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Short technical report

Development of 12 novel polymorphic microsatellite markers using a next generation sequencing approach for *Spiculopteragia spiculoptera*, a nematode parasite of deer

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ABSTRACT

Twelve novel polymorphic microsatellite markers were produced and characterized for *Spiculopteragia spiculoptera* (Nematoda, Trichostrongyloidae) a common parasite of abomasum of Roe and Red deer, using next generation sequencing approach, and two multiplexes PCR were developed with these markers. Polymorphism of each locus was tested in 40 individuals of this species from diverse wild populations of cervids, and was tested for crossed-amplification on four other species of nematodes, close to *S. spiculoptera* among the Trichostrongyloidea: 20 *Spiculopteragia houdemeri*, 34 *Ostertagia leptospicularis*, 16 *Ashworthius sidemi*, and 25 *Trichostrongylus* spp. Our new microsatellite markers seem to be specific to *Spiculopteragia spiculoptera* since no amplifications were obtained for the four other species. The number of alleles per locus ranged from 2 to 12, the average observed and expected heterozygosity per locus ranged from 0.025 to 0.641 and from 0.049 to 0.664, respectively. Four of the 12 microsatellite loci showed significant deviations from Hardy–Weinberg equilibrium (which two slightly significant). One locus pair showed significant linkage disequilibrium (Sspi4 vs. Sspi8). Neither evidence of scoring error due to stuttering nor evidence of large allele dropout was found at all of the 12 loci, but evidence of null alleles was indicated at three loci because of general excess of homozygotes for most allele size classes. These polymorphic loci will be useful markers to study population genetics structure of *Spiculopteragia spiculoptera* in order to understand transfer and to explain the relationships between deer populations.

1. Introduction

Follow movement of wild species and understand their population structure is not trivial. The first requires radio-tracking study and the other a population genetic analysis using a large sample, which means temporary captures and DNA sampling of many individuals of the target species [1]. All these projects require a lot of resources, and can be difficult to realize according to the species, sometimes hard to observe, and according to their type of habitat. Thus, we decided to track parasites to study movements, contacts, relationships, and to have at least a clue of population genetics structure of their related hosts [2]. Indeed, animals, especially the wild one, are bearing a lot of various ecto and endo-parasites with a direct cycle, and requiring contact between their hosts or to share the same habitat to be transferred. Hence, sampling is easier since in one host, we can sample many parasites for molecular analysis. Moreover, because lifespan of parasites is usually shorter than that of their host, the turnover of their generations is faster [3], allowing us to observe more quickly population structure modifications of their hosts, due to environment modifications (habitat fragmentation, urbanization).

Among Strongylids, *Spiculopteragia spiculoptera* (Gushanskaya, 1931) [=*S. boehmi* Gebauer, 1932] is a widespread nematode parasite belonging to the Trichostrongylids and localized on the abomasum of wild ungulates. Primarily considered as a pathogen of wild ruminants, it is rarely reported as a parasite of cattle, goats and sheep [4–7]. Since these parasites are abundant in cervids, it was selected to indirectly, through a population genetics structure analysis of this parasite, highlight deer social network. Hence, for comprehensive analyses of population structure, microsatellite loci are currently still the marker of choice on diverse organisms [8–13]. Widely common in ecology, conservation biology and evolutionary studies from the individual to the population level [14], microsatellites (also known as simple sequence repeats or short tandem repeats) are rare in helminthology and were developed for only few species among ruminant nematode parasites, especially for

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Haemonchus contortus, *Teladorsagia circumcincta*, *Nematodirus* sp., *Strongyloides ratti* and *Trichinella pseudospiralis* [15–21]. However, since few years, next generation sequencing (NGS) technologies provide an effective platform for the development of genetic markers, useful to provide insight into population processes and the evolutionary history of species [22]. Moreover, NGS has been widely used for the rapid and cost effective isolation of nuclear microsatellite markers. Indeed, these approaches result in tens of thousands of sequence reads, which are expected to contain a large amount of suitable microsatellite loci [23]. Among the current NGS approaches, the GS-FLX Titanium (Roche, 454 Life Sciences, Branford, CT, USA; also known as 454) is the most suitable for microsatellite development because of the larger average fragment size obtained (400 bp). Hence, the possibility to identify flanking regions on both sides of the microsatellite motif suitable for primer design increases with the size of the read [24].

Hence, to follow transmission and infection path of this nematode parasite and be able to realize in the future a population genetics study, our aim was to develop new polymorphic microsatellite markers. We chose to use a 454-sequencing approach with enriched libraries to develop highly polymorphic di/tri/tetra-nucleotide microsatellite loci specific for *Spiculopteragia spiculoptera*.

