Molecular identification of helminth parasites of the Heterakidae and Ascarididae families of free-ranging chickens from selected rural communities of KwaZulu-Natal province of South Africa

Silindokuhle Mlondo ,* Danisile Tembe,* Mokgadi P. Malatji,*,† Zamantungwa T. H. Khumalo,‡,§ and Samson Mukaratirwa *,#,1

*School of Life Science, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Durban 4001, South Africa; †Foundational Research and Services, South African National Biodiversity Institute, Pretoria 0001, South Africa; ‡Clinvet International, Study Operations, Bloemfontein, 9338, South Africa; §Department of Veterinary Tropical Diseases, University of Pretoria of Pretoria, Pretoria, 0110, South Africa; and #One Health Centre for Zoonoses and Tropical Veterinary Medicine, Ross University School of Veterinary Medicine, Basseterre, St Kitts, West Indies

ABSTRACT Free-range chickens are predisposed to diverse parasitic infections during scavenging. Accurate identification of these parasites using morphological characters has been a challenge. Therefore, this study aimed to identify nematodes from the Heterakidae and Ascarididae family infecting free-ranging chickens from KwaZulu-Natal province of South Africa using a combination of morphological and molecular techniques. Forty-two free-ranging adult indigenous chickens were purchased from randomly selected households in Shongweni (n=12), Umzinto (n=10), Gingindlovu (n=10) and Ozwathini (n=10) rural villages and examined for nematodes of the Heterakidae and Ascarididae family. Collected specimen were identified morphologically and confirmed using mitochondrial and nuclear ribosomal markers. Results showed that *Ascaridia galli* was common, occurring at all sampling locations with an overall prevalence of 58.3%, while *Heterakis gallinarum* and *H. beramporia* occurred in three locations. *Ascaridia galli* had high prevalence in Shongweni (58.3%), followed by Gingindlovu (40%), Ozwathini (20%) and Umzinto (10%). *Heterakis gallinarum* infection was prevalent in three locations, with an overall prevalence of 90% in Gingindlovu, 80% in Ozwathini and 58.3 % in Shongweni. *Heterakis gallinarum* and *H. beramporia* were not recorded in Umzinto. *Heterakis beramporia* was recorded in low prevalence in Gingindlovu (20%), Ozwathini (10%) and Shongweni (8.3%) villages. Mixed infections of *A. galli* and *H. gallinarum* were recorded in Gingindlovu, Ozwathini and Shongweni, and *H. gallinarum* and *H. beramporia* in Gingindlovu. Molecular analysis confirmed identification of *A. galli*, and further showed close relationship with the GenBank-derived South African isolates. Haplotype network further confirmed their ancestral history, where all South African *A. galli* isolates formed five novel haplotypes corresponding with the structure of the phylogenetic tree. Similar structure was observed with *Heterakis* isolates, where analysis of the *cox1* gene showed that *H. gallinarum* formed a well-supported monophyletic clade with other *Heterakis* species. The ITS marker identified three specimens from Gingindlovu, Ozwathini and Shongweni as *H. beramporia*, which formed strongly supported sister clade to *H. indica* and this is the first report confirming the occurrence of *H. beramporia* in South Africa.

Key words: free-range chickens, gastrointestinal helminth, Ascarididae, Heterakidae, South Africa

2022 Poultry Science 101:101979
https://doi.org/10.1016/j.psj.2022.101979

INTRODUCTION

Poultry significantly contributes to food security, poverty reduction, and ecological utilization of natural resources (Guéye, 2003). It has been estimated that of the 14.718 billion of poultry reared globally, approximately 11.038 billion of this population is found in developing countries (Minga, 1989; FAO, 2004). According to

Received December 20, 2021.
Accepted May 25, 2022.

[1]Corresponding author: smukaratirwa@rossvet.edu.kn

Melewas (1989), a large proportion of these poultry populations are kept under a traditional free-range production system (Maxwell et al., 2016), where they play a significant role in pest control and socioeconomic functions in traditional and religious rituals of the rural households (Mafu and Masika, 2003; McAinsh et al., 2004; Mtileni et al., 2012).

Saha (2003) reported that free-range chickens have poor productivity, low economic returns, improper housing, poor marketing strategies, and veterinary care. Through scavenging, free-range chickens feed on what they obtain from the environment such as insects, seeds, and other types of offal (Permin and Pederson, 2002). As a result, they become exposed to a wide range of bacteria, viral, and parasitic pathogens (Oniye et al., 2001; Swatson et al., 2002). Although high morbidity rates in free-range chickens are due to parasitic infections (Maxwell et al., 2016), viral, bacteria, and protozoan infections remain more economically significant to poultry farmers (Phiri et al., 2007) and the most studied as compared to parasitic infections (Ssenyonga, 1982; Katoch et al., 2012).

Parasitic infections in free-ranging chickens have an estimated prevalence of up to 100% (Permin and Hansen, 1998; Maxwell et al., 2016). The most reported gastrointestinal parasites include coccidia, cestodes and nematodes (Mwale and Masika, 2011), and mixed infections of 2 or more species of parasites in free-ranging chickens are very common (Permin and Pedersem, 2002; Maxwell et al., 2016). Nematodes are the most significant gastrointestinal parasites due to the large number of species and their wide geographical distribution (Bahz, 2013). In South Africa, Mukaratirwa and Khumalo (2010) reported a high prevalence of *Heterakis gallinarum* followed by *Ascaridia galli* with a prevalence range of 80.0 to 94.4% and 22.2 to 43.8% from free-range chickens in selected rural communities of KwaZulu-Natal province, respectively. Furthermore, Mwale and Masika (2011) reported an overall prevalence of *H. gallinarum* (Qolora = 25.72%, Nontshinga = 27.14%) and *A. galli* (Qolora = 14.28%, Nontshinga = 31.43%) in 2 villages of the Eastern Cape province of South Africa.

