ocellata*, both *M. cantiana* and *M. cartusiana* have two glands. In *M. cantiana* these are inserted on the medial vagina whereas in *M. cartusiana* they are inserted on the proximal vagina. *Monacha ocellata* also has a longer, more evenly tapering appendicula than *M. cantiana* and *M. cartusiana*, and differs in the absence of a vaginal sac. The internal canal of the penial papilla (PP) in transverse section shows a lateral projection (Fig. 6:e, f) which *M. cantiana* and *M. cartusiana* do not possess (Giusti & Manganelli, 1987: 124–128, fig. 1A–G, fig. 2A–D, fig. 3A–F).

A major difference is that *M. ocellata* has a large, tongue-like structure (TLS) embracing (in specimens fixed after drowning, as here) the apex of the penial papilla, on the internal wall of the distal penis. This most probably corresponds to the "roll" described by Hausdorf (2000: 94, fig. 31). This structure is absent even in closely related species of *Monacha* e.g. *M. syriaca* (see: Hausdorf, 2000: 103, figs 33, 40) and may be categorized as autapomorphic, providing a powerful distinguishing feature for the species.

Molecular analysis

The sequences of all analysed gene fragments obtained from a specimen of the Tilbury population were very similar to the sequences for *M. ocellata* from the Citadel of Constantinople fortress (along railway on grass, Istanbul, Turkey, 41°00'00" N, 28°59'00" E). The *COI* sequence MG918127 of the specimen from Tilbury population differs by only two nucleotides from KX507220 of the Istanbul population (K2P distance 0.2%). Similarly only two nucleotides separate the *16SrDNA* MG918128 sequence from Tilbury and the KX495409 sequence from Istanbul (K2P distance 0.7%). With *ITS2* sequences the difference between MG918129 of Tilbury and KX495462 of Istanbul, is one nucleotide only (K2P distance 0.3%).

Analyses of *COI*, *16SrDNA* and *ITS2* reveal the distinctness of *M. ocellata* from the other two *Monacha* species occurring in the UK, i.e. *M. cartusiana* and *M. cantiana*. This is strongly confirmed by K2P distances between the particular gene sequences of *M. ocellata*, *M. cartusiana* and *M. cantiana* (Table 1). Combined sequences of *COI*, *16SrDNA* and *ITS2* genes (Table 2) compared in

