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Distribution and Genetic Diversity of the Terrestrial Slugs *Arion lusitanicus* Mabille, 1868 and *Arion rufus* (Linnaeus, 1758) in Poland Based on Mitochondrial DNA

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The slugs *Arion lusitanicus* and *Arion rufus* inhabit ecologically degraded areas and are serious vegetation pests. In recent years, new localities of these species have been found in various parts of Poland. Here we study the morphology of 90 specimens from 9 populations of slugs. The morphology of the genital system allowed for the identification of 60 *A. lusitanicus* specimens from 6 populations and 30 *A. rufus* individuals from another 3 localities. In order to describe their genetic diversity at the level of the individual, population, and species, we compared sequences of the mitochondrial cytochrome oxidase subunit I (*cox1*) gene. The morphological analysis revealed that each of the studied populations comprised a single species, which was also confirmed by the molecular assay. We obtained 674-bp sequences of the *cox1* gene for each species that showed a total of eight haplotypes. The genetic diversity of *A. lusitanicus* individuals ranged from 0.5% to 2.1%, whereas that of *A. rufus* was twice as low: 0.4-1.0%. The difference between the two species within the *cox1* gene was at the level of 12%. Three *A. lusitanicus* and two *A. rufus* populations were found to be monomorphic. Large inter-population variability was found within each of the studied species, which suggests that the Polish populations of *A. lusitanicus* may have originated from repeated, separate introductions arriving from various parts of Europe.

Key words: *Arion lusitanicus*, *Arion rufus*, distribution, genetic structure, mtDNA sequences, *cox1* gene.

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Two of the terrestrial slug species of the genus *Arion* Férussac, 1819, that are found in Poland have gained economical importance recently, i.e. *Arion lusitanicus* Mabille, 1868 and *Arion rufus* (Linnaeus, 1758). The species *A. lusitanicus*, which was brought to Poland at the end of the 1980s, is a native slug of the Iberian Peninsula. Its original distribution covered Spain and Portugal; in the mid-1980s, however, the species was also discovered in Great Britain (QUICK 1952, 1960; ELLIS 1965; DAVIES 1987). Since then, the slug has spread over the territories of many countries of western and central Europe (VAN REGTEREN ALTENA 1955; SCHMID 1970; WIKTOR 1983; REISCHÜTZ 1984; DAVIES 1987; RISCH & BACKELJAU 1989; DE WINTER 1989; VON PROSCHWITZ 1992).

This synanthropic species is a serious pest of many vegetables, ornamental plants, field crops, fruit trees, and herbs (KOZŁOWSKI 2005). The first encounter of this species in Poland was reported in the southern part of the country, in the village of Albigowa, Subcarpathian Region, then also from Łańcut and Rzeszów (KOZŁOWSKI & KORNOBIS 1994, 1995). In the last decade it was also found in other southern regions, i.e. Opole Region, Małopolska Upland, and Silesia (KOZŁOWSKI 2000, 2001).

A. rufus inhabits mainly western and central Europe, it is, however, also found in northern Europe, on the British Isles and in Scandinavia. Its distribution reaches the Alps to the south and the Pyrenees to the west (RIEDEL & WIKTOR 1974). The first unconfirmed reports of the presence of *A. rufus* in Poland were published at the end of the 19th century (RIEDEL & WIKTOR 1974). The species is native to the western part of Poland, i.e. Lower Silesia, West Poland, and Pomerania,

reaching as far as the mouth of the Vistula. It can be also found in other regions, such as Lesser Poland and the Subcarpathian Region, where it occurs in large numbers, usually as a synanthropic organism (KOZŁOWSKI & KORNOBIS 1995; WIKTOR 2004). Found mainly in lowlands and mountain valleys, the species inhabits about 25% of the area of Poland, where the border of its natural range is also located. Large populations of this slug can be found both on arable and non-arable land (WIKTOR 2004). Originally, *A. rufus* was a typical dweller of sea-side forests and avoided developed areas (RIEDEL & WIKTOR 1974). Like *A. lusitanicus*, *A. rufus* causes serious damage to crops, especially in gardens (KOZŁOWSKI 2003).

Since there are virtually no differences in the individual appearance of the two species, the slugs are identified based on their morphological characteristics, mainly the structure of the genital system. As in other slugs, knowledge on differences in the morphology of all life stages, best if specified for the particular area and time of the year, is needed for species identification. Moreover, considerable individual variation makes identification of arionid species highly difficult. This also underlies the controversies over the taxonomic classification of the genus. Various authors assign the *Arion* species to two (SIMROTH 1885; BACKELJAU & DE BRUYN 1990), three (DAVIES 1987), or five subgenera (HESSE 1926; RIEDEL & WIKTOR 1974; WIKTOR 2004). WIKTOR (2004), based on the morphology of the reproductive system and other characteristics, distinguished five subgenera within the genus *Arion* Férussac, 1819, i.e. *Arion sensu stricto*; *Mesarion* Hesse, 1926; *Carinarion* Hesse, 1926; *Kobeltia*, Seibert, 1873, and *Microarion*, Hesse, 1926. According to this taxonomic division, *A. lusitanicus* and *A. rufus* belong to the subgenus *Arion s. str*. (WIKTOR 2004). They belong to the group of European species spread over extremely diverse geographic regions and the ranges of their distributions are poorly recognized (QUINTEIRO *et al.* 2005). Both species often occur in large numbers and spread to new habitats. This mainly pertains to *A. lusitanicus*, whose distribution, both in Poland and other European countries, is expanding from year to year. In recent years, we have observed a considerable increase in the spatial diversity of both species in Poland. Field observations carried out to date indicate that *A. lusitanicus* and *A. rufus* probably occupy different habitats.

