

Helix lucorum Linnaeus, 1758 (Gastropoda: Helicidae) – the morphological and molecular analysis of a new species to the Polish malacofauna

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The native range of the Turkish snail, *Helix lucorum* Linnaeus, 1758 includes the Caucasus, Anatolia and probably the Balkan region. However, this species has intensively increased its distribution range to other areas. Today, it is widely distributed throughout Europe. In this study, we characterise the first noted population of *H. lucorum* in Poland by means of integrative taxonomy. The population is located in the centre of Warsaw city (Poland), where both live individuals (adults and juveniles) and empty shells of *H. lucorum* were collected. Phylogeographic analyses indicated that the specimens of *H. lucorum* from the Polish population show genetic similarities to populations from Turkey and Slovakia. It is crucial to evaluate the possible consequences of *H. lucorum* on the local species in newly discovered areas and to consider implementing measures for its eradication, if necessary.

Key words: non-indigenous species, unintentional introduction, urban fauna, terrestrial molluscs.

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The Turkish snail, *Helix lucorum* Linnaeus, 1758 (Gastropoda: Eupulmonata: Helicidae) is a relatively large terrestrial gastropod, with a shell up to 60 mm in diameter, 35-60 mm in width and 25-45 mm in height (Horsák *et al.* 2010; Welter-Schultes 2012; Neubert 2014). *H. lucorum* is characterised by an essentially white shell, which is almost entirely covered with wide, fuzzy black-brown stripes with a reddish hue. The European form of the *H. lucorum* shell is characterised by some spiral lines on the upper whorls. The aperture is relatively smaller than other *Helix* species, and can be grey inside with a purple tinge and bands. The umbilicus is usually covered in

adult specimens, but is not always completely closed (Welter-Schultes 2012). Variability in the shell colouration and shape is observed between specimens of *H. lucorum*, and it is much higher than in other *Helix* species. Neubert (2014) discovered that specimens from Turkey and the Lesser Caucasus have the most conchologically variable shells. However, there are also populations that are characterised by a uniform appearance, such as those from Italy or the Balkans (Neubert 2014).

The native range of *H. lucorum* includes the Caucasus, Anatolia and probably the Balkan region (Korábek *et al.* 2018). Nowadays, this species has

become widely distributed across Europe and is found in Turkey, Georgia, Azerbaijan, Iran, Albania, Bulgaria, Bosnia and Herzegovina, Croatia, Greece, Macedonia, Serbia, Russia, Italy, Hungary, Romania, Ukraine, Czech Republic, France, Slovakia and Great Britain (Horsák *et al.* 2010; Palmer 2010; Balashov & Gural-Sverlova 2012; Peltanová *et al.* 2012; Welter-Schultes 2012; Balashov *et al.* 2013; Mumladze 2013; Čejka & Čačaný 2014; Egorov 2017; Korábek *et al.* 2018). The species, like other terrestrial gastropods, increased its distribution range through dispersion processes that were additionally facilitated by e.g. climate changes or human activity as *Cornu aspersum* (Müller, 1774), *Massylaea vermiculata* (Müller, 1774), *Cepaea nemoralis* (Linnaeus, 1758), *Monacha cartusiana* (Müller, 1774), *Arion vulgaris* Moquin-Tandon, 1855, *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt, 2011, *D. reticulatum* (Langner 2003; Peltanová *et al.* 2012; Hutchinson *et al.* 2014; Zemanova *et al.* 2016; Zajac *et al.* 2020a; Zajac & Stec 2020).

This synanthropic species occurs mainly in habitats ranging from open woodlands to sub-arid steppe-like areas (Neubert 2014). It can be also found in biotopes characterised by moderate humidity. In addition, it is reported in cultivated areas, shrubs and other places influenced by human activity (Welter-Schultes 2012). New populations of *H. lucorum* are usually noted in city centres, presumably due to higher temperatures that are closer to the physiological requirements of this species (Korábek *et al.* 2018). Like other *Helix* taxa, *H. lucorum* is a hermaphrodite, but little is known about its biology and ecology. Staikou *et al.* (1988) reported that the individuals from Greek populations exhibit a high growth rate during spring, with egg laying and hatching occurring within two months in July and August. These individuals reach sexual maturity three years after hatching, with a minimum shell length of 35 mm.

