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Molecular detection of natural infection of *Lymnaea* (*Pseudosuccinea*) *columella* (Gastropoda: Lymnaeidae) with *Fasciola gigantica* (Digenea: Fasciolidae) from two provinces of South Africa

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Research Paper

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Abstract

The main intermediate host of *Fasciola gigantica* in sub-Saharan Africa is *Lymnaea* (*Radix*) *natalensis*. *Lymnaea* (*Pseudosuccinea*) *columella* is capable of transmitting both *F. gigantica* and *F. hepatica* and has been reported to be present in South Africa. To date, no natural infection with *F. gigantica* has been reported despite the wide distribution of the snail. The aim of this study was to confirm whether *L.* (*P.*) *columella* was transmitting *F. gigantica* and/or *F. hepatica* in selected locations of KwaZulu-Natal and Eastern Cape provinces of South Africa. *Lymnaea* (*Pseudosuccinea*) *columella* snails were collected from two locations in two provinces of South Africa and screened for cercariae shedding. This was followed by humanely sacrificing the screened snails, and whole tissue of each individual snail was homogenized and amplified using primers designed to amplify the ITS-1 region of *Fasciola* spp. No cercariae were shed from the screened snails and molecular analysis showed that snails from the two locations were infected with *F. gigantica*. This study confirms natural infection of *L.* (*P.*) *columella* with *F. gigantica* in South Africa, where *F. gigantica* and *F. hepatica* have already been reported to coexist. Although *L.* (*P.*) *columella* is able to transmit the two species, surprisingly no infection with *F. hepatica* was detected from the screened snails. The natural intermediate host of *F. gigantica* in southern Africa, including South Africa, is *Lymnaea* (*Radix*) *natalensis* and comparative studies are needed to determine the competence of the two snail species in the transmission of *F. gigantica*.

Introduction

Fascioliasis is an important zoonotic parasite infection caused by digenean trematodes of the genus *Fasciola*, and the common species are *Fasciola hepatica* Linnaeus (1758) and *F. gigantica* Cobbold (1856) (Mas-Coma *et al.*, 2009, 2018; Mochankana and Robertson, 2016). The disease is distributed worldwide (Hurtrez-Boussès *et al.*, 2001; Mas-Coma, 2005), affecting mainly domestic and wild ruminants, but also humans (Walker *et al.*, 2008; Robinson and Dalton, 2009; Phalee *et al.*, 2015; Mas-Coma *et al.*, 2018), causing economic loss in livestock production globally (Khan *et al.*, 2013). Of the two *Fasciola* spp., *F. hepatica* has a cosmopolitan distribution, with human infection reported in five continents (Hurtrez-Boussès *et al.*, 2001; Mas-Coma, 2005; Mas-Coma *et al.*, 2009, 2018), whereas *F. gigantica* is more limited to Africa and Asia (Mas-Coma *et al.*, 2009, 2014, 2018; Abebe *et al.*, 2010), occurring to a lesser extent in southern parts of Europe, Turkey, the Near East and some southern states of the old USSR, Armenia in particular (Mas-Coma, 2005), and South America (Hurtrez-Boussès *et al.*, 2001). Furthermore, distribution of both species also overlaps in many areas in Africa and Asia (Mas-Coma *et al.*, 2005).

Fasciola spp. require freshwater snails as the intermediate host (IH) to complete their life cycle, and mammals, mainly herbivores, including humans, act as the definitive hosts (Mas-Coma *et al.*, 2005). Freshwater snails of the family Lymnaeidae act as the IHs of *F. hepatica* and *F. gigantica* (Bargues and Mas-Coma, 2005; Caron *et al.*, 2014), and play a significant role in determining the distribution and epidemiology of fascioliasis (Mochankana and Robertson, 2016). Hence, the distribution pattern and abundance of the two species is equally associated with and influenced by the global distribution of their lymnaeid intermediate snail host (Mas-Coma *et al.*, 2005). *Fasciola gigantica* utilizes *Radix* species (Mas-Coma, 2005), particularly species belonging to the *Lymnaea* (*R.*) *auricularia* (Linnaeus, 1758) complex (Hubendick, 1951) as IHs (Brown, 1994), whilst *Lymnaea* (*Radix*) *natalensis* Krauss (1848) is considered to be the main IH of *F. gigantica* in Africa (Walker *et al.*, 2008). In contrast, *F. hepatica* uses snail species from the genus *Galba*/*Fossaria* as IHs (Mas-Coma, 2005).

