A comparison of different DNA extraction methods for slugs (Mollusca: Pulmonata)

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This paper presents the results of a comparison of three DNA extraction methods for slugs of the genus *Arion* Férussac, 1819. The total of 69 individuals were examined and the extracted DNA was tested by different molecular techniques (PCR and RAPD) in 2001. The results allow us to propose for DNA isolation the DNeasy Tissue Kit (Qiagen) according to the procedure recommended by Qiagen. It is the simplest method omitting chloroform/phenol steps, quick and most effective: after testing by PCR technique for different genes the efficiency varied from 48% to 64%.

Key words: DNA, extraction, molecular technique, *Arion*, slug

INTRODUCTION

Morphological and anatomical species differentiation of slugs of the genus *Arion* Férussac, 1819 is a difficult venture because of colour polymorphism (Jordaens et al., 2001) and lacking obvious differences. Therefore various authors use different methods to recognise the species and to divide them into different species groups. For example, Hesse's classification (Hesse, 1926) was based on the analysis of anatomical characters, especially the reproduction system, in which the author divided five subgenera: *Lochea* M.- Tandon, 1855; *Mesarion* Hesse, 1926; *Carinarion* Hesse, 1926; *Kobeltia* Seibert, 1873; *Microarion* Hesse, 1926. Earlier Simroth (Simroth, 1885) divided the genus *Arion* into two groups, *Monoatriidae* and *Diatriidae*, according to the structure of the genital atrium. However, Davies (Davies, 1987), on the grounds of the spermatophor forms and egg structures, singled out three subgenera: *Mesarion* & *Arion*; *Kobeltia* & *Microarion; Carinarion*. The last Backeljau and De Bruyn classification (Backeljau, De Bruyn, 1990) of the genus *Arion* ssp. is based on a complex of characters, such as morphological, anatomical, ecological (mating behaviour), genetic (chromosome number) and biochemical (enzymatic polymorphism). They distinguished only two subgenera or chromosome groups, *Lochea* (*Mesarion* & *Arion* s.s) and *Prolepis* (*Kobeltia* & *Carinarion)* (Backeljau, De Bruyn, 1990).

Application of molecular biology methods offers new possibilities of dealing with taxonomic problems (Davis, 1994). The analysis of nucleotide sequences of nuclear and mitochondrial DNA provides us with a basis for taxonomic and phylogenetic considerations. The first step of the majority of molecular methods such as PCR (Polymerase Chain Reaction), RAPD (Random Amplified Polymorphic DNA) or PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is DNA extraction.

Slugs, like all land snails, have enormous quantities of mucopolysaccharides which make easy DNA extraction problematic. Some current extraction techniques designed to remove the mucus of snails use reagents that are hazardous to the environment (Douris et al., 1998; Winnepenninckx et al., 1993) or expensive (Armbruster, 1997) or need complete snails (Van Moorsel et al., 2000). Slug identification is always a complicated action and always needs to preserve the remains such as genitalia, body wall, intestinal tract in 70% ethanol for verification.

The aim of this study was to compare the known DNA extraction methods for snails and to find the best one for slugs of the genus *Arion* Férussac, 1819.

MATERIALS AND METHODS

Several individuals of the genus *Arion* spp. were collected in Lithuania and Poland in 2001. Table 1 presents species, numbers of individuals and their collection localities and date. Species identification was based on the reproductive system characters (Kerney et al., 1983; Riedel, Wiktor, 1974). DNA was isolated from frozen somatic slug tissue. The remaining parts were preserved in 75% ethyl alcohol for anatomical verification and are preserved in Zoological Museum of Vilnius University.

Three DNA extraction methods were used for DNA isolation from the frozen liver of *Arion* spp. slugs. Table 2 presents methods, stages, time and quantity of the main reagents used for DNA extraction. From each probe, 5 μl of final DNA extract were loaded into a 1% agarose gel stained with ethidium bromide (0.013 μl/ml) and then electrophoresed for 30 min at 70 V. Viewed under ultraviolet light, the gel revealed

Table 1. **Collected and examined material of slug species, their number and locality in Lithuania and Poland**