This contribution describes the first noted population of the Turkish snail, *H. lucorum* in Poland, confirmed by an integrative taxonomy approach with a combination of morphological and anatomical features supported by a molecular identification of the studied individuals.

Materials and Methods

The population of *H. lucorum* was discovered during faunistic research conducted in September 2020 in Warsaw (Poland) (Zajac *et al.* 2023). In total, 300 individuals were visually noted and collected by one person in a period of two hours. The description

of the location and habitat included characteristics of the plant community that occurred at the sampling site.

Morphological taxonomic features

The qualitative and quantitative characteristics of the snail shells were analysed. For this purpose, 50 individuals of *H. lucorum* were collected and qualitative characteristics of the shell such as the overall shape, colouration, position of the opening of the shell (right and left-handedness), apex shape as well as the appearance of the umbilicus were analysed. The quantitative characteristics of the snail shells such as the aperture height and width, shell height and width, as well as the shell diameter were measured using digital Vernier callipers with a 0.1 mm precision. The values of these features are presented as descriptive statistics (mean \pm SD). The calculations were performed by using Statistica 13 for Windows, StatSoft Inc.

To confirm the species identification, anatomical sections of *H. lucorum* were also performed. For this purpose, specimens of *H. lucorum* were drowned in warm water for 24 hours. After that time, the soft body of the snail was removed from the shell. Individuals were identified based on the morphology of the internal organs observed under a Nikon SMZ1500 stereomicroscope.

DNA extraction, amplification and sequencing

A total of 15 adults of *H. lucorum* were selected for a molecular analysis to confirm the species identification using DNA sequences of the COI gene, according to the method described by Zajac *et al.* (2020b). Additionally, this marker as well as 16S rRNA and 28S rRNA markers were used to detect the genetic variability within this newly detected Polish population of the species. Small pieces of body tissue from each snail were added to 1.5 ml tubes with 99.8% ethanol. DNA was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. To obtain DNA sequences of the COI gene, PCR reactions were performed using the universal set of primers, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994). In order to amplify the 16S rRNA and 28S rRNA markers, sets of the following primers: 16S-Agr-Ff₂ (5'-TTTGACTGTGCTAAGGTAGC-3') plus 16S-Agr-Rr₂ (5'-CTGAACTCAGATCAYGTAG-3') and LSU-2 (5'-GGGTTGTTTGGGAATGCAGC-3') plus LSU-5 (5'-GTTAGACTCCTTGGTCCGTG-3') were used, respectively (this study, Wade *et al.*

2006). A PCR cocktail and profiles/conditions were used, according to Zajac *et al.* (2020b). In the case of the 16S rRNA and 28S rRNA amplification, the annealing temperature was changed to 53°C for 16S rRNA and 55°C for 28S rRNA. To verify the DNA quality, a 3 µl sample of the PCR product was run on a 1.5% agarose gel for 30 min at 100 V. The PCR products were cleaned by enzyme purification with EPiCC Fast (A&A Biotechnology, Poland). A sequencing reaction was performed in a 10 µl reaction mixture, consisting of 2 µl of PCR product, 0.15 µl of primer, 1 µl of sequencing buffer, 5.85 µl of ddH₂O and 1 µl of the BrilliantDye Terminator Sequencing Kit (Nimagen, The Netherlands). The sequencing programme consisted of four steps: initial denaturation at 96°C, followed by a 10 s denaturation at 96°C, annealing at 5 s at 55°C, and a 4 min elongation at 60°C for 25 cycles. The sequencing products were cleaned up using ExTerminator (A&A Biotechnology, Poland) and were sequenced in Genomed (Warsaw, Poland). The obtained sequences were deposited in GenBank with the following accession numbers: COI: MW303319-23, OR136398-404, 16S rRNA: OR136410-24, 28S rRNA: OR136439-54.

Comparative and phylogenetic analysis

The obtained sequences were assembled and put together in BioEdit 5.0.0 (Hall 1999). After that, sequences were aligned for each marker separately using the Auto strategy implemented in MAFFT version 7 (Katoh *et al.* 2002; Katoh & Toh 2008). The number of haplotypes within each marker were calculated in DnaSP (Librado & Rozas 2009). Uncorrected *p*-distances between the haplotypes were calculated in MEGA version 7 (Kumar *et al.* 2016).