According to Oliveria et al. (2011), morphological approaches have been used vastly in the taxonomy of gastrointestinal nematode species. However, identifying nematodes to species level using morphological techniques alone is challenge due to their varying microscopic size, morphological similarity, finite number of distinguishable classification characters, and intersecting morphometry, which may lead to misidentification (Madsen, 1950; Tarbiat et al., 2015). Therefore, molecular based approaches have been developed and increasingly used to overcome these challenges and provide an avenue to understand the epidemiology, phylogeny, and population genetic structure of gastrointestinal nematodes (Zhou et al., 1998; Newton et al., 2002; Gasser, 2006; Bazh, 2013) at the same time ensuring correct identification of species.

Although several gastrointestinal nematodes of free-range chickens have been identified in different regions of South Africa including *A. galli, H. gallinarum*, and *Capillaria* species (Mwale and Masika, 2011; Thekisoe et al., 2003; Malatji et al., 2016), these findings cannot be used to reference beyond region of their study sites/area. This is because gastrointestinal nematode species differ across geographic areas. Furthermore, there is scarcity of gastrointestinal nematodes studies utilising molecular identification to species level (Malatji, 2017). The intergeographic diversity of nematodes and the paucity of molecular studies are main hindrances for correct control measures. Therefore, molecular characterization of the gastrointestinal nematodes from different ecological zones is a necessity and can be useful in development of effective control measures within South African provinces and across other countries (Malatji et al., 2016). Against this background, this study aimed at identifying gastrointestinal nematodes of free-range chickens from the Heterakidae and Ascarididae family occurring in four rural localities of KwaZulu-Natal province using a combination of morphological and molecular approaches.

MATERIALS AND METHODS

Study Areas and Sample Collection

Free-range chickens were purchased from four rural localities of KwaZulu-Natal, South Africa, namely, Shongweni, Umzinto, Ozwathini, and Gingindlovu (Figure 1). Shongweni and Umzinto are situated at the South Coast, whereas Gingindlovu and Ozwathini are located at the North Coast of KwaZulu-Natal. The south and north coast receive an average annual rainfall of 864 mm and 823 mm, respectively, with most rainfall occurring mainly during mid-summer (January to February). The chickens were reared by rural livestock and crop farmers for subsistence purposes.

A total of 42 free-range adult chickens were randomly purchased from households in Shongweni (n = 12), Umzinto (n = 10), Gingindlovu (n = 10) and Ozwathini (n = 10). Chickens were caged and transported to the Biomedical Research Unit (**BRU**) of the University of KwaZulu-Natal, Westville Campus, South Africa. Chickens were housed for 5 d after purchase in the BRU, with room temperature maintained at 25 ± 1°C and fed with chicken feed (Epol (PTY) Ltd, South Africa) and water ad libitum. They were maintained in their cages with flooring covered with sawdust for moisture absorption. The chickens were guillotined under supervision of a veterinarian to minimize pain and allow easy recovery of parasites from the gastrointestinal tract.

Isolation of Parasites

After slaughter the chickens were eviscerated and the small intestines and ceca regions were removed from the gastrointestinal tract using a thumb forceps, scalpel blades, and a pair of scissors. The small intestines were slit open, and contents washed with distilled water through a metal sieve with an aperture of 63 μm to recover helminth

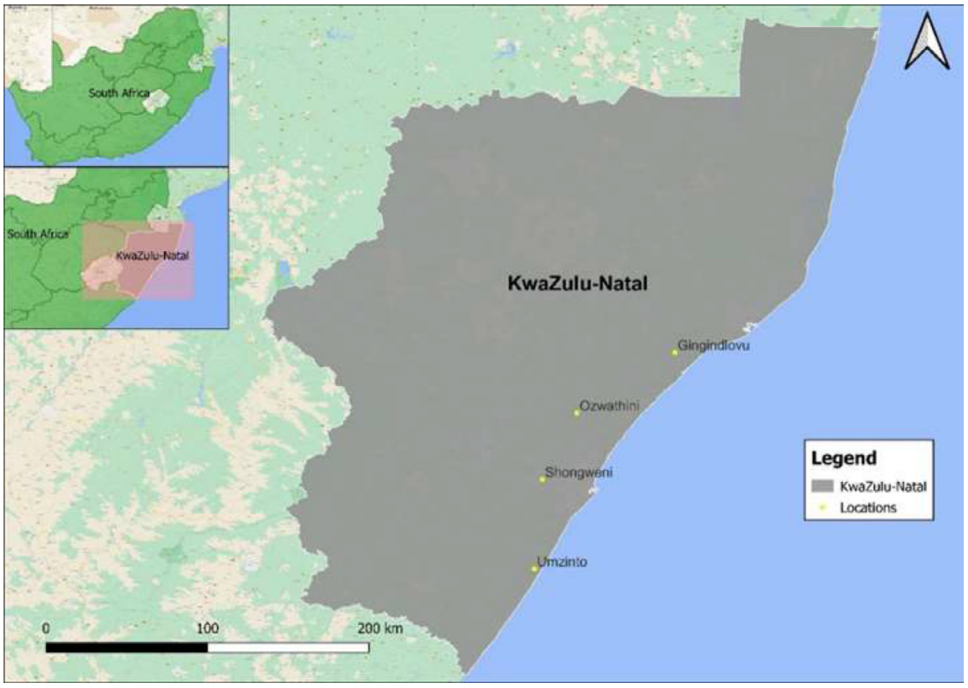

Figure 1. Map showing sampled rural communities in KwaZulu-Natal province of South Africa.

parasites. Parasites were then preserved in 70% ethanol for morphological and molecular analysis.