The first attempts to shed light on taxonomic and phylogenetic issues using genetic methods, allozyme electrophoresis, and then molecular techniques, date back to the 1960s. The methods have been applicable especially to species that defy identification by morphology, or to those that exhibit a high level of age- or habitat-related morphological variability.

At the end of the 1980s, allozyme electrophoresis allowed for the distinction between two freshwater bivalve species, *Dreissena polymorpha* (Pallas 1771) and *D. bugensis* (Andrusov 1897), occurring sympatrically in the North American Great Lakes. The two species differ in shell colour and shape, but also occupy habitats differing in water depth and temperature gradient (MAY & MARSDEN 1992; MILLS *et al.* 1993; SPIDLE *et al*. 1994). Further molecular studies and environmental tolerance analyses suggested that *D. bugensis* and *D. rostriformis* (Deshayes 1838) may represent two distinct races of the same species, since only a single base pair difference (0.23%) between the species occurred in the 16S gene and 2-3 bp differences (0.36-0.54%) were found in the *cox1* gene (THERRIAULT *et al*. 2004).

The genus *Marstoniopsis* (Gastropoda: Rissooidea) has long been represented by two species: *M. scholtzi* (A. Schmidt 1856) and *M. insubrica* (Küster 1853), partly due to their allopatric distribution patterns, although no morphological differences between the species seem to exist. Sequence analysis of a fragment of the mitochondrial *cox1* gene revealed extremely low genetic divergence (0.152%-0.304%) between populations of these two species (FALNIOWSKI & WILKE 2001). FALNIOWSKI and WILKE (2001) conclude that all the populations they studied comprised the same species, whose proper name – because of the priority rule – is *M. insubrica*.

Several research projects have been completed on arionid slugs, mainly concerned with population genetics, such as determination of the number of chromosomes, polymorphic enzyme loci, or random amplification of polymorphic DNA (BACKELJAU & DE BRUYN 1990; DAVIS 1994; NOBLE & JONES 1996; BACKELJAU *et al.* 1997; VOSS *et al.* 1999; SKUJIENE & SOROKA 2003). The most recent molecular studies on the taxonomy and phylogeny of *Arion* slugs were based on analyses of the mitochondrial genes, rDNA and ND1, as well as the nuclear sequence of ITS-1 (PINCEEL *et al.* 2004, 2005a,b; QUINTEIRO *et al.* 2005).

The aim of this study was to trace the current distribution and directions of expansion of the slugs *A. lusitanicus* and *A. rufus* in Poland, as well as to describe their genetic structure based on an analysis of the mitochondrial cytochrome oxidase subunit I (*cox1*) gene.

Material and Methods

The material comprised slugs collected in 2006, preserved in 70% methyl alcohol. Prior to immersion in the preservation solution, a fragment of the foot of each specimen was dissected and frozen for

Table 1

Specimens of *A. lusitanicus* and *A. rufus* slugs collected in various localities in Poland (S – South, SE – South-East, SW – South-West and NW – North-West Region of Poland) Taxonomy of the slugs according to WIKTOR (2004)

Slug species	Specimens	Locality	UTM grid	Date of collection (dd-mm-yy)	Coordinates
Arion (*Arion*) *lusitanicus* Mabille, 1868	1ALŁ – 5A LŁ	Łańcut, Subcarpathian – SE	EA 95	10-05-06	50°04'25,1''N 22°13'29,4''E
	6ALP – 10ALP	Poznachowice, South Poland – S	DA 43	28-04-06	49°48'24,3''N 20°06'01,5''E
	11ALM – 15ALM	Małujowice, Opole Region – SW	XS 75	12-06-06	50°50'52,1''N 17°22'53,7''E
	16ALR – 20ALR	Rzeszów-Słocina, Subcarpathian Region – SE	EA 85	18-07-06	50°01'23,6''N 22°02'22,8''E
	31ALZ – 35ALZ	Zawadka, South Poland – S	DA 62	08-08-06	49°44'17,9''N 20°17'19,6''E
	36ALB – 40ALB	Bielsko-Biała, Carpathians	CA 73	28-08-06	49°48'45,9''N 19°02'19,1''E
Arion (*Arion*) *rufus* (Linnaeus, 1758)	21ARM – 25ARM	Mielno, West Pomerania – NW	WA 72	14-07-06	54°15'36,3''N 16°03'39,7''E
	26ARL – 30ARL	Limanowa, South Poland – S	DA 61	08-08-06	49°42'02,7''N 20°25'36,3''E
	41ARS – 45ARS	Szalejów Górny, Lower Silesian – SW	XR 19	30-08-06	50°25'46,6''N 16°33'53,0''E

further molecular analyses. The collected material consisted of 10 individuals from each of 9 populations inhabiting distinct localities within the territory of Poland (Table 1). Notes were taken on each locality concerning its geographic position and habitat characteristics. The distribution of the localities is presented on a map of Poland divided by a 10 x 10 km square coordinate grid (Fig. 1) (BOGUCKI & STĘPCZAK 1974). The grid of the map is based on the Universal Transverse Mercator (UTM) grid and was adjusted to the European system of faunistic surveys. The species of the collected slugs were identified by an analysis of the morphology of the reproductive system, as well as by other diagnostic characters, and by comparing the resulting data with available references (RIEDEL & WIKTOR 1974; WIKTOR 2004).