Haplotype networks for the COI and 16S rRNA markers were prepared by using PopART with the implementation of the Median-Joining method (Bandelt *et al.* 1999). For this purpose, all the sequences obtained in this study, as well as all the genetic data for *H. lucorum* available in the GenBank database were used (Abdulmawjood & Bülte 2001; Korabek *et al.* 2015, 2018; Neiber & Hausdorf 2015; Fiorentino *et al.* 2016). Detailed information about the accession numbers of the sequences that were used for the construction of the haplotype networks are given directly in the figures, next to the haplotype circles. The sequences were aligned as described above. The dataset for the COI marker was comprised 68 sequences (573 bp length of alignment), whereas the dataset for 16S rRNA was built on the basis of 92 sequences (295 bp length).

Results

Description of the location and habitat

The *H. lucorum* population was detected in Warsaw, Poland (coordinates: 52°15'42.3" N, 20°58'14.1" E). The area is situated in the centre of the city and is divided by a local road in the middle; thus, it consists of the two parts (Fig. 1). The snails were observed in a shaded plant community dominated by *Taxus baccata*, *Urtica dioica*, *Robinia pseudoacacia* and *Parthenocissus quinquefolia*.

In the first part of the locality only adult individuals were observed, whereas in the second, juveniles were also noted. Moreover, in the second part of the habitat individuals of the common Roman snail, *H. pomatia* (Linnaeus, 1758), co-occurred. In both parts of the locality, a common species *Cepaea nemoralis* (Linnaeus, 1758) was also present.

Species identification

Most of the collected individuals (36 out of 50) possessed the typical brown shell for *H. lucorum*, characterised by a compressed, spherical shape and a finely-striped shell surface (Fig. 2). The aperture was grey inside with a violet hue and with bands, and the apex was blunt and distinct, whereas the umbilicus was small, covered and not completely closed in adults. The shells of the remaining specimens exhibited a greater variability in the characteristics analysed. Most specimens had a typical brown colouration with a brighter band on the shell (Fig. 2). All of the *H. lucorum* had right-handed shells. There were no differences ($p < 0.05$) in the appearance of the umbilicus, which was covered but not completely closed in the adult specimens. The data on the morphometric characteristics of the snail shells is presented in Table 1.

All adult individuals were also identified as *H. lucorum* species based on the morphology of their reproductive system which, in males, is characterised by: a club-shaped penis, an epiphallus reaching the same length as the penis, an extremely long flagellum and a musculus retractor penis, which is attached in a central to distal position on the epiphallus. The female reproductive system is composed of a very short vaginal tube and a dart sac, which is strongly developed (Neubert 2014).

The COI sequence analysed for the 15 individuals revealed the existence of four distinct haplotypes. The verification in BLAST resulted in a fitting to *H. lucorum* from Russia (MG709115 (Korabek *et al.*



Fig. 1. *H. lucorum* locality in the city of Warsaw, Poland.



Fig. 2. A shell of *H. lucorum* from the Polish population. Scale bar is 1 cm.

Table 1

Measured characteristics of the shells of 50 *H. lucorum* individuals (in mm)

Character	Min	Max	Mean	SD
Aperture height	18.79	24.96	21.42	1.38
Aperture width	16.29	22.86	18.52	1.36
Shell height	31.04	42.03	34.82	2.05
Shell width	33.22	41.27	37.12	1.70
Shell diameter	34.22	43.52	38.00	1.98

2018); Query cover: 97-98%; Percentage identity from 99.51 to 99.68%), which was consistent with the species identification of the investigated individuals according to the morphological features. The p-genetic distances between the discovered haplotypes were less than 0.3%.

Other molecular markers

The analysis of the 16S rRNA sequences indicated two distinct haplotypes. The verification in BLAST showed that the obtained sequences fit the best to the *H. lucorum* from Slovakia (MG709101 (Korabek et al. 2018); Query cover: 100%; Percentage identity from 99.66 to 100%). The p-genetic distances between the discovered haplotypes were less than 0.4%. The analysis of the 28S rRNA sequences resulted in obtaining only a single haplotype.