Microscopic Examination of Parasites

Gastrointestinal nematodes recovered from the chickens were rinsed with tap water to remove excess ethanol from the preservation. Nematodes were observed under a light microscope at ×10 magnification to distinguish between families and genera following the identification keys described by Anderson et al. (1974) and nematodes were counted for each individual chicken to determine the intensity of infection. Nematodes were then fixed in 3% buffered glutaraldehyde for 1 h in preparation for scanning electron microscope (**SEM**). GIT nematodes were then processed following instructions described by Lalchhandama (2010) and Lalchhandama (2011). Specimens were dehydrated in ascending series of graded ethanol (50, 70, 80, 90, 100%), for 10 min for each concentration, followed by subjecting them to Critical Point Drying (**CPD**). During CPD, ethanol was replaced with liquid CO_2, which was heated and pressurized to its critical point, at which the liquid was converted to gas without damaging effects of surface tension of the samples. This resulted in dry and intact specimens which were mounted onto SEM stubs. The specimen stubs were transferred to the sputter coater, to make them conductive to the electron beam. The coated samples were viewed with the SEM and morphological characters such as spicules, number of pairs of papillae, and postanal suckers were examined and measured. Identification using morphological characters was done using combination of primary literature and standard taxonomic references including Anderson et al. (1974), Chabaud (1978), and Soulsby (1982).

DNA Extraction and Amplification

DNA was extracted from individual *Heterakis* spp. and *Ascaridia* sp. specimens using the Genomic DNA Tissue MiniPrep kit (Zymo Research Corporation, Irvine, CA 92164, USA) according to the manufacturer's instructions. The mitochondrial *cox1* region of *Ascaridia* sp. was amplified using species-specific primers *gcox1* (4F: 5'-ATTATTACTGCTCATGCTATTTTGATG-3′ and 4R: 5-CAAAACAAATGTTGA AAAT-CAAAGG-3') (Katakam et al., 2010) under the following conditions: 10 min initial denaturation step at 95°C, followed by 35 cycles of 30 s of denaturation at 95°C, 40 s of annealing at 55°C, 30 s of polymerization at 72°C and 10 min of final polymerization at 72°C. Thermal cycling of *Heterakis* species were amplified based on the mitochondrial and ribosomal nuclear markers using the primers *cox1* (F: 5'-TTTCATACAGAATAAATAT-CAGGA-3' and R: 5'-AGTTCTAATCATAAGGA-TATTGGGA-3') (Amor et al., 2018) and ITS (ITS-1F: 5′-TTTCCGTAGGTGAACCT-3′ and ITS-2R: 5′-TCCTCCGCTTAGTGATA-3′) (Davidson et al., 2012), respectively. Thermal cycling of the ITS was set at 3 min of initial denaturation at 94°C, followed by 35 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 55°C, 1 min polymerization at 72°C, and lastly final polymerization for 10 at 72°C. Thermocycling conditions for the *cox1* included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 45 s, and lastly final extension of 72°C for 10 min. PCR amplification reactions were performed in a 25 μL volume, containing 12.5 μL of 2X DreamTaq PCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA), 5 μL of DNA, 1 μL of each primer (forward and reverse) and 5.5 μL of nuclease-free water.

Fragment was separated on a 1.5% agarose gel stained with ethidium bromide. Successful amplicons were sent to Inqaba Biotechnical Industries, Pretoria (South Africa) for Sanger sequencing of both forward and reserve directions using the same amplification primers.

Sequence and Phylogenetic Analysis

Sequences were assembled, edited, and aligned with homologue sequences obtained from GenBank database using BioEdit program (Sequence Alignment Editor version 7.2; Hall, 1999). Aligned sequences were trimmed to common length of 393 bp for *gcox1* (*Ascaridia*), 600 bp for *cox1* (*Heterakis*), and 370 bp for the ITS gene (*Heterakis*). The appropriate model of nucleotide substitution to use in maximum likelihood and neighbor-joining analyses were selected using MEGA v7.0.26 software (Kumar et al., 2016). Hasegawa models: HKY+G (*gcox1*), HKY+G (*cox1*), and HKY (ITS) were selected as the best fit models of nucleotide substitution to use for analyses. Maximum likelihood and Neighbor-joining trees were generated using MEGA (Kumar et al., 2016). For both methods, the phylograms were 50% majority-rule consensus trees and the nodal support was estimated using 1,000 bootstrap pseudo-replicates. DnaSP (v 5.10.1) (Rozas et al., 2003) was used to determine the number of haplotypes generated by the dataset and estimate the haplotype diversities. Haplotype networks were constructed using Network 5 (Bandelt et al., 1999) and the median joining rooting method under default (10) weight and with epsilon (ε) set to 0.

Data Analysis

Data of the collected parasites was recorded on Microsoft Excel. Prevalence of infection (%) was calculated using the following formula: Prevalence = (Total number of infected chickens by a species of parasite/total number of chickens examined) × 100. Intensity of infection was estimated as the number of worms collected for each species on each chicken.

RESULTS

Prevalence of Gastrointestinal Nematodes

Twenty-eight of 42 (66.7%) chickens were infected by gastrointestinal helminth species from the family Ascarididae and Heterakidae. *Heterakis gallinarum* had the highest overall prevalence by species (57.1%), as compared to *A. galli* (33.3%) and *Heterakis beramporia* (9.5%; Table 1). Results showed that *A. galli* was the most common nematode species, found in all 4 sampling locations with prevalence of 58.3% in Shongweni, 40% in Gingindlovu in the North Coast, 20% in Ozwathini, and 10% in Umzinto (10%) in the South Coast region (Table 1). *Heterakis* species infections were recorded in 3 locations, with highest prevalence of *H. gallinarum* infections recorded in Gingindlovu (90%), followed by Ozwathini (80%) in the North Coast and Shongweni (58.3%). Prevalence of *H. beramporia* infection was high in Gingindlovu (20%), followed by Ozwathini (10%) and the least prevalence was recorded in Shongweni (8.3%). There was no *Heterakis* species infections recorded from Umzinto village. Mixed infections by Heterakidae and Ascarididae species accounted for 23.8% (10/42) as compared to 42.8% (18/42) single infections by each species. Mixed infections of *A. galli* and *H. gallinarum* were recorded in Shongweni (3/12, 25%), Gingindlovu (4/10, 40%), and Ozwathini (5/10, 50%). Mixed infection between the three parasites was recorded in only 10% (1/12) from Shongweni.

