BRAIN-be
Belgian Research Action through Interdisciplinary Networks

PIONEER PROJECTS

TRojan snAILs: the role of gastropod snails in disease transmission revealed by state-of-the-art molecular techniques (TRAIL)

CONTRACT - 165/PI/TRAIL

FINAL REPORT

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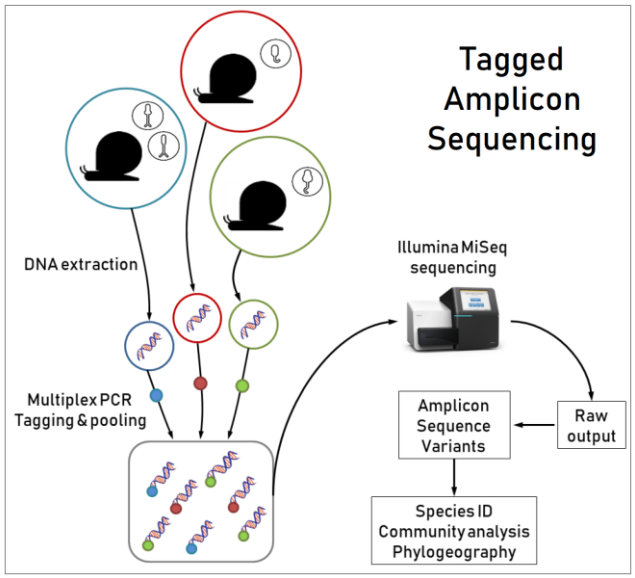
Promotors
Tine Huyse (Royal Museum for Central Africa, Leuvensesteenweg 13 - 3080 Tervuren)
Partners:
Bert Van Bocxlaer & Dirk Verschuren (Ghent University)
Christian Albrecht (Justus Liebig University, Germany)

Authors
Cyril Hammoud, Royal Museum for Central Africa / Ghent University
Tine Huyse, Royal Museum for Central Africa



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WTCIII
Simon Bolivarlaan 30 bus 7 / Boulevard Simon Bolivar 30 bte 7
B-1000 Brussels
Belgium
Tel: +32 (0)2 238 34 11
http://www.belspo.be

Contact person: Georges JAMART
+32 (0)2 238 36 90

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Context

Snail-borne diseases affect more than 300 million people worldwide and lead to economic losses and mortality in livestock. Mainly communities in developing countries are affected, but due to globalization and climate change the prevalence and distribution of snail-borne diseases are changing. Because the distribution of host snail species determines where snail-borne diseases can occur, updated information on snail distribution and their role in parasite transmission is highly needed. However, acquiring these insights was thus far hampered by the ambiguous taxonomic status of many involved gastropod species and a lack of associated ecological and parasitological data. Additionally, traditional techniques to identify snail infection are time-consuming and prone to missing very recent infections while experimental work cannot account for confounding factors like co-infection and strain variability.

Objectives

This project aimed to surmount these difficulties by developing an efficient, sensitive and robust monitoring tool that simultaneously allows to genotype snails and their associated parasites. After designing the tool, we aimed to test its sensitivity on controlled cases of infected snails, and subsequently to deploy it to study new and existing museum collections to unravel important ecological factors in the distribution and spreading of snail-borne diseases.

Conclusions

We designed a new workflow based on high-throughput amplicon sequencing (HTAS) to analyze entire communities of trematodes within populations of their snail hosts. By testing this workflow on artificially and naturally infected snails, we showed that it yields reliable and robust diagnosis, and it enables the simultaneous molecular characterization of both hosts and parasites. Our workflow successfully allowed the molecular characterization of snails belonging to 6 different species from 3 genera (superorder Hygrophila), and of parasites belonging to 16 different species from 8 families (class Trematoda), demonstrating its broad taxonomic versatility. In conclusion, we now have a powerful tool to describe snail-borne trematode communities and analyze the processes shaping their diversity and structure in natural snail populations, shedding light on disease dynamics in nature. We are currently applying this tool on Ugandan and Senegalese snail populations in the frame of two new projects that build upon this BRAIN Pioneer project. The abovementioned methodological advance is thus helping us now to better understand the natural and anthropogenic factors driving the distribution and transmission of snail-borne diseases affecting humans, livestock and wildlife.

