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# GENETIC STUDIES ON THE INVASIVE SLUG *ARION LUSITANICUS* MABILLE, 1868 (GASTROPODA: PULMONATA) IN POLAND

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ABSTRACT: Within the last decades the slug *Arion lusitanicus* has expanded its range over wide areas of Europe, in most of them it is now a serious pest. Poland has been invaded relatively recently (since the late 1980s). Considering the ecological importance of the slug, very little is known yet about the mechanism of invasion, establishment of new populations and influence on the native fauna and flora. The analysis of nucleotide sequences of mitochondrial cytochrome oxidase subunit 1 gene (*cox1*) revealed a great inter- and intrapopulation variation in the Polish populations of *A. lusitanicus*. The differentiation of all the studied Polish populations of *A. lusitanicus* is 0.2–2.2%, while two analysed Belgian populations are monomorphic and moderately genetically diverse at 0.8%. This indicates a heterogeneous origin of the Polish populations, probably resulting from multiple independent introduction events. The genotype found in the first four Polish populations (S. Poland) suggests that their origin is different from the remaining populations.

KEY WORDS: Pest species, migration routes, DNA, mitochondrial gene *cox1*

## INTRODUCTION

The original distribution range of *Arion lusitanicus* Mabille, 1868 (Gastropoda: Pulmonata: Arionidae) includes Spain and Portugal (SIMROTH 1891, QUICK 1952, 1960, VAN REGTEREN ALTENA 1971, CHEVALLIER 1972). Within the last 50 years the slug has spread to many other European countries where it is both a serious pest and an invasive species with a negative effect on the biodiversity of native ecosystems (REISCHÜTZ 1984, DAVIES 1987, VON PROSCHWITZ 1994, FRANK 1998, KOZŁOWSKI & KOZŁOWSKI 2011).

*A. lusitanicus* probably appeared in Poland at the end of the 1980s (KOZŁOWSKI & KORNOBIS 1994, 1995). Initially it occurred only in one locality, in the village of Albigowa near Rzeszów (Subcarpathian region), in subsequent years it spread over a considerable area in that region, south-east of Rzeszów. In 1997–2007 it was recorded from over 100 localities in Poland (KOZŁOWSKI 2000, 2001, 2008, KOZŁOWSKI & SIONEK 2000, KOZŁOWSKI & KOZŁOWSKI 2011). In Poland the slug is synanthropic and most often occurs in large numbers in gardens and cultivated fields located near buildings, among shrubs, in parks, cemeteries and in wasteland. It is a serious pest of many species of vegetables, ornamental plants, agricultural crops, orchards and herbs, and it readily feeds on various species of wild plants (KOZŁOWSKI 2005, 2008).

*A. lusitanicus* is often confused with the native slug *A. rufus*, especially when juveniles are concerned. Using molecular techniques combined with anatomical methods makes it possible to avoid misidentification. In our earlier studies we estimated the level of genetic differences within the mitochondrial gene *cox1* between the Polish specimens of *A. lusitanicus* and *A. rufus* as ca. 12% (SOROKA et al. 2009). It is thus

impossible to confuse the two species based on the *cox1* sequence.

KOZŁOWSKI & KOZŁOWSKI (2011) in their study on the distribution of *A. lusitanicus* in Poland gave a detailed description of field observations on particular sites of the species and the damage caused by its appearance. Genetic studies on Polish populations of *A. lusitanicus* may help ascertain their origin and routes of invasion. Such information is very important for the monitoring of invasive gastropod species and for explaining phylogeographic relationships among the populations. The previous studies of the Polish populations of *A. lusitanicus* revealed three monomorphic and three polymorphic populations, with high genetic diversity (SOROKA et al. 2009). This study was aimed at mapping the distribution of *A. lusitanicus* in Poland and recognising the intraspecific variation and probable migration routes of the populations. Specimens from two Belgian populations were used to compare the genetic structure of distant populations (Poland and Belgium).