Haplotype networks

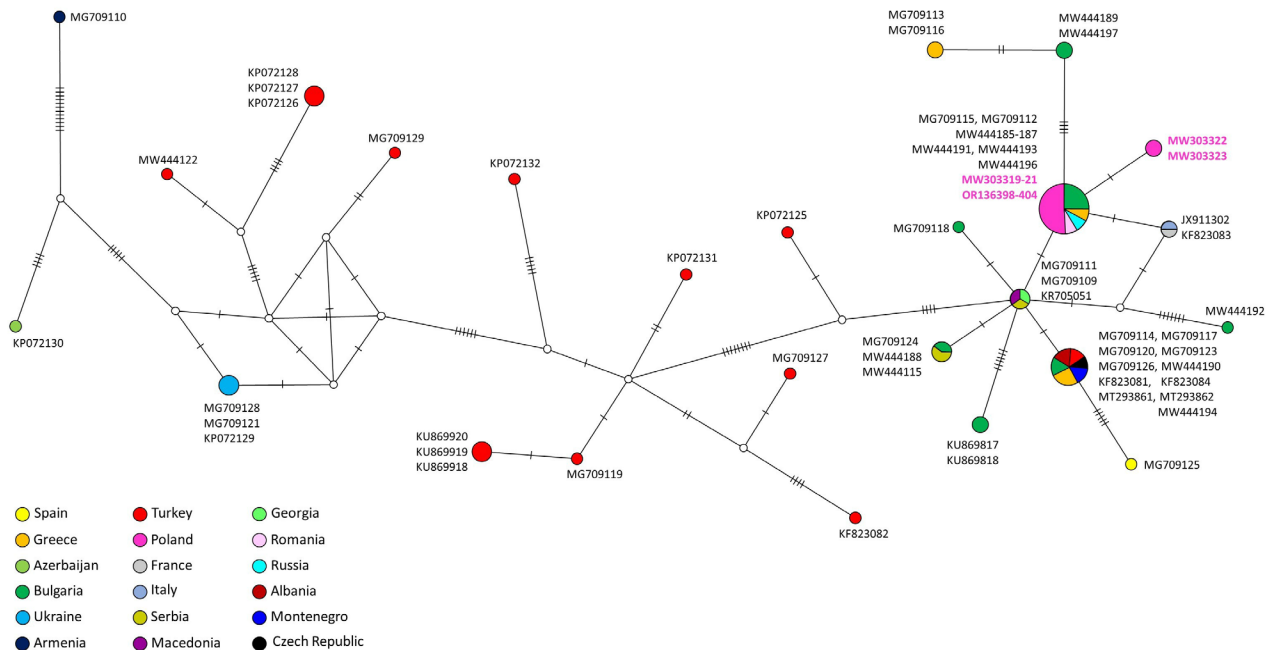
The analysis of the COI sequences obtained in this study, as well as those downloaded from GenBank, revealed a pattern of the haplotype distribution (Fig. 3). Haplotypes on the left side of the network include *H. lucorum* individuals from Turkey, Ukraine, Azerbaijan and Armenia. This area corresponds to the native range of the Turkish snail, which includes the Caucasus and Anatolia region. Within these detected haplotypes, there were also potential haplo-

types of *H. lucorum* that, to our knowledge, have not been discovered previously (marked in Figure 3 as white dots). The remaining haplotypes (right side in Figure 3) were mixed and consisted of snails from various localities where *H. lucorum* is considered as native, as well as an introduced species. Within this group, snails from the Polish population constitute a common haplotype together with gastropods from Bulgaria, Greece, Russia and Romania (Fig. 3). Similarly, a unique haplotype of *H. lucorum* from the population in Poland was detected, which has not previously been indicated during the detailed phylogeographic data of *H. lucorum* described by other researchers (Korabek et al. 2018).

A similar pattern of the haplotype distribution was revealed taking into account the analysis of the 16S rRNA dataset (Fig. 4). The first group of haplotypes consisted of snails from the native range of *H. lucorum*: Turkey, Ukraine, Azerbaijan, Iran and Armenia; whereas the second one was mixed and was comprised of both indigenous and non-native populations of the studied species.

