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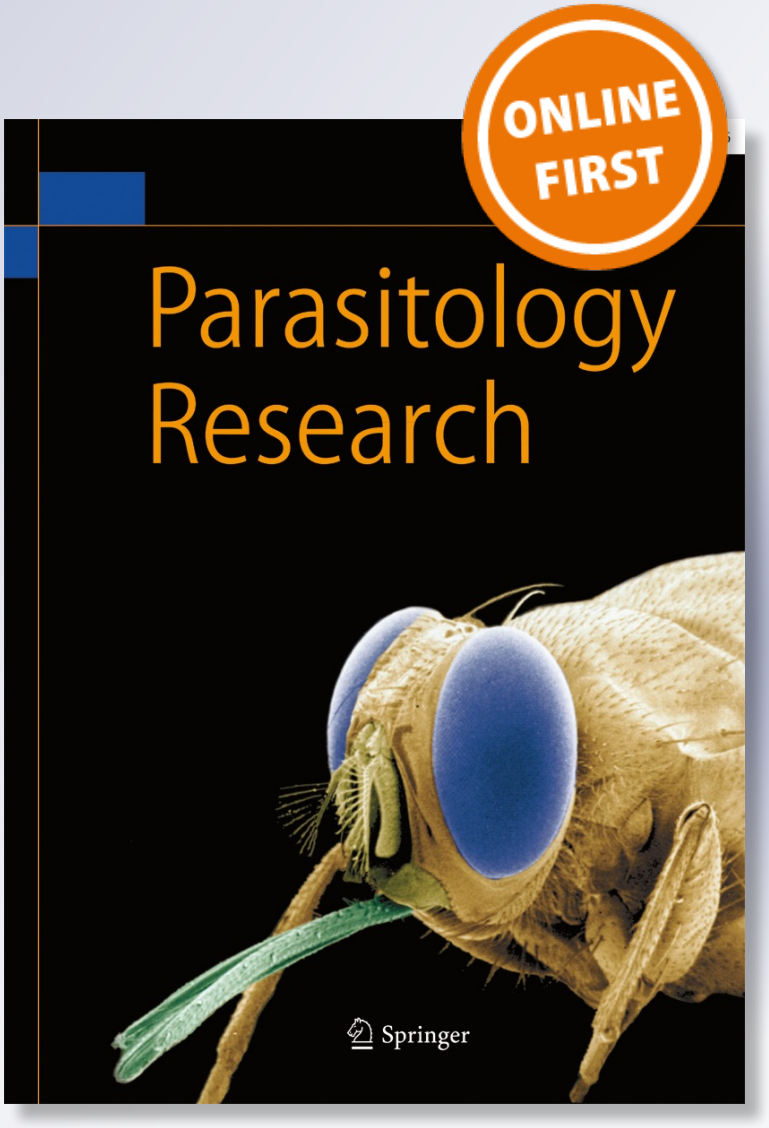

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ORIGINAL PAPER

Genomic diversity of cercarial clones of *Himasthla elongata* (Trematoda, Echinostomatidae) determined with AFLP technique

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Abstract The aim of this study was to reveal genomic diversity formed during parthenogenetic reproduction of rediae of the trematode *Himasthla elongata* in its molluskan host *Littorina littorea*. We applied amplification fragment length polymorphism (AFLP) to determine the genomic diversity of individual cercariae within the clone, that is, the infrapopulation of parthenogenetic progeny in a single molluskan host. The level of genomic diversity of particular cercariae isolates from a single clone, detected with *Eco*R1/*Mse*1 AFLP reaction, was significantly lower than the variability of cercariae from different clones. The presence of intraclonal genomic diversity indicates a nonsexual shuffle of alleles during parthenogenesis in the rediae of *H. elongata*. The obtained polymorphic AFLP fragments were long enough to detect the sequences that may be responsible for clonal genomic variability. Based on this, AFLP can be recommended as a tool for the study of genetic mechanisms of this variability.