## MATERIAL AND METHODS

For our genetic studies we collected specimens from five Polish populations of *A. lusitanicus* (Fig. 1) located in West Poland: Leszno (51°50'N, 16°34'E, collection date – 18.08.2007) and Ochla (51°52'N, 15°26'E, collection date – 20.06.2008); Central Poland: Łódź (51°46'N, 19°27'E, collection date – 12.06.2007) and Podkowa Leśna near Warsaw (52°07'N, 20°44'E, collection date – 2.08.2007); and North Poland: Gronowo Górne (54°08'N, 19°27'E, collection date – 20.07.2007). Moreover, we analysed specimens collected from two Belgian populations located in (Fig. 1): Antwerp (51°13'N, 4°25'E, collection date – 17.07.2011) and Bruges (51°13'N, 3°14'E, collection date – 18.07.2007).

For genitalia-based identification (RIEDEL & WIKTOR 1974, WIKTOR 2004), 10 slugs were randomly selected from each population, with the exception of Łódź (only 6 specimens were identified anatomically), Ochla (4), Antwerp (6) and Bruges (3).

Genetic studies included comparisons of sequences within the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*) between specimens and populations. For molecular studies we used 21 specimens of *A. lusitanicus* selected for anatomical identification and representing the five Polish populations: Leszno (5 specimens), Gronowo Podgórne (5), Podkowa Leśna (5), Łódź (2) and Ochla (4). We also sequenced DNA obtained from eight Belgian speci-