Discussion

The Turkish snail, *H. lucorum* has been noted from countries bordering with Poland in the past including Slovakia, Czech Republic and Ukraine (Horsák



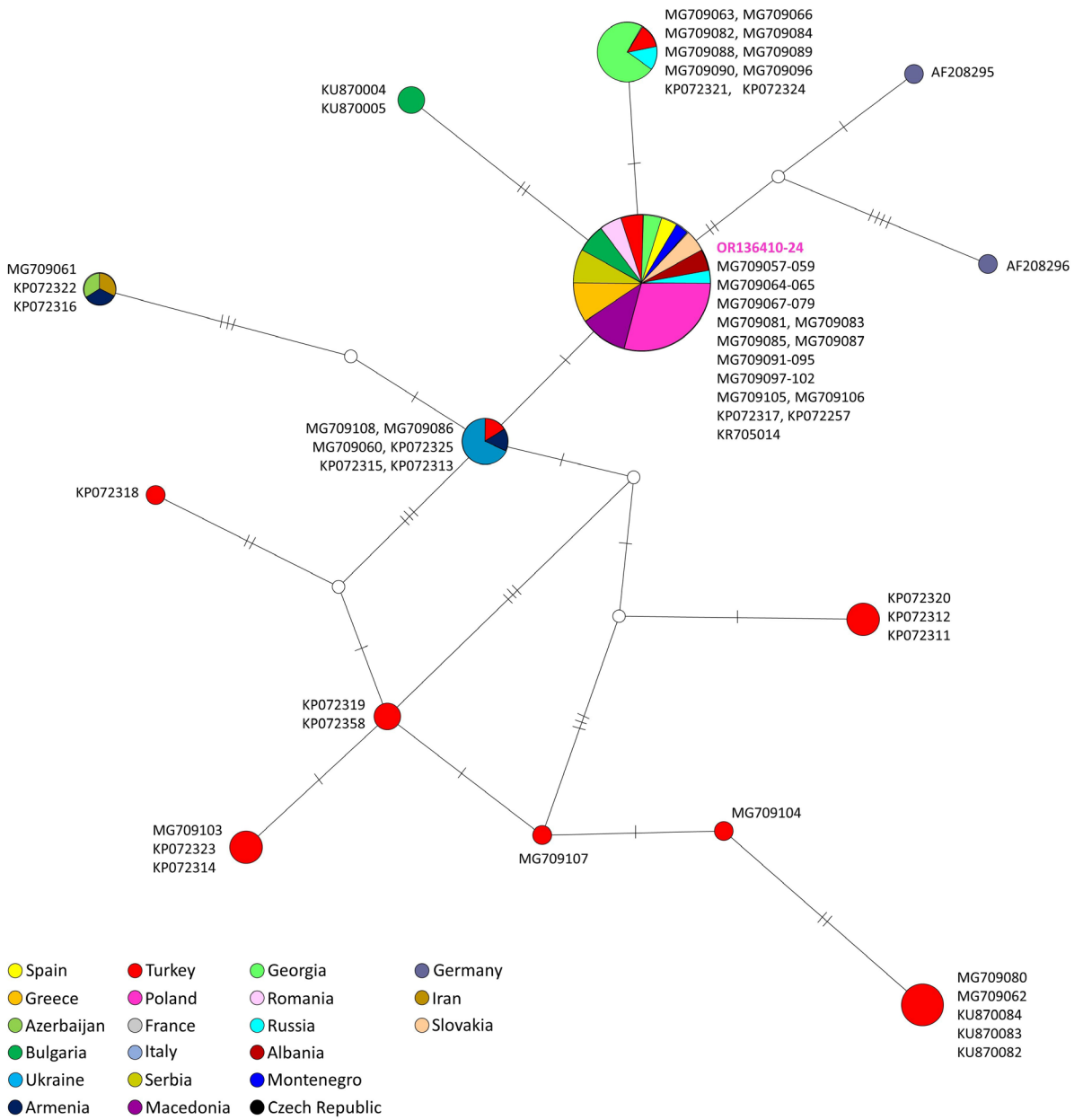


Fig. 4. Haplotype Median-Joining network of the mitochondrial 16S rRNA marker for *H. lucorum*. Haplotypes are labelled by coloured circles. The size of the circles is proportional to the number of sequences in a particular haplotype. White circles indicate a hypothetical intermediate haplotype linking the observed haplotypes of *H. lucorum*. Hatch marks in the network represent single mutations.

et al. 2010; Balashov & Gural-Sverlova 2012; Balashov *et al.* 2013; Čejka & Čačaný 2014). Thus, it is not surprising that this species has increased its distribution range and has now been found in Poland. In this study, we present detailed characteristic of the first population of the Turkish snail from Poland, in the area of Warsaw city. The presence of empty shells as well as live individuals – both adults and juveniles – suggests that this population is relatively stable. The snails that were found have typical conchological traits that are characteristic for the

H. lucorum species. However, it has been previously noted that this species may also exhibit a high variability of shell colouration compared to other populations (Neubert 2014).