Keywords

host-parasite interactions, zoonoses, monitoring, emerging diseases, global change

SAMENVATTING

Context

Meer dan 300 miljoen mensen wereldwijd worden getroffen door ziektes die overgedragen worden door zoetwaterslakken. Gerelateerde ziektes leiden ook tot economische verliezen in de veeteelt. Vooral ontwikkelingslanden worden getroffen, maar door de huidige globalisering en klimaatveranderingen verschuift de verspreiding van deze ziektes. Vermits de verspreiding van de zoetwaterslakken bepaalt waar deze ziektes voorkomen, is er dringend nood aan geactualiseerde informatie over de verspreiding van deze soorten en over hun exacte rol in het verspreiden van parasieten. Deze informatie was tot nog toe zeer moeilijk te verkrijgen omdat de taxonomie van deze soorten niet op punt staat, en er een gebrek is aan ecologische en parasitologische data. Bovendien zijn de traditionele methoden om infectie in slakken vast te stellen zeer tijdrovend, en missen ze bovendien recente infecties. Experimentele studies kunnen dan weer geen rekening houden met co-infecties en verschillen tussen geografische lijnen van zoetwaterslakken.

Doelstellingen

In dit project beoogden we om deze complicaties te overwinnen door de ontwikkeling van een efficiënte, gevoelige en betrouwbare techniek waarmee zowel de slakkensoort als de geassocieerde parasieten geïdentificeerd kunnen worden. Deze techniek wordt uitgetest op slakken met een gekende infectiegeschiedenis alvorens ze toegepast wordt op zowel nieuwe als bestaande museumcollecties. Dit moet ons uiteindelijk in staat stellen om de belangrijke ecologische factoren te achterhalen die de verspreiding van deze ziektes sturen.

Besluiten

We ontwikkelden een nieuwe methode gebaseerd op 'high-throughput amplicon sequencing' (HTAS) om volledige Trematoda parasietgemeenschappen te analyseren in populaties van hun slakkengastheren. Door deze techniek te testen op artificieel en natuurlijk geïnfecteerde slakken, toonden we aan dat het een betrouwbare en robuuste diagnose oplevert, en dat het de gelijktijdige moleculaire karakterisering van zowel gastheren als parasieten mogelijk maakt. We kunnen zoetwaterslakken behorende tot 6 verschillende soorten uit 3 genera (superorde Hygrophila), en parasieten behorende tot 16 verschillende soorten uit 8 families (klasse Trematoda) succesvol karakteriseren, wat de taxonomische veelzijdigheid onderstreept. We beschikken dus over een krachtig instrument om de parasietgemeenschappen in slakken te beschrijven en de processen te analyseren die hun diversiteit en structuur bepalen. Momenteel passen we deze techniek toe in twee nieuwe projecten die verder bouwen op dit BRAIN Pionier project. Dankzij deze techniek kunnen we vanaf nu de natuurlijke en menselijke factoren bestuderen die de verspreiding van deze ziektes sturen, en zo licht werpen op de dynamiek van infectieziektes in de natuur.

Trefwoorden

Gastheer-parasiet interacties, zoonoses, monitoren, opkomende ziektes, globale verandering

RESUME

Contexte

Les maladies transmises par les escargots affectent globalement plus de 300 millions de personnes et sont une source importante des dégâts économiques et de mortalité pour le bétail. Les communautés des pays en voie de développement sont les plus affectées, mais la prévalence et la distribution de ces maladies est en train de changer du fait de la globalisation et du changement climatique. Etant donné que la distribution des différentes espèces de gastéropodes qui fonctionnent comme hôte intermédiaire détermine les aires de répartition de ces maladies, il est essentiel de disposer d'informations précises et actuelles concernant leur distribution et leur rôle dans la transmission des parasites trématodes. Cependant, l'acquisition de ces données est actuellement prévenue par la confusion qui règne autours de la taxonomie de nombreuses espèces de gastéropodes impliquées, et par le manque de données écologiques et parasitologiques. De plus, les techniques traditionnellement utilisées afin d'identifier l'infection des escargots sont chronophages et ne détectent que rarement les infections précoces, tandis que le travail expérimental ne peut prendre en considérations des situations complexes tel que des cas de co-infection ou de variabilité des souches de parasites.

Objectifs

Nous proposons ici de surmonter ces difficultés en développant un outil de monitoring efficace, sensible et rigoureux qui permettra de génotyper simultanément les escargots et parasites qui leur sont associés. Une fois cet outil conçu, nous avons testé sa sensibilité sur des cas d'infections contrôlées, avant de l'employer sur des collections de musée anciennes et nouvelles afin de mettre en évidence les facteurs écologiques déterminants pour la distribution et la dispersion des maladies transmises par les trématodes.