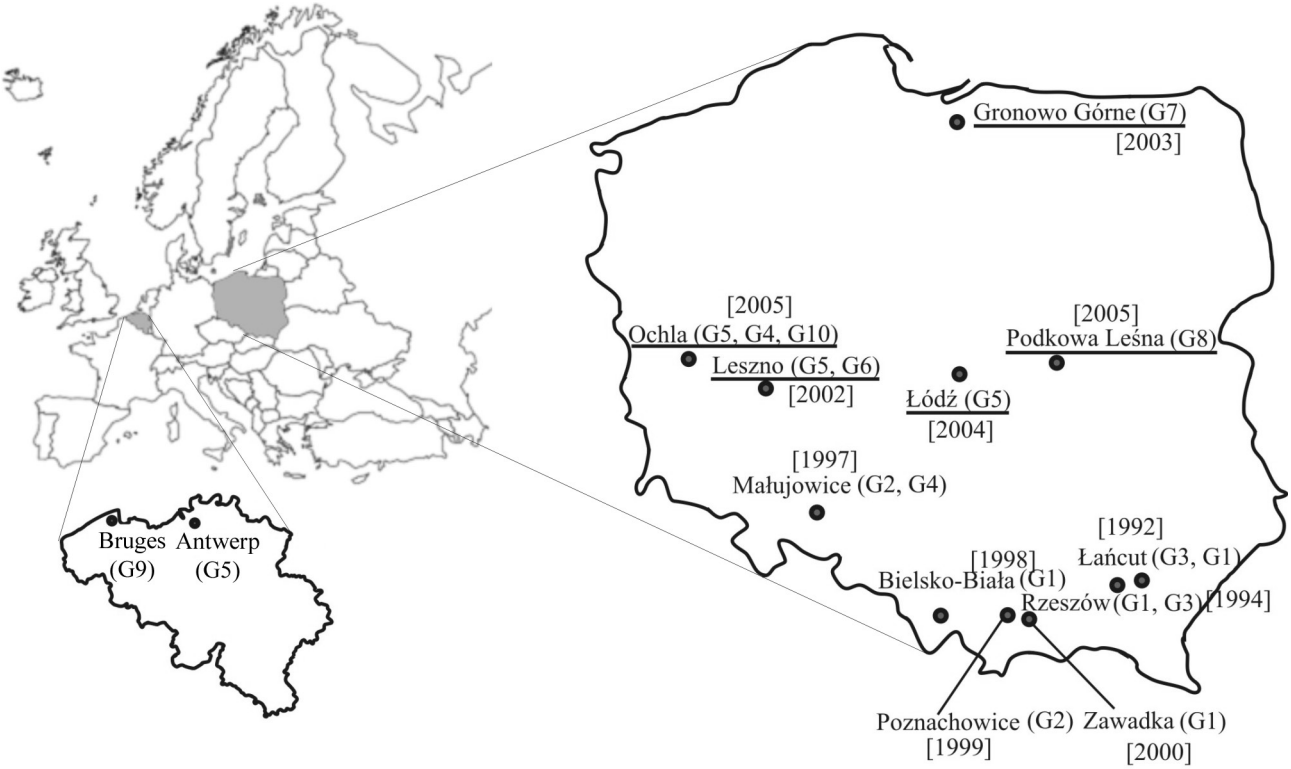


Fig. 1. Collecting sites of *Arion lusitanicus* in Poland and Belgium. Polish populations genetically analysed for the first time in this paper are underlined, genotypes are listed in order of frequency, the year of first appearance of *A. lusitanicus* in the studied sites is given in square brackets

mens from Antwerp (5) and Bruges (3) populations. The Qiagen set (DNeasy Tissue Kit, Germany) was used for DNA isolation. Polymerase chain reaction (PCR) with universal primers LCO1490 and HCO2198 (FOLMER et al. 1994) was used in order to obtain the fragment of the mitochondrial gene *cox1*. Detailed information on isolation and PCR reaction was presented in SKUJIENE & SOROKA (2003) and SOROKA & GRYGIEŃCZO-RAŹNIEWSKA (2005). Products of amplification of the *cox1* fragment were visualised in 1% agarose gel in UV light following electrophoresis. The size of the resulting PCR products was analysed with the use of BioCapt and Bio1D programmes (Vilbert Lourmat, France).

Sequence analyses for the PCR-obtained fragments of *cox1* gene were performed at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw (www.oligo.pl) or at the Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University in Poznań, Poland.

For comparison, we also used genetic data obtained earlier (SOROKA et al. 2009) for the following six Polish populations of *A. lusitanicus* (Fig. 1): South-East Poland: Łańcut (50°04'N, 22°13'E, collection date – 10.05.2006) and Rzeszów (50°01'N, 22°02'E, collection date – 18.07.2006); South Poland: Poznachowice (49°48'N, 20°06'E, collection date – 28.04.2006), Zawadka in the Gorce Mts (49°44'N, 20°17'E, collection date – 8.08.2006) and Bielsko Biała (49°48'N, 15°26'E, collection date – 28.08.2006); South-West Poland: Małujowice near Opole (50°50'N, 17°22'E, collection date – 12.06.2006).

Comparative analysis of the obtained sequences, identification of polymorphic sites and estimate of genetic distances according to Kimura's two-parameter method (KIMURA 1980) were done with the use of computer programme MEGA4 (TAMURA et al. 2007). The same programme was used to estimate the nucleotide diversity within the populations and to focus genetic relations between the populations and genotypes using UPGMA tree. This tree-making method assumes that the rate of evolution has remained constant throughout the evolutionary history of the included taxon.

## RESULTS

The sequence analyses of the mitochondrial *cox1* gene were always in agreement with the anatomical species identification of our specimens as *A. lusitanicus*. For all the studied specimens of *A. lusitanicus*, we obtained sequences of *cox1* gene which were 674 base pairs long. We found seven genotypes of which six were new (G5-10, G9 only from Belgium). Together with the genotypes recognised earlier (SOROKA et al. 2009), based on the observed variation in 21 polymorphic sites in that gene, ten genotypes recorded from Poland and Belgium were submitted to GenBank (accession numbers EF520640-EF520643, EU734823-EU734828 and GQ166169) (Table 1).

The genetic differentiation among the ten haplotypes ranged from 0.2% (between haplotypes G5 and G4, G5 and G6, G5 and G7 ) to 2.2% (between G1 and G2) and involved 1 and 14 nucleotide substitutions within the 674 compared nucleotides, respectively (Table 1). The intraspecific frequency of individual haplotypes for *A. lusitanicus* in Poland and Belgium ranged from 0.254 (G1) to 0.034 (G4, G6 and G10) and within populations it ranged from 1.0 to 0.2 (Tables 1 and 2).

