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Species identification of slugs of genus *Arion* Férussac, 1819 (Mollusca, Pulmonata) on the basis of genetics studies

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Species identification is the first task on purpose to improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity – one of goals (C) of the Strategic Plan for Biodiversity for the 2011–2020 period implementing The Convention on Biological Diversity. Slugs need a modern taxonomic revision thus RAPD-PCR (random amplification of polymorphic DNA by the polymerase chain reaction) technique and sequences analysis of the mitochondrial cytochrome oxidase subunit I (*COI*) gene were used in order to design molecular markers for 5 species of genus *Arion* Férussac, 1819 obtained from Lithuania and Poland. Six used RAPD primers revealed species-specific bands for all studied *Arion* species. Results with the use of RAPD primers and sequences analysis of *COI* gene fragment (655 bp) showed that Lithuanian '*A. hortensis*' belong not to *A. hortensis* but to *A. fuscus* and there is no variability among them. It is concluded that all reports about *A. hortensis* in Lithuania were made based on the external morphology without the anatomical analysis of reproductive organs or the use of molecular methods, hence all were erroneous. All slugs, determined as *A. hortensis* or *A. subfuscus* in Lithuania, according to novel genetic data belong to *A. fuscus*. It was specified that all three *Carinarion* species (*A. silvaticus, A. circumscriptus* and *A. fasciatus*) live in Lithuania.

Key words: DNA-fingerprinting, DNA sequencing, *COI* gene, slugs, *Arion*

INTRODUCTION

The assessment of biodiversity, monitoring changes, sustainable exploitation of biodiversity, and much legislative work depend upon validated knowledge of its components: the taxonomic diversity. Species identification is the first task on purpose to improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity – one of goals (C) of the Strategic Plan for Biodiversity for the 2011–2020 period implementing The Convention on Biological Diversity (http://www.cbd.int/sp/targets/). According to the Check-List of European Continental Mollusca (CLECOM), the European fauna consists of ca. 3600 species and subspecies of land and fresh molluscs (Bank et al., 2001) but this fauna is still incompletely studied. These data are regularly being updated in website of The Fauna Europaea project (www.faunaeur.org).

As Backeljau & De Bruyn (1990) have reported species identification of land slugs has been and still is troublesome and problematic because of colour polymorphism and the lack of obvious differences in morphological and anatomical characteristics. A major disadvantage in identification of slugs is the fact that some genital traits may only be observed during particular stages of life cycle and that body colour is clearly affected by age, food and climate (Jordaens et al., 2001). Nowadays, various authors use different methods to distinguish the species of *Arion* Férussac, 1819 genus. The most common is Hesse (1926) classification, based on the analysis of anatomical characteristics, especially the reproductive organs. This author divided the genus into five subgenera: *Lochea* M.-Tandon, 1855; *Mesarion* Hesse, 1926; *Carinarion* Hesse, 1926; *Kobeltia* Seibert, 1873; *Microarion* Hesse, 1926. However, later Davies (1987), on the basis of spermatophor forms and egg structures, divided the genus only into three subgenera: *Mesarion* & *Arion*; *Kobeltia* & *Microarion*; *Carinarion*. The most modern is the classification described by Backeljau & De Bruyn (1990) based not only on morphological and genital characteristics but also on breeding systems and mating behaviour as well as chromosome numbers and enzymatic polymorphism. These authors distinguished only two subgenera or chromosomal

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groups, *Lochea* (n ≤ 27) and *Prolepis* (n ≥ 27). *Lochea* contains species-groups *Mesarion* and *Arion* s. s., while *Prolepis* contains species-groups *Kobeltia* and *Carinarion*. The molecular studies suggest that North American *Carinarion* species (*Arion fasciatus, A. silvaticus* and *A. circumscriptus*) are inconsistent with the biological species concept because of inter-specific hybridization in places where these predominantly self-fertilizing slugs apparently outcross (Geenen et al., 2006).

As many authors (Backeljau & De Bruyn, 1990; Jordaens et al., 2001; Davis, 1994) have noted application of methods of molecular biology created new possibilities of dealing with taxonomic problems. Studying DNA sequence variation in nuclear (ITS-1) and mitochondrial (16S rRNA) genes it was found that there are five evolutionary lineages of *A. subfuscus* and two main evolutionary lineages of *A. fuscus* in Europe. Random amplification of polymorphic DNA by the polymerase chain reaction (RAPD-PCR) can be useful for the assessment of taxonomic genetic markers and can generate very sensitive measures of genetic relatedness within populations of organisms (Williams et al., 1990). The analyses of RAPD-PCR have been used for species identification and establishing of taxonomic relationships within some gastropod genera such as *Bulinus* (Stothard & Rollison, 1996; Stothard et al., 1997), *Cochlicopa* (Armbruster, 1997), *Biomphalaria* (Langand et al., 1999; Vernon et al., 1995) and subgenus *Stagnicola* (Rybska et al., 2000). As Voss et al. (1999) have reported this technique is also a suitable molecular tool to distinguish the slug species *A. distinctus, A. fasciatus* and *Deroceras reticulatum* on the species and genus level.

The aim of the present paper is to estimate the differentiation between some *Arion* species by finding molecular RAPD markers for them. Additionally, with the use of several primers, we would like to objectively establish the species identification of some individuals of Lithuanian slugs (called '*A. hortensis*' in this article), which were previously classified, depending on the used criterion (morphological or structure of reproductive organs), to the species *A. hortensis* or *A. subfuscus*. DNA sequences analysis of a fragment of the mitochondrial cytochrome oxidase subunit I gene (*COI*) was used in order to determine the taxonomic identity of questionable slugs. The *COI* gene encodes a conservative protein and evolves very slowly, thus its DNA sequences analysis enables comparison between species, genera, or even organisms that phylogenetically are very distant (Brown, 1985).

