FIRST MOLECULAR EVIDENCE OF *ALARIA ALATA* DIFFERENT DEVELOPMENT STAGES IN LATVIA

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Abstract. The genus *Alaria* includes seven species but only *Alaria alata* is widespread throughout all the Europe. In present research 43 adult samples, 20 mesocercaria samples and ten egg samples were analyzed utilizing molecular biology methods. *QIAamp DNA Mini Kit* (Protocol: DNA purification from tissues, Qiagen, Germany) and *DNeasy Blood & Tissue Kit* (Protocol: Purification of total DNA from animal tissues, Qiagen, Germany) spin-column protocols were approbated and modified to find out the suitable one for all development stages of *A. alata*. Extracted DNA was amplified and sequenced using primers used in previous studies. Two new primer pairs were generated based on obtained sequences and data from GeneBank database.

Scientific impact:

1. *This is the first try to extract A. alata DNA from their eggs.*
2. *Dneasy Blood & Tissue Kit procedure with increased amount of proteinase K (25µl) and increased incubation time (15h) were most suitable for DNA purification from different A. alata development stages.*
3. *With newly designed primers 28.1% more sequences were obtained.*
4. *This study demonstrate the necessity to improve the efficacy of the DNA extraction technique prior further studies*

INTRODUCTION

The genus *Alaria* includes seven species but only *Alaria alata* is widespread in Europe. This trematodes definitive hosts are carnivores that inhabit wetlands where first and second intermediate hosts (snails and amphibians) are found. Life cycle may include also omnivores as paratenic hosts (Möhl *et al*. 2009).
Findings of different life cycle stages of trematode in various hosts, as well as, usage of molecular methods to differentiate these stages from other parasites have an important role of *Alaria* genus studies which gives an understanding of *Alaria* sp. distribution and evolutionary relationship in Europe. However, there is a necessity to optimize molecular biology techniques and to obtain sufficient data regarding the true occurrence of *A. alata* in different hosts (Riehn *et al.* 2014).
The aims of present study were to optimize a DNA extraction protocol for different development stages and to develop new primers to analyze *A. alata* in Baltic region.

MATERIALS AND METHODS

During previous studies mesocercariae of *Alaria* spp. were collected from wild boar muscles and adult trematodes were collected from small intestines of raccoon dogs and red foxes. Eggs were collected from feaces of raccoon dogs and red foxes. For DNA purification 43 adult samples, 20 mesocercaria samples and ten egg samples were used. One sample contained five specimens of adults and mesocercaria. One sample of eggs contained 50 eggs to increase the yield of extracted DNA. Two spin-column DNA extraction kits - *QIAamp DNA Mini Kit* (Protocol: DNA purification from tissues, Qiagen, Germany) and *DNeasy Blood & Tissue Kit* (Protocol: Purification of total DNA from animal tissues, Qiagen, Germany) – were used for

DNA extraction from different development stages. These protocols were selected based on previous studies by Riehn *et al.* 2011, Portier *et al.* 2012, Rentería - Solís *et al.* 2013, Franssen *et al.* 2014, Riehn *et al.* 2014. Selected protocols were approbated and modified to find out the most suitable for all development stages of *A. alata*. Nine different modifications were used for adult specimens, further three suitable modifications were selected and used for mezocercariae samples. For DNA extraction form egg samples only one modification was chosen after results from DNA extraction from adult specimens and mesocercariae samples. DNA quality and quantity were detected using spectrophotometr (NanoDrop1000, Thermo Scientific, ASV).

Extracted DNA was amplified and sequenced with primers developed in previous studies (Riehn *et al.* 2011, Portier *et al.* 2012, Murphy *et al.* 2012). Five separate sets of PCR amplifications were carried out – three described in other studies (Riehn et. al. 2011, Portier et al. 2012, Murphy et al. 2012) and two aligned using SeqScape version 2.5 in this study. New primer pairs were generated based on obtained sequences and data from GeneBank database. Forward, reverse and sequencing primers were designed utilizing PyroMark Assay Design (Qiagen, Germany) software and developed for 28S ribosomal RNA gene region and COX I gene region. PCR products were separated using „submarine" gel electrophoresis and visualized on a High Performance 2 UV Transilluminator (UVP, USA). Newly developed primers were used for *A. alata* DNA amplification in previously described 72 samples. Insufficient PCR products were reamplified with changes in thermal profile. Purified PCR protocols were sequenced using Sanger sequencing technique. All sequences were aligned using SeqScape version 2.5 (Applied Biosystems, USA) with default settings and were identified by the online BLAST search tool.