The molecular analyses consisted of comparing the sequences within the mitochondrial cytochrome oxidase subunit I (*cox1*) gene between the individuals and the populations of slugs. We used foot fragments stored at -30°C from 5 individuals from each population (Table 1). DNA was extracted from a total of 45 specimens (30 *A. lusitanicus* and 15 *A. rufus*) using the DNeasy Tissue Kit (Qiagen, Germany). PCR was carried out in order to obtain a fragment of the mitochondrial *cox1* gene using the universal primers, LCO1490 and HCO2198 (FOLMER *et al.* 1994). A detailed description of DNA isolation and the PCR conditions was presented in SKUJIENE and SOROKA (2003) and SOROKA and GRYGIEŃCZO-RAŻNIEWSKA (2005). The *cox1* gene fragment amplification products were separated using 2-% agarose gel electrophoresis and visualized under UV light. The images were stored and the PCR product weights were analysed by means of the BIO-CAPT and BIO-1D software packages (Vilber Lourmat, France).

The PCR products were sequenced at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw (www.oligo.pl).

A comparative sequence analysis was carried out using the DNAMAN 5.2.9 program (Lynnon BioSoft, Canada). Genetic distances between the sequences were estimated using observed divergence and Kimura's two-parameter distance model (KIMURA 1980).

Results

Species identification

Identification of arionid species is based on the morphology of the reproductive system (RIEDEL & WIKTOR 1974). The main distinction between *A. lusitanicus* and *A. rufus* arises from the appearance of the genital organs and related additional

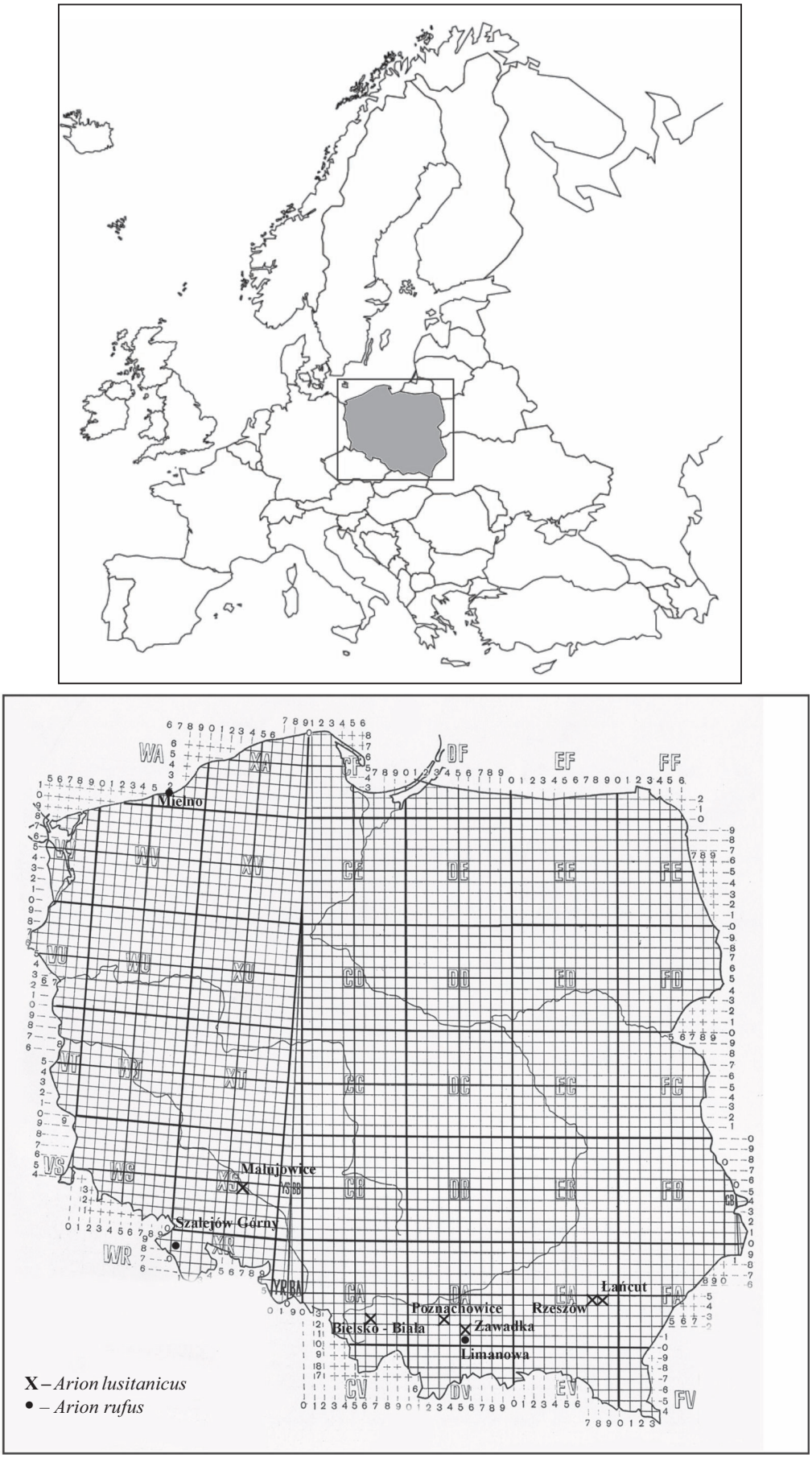

Fig. 1. Distribution of *Arion lusitanicus* and *Arion rufus* populations in Poland.