Table 1. Genetic diversity among 10 genotypes of *A. lusitanicus* using Kimura's two-parameter (KIMURA 1990), their GenBank accession numbers and frequencies in species

| Accession Number | Frequencies in species | | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EF520640 | 0.254 | G1 | 0 | | | | | | | | | |
| EF520641 | 0.152 | G2 | 0.022 | 0 | | | | | | | | |
| EF520642 | 0.085 | G3 | 0.018 | 0.003 | 0 | | | | | | | |
| EF520643 | 0.034 | G4 | 0.009 | 0.018 | 0.015 | 0 | | | | | | |
| EU734823 | 0.186 | G5 | 0.008 | 0.017 | 0.014 | 0.002 | 0 | | | | | |
| EU734824 | 0.034 | G6 | 0.009 | 0.018 | 0.015 | 0.003 | 0.002 | 0 | | | | |
| EU734825 | 0.085 | G7 | 0.009 | 0.018 | 0.015 | 0.003 | 0.002 | 0.003 | 0 | | | |
| EU734826 | 0.085 | G8 | 0.011 | 0.020 | 0.017 | 0.005 | 0.003 | 0.005 | 0.005 | 0 | | |
| EU734827 | 0.051 | G9 | 0.012 | 0.009 | 0.006 | 0.009 | 0.008 | 0.009 | 0.009 | 0.011 | 0 | |
| GQ166169 | 0.034 | G10 | 0.009 | 0.018 | 0.015 | 0.002 | 0.002 | 0.003 | 0.003 | 0.005 | 0.009 | 0 |

Table 2. Genotypes, their frequency and nucleotide diversity in the studied populations of *A. lusitanicus* in Poland and Belgium

| Country | Population | N | Number of genotypes | Genotypes and their frequencies | | Nucleotide diversity |
|---|---|---|---|---|---|---|
| POLAND | *Łańcut | 5 | 2 | G3 | 0.8 | 0.018 |
| | | | | G1 | 0.2 | |
| | *Poznachowice | 5 | 1 | G2 | 1.0 | 0.000 |
| | *Małujowice | 5 | 2 | G2 | 0.8 | 0.018 |
| | | | | G4 | 0.2 | |
| | *Rzeszów | 5 | 2 | G1 | 0.8 | 0.018 |
| | | | | G3 | 0.2 | |
| | *Zawadka | 5 | 1 | G1 | 1.0 | 0.000 |
| | *Bielsko-Biała | 5 | 1 | G1 | 1.0 | 0.000 |
| | Leszno | 5 | 2 | G5 | 0.6 | 0.002 |
| | | | | G6 | 0.4 | |
| | Gronowo Górne | 5 | 1 | G7 | 1.0 | 0.000 |
| | Podkowa Leśna | 5 | 1 | G8 | 1.0 | 0.000 |
| | Łódź | 2 | 1 | G5 | 1.0 | 0.000 |
| | Ochla | 4 | 3 | G4 | 0.25 | 0.002 |
| | | | | G5 | 0.5 | |
| | | | | G10 | 0.25 | |
| BELGIUM | Antwerp | 5 | 1 | G5 | 1.0 | 0.000 |
| | Bruges | 3 | 1 | G9 | 1.0 | 0.000 |
| | Total | 59 | 1 to 3 | 10 | | |