Out of 42 chickens examined, females accounted for 54.8% (23/42) and 45.2% (19/42) were males. In the South Coast, Umzinto did not show any association of sex and parasitic infection, however, females showed higher prevalence of *A. galli* (41.7%) and *H. gallinarum* (50%) as compared to males (10 and 8.2%) in Shongweni (Table 1). In the North Coast, infection of *A. galli* and *H. gallinarum* was more in males. The prevalence of *A. galli* in males was 30% compared to 10% in females in Gingindlovu. There was no observed difference in the prevalence of *A. galli* between sex in Ozwathini. The prevalence of *A. galli* infection in female and male chickens in this area was 10%. However, the prevalence of *H. gallinarum* infections in Gingindlovu was higher in males (60%) than in females (30%). In Ozwathini, the prevalence *of H. gallinarum* was also high in males (50%) than females (30%).

Table 1. Prevalence of Heterakidae and Ascarididae species from free-range chickens in rural communities of KwaZulu-Natal, South Africa.

			A. galli			*H. gallinarum*			*H. beramporia*		
Region/Coast	Study location	N	M (%)	F (%)	Overall (%)	M (%)	F (%)	Overall (%)	M (%)	F (%)	Overall (%)
South	Shongweni	12	1 (16.6)	6 (41.7)	7 (58.3)	2 (8.3)	5 (50)	7 (58.3)	0	1 (8.3)	1 (8.3)
South	Umzinto	10	1 (10)	0	1 (10)	0	0	0	0	0	0
North	Gingindlovu	10	3 (30)	1 (10)	4 (40)	6 (60)	3 (30)	9 (90)	1 (10)	1 (10)	2 (20)
North	Ozwathini	10	1 (10)	1 (10)	2 (20)	5 (50)	3 (30)	8 (80)	0	1 (10)	1 (10)
	Total	42			14 (33.3)			24 (57.1)			4 (9.5)

Abbreviations: F, female; *A. galli, Ascaridia galli; H. gallinarum, Heterakis gallinarum; H. beramporia, Heterakis beramporia*; M, male.

Intensity of Infection

Results shows that majority of the infected chickens had worm intensity range of 1 to 50 worms per chicken for *A. galli, H. gallinarum*, and *H. beramporia* (Table 2). The intensity for *A. galli*, was low (1−50) in 15 birds, with only one chicken which harbored worms ranging between 51 and 100. All chickens infected with *H. beramporia* showed a worm burden range of 1 to 50. The intensity of infection was the highest with *H. gallinarum*, where one chicken harbored more than 100 worms, while 4 chickens harbored worms ranging from 51 to 100 and 15 chickens had 1 to 50 worms.

Morphological Identification

Ascaridia Galli *Ascaridia galli* adult worms were observed as yellowish white and translucent nematodes. The males were shorter than females, measuring between 58 and 60 mm in length. The mouth of *A. galli* consisted of 3 conspicuous lips. The males had 2 unequal spicules at the posterior portion, with the right spicules measuring 1.30 to 1.70 mm in length and the left spicule measuring 1.10 to 1.50 mm. Males possessed a well-developed precloacal sucker that is oval and one pair of postanal papillae. The tail of males was curly with a terminal tip that was partially expanded at the base. Caudal papillae were located on the ventral side of the tail next to the anus and the extreme terminal tip was sharply pointed. These morphological features matched with the morphological structures of *A. galli* (Ramadan and Abouznada, 1992).

Heterakis Gallinarum *Heterakis gallinarum* adult worms were white in color and the male worm was longer than the female parasite. The anterior portion of the mouth of both female and male specimens was encompassed by 3 prominent lips. The lips were shrunk, collapsed, and depressed at the center. The male had unequal spicules, with the right spicule measuring 0.40 to 0.57 mm in length and the left spicule measuring 0.83 to 0.96 mm. The precloacal sucker of the male was round, well-developed, and encased in a chitinized ring. The tail of the male was slender and tapered toward the posterior portion of the worm. These characters corresponded to the description of *H. gallinarum* (Bobrek et al., 2019).

Heterakis Beramporia *Heterakis beramporia* and *H. gallinarum* are similar in size, length and were both isolated from the ceca. Both these species possessed identical features such as the colour, lips, precloacal sucker, and the tail, but differed in the size and shape of the spicules when examined under a microscope. This further confirms the importance of molecular diagnostics to distinguish species.

Table 2. Overall prevalence and intensity of *Ascaridia galli, Heterakis gallinarum* and *H. beramporia* infection in free-range chickens from rural communities of KwaZulu-Natal (n = 42).

	Intensity of infection (worms)			
Parasite species	1−50	51−100	>100	Prevalence (%)
A. galli	19	1	0	20 (47.62)
H. gallinarum	15	4	1	20 (47.62)
H. beramporia	11	0	0	11 (26.19)
Total	45	5	1	

Sequence, Phylogenetic, and Haplotype Analyses

Ascaridia Galli Amplification of these *Ascaridia* specimens was only successful with *gcox* primers. Molecular analyses confirmed the identification of all *Ascaridia* specimens as *A. galli*, showing a homology of 99 to 100% with isolates from South Africa (KT388440.1, KT388438.1) and were submitted to GenBank under the accession numbers OL457049-OL457072. Phylogenetic analysis showed that all *A. galli* isolates formed a well-supported monophyletic with GenBank *A. columbae* isolates from China (Figure 2). Within the *A. galli* clade, all isolates from this study formed a weakly supported clade with all *A. galli* isolates from GenBank from South Africa and China (KT613902.1), which is a sister clade to other GenBank *A. galli* isolates from Ghana (MW243594.1) and Italy (FM178545.1). Furthermore, analysis showed that our study isolates formed paraphyletic clades, which were not unique to locations. Molecular results confirmed the presence of *A. galli* in all 4 sampling sites; occurring as the only species in Umzinto area, and in conjunction with *Heterakis* species in the other three locations, where co-infections of *A. galli* + *H. gallinarum* and *H. gallinarum* + *A. beramporia* were also observed.