MATERIALS AND METHODS

Species of *Arion* genus were collected in Lithuania and Poland (Table 1). On the basis of morphological characteristics (Gurskas, 1997; Činikaitė, 1999) there were identified six species (Table 1). Verification was made by prof. dr. habil. A. Wiktor (Wrocław University, Poland) using anatomical characteristics (Riedel & Wiktor, 1974; Cameron et al., 1983) and some individuals earlier identified as '*A. hortensis*' were identified as the "dark form" of *A. fuscus* but in Table 1 they are left as '*A. hortensis*' as they were attributed in Lithuania (Gurskas, 1997; Činikaitė, 1999).

DNA was isolated from frozen somatic tissue (liver or muscle of leg) of an individual slug using DNeasy Tissue Kit (Qiagen), according to the procedure recommended by Qiagen. Amplification was performed in a total reaction volume of 20 µl, composed of 0.5 ng/µl of total template DNA, 1U Taq DNA polymerase (Qiagen), 0.4 µM of each primer or single primer, 200 µM of each dNTP and buffer PCR (Qiagen). Thermal cycling was performed with the use of two programmes depending on applied primers: PV – 5'-AATGGCGCAG – 3' (Voss et al., 1999), P2 – 5'-TGCACACTGA-3' (Klein-Lankhost et al., 1991) , P3 – 5'-TGGTGACTGA-3' (Klein-Lankhost et al., 1991), P5 – 5'-GTCCCGACGA-3' (Hosaka & Hanneman, 1994), P6 – 5'-CTCGTTTGGG-3' (Rybska et al., 2000), OPA7 – 5'-GAAACGGGTG-3' (Operon Technologies). For the primer PV amplification was performed for 40 cycles with the following profile: 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C and 10 min at 72 °C.

Table 1. **Collected and examined slug species, their numbers and localities in Lithuania and Poland**

Species	Number of individuals	Collection localities	Collection date
Arion (Mesarion) fuscus (Müller, 1774)	23, 24	Vilnius, Garden, Lithuania	May 2001
	62	Družiliai, Forest, Lithuania	September 2001
	33	Kariotiškės, Rubish dump, Lithuania	September 2001
'Arion (Kobeltia) hortensis' Férussac, 1819	21, 22, 61	Vilnius, Verkių Regional Park, Lithuania	May–June 2001
Arion (Kobeltia) distinctus Mabille, 1868	19, 20, 60	Szczecin, Park St. Żeromskiego, Poland	November 2001
	68	Wrocław, Botanic Garden, Poland	October 2001
Arion (Carinarion) fasciatus (Nilsson, 1822)	69, 71	Vilnius, Garden, Lithuania	May–June 2001
	64, 65	Jurbarkas, Garden, Lithuania	June 2001
Arion (Carinarion) circumscriptus Johnston, 1828	55, 56, 72	Vilnius, Verkių Regional Park, Lithuania	May–June 2001
	58, 73	Szczecin, Park St. Żeromskiego, Poland	September 2001
	66	Nemenčinė, Garden, Lithuania	June 2001
Arion (Carinarion) silvaticus Lohmander, 1937	37, 59	Vilnius, Verkių Regional Park, Lithuania	May 2001

Amplification for the remaining primers was performed with the following profile: 5 min at 95 °C, 50 cycles (each composed of three stages: 1 min at 92 °C; 2 min at 35 °C and 2 min at 72 °C) and the last extension at 75 °C for 5 min. Total genomic DNA isolated from particular slug species was used as the templates for PCR reactions with the following pairs of primers: P2+P6, P3+P5 and single primers: PV and OPA7. RAPD fragments originating from the same primer (or pair of primers) and migrating identically during the electrophoresis were assumed as homologous.

PCR products were separated in 2% agarose gel in 1 × TBE buffer during 3 hours together with DNA molecular weight marker. Gels were stained with ethidium bromide, and became visible in UV light, and documented using a BioCapMW system (Vilber Lourmat, France). Programs Bio1D (Vilber Lourmat, France) and DNAMAN version 5.2.9 (Lynnon BioSoft, Canada) were used to analyse the results (Tamura et al., 2007). The assessments of band value, the similarity index, according to Nei & Li (1979), were performed by comparison of the molecular weight of DNA obtained by the RAPD-PCR method, using the above mentioned program.

The 780 bp fragment of the mitochondrial *COI* gene was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994). Template PCR reaction was carried out in DNA Thermal Cycler 9600 (Perkin Elmer) in volume of 25 µl which included: 50 ng genomic DNA, 5 pmol each primer, 2.5 µl PCR buffer, 200 µM each dATP, dCTP, dGTP and dTTP and 1 U Taq DNA polymerase. The final concentration of $MgCl_2$ was 2.5–3.0 mM. PCR conditions were as follows: 5 min at 95 °C; 5 cycles, each – 30 s at 94 °C, 60 s at 45 °C, 90 s at 72 °C; then 27 cycles, each – 30 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C. In the final cycle the extension was carried out for 7 min.