Conclusions

Nous avons créé un nouveau protocole basé sur le séquençage haut-débit d'amplicons qui permettra d'analyser des communautés de trématodes au sein des populations de leurs escargots hôte. En testant cette méthode sur des escargots infectés artificiellement ainsi que naturellement, nous avons pu démontrer qu'elle produit un diagnostic robuste et fiable et qu'elle permet une caractérisation moléculaire simultanée de l'hôte et des ses parasites. Notre protocole a permis de caractériser des escargots de 6 espèces différentes, venant de 3 genres distincts (superorder Hygrophila), ainsi que des parasites appartenant à 16 espèces venant de 8 familles distinctes (classe Trematoda), ce qui témoigne de sa grande versatilité taxonomique. En conclusion, nous disposons maintenant d'une méthode puissante pour décrire les communautés de trématodes et analyser les processus influençant la diversité et la structure de ces communautés au sein de population naturelle de leurs escargots hôte, ce qui permet d'améliorer notre comprehension des dynamiques de transmissions de ces maladies. Nous appliquons actuellement cette méthode sur des populations d'escargots ougandaises et sénégalaises dans le cadre de deux nouveaux projets qui se basent sur ce projet BRAIN

Pioneer. Cette avancée méthodologique nous permettra d'identifier les facteurs naturels et anthropogéniques qui influencent la distribution et la dynamique de transmission des maladies par les escargots vers l'humains, les animaux domestiques et la faune sauvage.

Mots-clés

Interactions hôte-parasite, zoonoses, maladies émergentes, changement global

1. INTRODUCTION

Gastropod-borne **helminth diseases** affect more than 300 million people worldwide and cause mortality in humans and livestock, resulting in major health concerns and economic losses (Fig. 1). The most notorious disease in terms of prevalence and disease burden is human schistosomiasis. This poverty-associated, neglected disease affects more than 200 million people worldwide, with an additional 750 million people at risk, mainly in Africa (Gryseels et al., 2006). Schistosomiasis is also a very common in cattle in Africa and Asia with an estimated 165 million animals infected. Chronic infection in humans may lead to severe liver, intestinal and bladder complications, causing debilitating illness, sometimes leading to death. Especially developing countries are affected but due to globalization and climate change the distribution of snail-borne diseases is changing. New introductions of disease are linked with increasing human migration as illustrated by a recent outbreak of urinary schistosomiasis in Europe (Boissier et al., 2016). Successful establishment following introduction depends on the distribution of suitable snail hosts. **Knowledge** on the distribution of gastropod snails is however very **fragmentary**, which is linked with morphological plasticity, similarity between closely related species (Brown, 1994), but also with limited scientific interest (Adema et al., 2012). Given its crucial role in the transmission of so many diseases, it is hard to understand why the last decades saw so few publications on gastropod hosts. In this golden age of genomics, gastropod snails are conspicuous by their absence (Giannelli et al., 2016). The seminal work of Brown (1994) on the systematics of African freshwater snails and their medical importance has never been properly matched with molecular studies leaving us with no up to date **identification keys**. Taxonomic confusion therefore seriously hampers studies on the distribution, ecology and role of these snails in parasite transmission.

The full extent of the **parasite fauna** of gastropod snails is also **scarcely known**. Due to over-dispersion, only a small fraction of the snail population is typically infected (Loker et al., 1981; Eppert et al., 2002). Traditional snail-shedding experiments to verify snail infection are therefore time-consuming but they also miss 'prepatent' (immature) infections in the field. Moreover, the limited morphological characters of trematode cercariae preclude identification to species level. Molecular identification methods are mainly restricted to the medical important species like schistosomes, but even then genotyping single cercariae remains highly laborious (Van den Broeck et al., 2011). Therefore a **new tool** to accurately define the parasite fauna of snails is urgently **needed**. Co-infection of multiple strains of the same parasite species, or different parasite species within a single snail individual can lead to competitive exclusion and thus affect transmission patterns (Loker et al., 1981; Steinauer et al., 2010). Experimental snail infections have for example demonstrated predatory effects of amphistome rediae on schistosome sporocysts (Loker et al., 1981 and references therein). In order to understand the factors that drive co-infection patterns and how these change over time and space, a rapid and cheap tool is needed to monitor snail infection on a large scale.

This project aimed to develop a novel time- and cost-efficient technique to simultaneously address the problem of snail identification and to determine the presence of helminth parasites. Besides developing proof-of-principle, this project aimed to valorise museum collections and generate new data that will help to understand the biotic and abiotic factors influencing the dynamics of snail infection.

2. METHODOLOGY AND RESULTS

2.1. HTAS design

Using high-throughput amplicon sequencing we developed a new workflow to simultaneously genotype snail hosts and their infecting trematode parasites. In a nutshell, the methods consists of the following steps (figure 1): 1) the DNA of the snail hosts (and its infecting parasites) is extracted, 2) various markers are amplified from the snail and the parasite genomic DNA in a single-step multiplex PCR, 3) libraries are prepared by adding a sample-specific index to the amplicons and pooling them, 4) the libraries are sequenced using an Illumina MiSeq v3 sequencer (2 x 300 base paires overlapping), 5) using the dada2 pipeline, the sequenced reads are de-multiplexed, trimmed, filtered and inferred into functional Amplicon Sequence Variants (ASVs), 6) the ASVs' profile of each sample is used to molecularly characterize the snail and its parasite community.