*Population analysed in SOROKA et al. 2009

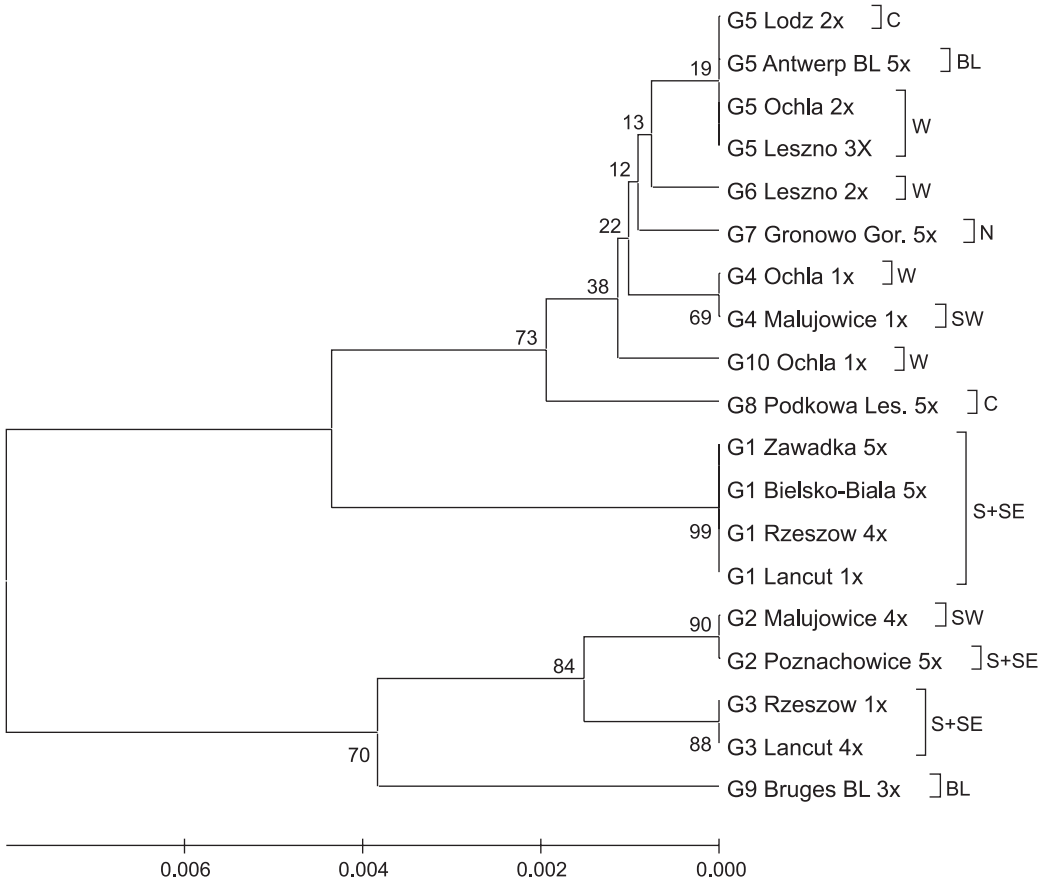


Fig. 2. UPGMA tree for genotypes and populations of *A. lusitanicus*. Abbreviations of regions: BL – Belgium, C – Central Poland, N – North Poland, S+SE – South and South-East Poland, SW – South-West Poland, W – West Poland

Among the eleven Polish populations, six were monomorphic, populations from Bielsko-Biała and Zawadka having identical haplotypes (G1), and the remaining ones had different haplotypes (G2, G5, G7 and G8) (Table 2). Each of the polymorphic populations except one had two haplotypes, one more frequent (0.8 – 0.6). Three haplotypes were found in the population from Ochla. The populations from Łańcut and Rzeszów had the same two haplotypes but at different frequencies (Table 2). Genotypes G1, G2 and G5 occurred in both monomorphic and polymorphic populations where they always constituted the most frequent haplotype, except for the Łańcut population. Rare genotypes G6 (0.034) and G10 (0.034) were found only in single polymorphic populations, in Leszno and Ochla, respectively. The nucleotide differentiation in the Polish polymorphic populations was up to 0.018 (Table 2). The relations among the populations and genotypes are shown in Fig. 2.

The two populations from Belgium (Antwerp and Bruges) were monomorphic and had genotypes G5 and G9, respectively; their similarity was 0.8% (Table 1 and 2). Genotype G5 was also found in three of the Polish populations: the polymorphic populations from Leszno and Ochla, where it was dominant and the monomorphic population from Łódź (Table 2, Fig. 2).