Keywords AFLP · *H. elongata* parthenitae · Clonal diversity · Monomiracidial infection

Introduction

Trematodes have complex life cycles with a successive change of hosts and an alternation of parthenogenetic and hermaphroditic generations. Parthenogenetic generations (mother sporocyst, rediae or daughter sporocysts–parthenitae) develop in the mollusk, the first intermediate host, after it has been infected by the miracidium. All parthenitae reproduce by apomictic (diploid) parthenogenesis (Dobrovolskij and Ataev 2003; Galaktionov and Dobrovolskiy 2003). Thus, a clonal group of parthenitae is formed in the molluskan host. All its members have the same genotype as the miracidium that infected the mollusk in the first place.

These traditional ideas have been shattered by recent data. A study of W1 satellite-DNA repeats (Grevelding 1999) has revealed that this sequence is not monotonously distributed in different individuals of the same parthenogenetic generation arising from a single miracidium (Bayne and Grevelding 2003). Random amplified polymorphic DNA (RAPD) genotyping also showed genome variability of parthenitae in species of *Schistosoma* and *Trichobilharzia* (Korsunenko et al. 2010). Several questions arise in this respect. How common is clonal variability in trematodes? What is the mechanism of its formation? Is it manifested phenotypically?

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If so, does this phenotypic manifestation play any role in the transmission processes? These questions remain open. To answer them, one needs to develop an efficient tool to reveal genetic variability.

PCR-based techniques, such as RAPD, are unlikely to be successful in this case. RAPD generates DNA fragments depending on the PCR primer sequences and the nature of the template DNA. Low primer annealing temperature leads to multiple fingerprints, whose accuracy is sensitive even to minor changes in the composition of reagents or sampling protocol (Thormann et al. 1994). Restriction enzyme DNA treatment, e.g., restriction fragment length polymorphism (RFLP), has a much higher fingerprinting accuracy (Botstein et al. 1980) but requires large amounts of DNA, which cannot be obtained in case of individual genotyping of tiny organisms. On the other hand, amplification fragment length polymorphism (AFLP) both is accurate and requires a low amount of DNA. PCR amplification of genomic restriction fragments produces DNA fragments of variable size. Their quantity can be adjusted by selecting certain restriction enzyme(s). In this way, one may obtain a repetitive and valid fingerprint set from DNA of any origin or complexity (Vos et al. 1995). Taking into account the small size of parthenitae and cercariae, AFLP seems to be the best tool for revealing their genetic variability.

The aim of this study was to assess the applicability of the AFLP in revealing inter- and intraclonal genomic diversity of the cercariae of *Himasthla elongata*, a model trematode species.

Material and methods

Model used for analysis

We used the trematode species *H. elongata* (Mehlis, 1831) Dietz, 1909 (Trematoda, Echinostomatidae), which is common in the coastal ecosystems of northern European seas. The system *H. elongata–Littorina littorea* is a popular model in immunological studies (reviewed in Loker 2010). There are many studies on the biology of cercariae of these trematodes and their transmission in marine ecosystems (e.g., Prokofiev et al. 2011; Tolstenkov et al. 2011; Levakin et al. 2013; Galaktionov et al. 2015).

Hermaphroditic adults of *H. elongata* in the intestine of gulls produce eggs, which are distributed in the environment. Miracidia, hatching from the eggs in seawater, penetrate the molluskan host (periwinkles *Littorina* spp.). There they transform into the first parthenogenetic generation, the mother sporocyst. It gives rise to successive parthenogenetic generations, the rediae, which form a redial clonal group in the molluskan host. The rediae produce dispersive larvae, cercariae (Fig. 1). The cercariae emerge from the infected periwinkles and penetrate the second intermediate host, blue mussels *Mytilus edulis*, where metacercariae develop. The final hosts (several species of gulls) become infected by ingesting the blue mussels containing infective metacercariae.

Cercariae and rediae of *H. elongata* have a body length of about 500 μm. They are thus too small to provide enough DNA for the RFLP. Therefore, AFLP is an appropriate tool for the study of their genomic diversity (Mueller and Wolfenbarger 1999; Behura 2012; Frascaroli et al. 2013).