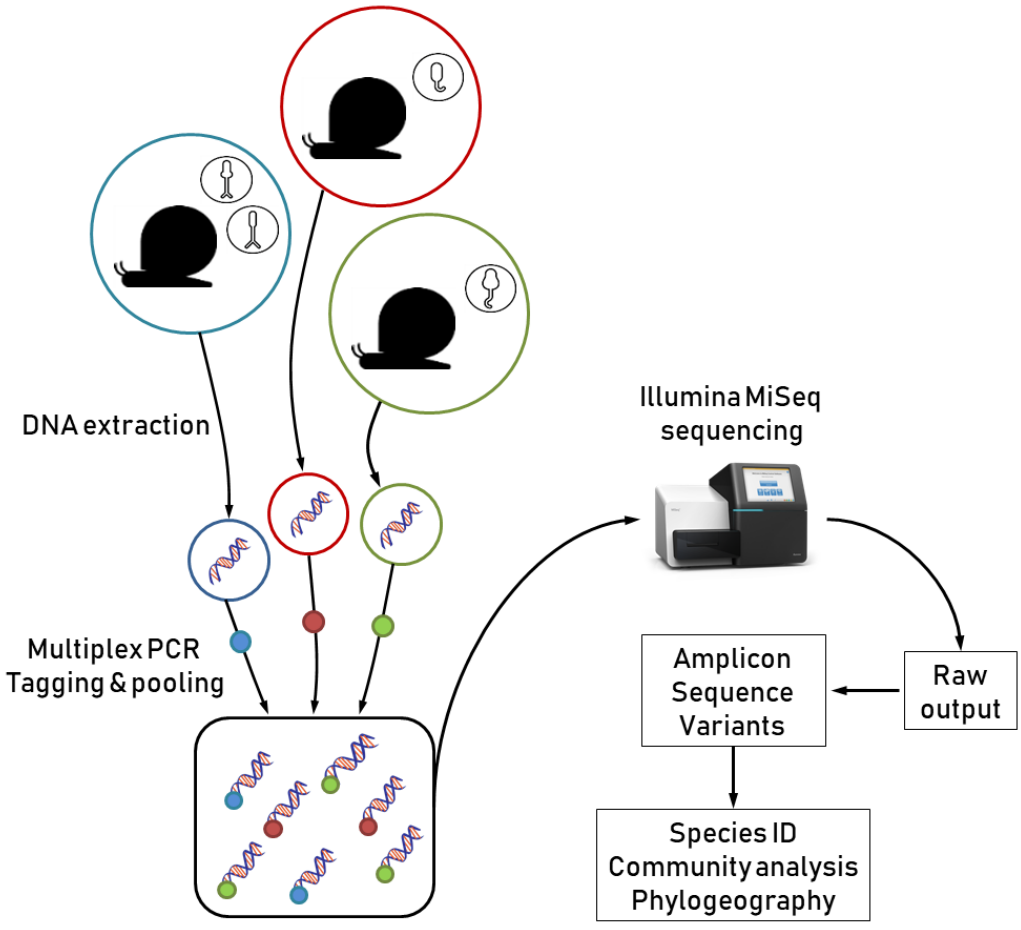
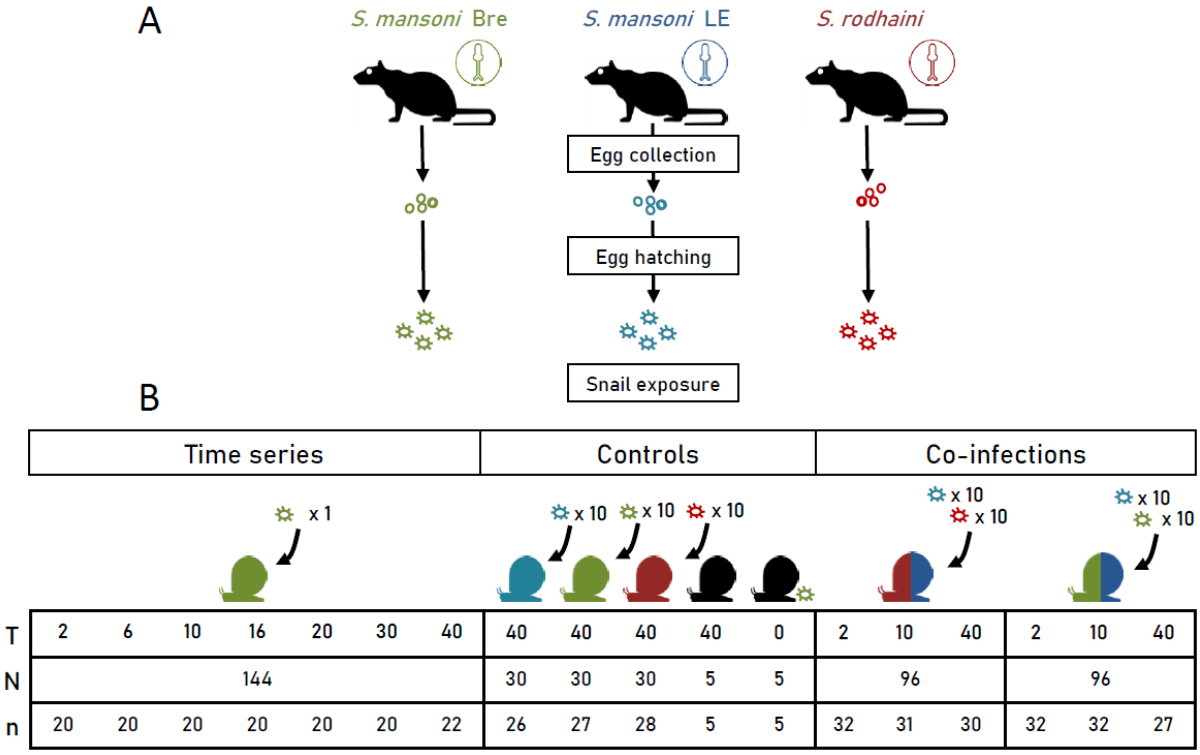