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However, the geographical distribution of *F. hepatica* is mainly linked to the geographical expansion of its original European lymnaeid IH *Lymnaea* (*Galba*) *truncatula* (Müller, 1774), the spread of the American species *Lymnaea* (*Pseudosuccinea*) *columella* (Say, 1817), and its adaptation to other lymnaeid species native to the newly colonized areas (Mas-Coma *et al.*, 2005). In South Africa, the presence of *G.* (*L.*) *truncatula* has been reported and has been assumed to be transmitting *F. hepatica* in areas where it has been found (De Kock *et al.*, 2003).

According to Quayle *et al.* (2010) and Mucheka *et al.* (2015), both *F. hepatica* and *F. gigantica* are present in South Africa, and *L.* (*G.*) *truncatula* and *L.* (*R.*) *natalensis* act as their IHs, respectively. Despite the overlap in the distribution of both *Fasciola* species observed by Mucheka *et al.* (2015) in KwaZulu-Natal and Mpumalanga provinces, both *F. hepatica* and *F. gigantica* seem to have different altitudinal distribution in accordance to the ecological requirements of their respective snail IHs in the country (Quayle *et al.*, 2010). *Galba* (*L.*) *truncatula* is found in cooler areas and is mostly abundant in high areas such as Lesotho and the northern parts of the Eastern Cape province of South Africa (De Kock *et al.*, 2003; Quayle *et al.*, 2010), whereas *L.* (*R.*) *natalensis* is one of the most widely distributed snail species according to the National Freshwater Snail collection (NFSC) records (De Kock *et al.*, 1989) but is not found in the cooler, drier regions of South Africa (Brown, 1994; Quayle *et al.*, 2010).

Lymnaea (*P.*) *columella* is a rapidly colonizing, heat tolerant species thought to have originated from Central America, the Caribbean, and the southern part of North America (Mas-Coma *et al.*, 2005). The species is considered to be invasive in southern Africa (Appleton, 2003), including South Africa, where it has been considered one of the most successful colonists of freshwater species in the country (De Kock *et al.*, 1989), and one of the most widespread freshwater snails by the NFSC, following *L.* (*R.*) *natalensis* and *Bulinus tropicus* (De Kock *et al.*, 1989). *Lymnaea* (*P.*) *columella* has been documented to act as an intermediate host of both *F. hepatica* and *F. gigantica* in many countries (Mas-Coma *et al.*, 2005; Grabner *et al.*, 2014). Although this invasive species has been presumed to be susceptible to both flukes in South Africa (Brown, 1980), the epidemiological role played by this species in the transmission of the two *Fasciola* species has not been proven. Hence, this study screened *L.* (*P.*) *columella* snails collected from selected locations of KwaZulu-Natal and Eastern Cape provinces of South Africa known to be endemic for fascioliasis in cattle and sheep to determine the infection status with *F. hepatica* and/or *F. gigantica* using molecular techniques.

Materials and methods

Snail collection and identification

Lymnaea (*Pseudosuccinea*) *columella* snails were collected in November 2017 and July 2018 from two locations at Hazelmere dam (HD1 and HD2) in Verulam area in KwaZulu-Natal province, at elevations ranging from 91 to 92 m above sea level, and from three locations at Lucingweni (LC1, LC2 and LC3) in Mthatha area in Eastern Cape province, at elevations ranging from 717 to 800 m above sea level, using a metal scoop (30 × 30 cm) (fig. 1). Snails were identified as *L.* (*P.*) *columella* following descriptions by Brown (1994). Immediately after collection, more than 50 snails were screened for cercariae shedding as described by Sharif *et al.* (2010). After screening, a total of 20 snails per location (n = 100) were selected randomly and fixed in 70% ethanol for molecular analysis.

Molecular identification of Fasciola *spp. in* L. (P.) columella *tissue*

DNA was extracted from the homogenized individual *L.* (*P.*) *columella* snail tissue using a Genomic DNA™ Tissue MiniPrep Kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions. In order to identify infected