Animals, DNA isolation, and genome restriction

At the White Sea, where our research was carried out, the periwinkle *L. littorea* (Gastropoda, Caenogastropoda) plays the role of the first intermediate host for *H. elongata*. The periwinkles infected with *H. elongata* rediae were collected in the intertidal zone in the vicinity of the Kartesh White Sea Biological Station of the Zoological Institute of the Russian Academy of Sciences (66° 33′ 69″ N, 33° 64′ 83″ E) in the summers of 2007–2010. The snails were collected from the population where the prevalence of *H. elongata* did not exceed 1.4 %. The infection of a snail by more than one miracidium was therefore unlikely. All studies where the development of parthenitae of more than one genotype has been shown in naturally infected snails (as the result of multimiracidial infection) have been carried out in the sites with a high prevalence (tens of percent) of the parasites in mollusks (Woolhouse et al. 1990; Minchella et al. 1995; Davies et al. 1999; Keeney et al. 2007).

The collected individuals were placed in 100-ml plastic jars filled with seawater (one periwinkle per jar) and left in indirect sunlight for 1 h. The periwinkles that had shed *H. elongata* cercariae were kept in containers filled with seawater under 15 °C and used as the source of cercariae. *H. elongata* cercariae shed by a single infected *L. littorea* were assumed to be a clone produced by the rediae in this individual host and to have originated from a single miracidium. The cercariae released from a single infected periwinkle were washed three times in sterile marine water (SMW). One hundred millimolars of $MgCl_2$ was added for their relaxation. Then cercariae were pelleted by 1.5 rpm centrifugation. To isolate cercariae from a single redia, the rediae were extracted from infected *L. littorea* and carefully dissected under a stereomicroscope after three times washing in SMW. The cercariae isolated from a single redia were processed as described above.

Marine invertebrates are rich in mucopolysaccharides, which inhibit enzymatic reactions. Therefore, we used cetyltrimethyl ammonium bromide (CTAB) buffer (2 % CTAB, 1.4 M NaCl, 0.1 M Tris Cl (pH 8.0), 50 mM EDTA) with 100 mM of proteinase K. Homogenized *H. elongata* cercariae in 55 °C prewarmed CTAB buffer were incubated for 1 h at 60 °C, treated with 1 V of chloroform, and centrifuged.

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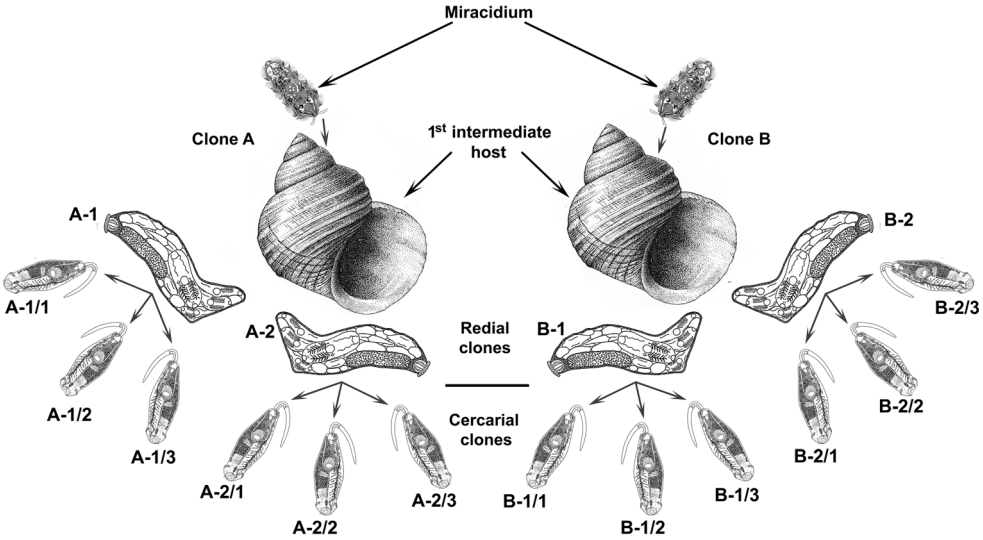

Fig. 1 Clone formation in *H. elongata* rediae and cercariae. A single miracidium, infecting the first intermediate host (*A*, *B*), gives rise to parthenogenetic generations of rediae (*A-1*…; *B-1*…). They reproduce expanding the infrapopulation of rediae and then produce the larvae, free-living cercariae (*dashed numbers*). As a result, the first intermediate host supports an infrapopulation of genetic clones of *H. elongata*

H. elongata genomic DNA (0.1 μg) was treated for 3 h at 37 °C with 5 U *Mse*I and 5 U *Eco*R in 30 μl of 10 mM Tris-HCl, 1 mM $MgCl_2$, 5 mM NaCl, 0.1 mM DTT, and 50 ng/μl BSA.