Haplotype analysis based on 393 nucleotides of *gcox1* primes yielded 9 haplotypes (Supplementary Table 1, Supplementary Figure 1), with a moderate haplotype diversity of 0.7892. The structure of the haplotype network resembled that of phylogenetic tree. Haplotype distribution showed that isolates from this study formed 5 novel haplotypes, which were unique to South Africa, but not specific to locality of study. Haplotypes (H_1, H_2, H_3, H_5) were the most common haplotypes, consisting of ≥3 isolates in each haplogroup. Haplotype H_1 consisted of 3 isolates; 2 isolates from Shongweni and Umzinto and one *A. galli* GenBank isolate from South Africa (KT388440.1). Haplotype H_2 consisted of 13 isolates; 2 isolates from Shongweni, 6 from Ozwathini, 4 from Gingindlovu and one GenBank isolate from South Africa (KT388438.1). Haplotype H_3 consisted of 2 isolates from Shongweni, one isolate from Umzinto, and 2 isolates from Gingindlovu. Haplotype H_5 was composed of 3 isolates from Umzinto and one isolate from Gingindlovu. Haplotypes H_7, H_8 and H_9 each contained one *A. galli* GenBank isolates from China (KT613902.1), Ghana (MW243594.1), and Italy (FM178545.1), respectively. Haplotype H_4 was the only haplotype consisting of one isolate, from Shongweni. This haplotype separated from the other South African haplotypes by 3 mutational steps from H_1 at positions 30, 117, 137; 5 mutational steps from H_2 at

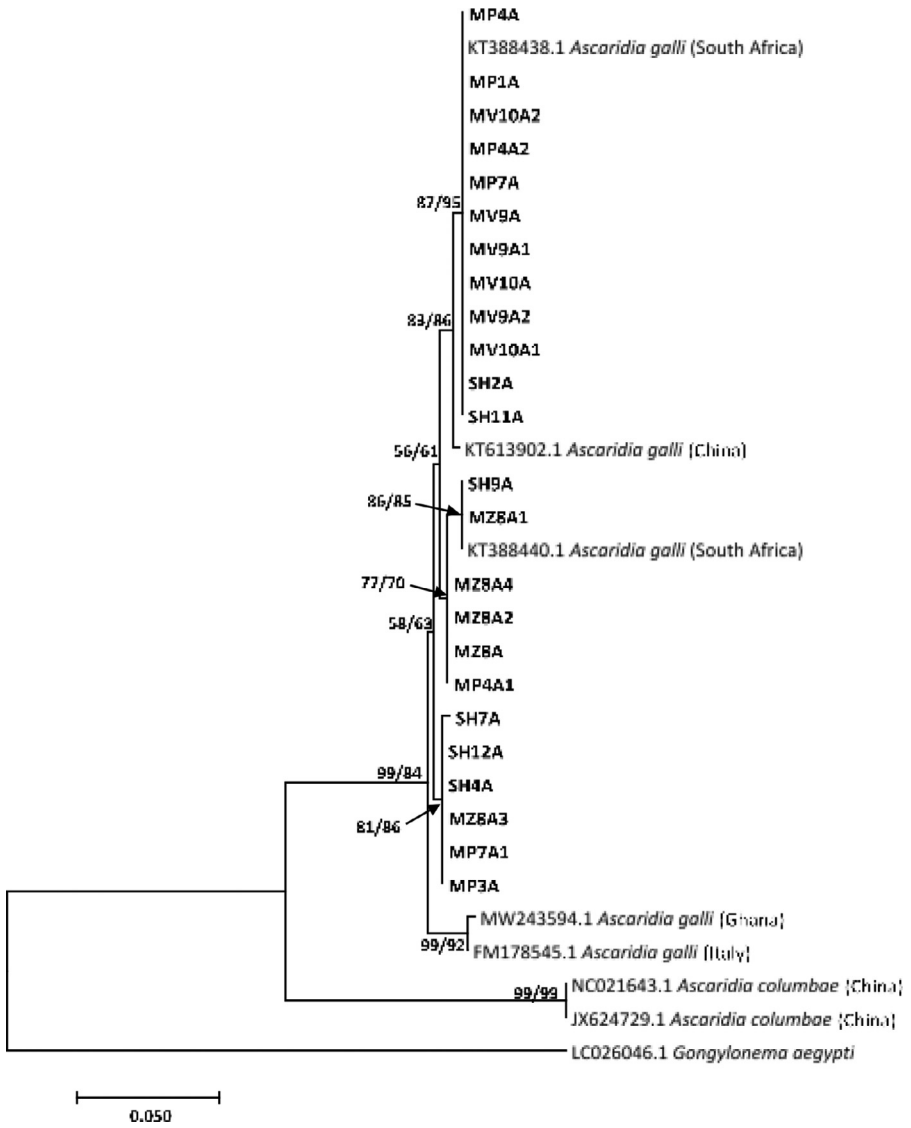

Figure 2. Neighbor-joining tree based on the 393 nucleotides of the mitochondrial GCOX gene illustrating the relationship between Ascaridia galli isolates from KwaZulu-Natal province of South Africa (in bold), and the closest matches from the NCBI GenBank. The nodal support values indicated in the order: maximum likelihood and neighbor-joining. Abbreviations: MP, Gingindlovu; MV, Mvoti; MZ, Mzinto; SH, Shongweni.

positions 7, 30, 117, 137, 228; three mutational steps from H_3 at positions 30, 132, 177, and three mutational step from H_1 at positions 30, 114, 177 (Supplementary Table 2). The most common mutation occurred on position 30, where Haplotype H_4 contained G, while the other 4 haplotypes contained A. None of the haplotypes contained isolates from all four sampling sites.