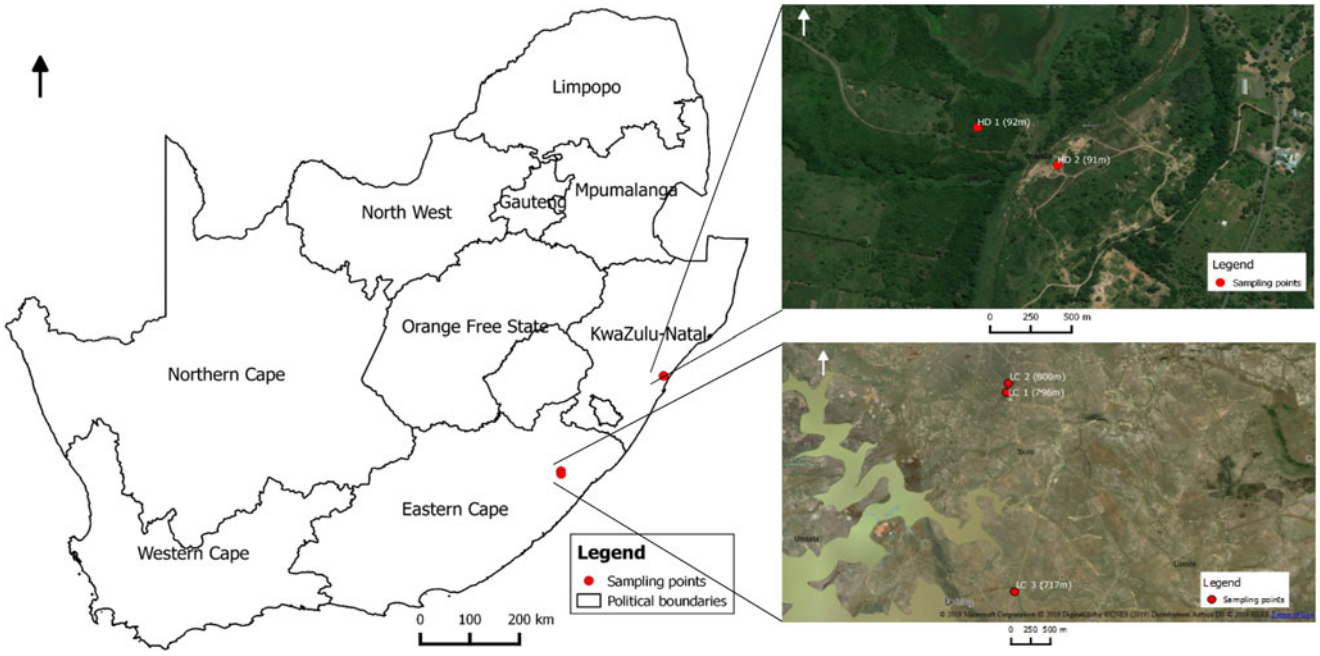

Fig. 1. Map showing locations were *Lymnaea (Pseudosuccinea) columella* were collected from KwaZulu-Natal and Eastern Cape provinces of South Africa.

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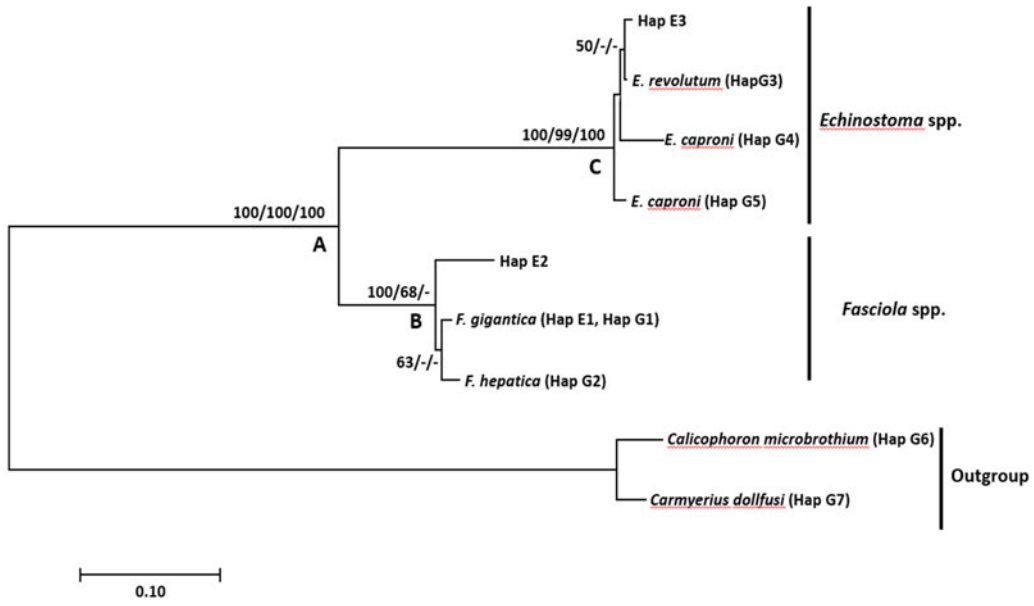