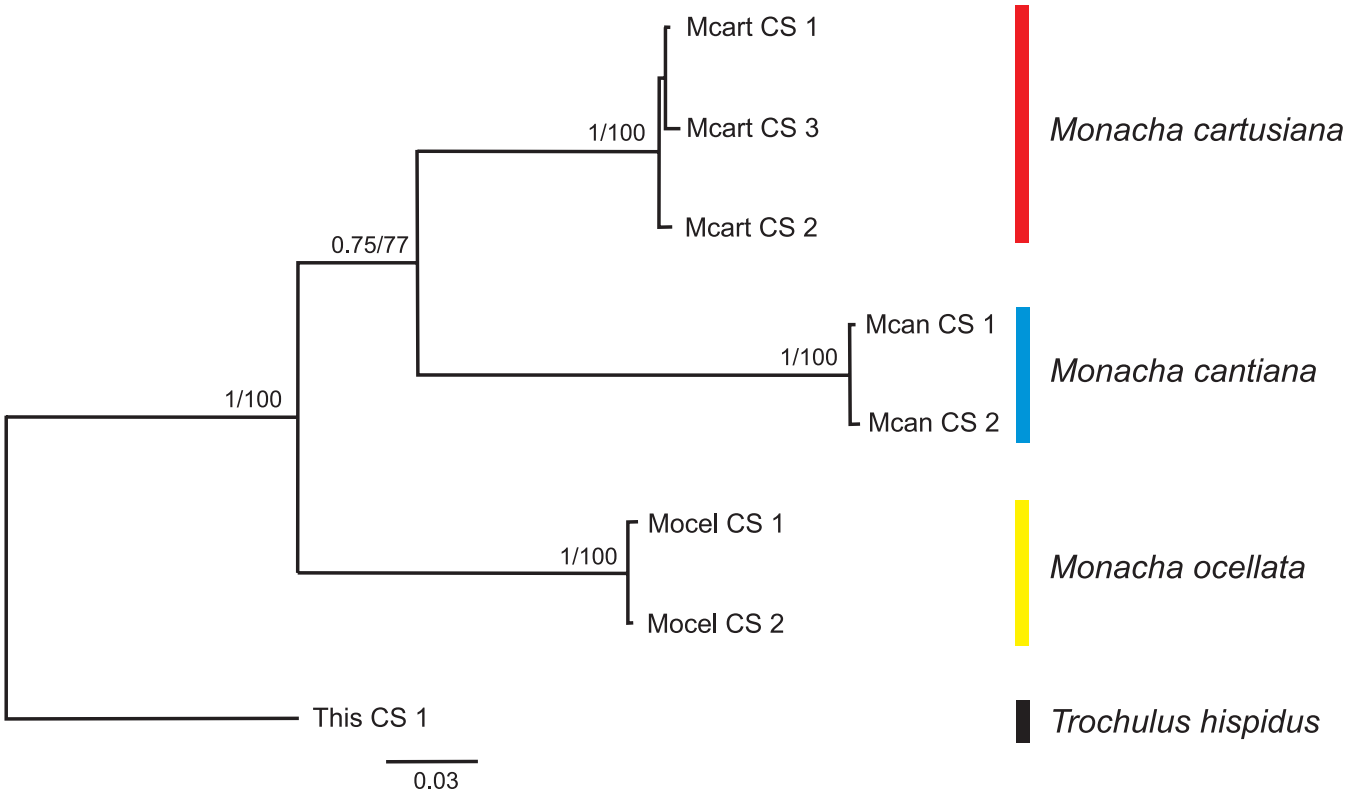

Figure 7 Bayesian analysis of combined sequences of *COI*, *16SrDNA* and *ITS2* genes. Values at nodes are Bayesian posterior probabilities / maximum likelihood bootstrap values.

Bayesian analysis show (Fig. 7) that the sequence characteristic of the Tilbury population is clustered together with the *M. ocellata* sequence of the Istanbul (type) population deposited in GenBank by Neiber & Hausdorf (2015).

Discussion

Molecular analysis strongly confirms that the *Monacha* population at Tilbury is conspecific with *M. ocellata*. Close proximity to a container port suggests that the species has probably been carried passively with container transport from its site of origin. However, it should be borne in mind that the earth bank on which the colony was found lies at the northern edge of an area of former coastal grazing marsh known as the Ferry Fields, which was developed in 1997 to provide parking on hard-standing for vehicles imported through the adjacent Port of Tilbury. It is thought that the bank was constructed using imported substrate, perhaps to screen the development from view. Although we have no information on the likely origin of this. This could, however, provide an alternative explanation for the presence of *Monacha ocellata* near Tilbury.

The strongest link, judging from molecular analysis, is with the type locality of *Monacha ocellata* at Istanbul and it seems unlikely that the Tilbury population could have originated far from this. There are several container ports in the Marmara to the west of Istanbul but the largest is at Ambarli, Istanbul Province. This might well be the source of the Tilbury population the vector being container traffic between Ambarli and Tilbury. If this is the case it raises the possibility of transport to other ports both within the Mediterranean and wider afield. Malacologists should be aware of the potential for this species to be found in other localities away from its native range in western Turkey.

Regarding the Tilbury population, there is a further issue. It can be assumed that it was established in a very small geographic area rather recently, however due to very large conchological similarity of *Monacha* species especially between *M. ocellata* and *M. cartusiana,* populations of the latter should be checked (as *M. cartusiana* was previously misidentified, see: Lesicki & Koralewska-Batura, 2007 vs. Pieńkowska *et al.*, 2015). The potential for spread of *M. ocellata* is at present unexplored. Because it is an alien species

to the British malacofauna, it would be desirable to monitor the Tilbury population to see if it persists and spreads.

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