AFLP

Double-stranded adapter sequences were prepared by adding equimolar amounts of both strands. They consisted of a primer-specific core sequence and an enzyme-specific 3′ sticky end (Table 1, Fig. 2). Five picomolars of *Eco*RI and 50 pM *Mse*I adapters were ligated by T4 DNA ligase (Fermentas, Lithuania) in T4 ligase buffer provided by the manufacturer, for 3 h to the 15× diluted genomic DNA digests. After ligation, the reaction mixture was 5× diluted by TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) and stored at −20 °C.

AFLP primers consisted of three parts: a core adapter-specific sequence, an enzyme-specific sequence (Table 1, uppercase bold), and variable selective nucleotides (Table 1, lowercase bold). The selective nucleotides tagged the primers in order to increase binding specificity and reduce the number of PCR fragments. *Eco*R1 primer, pr*Eco*RI+cag, was 5′ end labeled using [γ-32P] ATP by T4 polynucleotide kinase. The 50-μl reaction mix contains 500 ng of pr*Eco*RI+cag, 150 μCi [γ-^{32}P] ATP, 0.5× T4 polynucleotide kinase buffer (Sileks, Russia), and 5 U of T4 polynucleotide kinase (Sileks, Russia).

The AFLP mastermix contained 5 ng *Eco*RI primer and 50 ng *Mse*I primer. The preamplification (Fig. 2, step 1) reaction contains 5 ng of DNA templates (*Eco*R1/*Mse*1 or

Table 1 Primers and adapters. Adapters and selective primers used for AFLP reaction

Name	Source	Sequence 5′ to 3′	Length, bp
Adapters			
Ad*Eco*RI	Vas et al. 1995	5′-CTCGTAGACTGCGTACC |||||||||||| ATCTGACGCATGG**TTAA**-5′	17 18
Ad*Mse*I		5′-GACGATGAGTCCTGAG ||||||||||| TACTCAGGACTC**AT**-5′	16 14
Primers			
pr*Eco*RI+c	Vas et al. 1995	GACTGCGTACC**AATTC**c	17
pr*Eco*RI+cag		GACTGCGTACC**AATTC**cag	19
pr*Mse*I+c		GATGAGTCCTGAG**TAA**c	17
pr*Mse*I+cag		GATGAGTCCTGAG**TAA**cag	19

*Eco*R1 and *Mse*1 recognition sites marked in bold capital letters; selective nucleotides marked in bold lowercase letters

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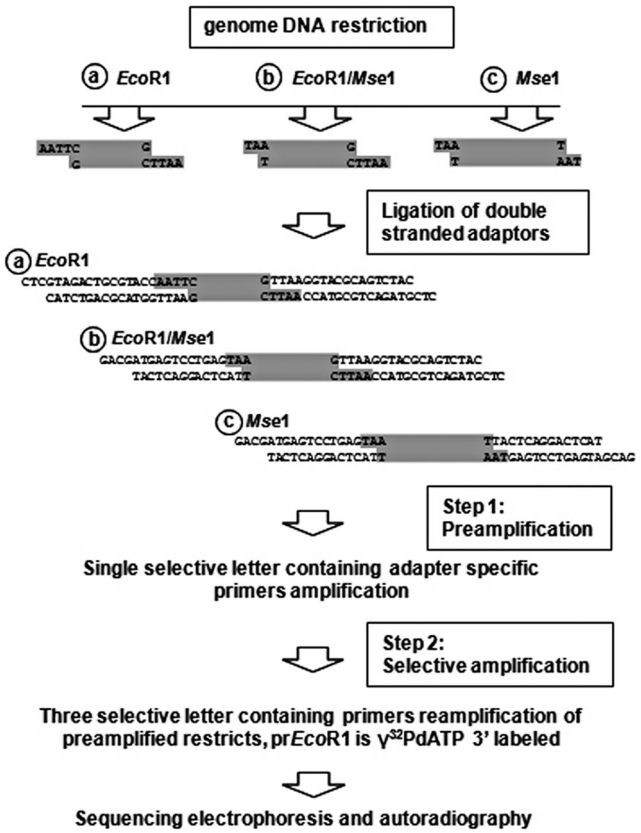