Fig. 2. Neighbour-joining tree based on the nuclear ribosomal ITS-1 region illustrating the relationship between *Fasciola gigantica, F. hepatica and Echinostoma* spp. from *Lymnaea (Pseudosuccinea) columella* (Hap E 1–3) collected from Eastern Cape and KwaZulu-Natal provinces of South Africa, and the close matches from the NCBI GenBank and outgroups (G 1–7). Nodal support values are shown in the order of neighbour-joining bootstrap value, Bayesian inference probability and maximum likelihood bootstrap value.

snails, and distinguish *Fasciola* species within the snails, the ITS-1 region of *Fasciola* spp. was amplified using the primers FascF: 5′-ACCGGTGCTGAGAAGACG-3′ and FascR: 5′-CGACG TACGTGCGTCCA-3′ (Rokni *et al.*, 2010). Polymerase chain reaction (PCR) amplification was performed in a 25 µl reaction volume, each containing 9.5 µl sterile water, 12.5 µl PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µl (10 µM) of each primer and 2 µl of genomic DNA. Amplification was performed under the following thermocycling conditions: 95°C for 5 minutes as initial denaturation, followed by 40 cycles of (denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 minute), and lastly 72°C for 7 minutes as final extension. Fragments were separated by 2% agarose gel electrophoresis stained with ethidium bromide, then visualized and photographed in a transilluminator (BIORAD).

Sequencing and sequence analysis

To confirm the identity of *Fasciola* spp., amplicons from positive snails were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The obtained sequences were assembled, edited and aligned with rDNA homologue sequences obtained from the GenBank database with Clustal W (Thompson *et al.*, 1997) using the BioEdit program (Hall, 1999). DnaSP (v 5.10.1) (Rozas *et al.*, 2003) was used to generate and determine the number of haplotypes generated by the dataset. The most appropriate model test of nucleotide substitution for neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference analyses were selected using jModeltest (Posada, 2008). The SYM model was selected under the AIC information criterion. Neighbour-joining and maximum likelihood trees were generated using PAUP* 4.0 (Swofford, 2002). The nodal support for both methods was estimated using 1000 bootstrap pseudo-replicates. Bayesian inference was carried out in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Four Markov chains were run for 5 million generations to ensure that the standard deviation of the split frequencies was < 0.01. The first 500,000 trees were discarded as burn in, after an initial run to check that this was sufficient to achieve stationarity.

Results

All 100 snails from the five locations screened for infection by shedding of cercariae were negative. When PCR was conducted based on an ITS-1 marker designed specifically for identification of *Fasciola* spp., all the 100 snails (20 per site) were positive (table 1), and positivity was denoted by a band of approximately 460 base pairs. Blast analysis of five sequenced samples per location (n = 25) showed that sequences from snails collected from the LC1, LC2 and LC3 locations in Mthatha area gave a 99% homology with a complete appropriate region of the ribosomal nuclear sequence of *F. gigantica* (accession number MG201868.1) (table 1). Furthermore, four snails from the HD1 and HD2 locations in Verulam area harboured *F. gigantica* and one snail in each location had *Echinostoma* spp., with 93–99% homology for *F. gigantica* (accession number MG201868.1) and 99% homology with *Echinostoma revolutum* (table 1).

The *F. gigantica* sequences from this study were 420 nucleotide bases long and homogenous except for one *Fasciola* species from HD2 in Verulam area, KwaZulu-Natal province. The haplotype analysis yielded nine haplotypes consisting of the samples derived from GenBank and isolates from our study (table 2). Haplotype one (Hap E1) was the most common, comprising 22 isolates from this study and six *F. gigantica* isolates from GenBank (table 2). Phylogenetic analysis showed that the outgroup formed a strongly supported monophyletic clade (clade A) with the ingroup samples (fig. 1). Clade A further showed a distinction between the *Fasciola* species (clade B) and *Echinostoma* species (clade C). Clade B, which was strongly supported by neighbour-joining and moderately supported by maximum likelihood,

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Table 1. Percentage similarities of *Fasciola* and *Echistostoma* species obtained intra *Lymnaea (Pseudosuccinea) columella* from selected locations in KwaZulu-Natal and Eastern Cape provinces, and their closest GenBank-derived isolates.