PCR product was purified on Microcon-100 (Amicon) according to manufacturer protocol. Standard procedures and an ABI PRISM 377 DNA Sequencer (Applied Biosystems) were used for the *COI* gene fragment sequencing. MEGA4 program (Tamura et al., 2007) was used for comparing the obtained sequences with other sequences available in the GenBank. The species used in the comparative analyses are presented in Table 2. Ten individuals (*A. fuscus* – No. 21, 23, 24 and 33, '*A. hortensis*' – No. 22 and 61, *A. distinctus* – No. 19, 20, 60 and 68) were sequenced and the obtained sequences are deposited in the GenBank (AY094597, DQ647391, AY094599, DQ677393, DQ647394).

Table 2. **List of *Arion* species with their GenBank accession number and length of sequence in bp in parentheses for the *COI* DNA sequences used**

Species	GenBank accession number	Source referred in the GenBank
Arion fuscus (Müller, 1774)	AJ 809425 (400)	Pinceel et al., 2005
	AJ809426 (400)	
	AJ809434 (400)	
Arion subfuscus (Draparnaud, 1805)	AY987906 (484	Pinceel et al., 2006 (unpublished)
	AY987907 (484)	
	AY987908 (562	
	AY987915 (484)	
	AY987916 (562)	
Arion hortensis (Férussac, 1819)	AY423673 (576)	Dodd et al., 2005 (unpublished)
	AY423682 (576)	
	AY423683 (576)	
	AY423691 (576)	
Arion distinctus (Mabille, 1868)	AY423695 (576)	Dodd et al., 2005 (unpublished)
	AY423700 (576)	

RESULTS

PCR products after amplification with the use of the PV primer revealed between two and seven distinct bands from 1500 bp to 70 bp in individuals of *Arion* spp. (Table 3). The largest number of bands was observed in individuals of '*A. hortensis*' and *A. fuscus*. These two species shared bands 1250, 800 & 350 bp. Two bands 700 bp & 400 bp were specific to *A. distinctus*. The band 420 bp was typical of *A. circumscriptus*, 550 bp of *A. fasciatus* and 230 bp of 3 species (Table 3).

Table 3. **Numbers of individuals (No.) and values of bands (in bp) derived from PV primer for *Arion* species. Boldface figures denote bands that are specific for species**

A. distinctus		*'A. hortensis'*		*A. fuscus*			*A. circumscriptus*			*A. silvaticus*	*A. fasciatus*		
No. 19	**No. 20**	**No. 21**	**No. 22**	**No. 24**	**No. 33**	**No. 62**	**No. 56**	**No. 66**	**No. 73**	**No. 59**	**No. 64**	**No. 69**	**No. 65**
700	1300	**1250**	**1250**	1500	**1250**	**1250**	**420**	**420**	**420**	300	1000	**550**	**550**
500	1000	**800**	**800**	**1250**	**800**	**800**	350	300	**230**	**230**	**550**	350	350
400	800	500	500	**800**	500	500	300	**230**		70	300	**230**	**230**
220	**700**	**350**	**350**	530	**350**	**350**	**230**				**230**		100
	600	280	290	450	200	200					110		
	400	200	200	**350**	130	130							
	320	130	130	160									

The highest values of genetic intraspecific similarity were demonstrated in *A. circumscriptus, A. fuscus* and '*A. hortensis*'. It ranged from 1.00 to 0.60 (Table 4). A slightly lower coefficient was recorded in the case of *A. fasciatus* specimens. Very high values of interspecific similarity, i. e. 1.00–0.60, were noticed between individuals of *A. fuscus* and '*A. hortensis*'. Between other species this parameter was 0.06 (min 0.00 and max 0.33). The highest value (0.50) was recorded in one case between *A. circumscriptus* (No. 56) and *A. fuscus* (No. 24) (Table 4).

After amplification using OPA7 primer three or four bands were revealed in *A. fuscus, 'A. hortensis', A. silvaticus* and *A. distinctus*, with the values from 3600 bp to 150 bp (Table 5). The band 460 bp was specific to two species, *A. fuscus* and '*A. hortensis*', and 1650 bp to *A. distinctus*. All tested individuals of *A. circumscriptus*, *A. fasciatus* and *A. silvaticus* had the bands at approximately 3400, 3500 and 3600 bp, respectively. Only few bands (very heavy and light) were generated in *A. circumscriptus*.

Each of analyzed species revealed species-specific bands using OPA7 primer except *A. fuscus* and '*A. hortensis*' that shared band with value 460 bp (Table 5). *A. distinctus*, '*A. hortensis*', *A. circumscriptus*, *A. fasciatus, A. silvaticus* showed the highest values of genetic intraspecific similarity ranging from 0.57 to 1.00 (Table 6). The highest values of genetic differences (from 0.00 to 0.75) were observed between individuals of *A. fuscus*. Specimens of *A. fuscus* showed higher genetic similarity to '*A. hortensis*' individuals (0.29–0.75) than within its own species except No. 24 of *A. fuscus* that displayed the highest genetic diversity (Table 6). Some genetic similarity between species *A. fasciatus* and *A. silvaticus* (from 0.33 to 0.50) and higher similarity between *A. fasciatus* and *A. circumscriptus* (from 0.50 to 0.67) were recorded (Table 6). Species *A. distinctus* presented genetic similarity only to *A. silvaticus*, ranging from 0.29 to 0.33 (Table 6).

For *A. distinctus, A. fuscus* and '*A. hortensis*' PCR amplification products derived from P3+P5 primers revealed between two and five distinct bands (Table 7). The bands from 2000 bp to 80 bp were noticed. The band of 300 bp appeared in all individuals of *A. fuscus* and '*A. hortensis*' (Table 7).