Figure 1: Schematic representation of the HTAS workflow.

We designed primers to amplify 4 snail and 5 trematode markers in a single multiplex PCR to optimize time- and cost-efficiency. Whereas also applicable to other genera, we focused on medically and economically important snail genera within the Superorder Hygrophila and targeted a broad taxonomic range of parasites within the Class Trematoda. We tested the workflow using 417 *Biomphalaria glabrata* specimens experimentally infected with *Schistosoma rodhaini*, two strains of *Schistosoma mansoni*, and combinations thereof (figure 2). Using this setup, we were able to evaluate the reliability of infection diagnostics, the robustness of the workflow, its specificity related to host and parasite identification, and the sensitivity to detect co-infections, immature infections, and changes of parasite biomass during the infection process. Finally, we investigated its applicability in wild-caught snails of other genera naturally infected with diverse trematode assemblages.



T	2	6	10	16	20	30	40	40	40	40	40	0	2	10	40	2	10	40
N	144							30	30	30	5	5	96			96		
n	20	20	20	20	20	20	22	26	27	28	5	5	32	31	30	32	32	27

Figure 2: A) Workflow of our controlled infection experiments, up to parasite exposure. Trematode eggs were obtained from infected mice and used to hatch miracidia to which snails were exposed. B) Graphic representation of the infection experiments, showing the number of snails exposed to the various parasite strains (N) and the number of individuals sampled (n) at different times (T, in days).

Our results show that, after stringent quality control on the output reads and ASVs, the workflow allows the simultaneous identification of snails to species level, and of trematodes to taxonomic levels ranging from family to strain; which represents a massive improvement compared to other existing techniques. Moreover, it is sensitive to detect immature infections (starting at 6 days after infection for the *B. glabrata – S. mansoni* system) and changes in parasite biomass comparable to those described previous experimental studies (see figure 3). Co-infections by parasites of different species, or by parasitese from different strains were successfully identified. This feature represents a major improvement over traditional shedding, genotyping using Sanger sequencing or RD-PCR (Eppert et al. 2002; Thiele & Minchella 2013; Bakuza et al. 2017; Schols et al. 2019) and will greatly facilitate the study of inter- and intraspecific parasite interactions within populations of their snail hosts, which is essential given the potential impact of parasite-parasite competition within the snail on the dynamics of transmission to final hosts (e.g. Laidemitt et al. 2019). Altogether, these results demonstrate that our workflow provides a powerful tool to analyse the processes shaping trematode communities within natural snail populations.