***Heterakis* Species** Amplification of *Heterakis* specimens was only successful with *cox1* and ITS markers. The *cox1* primers identified specimens from 12 chickens as *H. gallinarum* and are deposited into GenBank under the accession numbers OL457523-OL457534.These isolates showed a homology of 98 to 100% with GenBank isolates from China (KP308362.1; KP308363.1) and United States (MN732842.1). Phylogenetic tree further supported the classification of all the isolates from this study along with the GenBank *H. gallinarum* isolates by forming 2 sister clades (Figure 3). Eight isolates from Shongweni (SH), Gingindlovu (MP), and Ozwathini (MV) showed a close relationship with the isolates from China by forming, though moderately supported, a clade with these GenBank isolates. The remaining 4 isolates from the north coast formed a weakly supported clade with isolates from Tunisia and United States. The *H. gallinarum* clade formed a moderately supported monophyletic clade with *Heterakis* sp. and *H. isolonche.*

Heterakis isolates based on the *cox1* gene produced 11 haplotypes (Supplementary Table 3, Supplementary Figure 2) and they showed a higher diversity between each other (Hd = 0.806). There was no novelty in the haplotypes produced from these isolates. Eight isolates formed a clade with Chinese GenBank isolates formed a haplogroup (H_6) with 2 isolated from China (KP308362.1, KP308343.1). This haplotype diverged from H_7, which included one GenBank isolate from China (KP308363.1) by one mutational step at position 404. The remaining 4 isolates formed in 3 haplotypes: H_2 consisting of 2 isolates from Gingindlovu (MP4H) and Ozwathini (MV5H), H_3 and H_4 consisting of individual isolates MV4H and MP1H respectively. Other African isolates from Tunisia (MF066712.1;

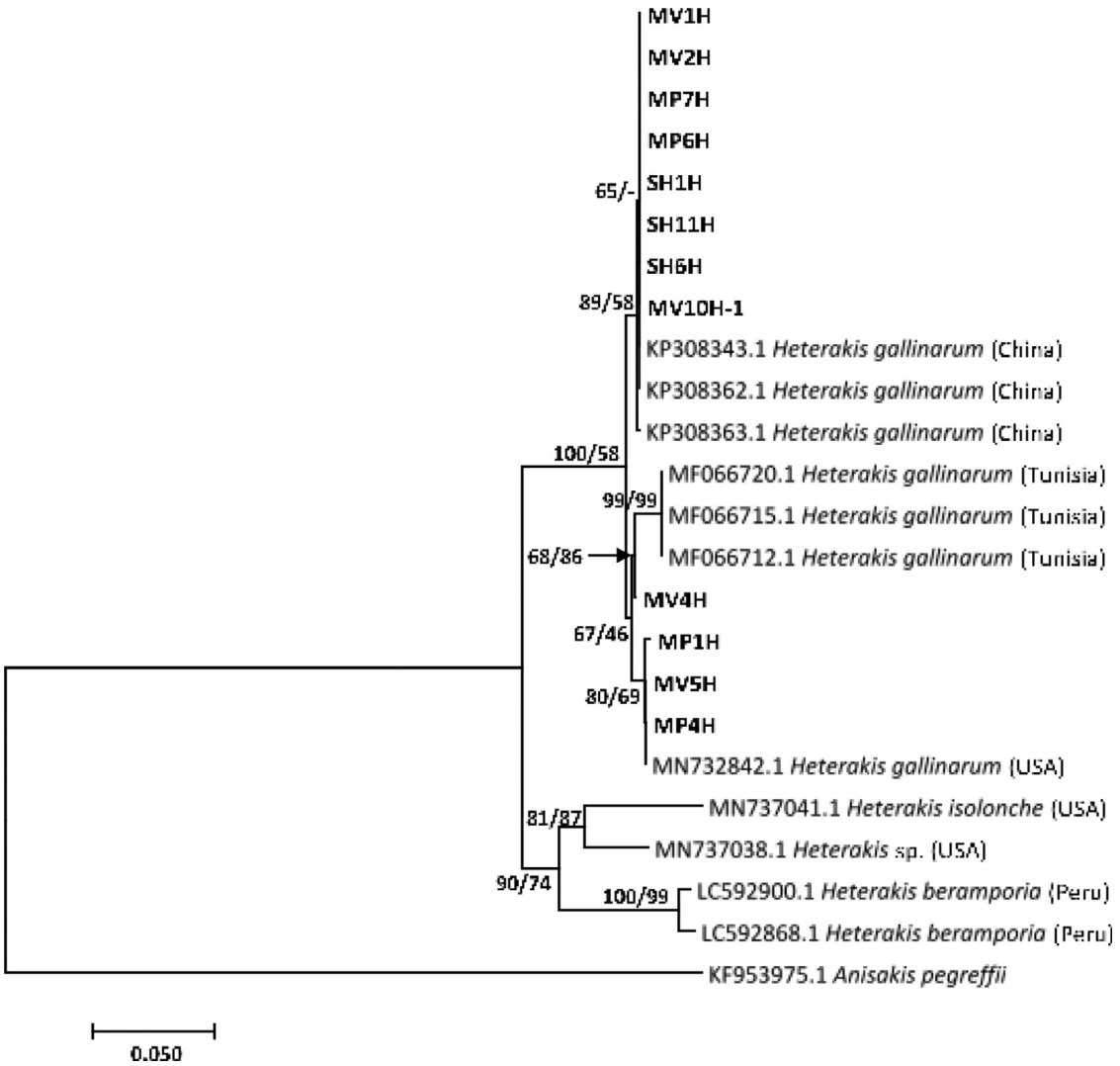

Figure 3. Neighbor-joining tree based on the 600 bp nucleotides of the mitochondrial Cytochrome c oxidase subunit 1 (*cox1*) gene illustrating the relationship between *Heterakis* isolates from KwaZulu-Natal province of South Africa (in bold), and the closest matches from the NCBI GenBank. The nodal support values indicated in the order: maximum likelihood and neighbor-joining. Abbreviations: MP, Gingindlovu; MV, Mvoti; MZ, Mzinto; SH, Shongweni.