The highest intraspecific genetic similarity was observed in '*A. hortensis*' and then in *A. distinctus*, which is visible in Nei and Li (1979) similarity coefficient assumed values: 1.00 and 0.29–0.57, respectively (Table 8). Remarkable variation (similarity coefficient from 0.00 to 0.50) was found

Table 4. **Pair wise distance matrix calculated by Nei & Li (1979) similarity coefficient on the basis of PCR products using PV primers. Abbreviation: *A. sv.* – *Arion silvaticus***

	A. distinctus		*'A. hortensis'*		*A. fuscus*			*A. circumscriptus*			*A. sv.*	*A. fasciatus*		
	No. 19	No. 20	No. 21	No. 22	No. 24	No. 33	No. 62	No. 56	No. 66	No. 73	No. 59	No. 64	No. 69	No. 65
No. 19	1.00													
No. 20	0.44	1.00												
No. 21	0.29	0.20	1.00											
No. 22	0.29	0.20	1.00	1.00										
No. 24	0.00	0.17	0.60	0.60	1.00									
No. 33	0.29	0.20	1.00	1.00	0.60	1.00								
No. 62	0.29	0.20	1.00	1.00	0.60	1.00	1.00							
No. 56	0.00	0.00	0.33	0.33	0.50	0.33	0.33	1.00						
No. 66	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.67	1.00					
No. 73	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.67	1.00	1.00				
No. 59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00			
No. 64	0.00	0.25	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	1.00		
No. 69	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.50	1.00	
No. 65	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.50	1.00	1.00

Table 5. **Numbers of individuals (No.) and values of bands (in bp) derived from OPA7 primer for *Arion* species. Boldface figures denote bands that are specific to species. Abbreviation: *A. cir.* – *Arion circumscriptus***

A. distinctus		*'A. hortensis'*			*A. fuscus*				*A. cir.*		*A. silvaticus*		*A. fasciatus*		
No. 19	No. 20	No. 21	No. 22	No. 61	No. 23	No. 24	No. 33	No. 62	No. 55	No. 56	No. 37	No. 59	No. 64	No. 65	No. 69
1650	**1650**	950	950	950	**460**	1500	2300	2400	**3400**	**3400**	**3600**	**3600**	**3500**	**3500**	**3500**
750	500	**460**	**460**	**460**	330	**460**	950	950	300	300	420	200		1200	
200	200	300	250	300	250	300	**460**	**460**			270	150			
				250		250	250	250			200				

Table 6. **Pair wise distance matrix calculated by Nei & Li (1979) similarity coefficient on the basis of PCR products with the use of OPA7 primer. Abbreviations: *A. cir. – Arion circumscriptus, A. dis. – A. distinctus, A. fas. – A. fasciatus* and *A. sv. – A. silvaticus***

	A. dis.		*'A. hortensis'*				*A. fuscus*			*A. cir.*			*A. sv.*		*A. fas.*	
	No. 19	**No. 20**	**No. 21**	**No. 22**	**No. 61**	**No. 23**	**No. 24**	**No. 33**	**No. 62**	**No. 55**	**No. 56**	**No. 37**	**No. 59**	**No. 64**	**No. 65**	**No. 69**
No. 19	1.00															
No. 20	0.67	1.00														
No. 21	0.00	0.00	1.00													
No. 22	0.00	0.00	0.67	1.00												
No. 61	0.00	0.00	0.57	0.57	1.00											
No. 23	0.00	0.00	0.33	0.67	0.29	1.00										
No. 24	0.00	0.00	0.29	0.29	0.00	0.29	1.00									
No. 33	0.00	0.00	0.57	0.57	0.75	0.29	0.00	1.00								
No. 62	0.00	0.00	0.57	0.57	0.75	0.29	0.00	0.75	1.00							
No. 55	0.00	0.00	0.40	0.00	0.00	0.00	0.33	0.00	0.00	1.00						
No. 56	0.00	0.00	0.40	0.00	0.00	0.00	0.33	0.00	0.00	1.00	1.00					
No. 37	0.29	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00				
No. 59	0.33	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57	1.00			
No. 64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.67	0.40	0.50	1.00		
No. 65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.50	0.33	0.40	0.67	1.00	
No. 69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.67	0.40	0.50	1.00	0.67	1.00

Table 7. **Numbers of individuals (No.) and values (in bp) of bands derived from P3+P5 primers for *Arion* species. Boldface figures denote bands that are specific to species**

A. distinctus				*'A. hortensis'*		*A. fuscus*		
No. 19	**No. 20**	**No. 60**	**No. 68**	**No. 21**	**No. 22**	**No. 24**	**No. 33**	**No. 62**
2000	2000	1900	2000	1600	1700	1600	1444	1700
1444	1370	1500	1700	900	1000	750	900	1000
900	1050		600	**300**	**300**	**300**	600	**300**
500	200			100	100	80	**300**	80
							100	

Table 8. **Pair wise distance matrix calculated by Nei & Li (1979) similarity coefficient on the basis of PCR products with the use of P3+P5 primers**

	A. distinctus				*'A. hortensis'*		*A. fuscus*		
	No. 19	**No. 20**	**No. 60**	**No. 68**	**No. 21**	**No. 22**	**No. 24**	**No. 33**	**No. 62**
No. 19	1.00								
No. 20	0.57	1.00							
No. 60	0.33	0.40	1.00						
No. 68	0.29	0.33	0.40	1.00					
No. 21	0.00	0.00	0.50	0.40	1.00				
No. 22	0.00	0.40	0.00	0.40	1.00	1.00			
No. 24	0.00	0.00	0.50	0.40	0.50	0.50	1.00		
No. 33	0.29	0.33	0.00	0.33	0.40	0.40	0.00	1.00	
No. 62	0.00	0.40	0.00	0.40	1.00	1.00	0.50	0.40	1.00

between individuals of *A. fuscus*. The difference was mainly due to specimen No. 24 from Vilnius, Garden that was strikingly different and more similar to '*A. hortensis*' individuals (Table 8).