structures. *A. rufus* has a large, asymmetrical genital atrium formed by two parts, the posterior – wide, and anterior – narrow. The posterior part of the atrium is nearly twice as long as its anterior part. Inside the atrium, in its anterior part, next to the exit of the oviduct, the ligula is present. During copulation, the ligula everts with the entire atrium. The oviduct is thin and short in relation to the rest of the genitals, while the spermatheca is oval in shape. In contrast, *A. lusitanicus* has a single atrium, almost symmetrical in shape. The oviduct is large and thick, with a short and thin posterior part and with an abruptly widening, thick-walled, pipe-shaped anterior part. Inside the anterior part of the oviduct, two oblong fringes joining to the front are located just near the outlet to the atrium. As in *A. rufus*, the spermatheca sac is oval in shape. In general, the atrium in *A. lusitanicus* is small and short compared with the very large and thick oviduct, which is in contrast to that of *A. rufus*. According to these diagnostic characters, the material collected from 9 localities was composed of 60 *A. lusitanicus* specimens and 30 *A. rufus* individuals. We did not detect the presence of both species at the same locality. *A. lusitanicus* was found in 6 populations at the following localities: Łańcut, Rzeszów (Subcarpathian Region), Poznachowice, Zawadka (South Poland), Małujowice (Opole Region), and Bielsko-Biała (Carpathians); *A. rufus* was identified in 3 populations in Mielno (West Pomerania), Szalejów Górny (Lower Silesia), and Limanowa (South Poland) (Fig. 1).

Characteristics of localities and populations of slugs

The *A. lusitanicus* specimens used for the morphological and genetic studies were collected at 6 separate localities (Table 1, Fig. 1). In the towns of Łańcut (1ALŁ -5ALŁ) and Rzeszów (16ALR-20ALR), the slugs were picked from gardens which they had infested in large numbers and where they inflicted serious damage to cultivated plants. The slugs belonged to the populations distributed over a large part of the Subcarpathian Region. At present, *A. lusitanicus* occupies local habitats in this region, such as gardens, crop fields, parks, cemeteries, wastelands, shrubs, river banks, waste dumps, etc. Other specimens collected in the villages Małujowice (11ALM-15ALM) and Poznachowice (6ALP-10ALP) represented populations occurring on a few small (0.5-1.2 ha) gardens and farms. The first encounters of *A. lusitanicus* in these villages were reported in 1997 (Małujowice) and 1999 (Poznachowice). The habitats occupied by these slugs include gardens, crop fields, wastelands, shrubs, and ruined houses covered with vegetation. In gardens, the slugs are very numerous and seriously damage vegetables and flowers. The population of Bielsko-Biała (specimens: 36ALB-40ALB) is found in allotment gardens located in the city centre over an area of approx. 12 ha. The first reports on slugs from this area are dated to 1998. *A. lusitanicus* occurs in the area of the gardens and the neighboring housing estates. The specimens from the village of Zawadka (31ALZ-35ALZ) were collected from a population living in a single garden. According to the owner's communication, the slugs had probably been brought unintentionally with plant material from Bielsko-Biała in 2000. As in the aforementioned localities, the mollusks occur in large numbers and cause serious damage to many crop plants.

The *A. rufus* specimens were collected from three localities. Two of them, Mielno (21ARM-25ARM) and Szalejów Górny (41ARS-45ARS), are located within the natural range of the species. The specimens from Mielno were taken from a population found on meadows and shrubs growing on the shore of lake Jamno located on the Baltic Sea coast. The locality of *A. rufus* in the village of Szalejów Górny, on the other hand, was of an entirely different character. It comprised allotment gardens of a total area of 3.8 ha with diverse vegetation including vegetables, ornamental plants, as well as fruit bushes and trees. This habitat has been massively infested by the slugs for more than a decade, causing serious damage to plants, especially flowers. Most often, however, the slugs are found on compost heaps, where they feed on decaying plants. The third locality, i.e. the town of Limanowa (26ARL-30ARL), has been known for more than 30 years. It is situated outside the natural distribution area of *A. rufus* and has an insular character. The population predominantly inhabits grasslands located along the banks of a local stream (a Łosina river tributary) as well as some gardens on the periphery of the town. In recent years, the population of *A. rufus* found in this locality has not increased substantially. A black colour is characteristic of the slugs living in Limanowa; the specimens from the other studied populations were much lighter, most often dark-brown.