Species	Number of individuals	Collection localities	Collection date
Arion (*Mesarion*) *subfuscus* (Draparnaud, 1805)	9 1 10 4	Vilnius, Verkiai Regional Park, Lithuania Vilnius, Garden, Lithuania Družiliai, Forest, Lithuania Kariotiškės, Rubbish, Lithuania	May–June 2001 May 2001 September 2001 September 2001
Arion (*Carinarion*) *fasciatus* (Nilsson, 1822)	6 5 3 1 2	Vilnius, Verkiai Regional Park, Lithuania Vilnius, Garden, Lithuania Nemenčinė, Garden, Lithuania Jurbarkas, Garden, Lithuania Szczecin, Park St. Żeromskiego, Poland	April–May 2001 May–June 2001 June 2001 June 2001 November 2001
Arion (*Carinarion*) *circumscriptus* Johnston, 1828	13 2 1	Vilnius, Verkiai Regional Park, Lithuania Szczecin, Park St. Żeromskiego, Poland Wrocław, Botanic Garden, Poland	May–June 2001 September 2001 October 2001
Arion (*Carinarion*) *silvaticus* Lohmander, 1937	4	Vilnius, Verkiai Regional Park, Lithuania	May 2001
Arion (*Kobeltia*) *distinctus* Mabille, 1868	5 3	Szczecin, Park St. Żeromskiego, Poland Wrocław, Botanic Garden, Poland	November 2001 October 2001

Table 2. **Short descriptions of DNA extraction methods used for slugs**

Stage of isolation	Method I		Method II		Method III	
	Time of centrifugation or incubation	Main reagents	Time of centrifugation or incubation	Main reagents	Time of centrifugation or incubation	Main reagents
1	*2*	*3*	*4*	*5*	*6*	*7*
Lysis	12–24 h (–20 °C)	40 mg of liver 500 μl buffer A	1–3 h (+55 °C)	25 mg of liver 180 μl buffer ATL*	2 h (+56 °C)	25 mg of liver 500 μl buffer STE 100 μl 10% SDS
		35 μl proteinase K (20 mg/ml)		20 μl proteinase K		20 μl proteinase K (20 mg/ml)
	12 h (+37 °C)	25 μl 20% SDS	10 min (+70 °C)	200 μl buffer AL	5 min 15000 × g	500 μl phenol
	1 h (+37°C)	35 μl proteinase K (20 mg/ml)			5 min 15000 × g	250 μl phenol 250 μl chloroform
	10 min 15000 × g	200 μl NaCl (36g/l)			5 min 15000 × g	500 μl chloroform

Table 2 (continued)

1	2	3	4	5	6	7
Precipi- tation	10 min 15000 × g	1000 μl ethanol 96%	1 min 8000 × g	200 μl ethanol 96%	5 min 15000 × g	500 μl isopropanol
Wash	10 min	1000 μl ethanol 70%	1 min 8000 × g	500 μl buffer AW1	10 min 15000 × g	500 μl ethanol 70%
	10 min	1000 μl ethanol 70%	3 min 15000 × g	500 μl buffer AW2	5 min 15000 × g	500 μl ethanol 96%
Drying	15–30 min				15–30 min	
Elution		500 μl buffer TE (pH 8.0)	1 min (+18 °C) 8000 × g	200 μl buffer AE	2 h (+56 °C)	50 μl buffer TE (pH 8.0)
			1 min (+18 °C) 8000 × g	200 μl buffer AE		
Duration	~28–40 h		~1.2–3.2 h		~ 5 h	

bands of intact DNA and smears of degraded DNA (Fig. 1). Isolated DNA was applied in PCR technique using a PTC-200 MJ Research thermal cycler. PCR analyses were performed for gene cytochrome c oxidase subunit I (COI) using Folmer's primers (Folmer et al., 1994) and 16SrRNA using 16Scs1 and 16Sma2 primers (Chiba, 1999). Approximately 700 base-pair fragments were amplified via PCR from genomic DNA for COI gene and 900 base-pair fragments for 16SrRNA gene. The third method applied was RAPD analysis using the OPA7 primer (5'-GAAACGGGTG -3'). The products of the PCR were viewed under UV light after running into a 2% agarose gel stained with ethidium bromide (0.013 μl/ml) (Figs. 2–4).