PCR amplification products derived from P2+P6 primers revealed three or four bands for *A. distinctus* (1000 bp, 800 bp and 650 bp), *A. fuscus* (1000 bp, 700 bp, 500 bp and 400 bp) and '*A. hortensis*' (1000 bp, 700 bp and 500 bp). Three bands were shared by *A. fuscus* and '*A. hortensis*'. According to Nei and Li (1979) similarity coefficient, *A. distinctus* was different from *A. fuscus* and '*A. hortensis*' 0.29 and 0.33, respectively and value 0.86 was observed between *A. fuscus* and '*A. hortensis*'.

Ten sequences were obtained for *COI* gene fragment belonging to *A. fuscus* (No. 21, 23, 24, and 33), '*A. hortensis*' (No. 22 and 61) and *A. distinctus* (No. 19, 20, 60 and 68).

A. fuscus and '*A. hortensis*' revealed the same nucleotide sequences within 655 and 661 base pairs, except that one transversion T / G in the end of analysed sequence caused one substitution phenyloalanine (F) into valine (V) in protein within 220-aminoacid (accession no.: AY094597 and DQ647391). Both amino acids are polar, hydrophobic. In the mitochondrial genome of *Cepaea nemoralis* these sequences are located between position 7 and 663 (accession no.: U23045) in the *COI* gene. There is nucleotide composition bias towards T at all codon sites, and mean nucleotide frequencies (given in percent) for *A. fuscus* were as follows: *A* = 25.5, *C* = 16.2, *G* = 19.0, *T* = 39.4. 634 or 661 bp sequences were obtained for four individuals of *A. distinctus* (accession no.: AY094599, DQ647393 and DQ647394). In the alignment of this species partial *COI* sequences we identified 14 variable positions (1.5% of diversity). The mean nucleotide frequencies (given in percent) into this gene were: *A* = 25.9, *C* = 16.4, *G* = 18.5, *T* = 39.2.

These sequences were compared with other sequences available in the GenBank for these species and for *A. fuscus, A. subfuscus* and *A. hortensis* (Table 2 and Figure). Before this comparison there were selected 3–5 sequences from 39 *A. fuscus*, 12 *A. subfuscus*, 22 *A. hortensis* and 11 *A. distinctus* sequences available in the GenBank (Table 2). These selected sequences showed the largest intraspecific differences after primary comparing analysis made in each species according to Kimura's (1980) two-parameter genetic distances.

The phylogenetic tree based on *COI* sequences of *A. fuscus, A. subfuscus, A. hortensis* and *A. distinctus* was developed to provide higher resolution on the *A. subfuscus–A. fuscus* relationship (Fig. 1). According to Kimura (1980), genetic divergence between *A. subfuscus* and *A. fuscus* is over 23% (Table 9). The highest intraspecific difference in the *COI* gene fragment was observed within individuals of *A. subfuscus* to 18% (showing two clads on Fig. 1) and then for *A. fuscus* specimens to 2.1% whereas for *A. hortensis* and *A. distinctus* these parameters were approximately similar and carried out less than 1% (as measured by Kimura's (1980) distances) (Table 9). The interspecific differences between all analysed *Arion* species were from 23.3% (between *A. fuscus* and *A. subfuscus*) to 28.5% (between *A. fuscus* and *A. hortensis*) (Table 9).

DISCUSSION

As several authors (Claxton et al., 1997; Rybska et al., 2000; Roe et al., 2001; Therriault et al., 2004; Soroka, 2005; Soroka & Grygieńczo-Raźniewska, 2005) have noted using of