DNA sequence analysis: *Arion lusitanicus* and *Arion rufus*

We obtained 674-bp sequences of the *cox1* gene for 45 specimens of both species, in which 16 polymorphic sites were found for *A. lusitanicus* and 9 for *A. rufus* (Tables 2a,b). Three polymorphic sites detected at positions 261, 459, and 621 were common for both species. At each polymorphic site, the variation involved two bases, except for position 408 in *A. rufus*, where three different bases were observed (A, T, and G). Observed interspe-

Table 2

Haplotypes (with frequencies and accession numbers) and polymorphic sites in *Arion lusitanicus* (a) and *A. rufus* (b) within a 674-bp region of the *cox1* gene

a

Nucleotide	31	82	146	261	309	360	405	459	469	475	492	498	615	621	630	643
G1 0.500 EF520640	G	G	C	C	G	T	T	T	T	T	G	G	T	G	T	C
G2 0.300 EF520641	C	G	C	T	A	G	G	C	C	C	A	A	G	A	C	T
G3 0.167 EF520642	C	G	C	T	A	G	G	C	T	T	A	A	G	A	C	T
G4 0.033 EF520643	G	A	T	C	G	T	T	C	T	T	G	G	T	A	C	T

b

Nucleotide	33	192	219	261	384	408	459	618	621
G1 0.333 EF520644	G	G	G	C	C	A	C	A	A
G2 0.333 EF520645	T	G	G	T	T	T	T	A	C
G3 0.267 EF520646	T	C	A	T	C	G	C	G	C
G4 0.067 EF520647	T	G	G	T	C	G	C	A	C

cific divergence and KIMURA's distance (KIMURA 1980) were 11.6-12.6% and 12.7-14.0%, respectively (Table 3). The analysed gene fragment codes for 224 aminoacids of the protein cytochrome oxidase subunit I.

All dissimilar sequences obtained from both *Arion* species were submitted to GenBank (accession numbers EF520640 to EF520647, Tables 2a, b). For two specimens of *A. lusitanicus* (2ALŁ and 5ALŁ), we obtained identical 697-bp sequences of the *cox1* gene fragment representing the G3 haplotype, deposited in GenBank under a single accession number (EF535149).

Table 3

Observed pairwise divergence between fragments of the mitochondrial *cox1* gene of *A. lusitanicus* and *A. rufus* (below diagonal) and Kimura's two-parameter distance (above diagonal)

		A. lusitanicus				*A. rufus*			
		G1	G2	G3	G4	G1	G2	G3	G4
A. lusitanicus	G1		0.021	0.018	0.009	0.134	0.132	0.136	0.136
	G2	0.021		0.003	0.018	0.140	0.139	0.140	0.140
	G3	0.018	0.003		0.015	0.136	0.136	0.136	0.136
	G4	0.009	0.018	0.015		0.127	0.130	0.131	0.131
A. rufus	G1	0.121	0.126	0.123	0.116		0.009	0.010	0.006
	G2	0.120	0.126	0.123	0.119	0.009		0.009	0.004
	G3	0.123	0.126	0.123	0.119	0.010	0.009		0.004
	G4	0.123	0.126	0.123	0.119	0.006	0.004	0.004	

Arion lusitanicus

Among 30 *A. lusitanicus* specimens, we distinguished four haplotypes with frequencies between 0.500 (G1) and 0.033 (G4). Particular haplotypes differed by 2 (G2/G3), 6 (G1/G4), 10 (G3/G4), 12 (G1/G3 and G2/G4), and 14 (G1/G2) nucleotides (Table 2a). These changes most often involved the third position of the codon (71.4%) followed by the first position (28.6%). All changes in the third position of the codon were neutral and did not cause a change in the coded amino acid of the protein. On the other hand, 43.7% of the changes in the first position of the codon resulted in amino-acid substitutions, usually valine for leucine (57.1%), valine for isoleucine (28.6%), and glycine for isoleucine (14.3%). Genetic diversity between the four haplotypes ranged from 0.3% (between G2 and G3) up to 2.1% (between G1 and G2) and involved, respectively, 2 and 14 nucleotide substitutions within 674 compared nucleotides (Table 3). The observed differences were of transition type in 75% of cases, and of transversion type in 25% of cases (Table 2a).

Three populations were monomorphic, with the populations of Bielsko-Biała and Zawadka having

identical haplotypes (G1) and Poznachowice having a different one (G2). The remaining populations had two haplotypes each, one always being more frequent (frequency 0.8), the other being rare (0.2). The populations of Łańcut and Rzeszów had two identical haplotypes, however, with different frequencies (Fig. 2).

Arion rufus

We found four haplotypes for 15 specimens of *A. rufus* from three populations. The two most common haplotypes (G1 and G2) were found with the same frequencies, 0.333 each, whilst the rarest one, G4, was found only once, with a frequency of 0.067 (Table 2b). The haplotypes differed by 3 to 7 nucleotides, always in the third position of the codon, which only in 10.3% of cases caused substitutions in the coded amino acid and always involved the 408th nucleotide, resulting in a methionine-for-isoleucine substitution. Observed nucleotide substitutions were equally transition and transversion substitutions (Table 2b). The lowest genetic diversity was observed between haplotypes G2 and G4 and between G3 and G4 (0.4% each), while the highest was 1.0%, involving 7 nucleotide substitutions between haplotypes G1 and G3 (Table 3).

The slug population of Limanowa was the only polymorphic one and had haplotypes G3 and G4 with frequencies 0.8 and 0.2, respectively. The other populations, Mielno and Szalejów, were monomorphic and contained haplotypes G1 and G2, respectively (Fig. 3).