					Species identity		
Province	Sampling site(s)	N	No. positive	No. sequenced	Species	N isolates	% Similarity
KwaZulu-Natal	HD 1	20	20	5	*F. gigantica* *E. revolutum*	4 1	93–99% 99%
	HD 2	20	20	5	*F. gigantica* *E. revolutum*	4 1	93–99% 99%
Eastern Cape	LC 1	20	20	5	*F. gigantica*	5	99%
	LC 2	20	20	5	*F. gigantica*	5	99%
	LC 3	20	20	5	*F. gigantica*	5	99%

HD 1, Hazelmere dam location 1; HD 2, Hazelmere dam location 2; LC 1, Lucingweni location 1; LC 2, Lucingweni location 2; LC 3, Lucingweni location 3

Table 2. Haplotype status of *Fasciola* spp. and *Echinostoma* spp. from Verulam area in KwaZulu-Natal, and Mthatha area in Eastern Cape, and the GenBank-derived isolates.

Haplotype	Number of isolates	Samples	Species identity
Hap E1	22	E1.1, E1.2, E1.3, E1.4, E1.5, E2.1, E2.2, E2.3, E2.4, E2.5, E3.1, E3.2, E3.3, E3.4, E3.5,KZ1.1, KZ1.2, KZ1.3, KZ1.4, KZ2.3, KZ2.4,KZ2.5	*F. gigantica*
Hap E2	1	KZ2.1	*Fasciola* sp.
Hap E3	2	KZN 1.5, KZN 2.2	*Echinostoma* sp.
Hap G1	6	AJ853848.2, KT182349.1, KT182368.1, MH7903261.1, MF969015.1, JF496715.1	*F. gigantica*
Hap G2	3	KT182407.1, KT182400.1, KT182387.1, MG201868.1, MF969010.1	*F. hepatica*
Hap G3	1	KF425322.1	*E. caproni*
Hap G4	1	U58102.1	*E. revolutum*
Hap G5	1	U58098.1	*E. caproni*
Hap G6	1	KP639638.1	*Calicophorum microbrothium*
Hap G7	1	KP639636.1	*Carmyerius dollfusi*

E, experimental samples; G, GenBank-derived isolates; E, Eastern Cape province; KZ, KwaZulu-Natal province

included all *Fasciola* species. Within this clade (B), there was a distinction between *F. gigantica* and *F. hepatica* isolates, which formed a moderately supported monophyletic clade by neighbour-joining, with isolate Hap E2 (KZ2.1) from HD2 location in Verulam area branching out forming a sister clade to the two *Fasciola* species. A strongly supported clade C consisted of two isolates (Hap E6), each from HD1 and HD2 locations in Verulam area and three *Echinostoma* isolates from GenBank, namely *E. caproni* (KF425322.1, U58098.1) and *E. revolutum* (U58102.1).

Discussion

The current study demonstrated a high rate of natural infection of *L.* (*P.*) *columella* with *F. gigantica* from Mthatha area in Eastern Cape and Verulam area in KwaZulu-Natal provinces in South Africa. Based on reports elsewhere this invasive lymnaeid species acts as an intermediate host of both *F. gigantica* and *F. hepatica* (Bargues and Mas-Comas, 2005). Confirmation of presence of this snail species in South Africa led to the assumption that the observed increase in the prevalence of fascioliasis was due to the introduction of *L.* (*P.*) *columella* (De Kock *et al.*, 1989), and no information is available confirming natural infection of *L.* (*P.*) *columella* with *F. gigantica*. With both *Fasciola* species occurring in South Africa and *F. hepatica* being reported as the most prevalent species in KwaZulu-Natal by Mucheka *et al.* (2015), it is surprising that none of the *P. columella* snails collected from the two locations were infected with *F. hepatica*.

Lymnaea (*Pseudosuccinea*) *columella* is widely distributed in South Africa, ranking as the third most distributed freshwater snail in the country, surpassed only by *L.* (*R.*) *natalensis* and *Bulinus tropicus* (De Kock *et al.*, 1989). Furthermore, this species has been found to occur frequently in the same habitats as *L.* (*R.*) *natalensis* (De Kock *et al.*, 1989; Wolmarans and De Kock, 2006; Kemp *et al.*, 2016) and *B. tropicus* (De Kock *et al.*, 1989). The ability of *L.* (*P.*) *columella* to adapt and tolerate various ecological and climatic conditions and to coexist with *L.* (*R.*) *natalensis* (main IH of *F. gigantica*) allows the snail to transmit the two *Fasciola* species (De Kock *et al.*, 1989). The invasive nature of this lymnaeid species, and its ability to invade in areas with summer and winter rainfall, cold highveld regions such as the Transvaal area and Free State, and the hot subtropical regions of the Transvaal Lowveld and KwaZulu-Natal (De Kock *et al.*, 1989), gives this species advantage over the natural intermediate hosts of both *F. hepatica* (*G.* (*L.*) *truncatula*) and *F. gigantica* (*L.* (*R.*) *natalensis*). Hence, the high rate of infection of *L.* (*P.*) *columella* with *F. gigantica* as shown in this study is an indication of the significant role played by this snail species in extending the geographical distribution of the parasite in South Africa.