MF066720.1; MF066715.1) formed their own haplotype (H_1). Haplotype 5 included one isolate of *H. gallinarum* from United States (MN732842.1). The number of mutations and positions where the mutations occurred between all *H. gallinarum* isolates is outlined in Supplementary Table 4. Haplotype 8 represented a *H. isolonche* isolate from United States (MN737041.1) while Haplotype 9 represented an uncategorized *Heterakis* sp from United States (MN717038.1). Haplotypes 10 and 11 consisted of 2 *H. beramporia* isolates from Peru (LC592900.1; LC502868.1).

The ITS marker successfully amplified 6 *Heterakis* isolates from 4 locations. The sequences of these isolates were deposited into GenBank under the following accession numbers OL470970-OL470975. BLAST analysis identified 3 isolates as *H. beramporia*, showing a homology ranging from 97 to 100% with *H. beramporia* isolate from China (KU529974.1) and Bangladesh (LC592731.1). These isolates formed a strongly supported monophyletic sister clade with GenBank *H. indica* isolates from Bangladesh. Within the *H. beramporia* clade, isolate MP5H, a well-supported clade by neighbor-joining with other *H. beramporia* isolates (Figure 4). The pairwise distances show that this isolate separated with other *H. beramporia* isolates by genetic p-distance of 2.4 to 2.9%. Results show that specimen MP1H was successfully amplified with both *cox1* and ITS markers. The *cox1* gene identified the specimen as *H. gallinarum*, whereas the ITS marker identified this specimen as *H. beramporia* with a homology of 98 %. BLAST and molecular analysis of specimens SH6H and MV3H identified them *as H. gallinarum*. These isolates showed a homology of >99% with numerous *H. gallinarum* isolates, and further formed strong supported clade with all GenBank *H. gallinarum* isolates included in the analysis. The *H. gallinarum* isolates showed a genetic distance of >50% from the *H. beramporia* and *H. indica*.

The ITS gene generated 7 haplotypes from 18 isolates consisting of 6 isolates from this study, and 2 *H. indica*, 3 *H. beramporia*, and 7 *H. gallinarum* isolates from GenBank (Supplementary Table 5, Supplementary Figure 3). The haplotypes showed a haplotype diversity (Hd) of

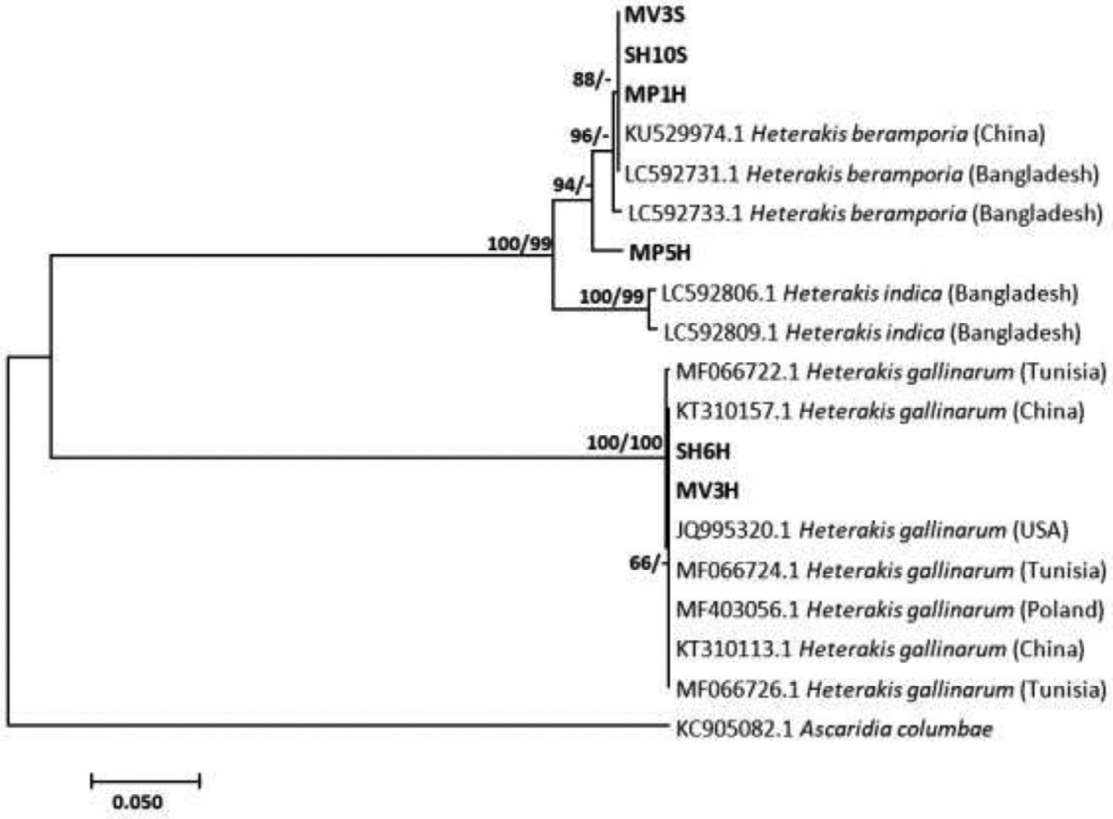

Figure 4. Neighbor-joining tree based on the 370 nucleotides of the ribosomal nuclear gene (ITS) gene illustrating the relationship between *Heterakis* isolates from KwaZulu-Natal province of South Africa (in bold), and the closest matches from the NCBI GenBank. The nodal support values indicated in the order: neighbor-joining and maximum likelihood. Abbreviations: MP, Gingindlovu; MV, Mvoti; MZ, Mzinto; SH, Shongweni.