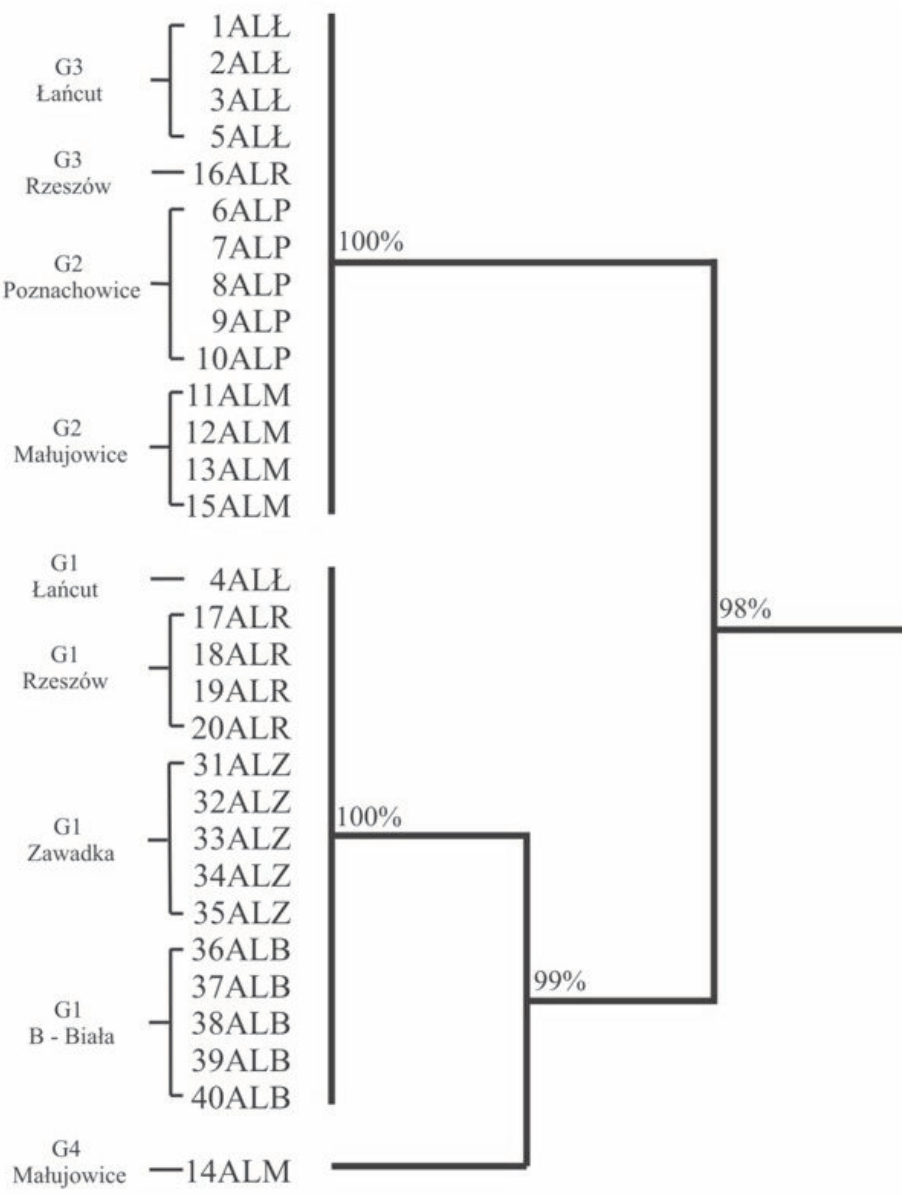

Fig. 2. Genetic similarity of individuals and populations of *A. lusitanicus*.

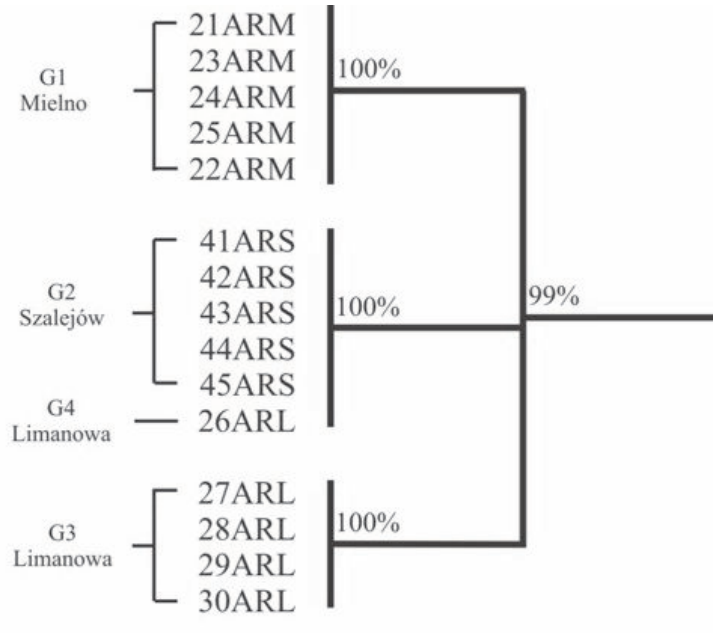

Fig. 3. Genetic similarity of individuals and populations of *A. rufus*.