Fig. 2 AFLP experimental workflow. *H. elongata* genomic DNA restriction by *Eco*RI/*Mse*I resulted in numerous fragments flanked by *Eco*RI-only (*a*), *Eco*RI/*Mse*I (*b*), and *Mse*I-only (*c*) sticky ends. Ligation of double-stranded adapter sequences enables the amplification of digests obtained. The preamplification has been performed by *Eco*RI and *Mse*I adapter-specific primers that contain the selective nucleotide (+c) that 5′ end overlaps genomic DNA. The selective amplification enables the reduction of the number of amplicons by adding three selective nucleotides (+cag) at the 5′ end to both *Eco*RI and *Mse*I primers. The *Eco*RI primer was 5′ end labeled by [γ-^{32}P] ATP. Amplicon visualization was accomplished by sequencing electrophoresis followed by autoradiography

single *Eco*R1 adapter-ended digests of *H. elongata* genomic DNA), 0.5 U Taq polymerase (Fermentas, Lithuania), 1.5 mM $MgCl_2$, and 1× PCR buffer (Fermentas, Lithuania). PCR was performed for 20 cycles with the following cycling profile: 95 °C (30″), 57 °C (40″), and 72 °C (1′).

The selective amplification mix (Fig. 2, step 2) contained 0.1 μl of a template from the preamplification reaction, 50 ng pr*Mse*I+cag, and 5 ng labeled pr*Eco*RI+cag. Touch-down PCR was performed in 36 cycles: 95 °C (30″), Ta (40″), and 72 °C (1′). The annealing temperature (Ta) was 65 °C in the first cycle, then was subsequently reduced for the next 11 cycles and remained 56 °C for the next 23 cycles (Vos et al. 1995).

Fingerprint visualization and analysis

The amplification products were separated on 5 % sequencing PAAG and visualized by autoradiography (Sambrook and Russell 2001). Images were processed in GelAnalyzer2010a (http://www.gelanalyzer.com), and band position data were then rendered into a binary matrix for further analysis. For valid representation of fuzzy bands, we employed pixel-by-pixel analysis. It resulted in the table where each band was assigned the number of "1"s (units) corresponding to its size with a step of 5 pixels. The table obtained was transmitted to PAST 3.07 software (Hammer et al. 2001).

The overall variation of the data visualized by nonmetric multidimensional scaling (MDS) 2-D plots was accomplished in PAST 3.07 employing the Jaccard index as a band-based measure of similarity between AFLP fingerprints of individual cercaria isolates. A significance test of the differences between clonal groups was performed by pairwise similarity analysis using the analysis of similarity (ANOSIM) test (Clarke 1993).

Results

The combination of a rarely cutting enzyme (*Eco*R1) with sites within A/T enriched sequences and frequently cutting enzyme (*Mse*1) with sites within G/C enriched sequences enabled coverage for both hetero- and euchromatin. Double (*Eco*RI/*Mse*I) digestion of *H. elongata* genomic DNA resulted in abundant fragments of 500 to 50 bp (Fig. 3, I). It is known that in case of double digestion most fragments are flanked by both enzyme recognition sites (Masiga et al. 2000).

The main body of the *Eco*R1/*Mse*1 AFLP fingerprints (Fig. 3, I) was shorter than 500 bp. AFLP revealed a difference in cercariae groups: the pattern of fragments in cercariae belonging to a single clone (isolates from a single periwinkle) was more obvious than in those belonging to a different clone (Fig. 3, I).

The MDS plot of *Eco*R1/*Mse*1 AFLP (Fig. 3, II) demonstrated that all the three clonal groups were differentiated. The test statistic for the ANOSIM (=0.734) was highly significant ($p = 0.0002$), confirming the differences of AFLP profiles among clones (Suppl. Table S1, I). All pairwise comparisons between cercariae individuals within one clone were also significant ($p < 0.03$ in individual comparisons).