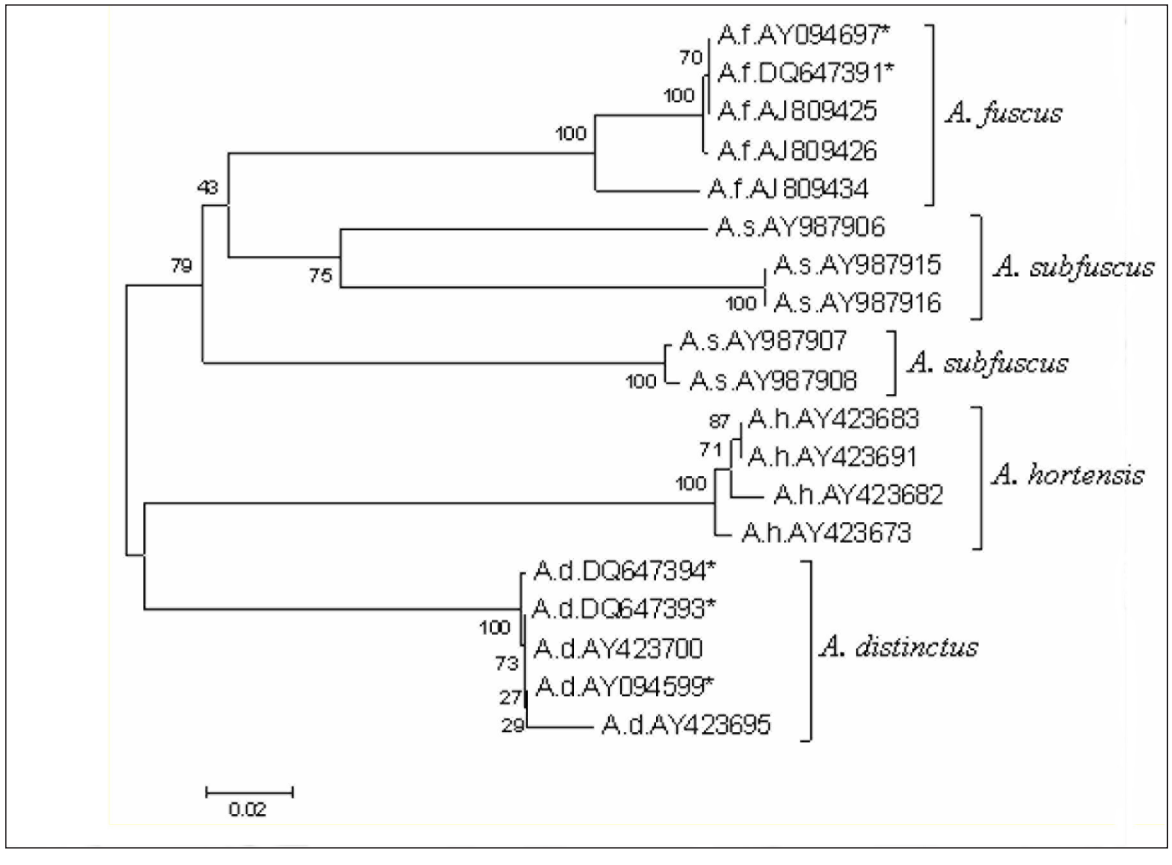

Fig. 1. Neighbour Joining Tree for four species of genus *Arion* based on the Kimura two-parameter model into partial *COI sequences* obtained by authors (marked by asterisk) and by other scientists from the GenBank (see Table 2). Values above branches represent bootstrap support (2000 replications)

Table 9. **DNA distance matrix of *Arion* species based on Kimura two-parameter model (1980) (asterisk marks accessions sequenced by the present authors). Abbreviations: A. s. – *A. subfuscus,* A. f. – *A. fuscus*, A. d. – *Arion distinctus* and A. h. – *A. hortensis***

	A.s AY987606	A.s.AY987607	A.s.AY987908	A.s.AY987915	A.s.AY987916	A.f.AY094697*	A.f.DQ647391*	A.f.AJ809425	A.f.AJ809426	A.f.AJ809434	A.d.AY094599*	A.d.DQ647393*	A.d.DQ647394*	A.d.AY423695	A.d.AY423700	A.h.AY423673	A.h.AY423682	A.h.AY423683	A.h.AY423691
A.s AY987606	0																		
A.s.AY987607	0.213	0																	
A.s.AY987908	0.212	0.005	0																
A.s.AY987915	0.185	0.250	0.250	0															
A.s.AY987916	0.185	0.250	0.250	0.000	0														
A.f.AY094697*	0.198	0.220	0.227	0.255	0.255	0													
A.f.DQ647391*	0.198	0.220	0.227	0.255	0.255	0.000	0												
A.f.AJ809425	0.198	0.220	0.227	0.255	0.255	0.000	0.000	0											
A.f.AJ809426	0.198	0.220	0.227	0.255	0.255	0.003	0.003	0.003	0										
A.f.AJ809434	0.201	0.237	0.244	0.265	0.265	0.049	0.049	0.049	0.052	0									
A.d.AY094599*	0.236	0.219	0.223	0.237	0.237	0.230	0.230	0.230	0.230	0.223	0								
A.d.DQ647393*	0.236	0.219	0.223	0.237	0.237	0.230	0.230	0.230	0.230	0.223	0.000	0							
A.d.DQ647394*	0.239	0.215	0.219	0.241	0.241	0.227	0.227	0.227	0.227	0.223	0.003	0.003	0						
A.d.AY423695	0.257	0.236	0.240	0.259	0.251	0.251	0.251	0.251	0.244	0.015	0.015	0.015	0.018	0					
A.d.AY423700	0.236	0.219	0.223	0.237	0.230	0.230	0.230	0.230	0.223	0.000	0.000	0.000	0.003	0.015	0				
A.h.AY423673	0.271	0.276	0.272	0.272	0.285	0.285	0.285	0.281	0.266	0.227	0.227	0.227	0.231	0.248	0.227	0			
A.h.AY423682	0.279	0.284	0.279	0.279	0.279	0.297	0.297	0.297	0.293	0.277	0.230	0.230	0.234	0.252	0.230	0.018	0		
A.h.AY423683	0.279	0.284	0.280	0.279	0.279	0.289	0.289	0.289	0.285	0.270	0.224	0.224	0.227	0.244	0.224	0.008	0.010	0	
A.h.AY423691	0.279	0.284	0.280	0.279	0.279	0.289	0.289	0.289	0.285	0.270	0.224	0.224	0.227	0.244	0.224	0.008	0.010	0.000	0

COI, 16S rRNA sequences and RAPD techniques in molecular studies resolve some taxonomic problems of molluscs and let us describe the level of genetic differentiation. For example, the recent molecular analyses based on *COI* sequence data in combination with environmental tolerance suggest that *Dreissena bugensis* is not a distinct species, but it appears to be only a race of *D. rostriformis* (Therriault et al., 2004).