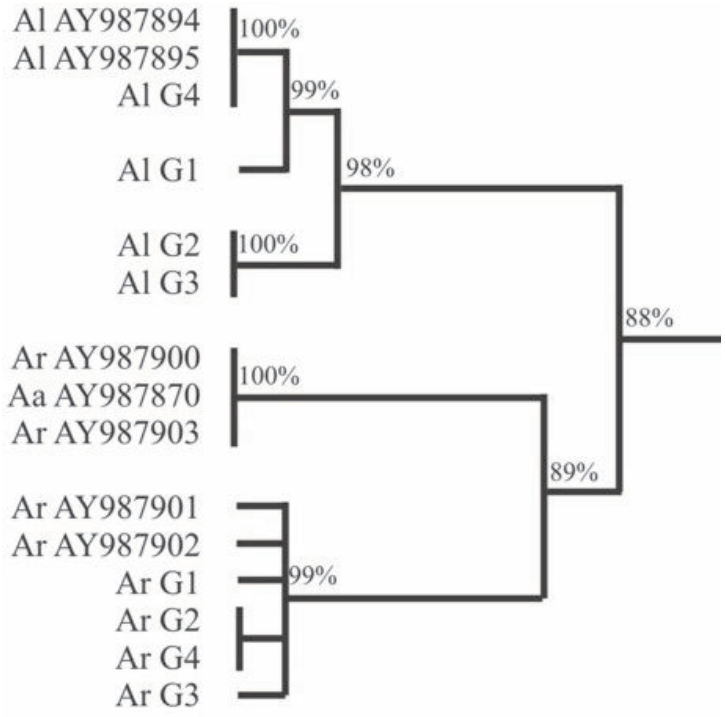

Fig. 4. Similarity of sequences within the fragment of the *cox1* gene for detected haplotypes and individuals obtained from GenBank (with accession numbers). Abbreviations: Aa, *A. ater*; Al, *A. lusitanicus*; Ar, *A. rufus*.

Discussion

The slugs *A. lusitanicus* and *A. rufus* are externally indistinguishable and their taxonomic affiliation can only be specified by a detailed morphological analysis of the genital organs in sexually mature individuals. Consequently, juvenile slugs defy taxonomic classification. Only expert researchers with long experience are able to determine the species of a juvenile specimen, although their decisions are often arbitrary. At present we can couple molecular techniques and morphological studies, enabling comprehensive and unbiased species identification (irrespective

of the developmental stage of a specimen) and allowing verification of the applied methods. Our analyses involved 90 *Arion* specimens belonging to 9 populations. Each population was comprised of only one species, which was confirmed by the molecular analyses. Thus, *A. lusitanicus* and *A. rufus* occupy separate areas. This was observed even for very close localities, as in the case of Zawadka, occupied only by *A. lusitanicus*, and Limanowa, inhabited exclusively by *A. rufus* – sites located 8 km away from each other. Not a single case of erroneous identification of the species was recorded, which differ from each other at a level of approx. 12% within the *cox1* gene sequence compared to 2.1% intraspecific variability in *A. lusitanicus* and 1% in *A. rufus*. The individuals of both species collected in Belgium, whose sequences were taken from GenBank, remain within the estimated ranges of their respective within species variability (Fig. 4). The exceptions are two *A. rufus* specimens (AY987900 and AY987903) that exhibit more than 10% variability in relation to the other species, which implies that they may belong to another arionid species, or that they may have been misidentified; this may also mean that *A. rufus* exhibits high intraspecific variation, similar to that of *Arianta arbustorum* (GITTENBERGER *et al.* 2004). A comparative genetic analysis revealed that these two sequences belong to the species *A. ater* (AY987870), which was based on their low level of variability (0.5%), characteristic of individuals belonging to a single taxon (Fig. 4). This comparison also reveals the extensive homogeneity of Polish *A. rufus* populations in relation to the Belgian one; on the other hand, the G4 haplotype of *A. lusitanicus*, rare in Poland (occurring only once per 30 assays), resembles Belgian rather than the other Polish sequences (Fig. 4).

The observed intraspecific variability within the *cox1* gene, 2% and 1% for, respectively, *A. lusitanicus* and *A. rufus* of either Polish or Belgian origin, is very low in relation to other slugs. The values reported in the literature range from 3.3%, for *A. fucus* (PINCEEL *et al.* 2005b), to 7.5%, for *Arianta arbustorum* (HASSE *et al.* 2003), to 18%, for *A. arbustorum* (GITTENBERGER *et al*. 2004). The variability *within* the last mentioned species is even higher than variability observed between the two species presented in this paper, which is 12%.

A. lusitanicus was introduced to Poland at the end of the 1980s. In 1993 the presence of the slug was documented based on specimens collected near Rzeszów, at the Albigowa (EA 95) and Markowa (EA 95) localities (KOZŁOWSKI & KORNOBIS 1994). *A. lusitanicus* slugs were then also found in Łańcut (EA 95) and, two years later, in the city of Rzeszów (EA 85). Over the years that followed, the species dispersed over nearly the entire area of the Subcarpathian Region (KOZŁOWSKI 2000). It was confirmed in Małujowice (XS 75), in 1997, and Poznachowice (DA 43), two years later (KOZŁOWSKI 2001). Novel localities of these slugs, i.e. Zawadka (DA 62) and Bielsko-Biała (CA 73), were confirmed in 2006 (Fig. 1).