Amplified *Eco*RI digests produced variable fragments between 1500p and 100 bp within cercariae isolated from a single redia (Fig. 4).

Most fragments congregate in the range of 1500p to 800 bp corresponding to the rare-cutting nature of *Eco*RI. In that range, arrays are highly heterogeneous between each other and only a few bands of the same mass are presented in all the clones. The fragments lower than 500 bp are less frequent and may be a result of artificial *Eco*RI digestion; hence, they were excluded from analysis.

The quantitative image processing of the bands comprising a region of 1500 to 800 bp revealed the discrepancy of the

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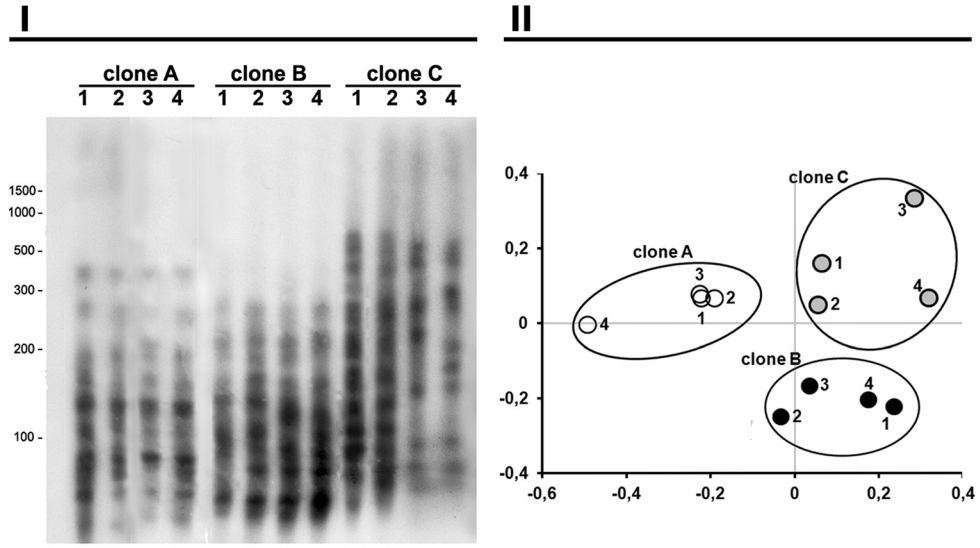

Fig. 3 *Eco*RI and *Mse*I AFLP fingerprints of individual *H. elongata* cercariae DNAs and graphical representation of their genomic polymorphism. *I* Autoradiography of AFLP fingerprints obtained from cercariae individuals belonging to the same and to different clones. *Eco*RI/*Mse*I treatment revealed an array of heterogeneous bands carrying conservative bands within the clone (*A*, *B*, *C*). Cercariae individuals (*1–4*) were categorized by clone-specific pattern of conservative bands, yet fingerings contained a number of zones polymorphic within the clone. *II* The *Eco*RI/*Mse*I AFLP graphical representation. Nonmetric multidimensional scaling (MDS) 2-D plots generated by band-based genetic similarity (Jaccard's coefficient) indicated the genetic variability of the cercarial clone. Clones *A*, *B*, and *C* (*ovals*) demonstrated a rate of interclonal variation (panel *I*). At the same time, the rate of heterogeneity of cercariae from the same clone (*1–4*) indicated their intraclonal genetic variability