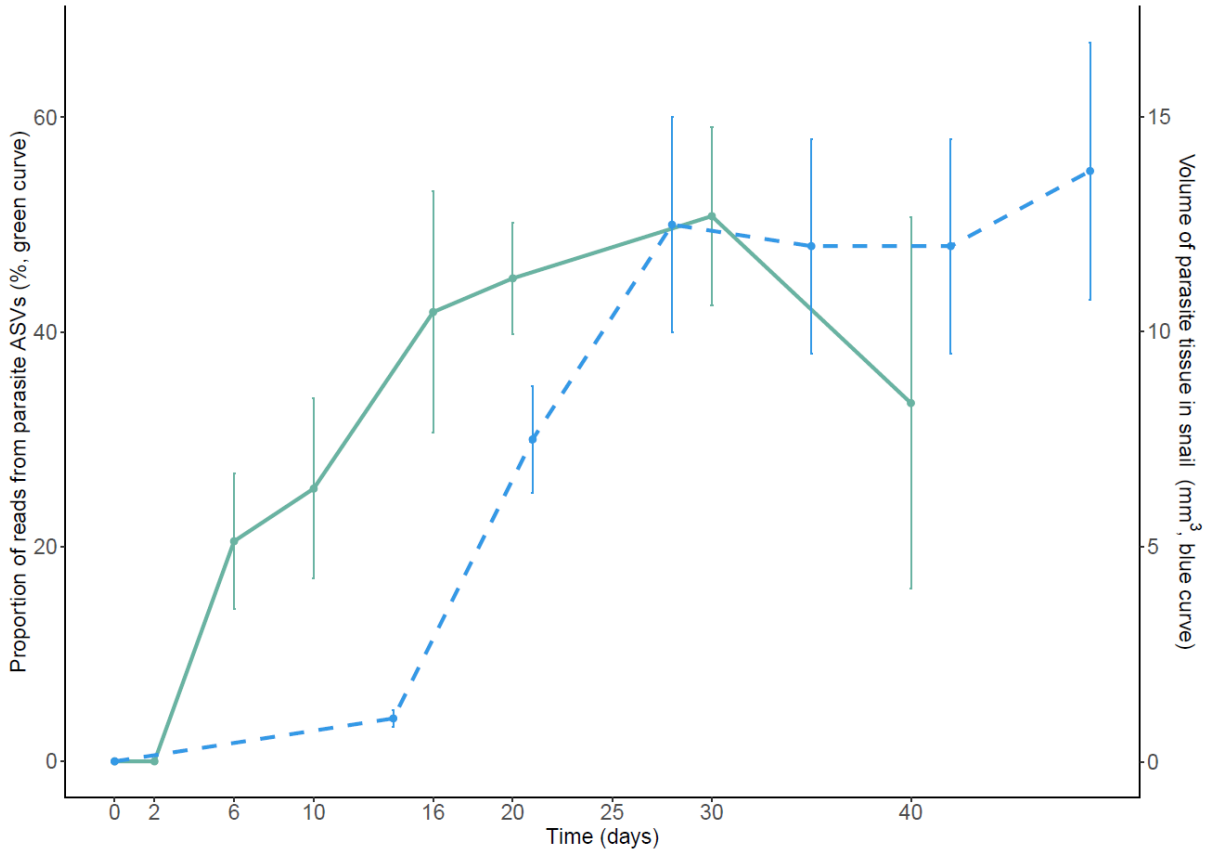


Figure 3: Comparison between the proportion of reads attributed to* Schistosoma mansoni *Amplicon Sequence Variants (ASVs) in infected* Biomphalaria glabrata *snails throughout the time-series experiment (solid line; error bars reflect standard deviation on the mean) and the volume of parasite biomass within the snail's digestive gland as reported by Théron et al. (1992; dashed line).

Finally, applying the HTAS workflow on wild-caught *Bulinus* and *Radix* snails with previously established infection status indicated that our method is applicable to a wide diversity of Hygrophila snail species and Trematoda. HTAS allowed the identification of most trematode infections detected previously and detected a few new ones, thus showing a slightly higher sensitivity compared to the combination of cercarial genotyping and RD-PCR. The taxonomic resolution was also generally higher, as was the sensitivity towards detecting co-infection by strains belonging to the same species. The manuscript describing our new HTAS workflow has been accepted in Molecular Ecology Resources (see below).

2.2. Using HTAS to test for an impact of trematode infection on snail morphology

For this research question we focused on a single sampling event in a single crater lake in order to minimise the effect of time and space on snail morphology. Integrating the diagnostic PCR (Schols et al., 2019), the new HTAS protocol (see above) and 2D geometric morphometrics on the shells of the genotyped *Bulinus truncatus* specimens, we managed to identify morphological traits that indicate or are suggestive of infection with trematode parasites. High-resolution photographs were taken of 257 shells using the focus-stacking system with the Zerene® stacker software (T2019-10-07-1410) as described in Brecko *et al*. (2014) after which 10 landmarks and 4 semi-landmark curves were digitized in TpsDig v. 2.31. The (semi-)landmark data was imported into CoordGen8 of the Integrated Morphometrics Package and subjected to Procrustes super-impositioning to remove variation in scale, orientation and position. The resulting dataset was imported in R v. 3.4.3 for further statistical analyses. A significant difference in morphology was found between infected and non-infected snail species. In order to rule out genetic variation, all snails were sequenced for a partial COI fragment. This confirmed that all snail specimens belong to the same haplotype group, strongly corroborating the hypothesis that morphological changes are linked with parasite infection. This is an exciting result, as covariation between morphology and infection status has not been demonstrated previously for *B. truncatus*, a widespread snail species that acts as intermediate host for many trematode parasites, causing snail-borne diseases like schistosomiasis. These data are currently being prepared for publication.