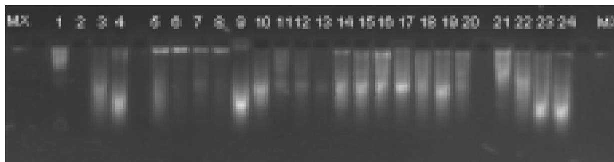

Fig. 1. Some probes after DNA isolation (5 μl of each DNA extraction). From left to right: MX – marker X (12.2 – 0.07 kbp, Boehringer Mannheim) shows two bands, 12200 bp (above) and 3000 bp (below), lanes 1–4 – method I, 5–20 – method II, and 21–24 – method III

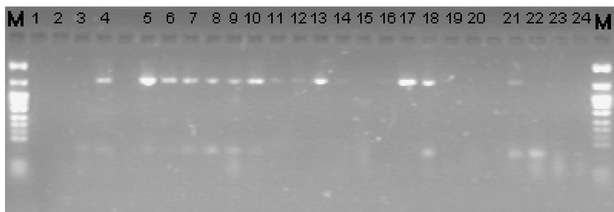

Fig. 2. PCR products after COI gene amplification (5 μl of each probe). From left to right: M – marker 1444 (1444, 736, 587, 476, 434, 298, 267, 174, 102, 80 bp, POLGEN S. C.); lanes 1–4 – method I, 5–20 – method II, and 21–24 – method III

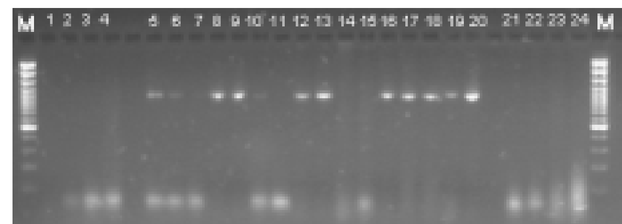

Fig. 3. PCR products after 16SrRNA gene amplification (5 μl of each probe). From left to right: M – marker 3000 (3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp, MBI Fermentas gene ruler 100 bp DNA ladder); lanes 1–4 – method I, 5–20 – method II, and 21–24 – method III

Fig. 4. An example of electrophoretic separation of PCR amplification products derived from OPA 7 primer. The first lane is marker (3000–100 bp, MBI Fermentas gene ruler 100 bp DNA Ladder). Numbers 33 and 37 are after method III isolation, all other numbers are after method II. Lanes 19, 20, 60, 68 illustrate individuals of *A. distinctus*; 21, 22, 61, 23, 24, 33, 62 – *A. subfuscus*; 55, 56, 72, 73 – *A. circumscriptus*; 37, 59 – *A. silvaticus,* and 64, 65, 69 – *A. fasciatus*

RESULTS AND DISCUSSION

Method I was described for slugs by Voss et al. (Voss et al., 1999). We took 10 probes that represented 4 species: *A. subfuscus* (5 individuals); *A. fasciatus* (1 individual); *A. circumscriptus* (2 individuals) and *A. distinctus* (2 individuals). Viewed under ultraviolet light, only some individuals on the gel revealed bands of intact DNA with smears of degraded DNA, and the efficiency of this method was 70% (Fig. 1, Table 3). Nevertheless, the efficiency of PCR analyses for genes COI and 16SrRNA was very low and equalled respectively to 14% for gene COI (Figs. 2, 3, Table 3). This extraction method was the longest in time comparing to the others. After cutting the tissue and adding it into the nucleus-lysis-buffer, the samples were stored overnight at –20 °C. After thawing the next day, the next lysing components were added into the samples and the latter were incubated in a shaking water bath at +37 °C for 13 h. After this stage the DNA extraction can be completed in 1.5–2 h. So, this method takes two or three days. It requires a great deal of proteinase K – 70 µl (20 mg/ml) per probe (Table 2). In the end DNA is eluted in buffer TE (pH 8.0), but not is ready for use (Table 1). Working dilution of extracted DNA needs to be prepared for each individual at a concentration of 50 ng μl^{-1} by addition of an appropriate amount of 10 mM Tris-HCl (pH 8.0).