One of the issues described in this work is the verification, with the use of genetic methods, of species identification of individuals previously classified (depending on used criteria) as two different species. Some individuals from the Lithuanian population were previously classified by Lithuanian scientists on the basis of morphological characteristics as *A. hortensis* (Gurskas, 1997; Činikaitė, 1999) while A. Wiktor (Wrocław University, Poland) on the basis of reproductive organs identified them as the "dark form" of *A. subfuscus*. According to De Winter (1984), after a revision of the data, *A. hortensis* occurs in more southern than Lithuania regions of Europe, so its occurrence in Lithuania seemed almost improbable. According to Hesse (1926), Davies (1987), Backeljau & De Bruyn (1990), *A. hortensis* belongs to *Kobeltia* subgenus and therefore it should show higher similarity to *A. distinctus* (which belongs to *Kobeltia* too) and not to *A. subfuscus* that belongs to *Mesarion* subgenus. According to Pinceel et al. (2004, 2005), after a comparison of the molecular, biochemical and anatomical data, all northern specimens (Lithuanian specimens too) earlier described as *A. subfuscus* belong to *A. fuscus*, so it was questionable whether specimens collected from Lithuania were *A. subfuscus* or *A. fuscus*.

The use of six RAPD primers and sequences analysis of mitochondrial *COI* fragment gene in our studies confirmed suggestions about it being only one species. Individuals of '*A. hortensis*' and *A. fuscus* shared some bands in all primers tested (Tables 3, 5, 7) and high values of similarity between them were recorded (Tables 4, 6, 8). The *COI* gene fragment sequences obtained in this study for two specimens of *A. fuscus* and '*A hortensis*' exhibit the same sequences of nucleotides in 655 bp (100% of genetic similarity). Thus we can conclude that all reports about '*A. hortensis*' in Lithuania were erroneous because all previous Lithuanian scientists used only morphological criteria for identification. Our results confirm that for identification of *A. hortensis* it is necessary to do anatomical analysis of reproductive organs or use new molecular tools. The same mistake happens in other countries too (Lupu, 1974).

Additionally, we have indisputable evidence that those questionable slugs and all studied *A. subfuscus* (according to confirmation by prof. A. Wiktor) from Lithuania belong even not to *A. subfuscus* but to *A. fuscus* after comparison of the obtained sequences with other sequences available in the GenBank by Pinceel et al. (2005). Recent molecular and morphological work on native European *A. subfuscus s. l.* showed that *A. subfuscus* and *A. fuscus* are not synonymous names but species complex (Pinceel et al., 2004, 2005). These two species – *A. subfuscus s. s.* (Draparnaud, 1805) and *A. fuscus* (Müller, 1774) show clear allozyme, mtDNA and gonad differentiation, but are indistinguishable by their external morphology (Pinceel et al., 2004). According to these authors, all slugs in North-West Europe that have been identified as *A. subfuscus* are *A. fuscus* and our results confirmed this fact on Lithuanian slugs. Thus we can conclude that comparing of sequences could be used not only in questionable cases of taxonomic identification but also as a regular method very useful in such kind of studies.

The highest genetic variation was observed in the *COI* gene fragment from *A. subfuscus*. The intraspecific differences in this species ranged from 0 to 25.0%, including the specimens analysed and other sequences from the GenBank (Table 9, Fig. 1) and this gave us a suggestion that *A. subfuscus* identification can be mistaken by the authors who gave data to the GenBank as the intraspecific differences usually are so high (equal to 25.0% or bigger) between different species (Dodd et al., 2005; Yu & Pauls, 1992).

The values of observed intraspecific variability within the *COI* gene reported in literature range from 3.3% for *A. fuscus* (Pinceel et al., 2005) to 7.5% or even to 18% for *Arianta arbustorum* (Gittenberger et al., 2004). The greatest intraspecific diversity was recorded in *A. fuscus* too in the case of primers OPA7 and P3+P5 for which the similarity indexes according to Nei & Li (1979) were respectively 0.00–0.75 and 0.00–1.00. In the case of primers PV and P2+P6 diversity was less (from 0.60 to 1.00).

The second issue described in this work is the verification, with the use of genetic methods, of species identification of individuals of group *Carinarion* previously classified by Lithuanian scientists in two ways: Činikaitė (1999) identified only *A. circumscriptus*, Gurskas (1997) – *A. circumscriptus*, *A. fasciatus* and *A. silvaticus*.

A. circumscriptus was characterised by large genetic homogeneity despite different origin of individuals – from Lithuanian and Polish populations (Table 1). The intraspecific similarity indexes according to Nei & Li (1979) for this species ranged from 0.67 to 1.00. *A. distinctus*, represented by two Polish populations (from Szczecin and Wrocław), was more diversified, 0.29–1.00 for 3 primers. In Lithuanian populations of *A. fasciatus* and *A. silvaticus*, the indexes of intraspecific similarity were respectively 0.50–1.00 and 0.57 (Tables 4, 6).