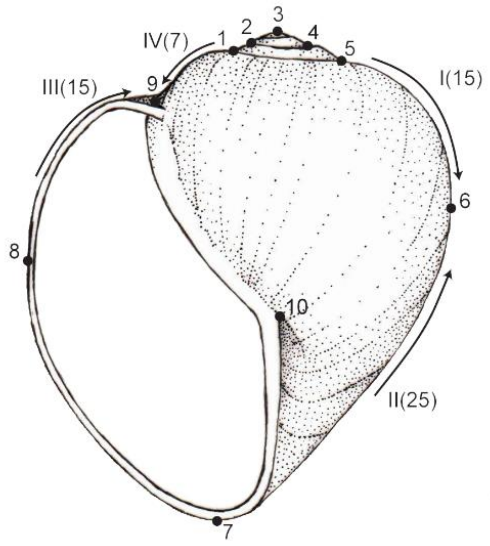


Figure 4: Illustration of a* Bulinus *shell with the landmarks (numbered from 1 to 10) and curves of semi-landmarks (numbered from I to IV, between brackets the resampling size) indicated.

2.3. Using HTAS to better understand the biotic and abiotic factors that influence the distribution of snail-borne diseases

This pioneer research project also led to securing a FWO fellowship for Cyril Hammoud. He will use the HTAS to study the factors that impact trematode communities of natural snail populations. More than 3000 snails from Ugandan crater lakes have been extracted by now and subjected to the RD-PCR protocol that was developed previously, followed by the TAS protocol on the MiSeq. The main focus is on *Bulinus truncatus* and *B. globosus*. The first results point to a very high infection rate with trematodes detected by RD-PCR, which was largely underestimated by traditional shedding in the field. For example, the infection prevalence in Lake Nyabikere was estimated around 4.2% using shedding and 34.5% using RD-PCR. The analysis of the TAS outcome is on-going, and these data will allow us to understand the role of these snail species in disease transmission to various final hosts. The combination of crater lakes in national parks and in agricultural settings will allow us to look at the link between anthropogenic factors and disease transmission, amongst others. For Senegal, the DNA of the snails has been sampled, but the HTAS protocol will be applied later this year in the frame of another project (see below).

3. DISSEMINATION AND VALORISATION

The results have been communicated with the scientific community by means of poster and oral presentations in various conferences (see below under Publications). The audience of these presentations ranged from scientific experts in the field of parasitology (e.g. at the Spring meeting of the British Society of Parasitology), to a mix of scientists from various fields (e.g. at the "One Health" multidisciplinary workshop organized by the Royal Academy for Overseas Sciences), but also included non-experts, during public Info Lunchs or PhD days at the Royal Museum for Central Africa.

TRAIL project Website:
https://www.africamuseum.be/nl/staff/1339/project_detail_view?prjid=669).

Also, apart from the FWO project of Cyril Hammoud, the developed protocol will be used by colleagues of JEMU (Joint Experimental Molecular Unit of the RMCA and the RBINS, http://jemu.myspecies.info/) and in another *BRAIN-be 2.0* project named 'MicroResist': *The influence of snail host microbiome in trematode parasite resistance*
https://www.africamuseum.be/nl/staff/1591/project_detail_view?prjid=723
Finally, thanks to this TRAIL project a new collaboration between RMCA and the MUST Uganda was established, leading to a successful application for a new project (ATRAP project).

4. PERSPECTIVES

We developed a new workflow using high-throughput amplicon sequencing to simultaneously genotype snail hosts and their infecting trematode parasites. This opens new and exciting avenues to efficiently and accurately study trematode communities within individual or pooled snail hosts. Applying this tool in large-scale monitoring will help to map transmission hotspots of snail-borne disease that affect humans, livestock and wildlife. Also, due the high sensitivity, this tool can depict parasite-parasite interactions within individual snail hosts. This will allow us to decipher the impact of specific combinations of parasite species within the snail host on infection outcome and thus disease dynamics. Also, characterizing parasite communities in snail populations from different environments will furthermore allow disentangling the relative role of anthropogenic factors on disease distribution.

The technique furthermore allows to uncover the hidden diversity of trematode parasites within snails. Due to the high diversity, and the fact that these African trematode species are understudied, there exists a huge barcoding gap, preventing us of identifying all trematodes to species level. Indeed, African trematodes are highly under-represented on the GenBank database. We therefore called for increased research efforts following standardized procedures to fill this gap (Schols et al., 2020). Our new TAS tool, which allows to sequence >500 samples in one sequencing run, will directly help to close this knowledge gap by generating many new sequences, which will allow us to generate a molecular reference database for snail-borne parasites.