Method II of DNeasy Tissue Kits uses an advanced silica-gel-membrane technology without organic extraction. Lysis takes only 1.5–3 h, and the rest of this procedure can be completed in 20 min. Simple centrifugation processing and two efficient wash steps remove contaminants and enzyme inhibitors. DNA is eluted in buffer, ready for use. We isolated in this way 28 probes from 5 species (Table 3): *A. subfuscus* (6 individuals); *A. fasciatus* (10 individuals); *A. circumscriptus* (7 individuals); *A. silvaticus* (1 individual) and *A. distinctus* (4 individuals). Viewed under ultraviolet light, most of individuals (89%) on the gel revealed strong bands of intact DNA and smears of degraded DNA. After testing by PCR technique, 16 probes (64% of all probes with good DNA isolation using this method) had positive results for COI gene and 12 probes (48%) for gene 16 SrRNA (Table 3). Method II was the most effective and gave the best results in PCR amplification, but it is the most expensive, too (the box for 50 probes costs 270 EURO).

Method III (phenol/chloroform, according to Skibinski et al. (Skibinski et al., 1994) was successfully applied for other molluscs. In our studies, by this method 31 probes from 5 species were isolated: *A. subfuscus* (14 individuals); *A. fasciatus* (5 individuals); *A. circumscriptus* (7 individuals) *A. silvaticus* (3 individuals) and *A. distinctus* (2 individuals). Method III consists in 2 h of lysing samples incubated in a shaking water bath at +56 °C. Nucleic acid was purified during 35 min by phenol/chloroform extraction, precipitated with isopropanol at room temperature, washed with 70% and then with absolute alcohol. After drying DNA pellet was dissolved in TE buffer (pH 8.0). All stages of this method take 5–7 h (depending on the numbers of probes). Viewed under ultraviolet light, the majority of individuals on the gel revealed bands of intact DNA with smears of degraded DNA, and the efficiency of this method was 81% (Fig. 1, Table 3). After testing by PCR technique only one probe had positive results for COI (4% of all probes with good DNA isolation using this method) and no probes had positive results for gene 16 SrRNA (Table 3). This kind of isolation is low-efficient and is limited to shorter products (ex. 700 bp for gene COI), nevertheless, it has no application in the amplification of longer DNA products such as 900 bp (Fig. 3). Applying the RAPD technique we obtained legible results using DNA by Method II isolation and only two probes by Method III (Fig. 4).

Table 3. **Results of DNA extraction and DNA testing in PCR of genus *Arion* Férussac, 1819 individuals**

Type of research	Method I DNA extraction results		Method I Testing of DNA in PCR results		Method II DNA extraction results		Method II Testing of DNA in PCR results		Method III DNA extraction results		Method III Testing of DNA in PCR results	
Type of the results	Negative	Positive	CO I gene	16Sr RNA gene	Negative	Positive	CO I gene	16Sr RNA gene	Negative	Positive	CO I gene	16Sr RNA gene
Number of individuals	3	7	1/7	0/10	3	25	16/25	12/25	6	25	1/25	0/25
Efficiency	30%	70%	14%	0%	11%	89%	64%	48%	19%	81%	4%	0%

Considering the presence of enormous quantities of mucopolysaccharides in all land snails, the extraction of DNA from them is very difficult. We cannot apply successfully the different methods that gave good results in other species or in other laboratories. Summarising, the best and universal way for isolation of DNA from the genus *Arion* spp. is a ready-made kit of DNeasy Tissue Kits. DNA isolated by this method can be successfully applied in different molecular methods.

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SKIRTINGŲ DNR IŠSKYRIMO METODŲ TAIKYMO ŠLIUŽAMS (MOLLUSCA: PULMONATA) PALYGINIMAS

S a n t r a u k a

Šliužų DNR išskyrimas nėra paprastas procesas, nes šliužų kriauklė redukuota ir jų epitelyje yra ypač daug gleivinių liaukučių, kurios gamina polisacharidines gleives. Daug šių medžiagų trukdo sėkmingai išskirti DNR. 2001 m. atlikti 69 *Arion* Férussac, 1819 genties šliužų DNR išskyrimo tyrimai. Naudotos trys DNR išskyrimo metodikos ir nustatyta, kad DNeasy Tissue Kit (Qiagen) metodika, rekomenduota Qiagen, yra paprasčiausia, greičiausia ir efektyviausia: 48–64% tirtų šiuo metodu individų buvo gauti teigiami PGR (polimerazės grandininė reakcija) rezultatai.

Raktažodžiai: DNR, išskyrimas, molekulinė metodika, *Arion*, šliužai