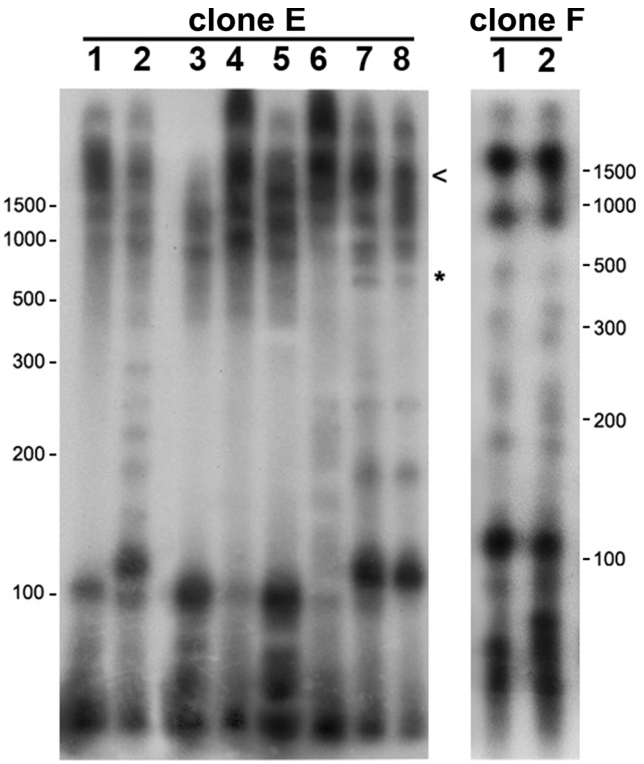

Fig. 4 *Eco*RI AFLP fingerprints of *H. elongata* cercariae isolates. An *Eco*RI produced mostly long fragments over 500 bp and short fragments less than 150 bp. Despite the fact that high fragments carried both conservative (*less-than sign*) and variable (*asterisk*) bands, *Eco*RI did not produce a reliable array to separate the cercariae individuals within as well as across the clone

bands was less reliable than that in case of double restriction (Suppl. Table S1, II).

Discussion

In this study, we showed for the first time that the AFLP technique can be used to study clonal genetic diversity of trematodes. Using this approach, we demonstrated the presence of genetic variability not only between clones (groups of rediae and cercariae in the same individual of the molluskan host) but also within a clone.

The level of genomic differences between *H. elongata* cercariae from the same clone was always considerably lower than that between the cercariae isolated from different molluskan hosts. This observation supports the initial proposition that the group of *H. elongata* rediae in each infected *L. littorea* individual involved in our analysis originated from a single miracidium (monomiracidial infection). However, a certain level of genomic variability was also revealed within cercariae isolated from a single *L. littorea* individual. This may indicate that the processes resulting in the cercarial intraclonal diversity could occur only during rediae parthenogenesis, which strongly suggests an asexual origin of such genome diversity.

Our data are consistent with the growing body of evidence of intraclonal genetic variability in parthenitae and

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cercariae (Grevelding 1999; Bayne and Grevelding 2003; Semyenova et al. 2007; Shalaby et al. 2011; Korsunenko et al. 2012). The *Schistosoma mansoni* W1 (Webster et al. 1989) and W2 (Drew and Brindley 1995) satellite-DNA elements used as probes to trace intraclonal variability were found to be heterogenic even across cercarial clones originating from monomiracidial snail infections. It has been suggested that mitotic recombination events occur within the molluskan host during *S. mansoni* sporocystogenesis (Grevelding 1999). Being a noncoding tandemly repeated DNA sequence, W clusters may be a template for recombination processes but they can hardly be a driving force of intraclonal variability.

The AFLP technique is not simply a fingerprinting. It is an enabling technology in genome research because it can bridge the gap between genetic and physical maps (Vos et al. 1995). AFLP, by itself, could tell nothing about the nature of the fragments amplified. The cloning and sequencing of AFLP fragments could shed light on the source of the clonal diversity. Our results show that *H. elongata* cercarial clones can be used to explore these questions. Even a single-enzyme DNA digestion AFLP produced fragments that were diverse and long enough to be sequenced.

Variable bands obtained previously from *H. elongata* genome by transposon display (Galaktionov et al. 2013; Solovyeva et al. 2013) and *Trichobilharzia szidati* genome by RAPD (Semyenova et al. 2015) techniques have revealed a large fraction of mobile elements. We expect that the vast majority of sequences within the variable bands obtained in this study would bear the similarity to different classes of mobile elements.

Recent studies on trematode cercariae including *H. elongata* revealed a significant level of inter- and intraclonal variability in cercarial morphology, expression of their behavior reactions such as photo- and georeactins, and infectivity to the second intermediate host (Koehler et al. 2011; Prokofiev et al. 2011; Koehler and Poulin 2012; Levakin et al. 2013). In our opinion, the AFLP technique may help to elucidate whether the variability reflects only a phenotypic plasticity (as a response to some environmental influences) or has a genetic basis.

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