Apart from diagnostic purposes, the molecular sequences that are generated by this approach can also be used for more fundamental research on host and parasite biogeography, which will be one of the aims of PhD student Cyril Hammoud. One of his chapters will focus on our last aim, namely reconstructing the historical biogeography of snail host-trematode relationships within the crater lakes of Uganda. Haplotype networks will be constructed for snails and parasites, using the COI, cytb and NAD1 sequences generated through the HTAS protocol. About 75% of those data have been collected within the timeframe of the TRAIL project.

Finally, the concept and protocol of the workflow are highly versatile for studying other snail-borne trematode communities. Depending on the specific target, primer concentrations in the multiplex PCR may be optimized to balance amplicon representation. Alternatively, depending on research interests, one may choose to remove snail or trematode markers to simplify the workflow and to increase the coverage of the targeted markers.

5. PUBLICATIONS

5.1. Publications in peer-reviewed international journals

Schols, R., Carolus, H., Hammoud, C., Mulero, S., Mudavanhu, A., Huyse, T. (2019). A rapid diagnostic multiplex PCR approach for xenomonitoring of human and animal schistosomiasis in a 'One Health' context. *Transactions of the Royal Society of Tropical Ledicine and Hygiene*, *113* (11), 722-729. doi: 10.1093/trstmh/trz067 Open Access

Schols, Ruben; Mudavanhu, Aspire; Carolus, Hans; Hammoud, Cyril; Muzarabani, Kudzai; Barson, Maxwel; Huyse, Tine; 2020. Exposing the barcoding void: An integrative approach to study snail-borne parasites in a One Health context. *Frontiers In Veterinary Science*; 2020; Vol. 7; pp

Maes, T., Hammoud, C., Volckaert, F. & Huyse, T. 2021. A call for standardised snail ecological studies to support Schistosomiasis risk assessment and snail control efforts. *Hydrobiologia* 848 (8): 1773-1793. doi: 10.1007/s10750-021-04547-4

Hammoud, C., Mulero, S., Van Bocxlaer, B., Boissier, J., Verschuren, D., Albrecht, C. & T. Huyse. Simultaneous genotyping of snails and infecting trematode parasites using high-throughput amplicon sequencing. *Molecular Ecology Resources*, accepted.

Hammoud C., Kayenbergh, A., Tumusiime, J. Verschuren D., Albrecht D., Huyse, T. & Van Bocxlaer B., Investigating the influence of trematode infections on the shell morphology of *Bulinus tropicus* using a combination of high-throughput sequencing and landmark-based geometric morphometrics, in prep.

5.2. Publications in abstract book of conferences

Hammoud C., Van Bocxlaer B., Verschuren D., Albrecht D., Huyse T., 2018, Deep amplicon sequencing as a tool to study community structure and speciation in trematodes. Oral presentation at the Speciation in Ancient Lake meeting (SIAL8) (Entebbe, Uganda).

Hammoud C., Van Bocxlaer B., Verschuren D., Albrecht D., Huyse T., 2018, Untangling the drivers of parasite diversity along gradients of natural and anthropogenic variables in a tropical crater-lake system (Kasenda, Uganda). Poster presentation at the Spring meeting of the British Society of Parasitology (UK, Asberyswyth).

Hammoud C., Van Bocxlaer B., Verschuren D., Albrecht D., Huyse T., 2018, Untangling the drivers of parasite diversity along gradients of natural and anthropogenic variables in a tropical crater-lake system (Kasenda, Uganda). Poster presentation at the Joint meeting for the Belgian Society of Parasitology and Protistology (BSPP), the Irish Society of Parasitology, the

British Association for veterinary parasitology and the European Veterinary Parasitology College (Brussels).

Hammoud C., Maes T., Van Bocxlaer B., Verschuren D., Albrecht D., Huyse T., 2019, The influence of anthropogenic activities on communities of snail-borne parasites of public and veterinary importance, "Biodiversity and Health in the Tropics" multidisciplinary workshop organized by the Royal Academy for Overseas Sciences (Brussels).

Hammoud C., Mulero S., Van Bocxlaer B., Boissier J., Verschuren D., Albrecht C., Huyse T., 2021. Simultaneous genotyping of snails and infecting trematode parasites using high-throughput amplicon sequencing. Parasites Online Meeting 2021 of the British Society for Parasitology (online).

6. ACKNOWLEDGEMENTS

We would like to wholeheartedly thank our colleagues at Mbarara University for Science and Technology (MUST) in Uganda for our great collaboration in the field, with a special thanks to Dr. Casim Tolo, Julius Tumusiime and Ainomugisha Naboth. The Uganda National Council for Science and Technology (UNCST) has reviewed and approved the research activities in this project (reference number: NS 639).

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