RESULTS (2000)

Two DNA extraction protocols were compared and optimized for DNA extraction from *A. alata* different development stages, and overall nine variations with changed amount of proteinase K and incubation time of DNA extraction protocols were estimated. The concentration (ng/µl), purity (ratios), number of sufficient PCR products, and quality of recovered sequences from amplicons were taken into the account for evaluation the protocols. Variations with incubation time increased up to 15h were more suitable for DNA purification of different *A. alata* development stages considering DNA product purity. Variations with increased amount of proteinase K to 25 µl, and/or added buffer ATL, and samples containing only one specimen in cases of different development stages were unsuitable for molecular evidence. There were no differences in purified DNA quality extracted by both used protocols for different stages of *A. alata*. Overall, 82.9% of 72samples from different *A. alata* development stages showed satisfactory purity and sequencing results with several DNA extraction kit variations.

Further 276 PCR products were selected for sequencing and 77.2% of sequences had good quality for further analyses. Obtained sequences showed one *Alaria* species in Latvia. However there is a need for an in-depth sequences analysis to determine the genetic diversity. Primers developed in present study were suitable for identification *Alaria* species at each life cycle development stage (Table 1.). The number of obtained sequences using these primers was 28.1% more than using primers from previous studies. Obtained sequences did not demonstrate the intraspecific polymorphism.

Table 1. Designed primers for analyzing *Alaria alata*.

Primer ID	Forward primer	Reverse primer	Sequencing primer	T_m
Aa28S	5`-ATA TGG GTT TGG TGG GTG CTA T-3`	5`-CAC GCA ACA TAT AAA TCC ACG AGA-3`	5`-GGT GAG TGT GTC ATG G-3`	55 °C
AaCOI	5`-TTC AGC TGG TGA GTG TGT CAT-3`	5`-TAC ACC CAA AGA CTG GAC AAG C-3`	5`-ATA CCA ATA ACC ATC GTA GT-3`	57 °C

DISCUSSION (2000)

Previously usage of molecular biology methods for studies of genetic diversity of *A. alata* is described for three development stages – cercariae (Portier *et al.* 2012), mesocercariae (Riehn *et al.* 2011) and adult trematodes (Murphy *et al.* 2012). Present study is the first study using molecular techniques for eggs of *A. alata*. Two spin-column DNA purification protocols were used for adults, eggs and mesocercariae development stages of *A. alata* in Latvia. These protocols were previously used in studies of *A. alata* cercariae in France (Portier *et al.* 2012) and mesocercariae in Germany (Riehn *et al.* 2011). Variations of DNA purification protocols differed with used reagents, volume of reagents and incubation period. In a study of adult trematodes in Ireland a modified Hi-Pure DNA purification protocol was used (Roche, UK). Authors increased the amount of proteinase K to 40 µl, grinded the adults of *A. alata* in pestla and incubated the cell samples 60 – 90 minutes (Murphy *et al.* 2012). In this study the amount of proteinase K was increased to 40 µl and cell samples of trematodes were incubated for 60 minutes. However, the results showed lower DNA concentration and high level of contaminants in purified DNA samples.

Present study demonstrates that the DNA quality and quantity of different stages of *A. alata* detection by PCR is affected by primers and extraction methods used. Previously researchers made high investments in molecular biology method development for *A. alata* (Riehn *et al.* 2011, Portier *et al.* 2012, Rentería - Solís *et al.* 2013, Franssen *et al.* 2014, Riehn *et al.* 2014). However present study demonstrate the necessity to improve the efficacy of the DNA extraction technique. There are limited data in DNA quality and quantity context, to discuss the suitably of the method.

Use of molecular techniques for studying *Alaria* species is getting more popular in recent years. Further studies, including phenotypical different isolates from different European regions, are necessary to show whether there is in fact a high level of similarity between *A. alata* populations in Europe (Riehn et al. 2011). Present study confirms that significant attention should be paid to extracted *A. alata* DNA qualitative and quantitative parameters and sufficient DNA extraction protocols should be approbated for different parasite development stages.

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