## DISCUSSION

This study revealed six new genotypes (G5–G10) in *A. lusitanicus*, compared to the earlier studies (SOROKA et al. 2009), but the level of intraspecific and interpopulation variability did not increase (Tables 1, 2). The genetic diversity among the six new genotypes was up to 1% whereas the previous estimate of more than 2% pertained to only four genotypes (SOROKA et al. 2009). The population genetic studies showed that six out of eleven analysed populations of *A. lusitanicus* in Poland were monomorphic, and for the polymorphic populations the genetic diversity was most often 0.018. Such populations had mostly two haplotypes for the five examined specimens in each locality. Some genotypes (G1, G2 and G3) were found only in the south of the country, the remaining ones (G4, G5, G6, G7 and G8) – in other regions, which would indicate different expansion routes (Fig. 2).

The four southern populations of *A. lusitanicus* in Poland (Łańcut, Rzeszów, Bielsko-Biała and Zawadka) are closer together than the remaining ones (20–230 km); they were among the first populations of the species found in Poland. They all occupy big areas, except the population from Zawadka, and each has a high density (mean 12.4–28.5 $m^{-2}$). Genotype G1 found in these four localities and only in the south of Poland, with a very high frequency in the species (0.254), suggests their common origin.

The populations from Poznachowice and Małujowice have the same genotype G2, which is genetically the closest to G3 found in the south of the country. Their genetic diversity at 0.3% involves two nucleotide substitutions. The rare genotype G4, found only twice in Małujowice and Ochla, shows the greatest genetic similarity to genotypes G5, G6 and G7, found outside the southern part of the country (Table 1). The genetic differentiation of co-occurring genotypes G2 and G4 in Małujowice is high (1.8%) and involves 12 nucleotide changes. It appears that the Małujowice population was founded by highly genetically diverse colonisers. A similarly diverse colonisation seems to be true of the other two southern polymorphic populations, Łańcut and Rzeszów, where G1 and G3 genotypes occur and are genetically differentiated at 1.8%. However, their frequency differs between the localities (Table 1, Fig. 2).

G1 genotype, as well as the other very differentiated genotypes G2 and G3 (Table 2), dominate in the first sites of occurrence of *A. lusitanicus* in Poland, suggesting that the founder individuals originated from different localities or another polymorphic population. Unfortunately, nothing is known about genetics of older European populations of *A. lusitanicus*. The Polish populations are rather young, only about 15 years old, hence the observed large genetic differentiation of up to 2.2% could not have resulted from in situ mutations. The genetically different individuals may have been introduced in the south of Poland with plant material. The polymorphic population from the region of Leszno in western Poland, with its genotypes G5 and G6, shows a small genetic differentiation (0.2%), the genotypes differ in one transition A/G (Table 2, Fig. 2). This indicates a uniform group of colonisers. The Polish populations from Leszno, Łódź and Ochla have the same genotype G5 as the monomorphic Belgian population (Antwerp) which points to a direct or indirect route of introduction of *A. lusitanicus* from Western Europe to these three localities in Poland. It is interesting to notice that these three Polish populations are situated along the main traffic route from Western Europe to Warsaw. The second Belgian population (Bruges), with its G9 genotype, shows the greatest similarity to the G2 and G3 genotypes found in the south of Poland and the similarity is 0.6 and 0.9%, respectively (Fig. 2).

The intraspecific differentiation of the *cox1* gene of up to 2.2% observed in *A. lusitanicus* is high compared to *A. rufus* (1.0%) and the mussel *Dreissena polymorpha* (1.1%) (THERRIAULT et al. 2004, SOROKA

et al. 2009, SOROKA 2010). The considerable variation of the gene may favour a great plasticity of *A. lusitanicus* when invading new territories. Another freshwater bivalve, *Sinanodonta woodiana*, introduced in Poland in the mid-1980s and found only in six localities, shows no differentiation within its mitochondrial gene *cox1* (KRASZEWSKI 2007, SOROKA 2008).

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