2. Materials and methods

2.1. Development of microsatellite markers

All samples used for this study were collected along a survey program which began in 1986, and also during the hunting season of 2012/2013 during IN SITU program. Hence, male worms were collected from abomasa of different roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) coming from diverse areas in France. The anterior and posterior parts of each male worm were cleared and preserved in Amman lactophenol (internal protocol H. Ferté) between slide and cover slide and identified using morphological features of *Spiculopteragia spiculoptera* according to Skrjabin et al. [25] and Dróżdż [4,26]. If specific identification of male is quite reliable, it is extremely difficult for female. To obtain useful diagnostic microsatellite markers, the 454 next generation sequencing platform was used on 40 adults of *Spiculopteragia spiculoptera* morph *spiculoptera*, and on four *Spiculopteragia spiculoptera* morph *mathevossiani* [26,27]. The middle part of the body of each worm was preserved in 95° ethanol and kept at −20 °C before DNA extraction. Ethanol-preserved material was soaked in distilled water and was then dried for 40 min in an oven at 37 °C. After crushing adult worms using a piston pellet (Treff, Dergersheim, Switzerland), genomic DNA of all samples was extracted using the Qiamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions, and sent to GenoScreen, Lille, France (www.genoscreen.fr) for the following steps. Then, 1 μg of twenty DNA samples was used for the development of microsatellites libraries through the Roche 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. [28]. Briefly, total DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeat motifs. Enriched fragments were subsequently amplified. PCR products were purified, quantified and GsFLX libraries were then carried out following manufacturer's protocols and sequenced on a GsFLX PTP.

Unique sequence contigs possessing microsatellite motifs were identified using QDD software version 2 [29]. This pipeline software deals with all steps of treatment of raw sequences: tag sorting, adapter/vector removal, elimination of redundant sequences, detection of possible genomic multicopies (duplicated loci or transposable elements), stringent selection of target microsatellites, customizable primer design (Primer 3), and also now, BLASTing potential markers against the nucleotide database of NCBI. Then, we tested 47 primer pairs for amplification success and subsequently 24 for degree of polymorphism and heterozygosity using template DNA from eight individuals. All PCR were performed in a 10 μl volume using 1 μl of extracted DNA solution (40 ng/μl), 10 pmol of the primer set, 37.5 pmol $MgCl_2$, 6 pmol of dNTP, and 0.5 unit of Taq polymerase (Fast Start, Roche), on a Mastercycler® personal (Eppendorf). Applied PCR parameters were as follows: an initial denaturation step at 95 °C for 10 min, 40 cycles of a denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min, and finished by an extension step at 72 °C for 10 min. At the end 12 polymorphic microsatellite markers were chosen, pooled into two groups based on observed locus specific allele size ranges containing four primer sets labeled with fluorophores (FAM, NED, VIC, PET), and co-amplified by multiplex PCR using the same cycling as for simplex PCR. Multiplex PCR was carried out on a 25 μl volume with 1 μl of extracted DNA solution (40 ng/μl), 37.5 pmol MgCl 2, 6 pmol of dNTP, and 1 unit of Taq polymerase (Fast start, Roche), and concentration of each primer set labeled are precised in Table 1. One μl of PCR product was added to 19 μL of Genescan 500-LIZ size standard (Applied Biosystems) prior to analysis on an ABI 3740 XL 96-capillary automated DNA sequencer (Applied Biosystems). Analysis of the microsatellites was performed using GENEMARKER software (version 1.95, SoftGenetics, State College).

Moreover, to test the specificity of our new microsatellite markers, we proceed to crossed-amplifications on four other species of nematode parasites phylogenetically close to *S. spiculoptera* among the Trichostrongyloidea: 20 *Spiculopteragia houdemeri*, 34 *Ostertagia leptospicularis*, 16 *Ashworthius sidemi*, and 25 *Trichostrongylus* spp.

2.2. Data analysis

Observed and expected heterozygosities were calculated using CERVUS 3.0 software [30,31]. Tests for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) at each locus were performed in GENEPOP version 4.0 [32]. Results of tests for linkage and Hardy–Weinberg disequilibria were corrected for multiple comparisons by applying sequential Bonferroni corrections [33]. The evidence of null alleles, scoring error due to stuttering, and large allele drop out for each locus were tested using CERVUS 3.0 software.

3. Results and discussion

We produced new polymorphic microsatellite loci using a next generation sequencing approach, useful and nonivasive tools to investigate the contamination path of this nematode parasite among cervids and thus, to build the social network of deer populations. At the end of the first step, we obtained 33,6396 reads containing 6958 potential microsatellite markers. Among those markers, PRIMER 3 software (integrated in QDD) proposed optimal primer set to amplify 94 microsatellites. Forty-seven of those markers were tested, whose 31 showed correct and clear amplification. Then, 24 of those markers were tested for polymorphism, whose 21 were. At the end, among the 21 polymorphic microsatellite markers, 12 were selected to develop two multiplexes PCR tested on 40 individuals from diverse origins. Three di-nucleotide, seven tri-nucleotide, and two quadri-nucleotide loci were isolated. Our new microsatellite markers seem to be specific to *Spiculopteragia spiculoptera* since no amplifications were obtain for the four other species tested. The characteristics of the innovative microsatellite loci are described in Table 1. Overall, the number of alleles per locus ranged from 2 to 12, with an average of 5.250 alleles per