Species *A. silvaticus*, compared to *A. circumscriptus* and *A. fasciatus*, was genetically more variable in these studies and this fact was also described in earlier allozyme analysis of these three species by several authors (Foltz et al., 1982; Backeljau et al., 1997; Jordaens et al., 1998, 2000). An electrophoresis survey of 13 allozyme loci in *A. circumscriptus* and *A. silvaticus* from Ireland confirmed that *A. circumscriptus* consists of one multilocus genotype (MLG), whereas

A. silvaticus yielded two MLGs (Foltz et al., 1982). Similarly a comparison in seven / eight polymorphic enzyme loci revealed 15/24 homozygous multilocus genotypes and gene diversity index (H_{exp}) = 0.130 in *A. silvaticus* while in *A. circumscriptus* only 2/9 MLGs were revealed and H_{exp} = 0.061 (Jordaens et al., 1998, 2000). In *A. fasciatus*, 13 stains were recorded and two of them were also shared by *A. silvaticus* (Jordaens et al., 1998). The latest data based on nucleotide sequences of the nuclear ribosomal internal transcribed spacer 1 (ITS-1) and the mitochondrial 16S rRNA of three North American *Carinarion* species suggest that *Carinarion* can be a single species-level taxon (Geenen et al., 2006). The three species have been introduced to North America by man and for this reason their populations are genetically impoverished compared to European population and may be not sufficiently representative with respect to the taxonomy of *Carinarion* (Geenen et al., 2006).

Random amplification of polymorphic DNA (RAPD) was described as a suitable molecular tool to distinguish the agriculturally important slugs such as *A. distinctus*, *A. fasciatus* and *Deroceras reticulatum*, by generating species-specific bands. Voss et al. (1999) obtained between six and ten distinct bands for *Arion* spp. using the primer 5'AATGGCGCAG 3' (called PV in present studies). The bands 680 & 560 bp were specific to *A. distinctus* and 1100 & 800 bp to *A. fasciatus*. In our studies we also received two bands specific to *A. distinctus* but their values were 700 & 400 bp, and only the 550 bp obscure band to *A. fasciatus* (Table 3). The band 520–550 bp in *A. fasciatus* was also obtained by Voss et al. (1999) but it was not distinguished as species-specific. Therefore, the significant differences between results obtained with the use of the same primers were recorded which was not surprising in the case of RAPD technique. As Yu & Pauls (1992) have reported the banding patterns generated by RAPD are often complex, making interpretation and standardization difficult. The low annealing temperature applied in RAPD analysis (due to the comparatively short and random primer) leads to some non-reproducible artifacts. Furthermore, mutations that inhibit primer annealing or that somehow prevent amplification are detected by absence (i. e. band loss) of DNA fragments found earlier (Pinceel et al., 2005). RAPD markers are generated on low-stringency PCR reactions and therefore are strongly dependent on amplification conditions such as PCR temperature profile, salt concentration, brand of thermo stable DNA polymerase applied and the strategy used for band separation and detection, factors that can cause low reproducibility within a laboratory and between laboratories (Valentini et al., 1996). Nevertheless, differentiation between the investigated species was achieved in all tested primers as it was shown by the occurrence of reproducible and species-specific bands (except *A. distinctus* in case of P3+P5 primer). They are marked as boldface figures in respective tables.

CONCLUSIONS

In conclusion, we are certain that RAPD method is a suitable molecular tool to identify the species and sometimes subgenera in *Arion* genus despite of some differences noticed in literature. However, each laboratory has to standardize this method to its own conditions and needs.

With respect to anatomical data our molecular analysis suggests that in Lithuania there are three species of *Carinarion: A. circumscriptus, A. silvaticus* and *A. fasciatus*.

It is concluded that all reports about *A. hortensis* in Lithuania were made based on the external morphology without the anatomical analysis of reproductive organs or the use of molecular methods, hence all were erroneous. All slugs, earlier determined as *A. hortensis* or *A. subfuscus* in Lithuania, according to novel genetic data belong to *A. fuscus*.

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ARION FÉRUSSAC, 1819 (MOLLUSCA, PULMONATA) GENTIES ŠLIUŽŲ RŪŠIŲ NUSTATYMAS GENETINIAIS METODAIS

S a n t r a u k a

Rūšių nustatymas yra pradinis uždavinys, norint įvykdyti bent vieną (C) Biologinės įvairovės konvencijos įgyvendinimo strategijos 2011–2020 m. tikslą – pagerinti biologinės įvairovės būklę, išsaugoti ekosistemų, rūšių ir genetinę įvairovę. Siekiant sukurti molekulinius žymenis 1819 *Arion* Férussac rūšių, pagautų Lietuvoje ir Lenkijoje, buvo panaudota RAPD-PGR (atsitiktinės amplifikuotos polimorfinės DNR polimerazės grandininė reakcija) ir mitochondrijų citochromo oksidazės I (*COI*) subvieneto sekų analizės metodika. Panaudojus šešis RAPD pradmenis, išskirti *Arion* rūšims specifiniai žymenys. Abiem metodais (naudojant RAPD pradmenis ir *COI* geno fragmento (655 bp) sekų analizę) nenustačius tarprūšinio kintamumo patvirtinta, kad anksčiau Lietuvoje klaidingai apibūdintos *A. hortensis* ir *A. subfuscus* rūšys yra *A. fuscus*. Nustatant klaidos priežastis remtasi tik išoriniais morfologiniais požymiais, neatlikta anatominė reprodukcinių organų analizė, nenaudoti DNR analizės molekuliniai metodai. Patikslinta, kad Lietuvoje gyvena visos trys *Carinarion* rūšys: *A. silvaticus*, *A. circumscriptus* ir *A. fasciatus*.

Raktažodžiai: DNR, pirštų atspaudų metodas, sekvenavimas, *COI* genas, šliužai, *Arion*