The history of the dispersal of *A. lusitanicus* in Poland is reflected by the level of genetic diversity observed within its populations. The first populations of this species noted in the Subcarpathian Region (Łańcut and Rzeszów), as well as those in the Opole Region (Małujowice), are polymorphic. The Subcarpathian localities comprise the same halpotypes (G1 and G3), however, they differ in frequencies (Fig. 2). The Opole Region locality, also polymorphic, has two other halpotypes (G1 and G4), which suggests that the founder population in this region was different from that of the Subcarpathian populations, and that the G4 haplotype may have originated in Belgium (Fig. 4). On the other hand, the young populations discovered during 1999-2006 (Poznachowice, Zawadka, and Bielsko-Biała) are monomorphic. The Bielsko-Biała and Zawadka populations share the G1 haplotype, which confirms our field observations that the Zawadka locality originates from Bielsko-Biała. Slugs from both localities have been brought from the Subcarpathian Region, most probably from the area of Rzeszów, where the G1 haplotype occurred with a high frequency (0.8). The Poznachowice population of *A. lusitanicus* has only the G2 haplotype, which implies that it originates from the Opole Region, where this haplotype at the Małujowice locality was found with a frequency of 0.8. Two interesting Southern localities, separated by a 40-km distance, host monomorphic populations and represent the haplotypes belonging to both of Poland's first populations. The Subcarpathian Region is the source of the Zawadka population with individuals having the G1 haplotype, while the Opole Region is the original locality of the Poznachowice population, with the G2 haplotypes.

This distribution analysis of the four *A. lusitanicus* haplotypes in Poland indicates two possible sites of origin. The Opole Region hosts slugs of the G2 and G4 haplotypes, whereas those of G1- and G3-haplotypes occur in the Subcarpathian Region. The fact that the G4 haplotype exhibits higher similarity to Belgian sequences rather than to Polish ones suggests that some specimens may be of Belgian origin. We presume, however, that this is not the main migration route of *A. lusitanicus* to Poland since the G4 haplotype has a low frequency (0.03) as compared to the other observed haplotypes (Table 2). The remaining haplotypes that we have detected come from rather uncertain places in Europe; high frequencies of the haplotypes

(0.17-0.50) may suggest that these were major directions of dispersal of the species in Poland. If we can identify these places in the future, we will also gain insight into the origins of the source populations and the main routes of *A. lusitanicus* introduction into Poland.

The results presented here concerning the differences in the types and frequencies of occurrence between the four *A. lusitanicus* haplotypes suggest that the species has a short-distance dispersal ability, illustrated by the process of colonizing novel, nearby localities in the area of Albignowa and Łańcut in 1993-1997 (KOZŁOWSKI 2000). Most often, however, the slugs do not migrate actively beyond the occupied area and their expansion is mostly due to human activity, particularly transport of plant material. For instance, gene flow between the Subcarpathian populations (Rzeszów and Łańcut), despite a 15-km distance between them, is limited, and since 1995 has not resulted in the uniformity of their genetic structures, with equal levels of adaptation of both haplotypes (G1 and G3). Similar low gene flow and colonization of large areas by single haplotypes were observed in *Arion fuscus* (PINCEEL *et al*. 2005).

The genetic diversity of the distinguished haplotypes in *A. lusitanicus* ranges between 0.3% and 2.1%. Haplotypes that occur in territorial pairs, i.e. G1 with G3 and G2 with G4, differ from each other at a level of 1.8%, for which 12 nucleotide substitutions are responsible for 1 and 2 changes in amino acids, respectively. The amino-acid substitutions involved valine-for-leucine and glycine-for-isoleucine substitutions. These four amino acids do not substantially alter the properties of the coded protein, since they all are non-polar and hydrophobic. This allows concluding that the discussed haplotypes exhibit equal adaptation value and the observed nucleotide- and, in consequence, amino-acid substitutions are not evolutionarily significant.

For the three *A. rufus* population living in distant localities (430 to 590 km away from each other) no common haplotypes were found. Two populations from Mielno and Szalejewo Górne, which are located within the natural distribution of the species in Poland, are monomorphic and have distinct haplotypes, G1 and G2, respectively (Fig. 3). The insular population of Limanowa, on the other hand, is polymorphic and also has distinct haplotypes in relation to the other analysed populations, suggesting that this group originated from other founder populations of this species. Genetic diversity of the four *A. rufus* genotypes was twice as low as that of *A lusitanicus*, up to 1%. Equally low values of this parameter (0.4) involved the territorial pairs of genotypes, G3 and G4 from Limanowa, as well as G2 and G4 from Szalejewo and Limanowa, respectively. The diversity involves three nucleotide substitutions each, while in the case of the haplotypes G2 and G4, also one amino acid substitution, methionine for isoleucine (Table 3). These amino acids are non-polar and hydrophobic in character, suggesting equal adaptive values.

Low genetic divergence (0.4%) between the rare G4 haplotype and the haplotypes G1 and G2, which come from monomorphic populations, indicate a Polish origin. On the other hand, the frequent G3 haplotype is characterised by a divergence of 0.9-1.0% in relation to the remaining haplotypes and may originate from source populations located in other countries.

The study has revealed that both species of slugs inhabiting Poland demonstrate high inter-population diversity. This suggests a heterogeneous origin of *A. lusitanicus* populations now found in Poland, which probably results from multiple independent introductions of this species from various localities in western Europe, also – directly or indirectly – from Belgium. This does not apply to *A. rufus*. The monomorphic populations (Mielno and Szalejów Górny), located within the natural range of this species in Poland, confirm the active character of past dispersal, without human interference. The insular polymorphic populations, on the other hand, are a consequence of various human-induced, casual introductions. These conjectures, however, should be verified in further studies on the genetics of the populations of *A. lusitanicus* and *A. rufus* inhabiting other localities both in Poland and other European countries.

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