Results showed that isolates of *F. gigantica* from our study formed one haplotype with the GenBank-derived isolates. The phylogenetic tree showed that *F. hepatica* formed a moderately supported sister clade to *F. gigantica*. This is similar to the

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findings of Mucheka *et al.* (2015), although these were strongly supported. The sequence analysis supported the identification of 22 isolates—15 from Mthatha and seven from Verulam (4 from HD1 and 3 from HD2)—as *F. gigantica*, and one isolate (Hap E7) from HD2 as *Fasciola* sp., which formed a separate subclade from *F. gigantica* and *F. hepatica*. Furthermore, the isolate is separated from *F. gigantica* and *F. hepatica* by a mean genetic distance of 4.61% and 6.58%, respectively. Although this isolate seemed to be closely related to *F. gigantica* based on BLAST analysis with genetic *p*-distance, eight mutations were found between the isolate and *F. gigantica* in positions 79, 133, 195, 196, 197, 368, 376 and 377 of the alignment. The formation of a separate subclade of this isolate from the two *Fasciola* species leads to the suggestion of the possibility of a hybrid between the two species.

Besides *F. gigantica*, *Echinostoma* sp. was also detected in two *L.* (*P.*) *columella* snails from HD1 and HD2 locations in Verulam, KZN. These isolates formed a strongly supported clade with *E. caproni* (KF425322.1, U58098.1) and *E. revolutum* (U58102.1). Within this clade, this isolate formed a weakly supported subclade with *E. revolutum*. Genetic distance between the two *Echinostoma* isolates from our study (Hap E3) and the GenBank isolates further confirmed the close relatedness between our isolates (Hap E3) and the GenBank isolates, which showed a genetic distance of 0.658% and 30.0% between our isolates (Hap3) and *E. revolutum* and *E. caproni*, respectively. The detection of *Echinostoma* sp. correlates with the report by Grabner *et al.* (2014), who detected natural infection of *F. gigantica*, *Echinostoma caproni* and unidentified *Echinostoma* sp. in *L.* (*P.*) *columella* in Egypt using PCR. Lymnaeids act as the first IH of *E. revolutum*, whereas *E. caproni* utilize *Biomphalaria* and *Bulinus* spp. as first intermediate hosts (Fried and Huffman, 1996), and most likely use *L.* (*P.*) *columella* as the second intermediate host (Fried and Huffman, 1996; Grabner *et al.*, 2014). Nonetheless, infection of *L.* (*P.*) *columella* with *Echinostoma* spp. has not been reported in South Africa and, to the best of our knowledge, this is the first report in the country.

In conclusion, our study confirmed that *L.* (*P.*) *columella* acts as an intermediate host of *F. gigantica* and *Echinostoma* sp. in South Africa. This is the first study reporting natural infection of *L.* (*P.*) *columella* with *F. gigantica* and *Echinostoma* sp. in South Africa. Although the susceptibility of *L.* (*P.*) *columella* to *F. gigantica* has been reported elsewhere, this is the first report confirming natural infection in South Africa. Given the invasive nature of *L.* (*P.*) *columella* (Appleton, 2003), further studies are needed to determine the role of this snail in extending the geographical distribution of *F. gigantica* and *F. hepatica* in South Africa. Furthermore, there is a need for a countrywide survey of this invasive species to assess the geographical distribution and infection status with *F. gigantica* and *F. hepatica* in various geographical regions of South Africa and also carry out laboratory-based experiments to compare the IH competence between *L.* (*P.*) *columella* and *L.* (*R.*) *natalensis* in areas where the two species coexist.

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Conflict of interest. None.

Ethical standards. Experimental protocols for this study were reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (Ref: AREC/044/016D) in accordance with the South African national guidelines on animal care, handling and use for biomedical research.

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