Staikou *et al.* (1988) discovered that *H. lucorum* reaches sexual maturity three years after hatching, at the diameter of the shell equal to or larger than 35 mm. The mean diameter of the *H. lucorum* shell in the newly discovered Polish population is 38 mm, which indicates the presence of adult specimens able to reproduce. This is supported by the presence

of juveniles in the studied locality, which may also suggest that the population is established. However, the use of the shell size as a maturity indicator in the case of terrestrial gastropods has been criticised and vastly discussed (e.g. Boman *et al.* 2018). Nevertheless, similarly to other snails, the mature individuals of *H. lucorum* cease their growth and usually build a thickened aperture margin, which is useful trait for the identification of maturity in gastropods (e.g. Choat & Shiel 1980).

The shell measurements obtained in this study were much lower than those presented by Mumladze (2013). It is possible that non-indigenous *H. lucorum* adapts to the local conditions in a new distribution area and the adults probably have smaller shells in anthropogenic environments due to several environmental factors, such as the micro-climate or altitude but also the population density (Mumladze 2013).

An important feature of the described population from Warsaw is the relatively different colouration patterns and shape of the shells. However, such differences in the external appearance of *H. lucorum* gastropod shells is characteristic for this species in comparison with other *Helix* species. Hybridisation events may be excluded, despite the fact that a second species, *H. pomatia*, co-occurs at the presented locality. Hybridisation was discovered among the other *Helix* taxa (Gomot-de Vaufleury & Borgo 2001; Korábek *et al.* 2016), but such a process between *H. lucorum* and *H. pomatia* is unlikely. This is due to the fact that *H. lucorum* and *H. pomatia* are distantly related to each other, and are placed phylogenetically quite far from each other in comparison to other *Helix* taxa (Korábek *et al.* 2015).

The co-occurrence of *H. pomatia* and *H. lucorum* in the same habitat may suggest the occurrence of competition between these species for habitat and food resources, which may lead to a gradual displacement of the native species. This is a common phenomenon when an invasive alien species appears in a new environment and competes locally for resources with the native species (e.g. Zając *et al.* 2017). Moreover, invasive species are mostly characterised by high fertility, rapid development, greater ecological tolerance, drought resistance, few natural enemies, good dispersal abilities, greater survival at higher temperatures, and by behavioural and greater phenotypic plasticity in comparison to the native species (Kolar & Lodge 2001; Simberloff 2001; Knop & Reusser 2012). The process of invasion can be divided into three to five stages, which end when the species reaches a high density and spreads further (Vermeij 1996; Kolar & Lodge 2001; Colautti & MacIsaac

2004). Such gastropod invasions were noted previously in the case of *Arion vulgaris* Moquin-Tandon, 1855 (e.g. Zając *et al.* 2017), *Theba pisana* (Müller, 1774) (e.g. Däumer *et al.* 2012), *Lissachatina fulica* (Bowdich 1822) (e.g. Thiengo *et al.* 2007) and many others.

The discovery of the first locality of *H. lucorum* in Poland does not seem to be surprising, considering the analysis of the DNA sequences obtained for the COI and 16S rRNA markers. Korábek *et al.* (2018) noticed that the Turkish snail successfully expanded from the Anatolia region to other areas of Europe, where it found favourable conditions to survive and reproduce. This pattern fits the Polish population of *H. lucorum* that is described in the present study. The Warsaw population of Turkish snail is most closely related to these populations, which were considered previously as introduced ones.

The studied population is characterised by a relatively large number of juveniles and adults that are evidently capable of reproducing, which shows that *H. lucorum* has favourable reproductive conditions in the described new locality. The ability of *H. lucorum* to live in synanthropic conditions may be the basis for the successful invasions of this species. Thus, detailed studies on the impact of this species on its native counterparts and the ecosystem functioning are needed. Moreover, population monitoring and eradication actions should be introduced with regard to this species.

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Author Contributions

Research concept and design: A.B.; Collection and/or assembly of data: K.S.Z., J.T.; Data analysis and interpretation: K.S.Z., A.B.; Writing the article: K.S.Z., A.B.; Critical revision of the article: K.S.Z., A.B.; Final approval of article: K.S.Z., J.T., A.B.

Conflicts of Interest

The authors declare no conflict of interest.

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