0.752. The structure of the haplotype network (Supplementary Figure 3) followed that of the phylogenetic tree (Figure 4). Three *H. beramporia* isolates from this study formed one haplogroup H_1, which included also 2 GenBank sequences from China and Bangladesh. Isolate MP5H, which formed a sister clade to these isolates formed its own haplotype (H_3), which separated from the other *H. beramporia* haplotypes H_1 and H_2 with 7 and 9 mutational steps in the positions as indicated in (Supplementary Table 6A), respectively. Haplotypes H_1 and H_2 diverged from each one with 2 mutational steps occurring in positions 158 and 159 (Supplementary Table 6A). The *H. gallinarum* isolates formed 2 haplogroups, H_4 and H_5). These haplotypes were separated by one mutational step which occurred at position 76 (Supplementary Table 6B). *Heterakis indica* isolates formed 2 individual haplotypes, H_6 and H_7, showing eight mutations occurring between them.

DISCUSSION

Several nematode species of free-range chickens have been recorded in other sub-Saharan African countries and elsewhere (Permin et al., 1999; Permin et al., 2002; Mungube et al., 2008; Kaufmann et al., 2011). This study identified *A. galli, H. gallinarum*, and *H. beramporia* from the Family Ascarididae and Heterakidae respectively, infecting free-ranging chickens in Shongweni, Umzinto, Ozwathini, and Gingindlovu in KwaZulu-Natal province of South Africa. This was not surprising as the free-ranging system exposes chickens to eggs with infective larval stages including arthropods that act as paratenic or intermediate hosts of gastrointestinal parasites (Pandey and Jiang, 1992; Opara et al., 2014).

The overall prevalence of GIT nematodes recorded in this study was similar to the 66.44% previously recorded by Malatji et al. (2016) in South Africa. Permin et al. (1999) reported 72.5% of *H. gallinarum* in laying hens reared under an organic free-range system, while Kaufmann et al. (2011) documented 48% of *H. gallinarum* in laying hens raised under the same system. Permin et al. (2002) observed 48.24% prevalence of *A. galli* in free-range chickens and an infection rate of 33.3% was recorded by Mungube et al. (2008). Their findings correspond with the prevalence observed in our study, where approximately 90, 80, and 58.3% of chickens were infected with of *H. gallinarum* in Gingindlovu, Ozwathini, and Shongweni, respectively. *Ascaridia galli* accounted for 53.8% in Shongweni and 40% in Gingindlovu. Mushi et al. (2000) also recorded a high prevalence of *H. gallinarum* and *A. galli* in indigenous chickens in Botswana, which were also identified in the present study. Mukaratirwa et al. (2001) also found 11 species of nematodes in chickens, with *A. galli* being the most frequently recorded species in Zimbabwe. However, this was in contrast to previous records of Malatji et al. (2016), where low infestations of *A. galli*

and *H. gallinarum* were observed in KwaZulu-Natal (17.65 and 8.82%) and Limpopo (1.12 and 6.64%) provinces, respectively.

The highest infection rate of *H. gallinarum* was encountered in Gingindlovu and Ozwathini, and these areas are located in the north coast of KwaZulu-Natal. This was not expected as the north coast is generally characterized by low humidity compared to the south coast (Shongweni and Umzinto) which is distinguished by high temperature, moisture, and humidity (Mukaratirwa and Khumalo, 2010). The high prevalence of *H. gallinarum* and *A. galli* may be linked to the presence of the paratenic hosts of these nematodes in the study areas and for this reason, the prevalence of *H. gallinarum* and *A. galli* was relatively high. Furthermore, another contributing factor may be that *H. gallinarum* and *A. galli* eggs can remain viable in the soil for several months, thereby lengthening the duration of contamination in the environment as chickens continuously consume eggs from the environment when scavenging (Taylor et al., 2007).

Ascaridia galli was present across the 4 rural communities. Previous studies have showed that *A. galli* is the most common and significant gastrointestinal nematode of poultry (Eshetu et al., 2001; Basit et al., 2014; Raza et al., 2019). The prevalence of infection by *A. galli* was higher in the south coast compared to the north coast. This corresponds with the findings of Mukaratirwa and Khumalo (2010), who recorded 22.2 and 22.6% in the north coast (Maphumulo and Mvoti) and 43.3 and 43.8% in the south coast (Port Shepstone and Shongweni), respectively.

There was a low prevalence of *H. beramporia* in Gingindlovu (20%), Ozwathini (10%), and Shongweni (8.3%). This species is native to Asia and other countries (Tran et al., 2015). This is the first record of *H. beramporia* in South Africa, and it is thought to may have been introduced into the country, especially in KwaZulu-Natal province by indentured Indian workers brought to Natal between 1860 and 1911 to develop the sugar industry in this province (Gupta, 1960). Furthermore, it is possible this species has been present in South Africa for decades, and might have been misidentified as *H. gallinarum*, as the 2 species are very similar morphologically with only difference in the size of the adult, where *H. gallinarum* is bigger than *H. beramporia* (Gupta, 1960). The absence of *H. gallinarum* and *H. beramporia* in Umzinto might be attributed to the differences in climatic conditions which might have influence in the life cycle of the parasite (Tarbiat et al., 2015).

According to Uhuo et al. (2013), sex and age of the chickens are some of the factors that can be linked with the rate of infection of gastrointestinal parasites. Zuk et al. (1998) observed that the differences in physiology and behavior between male and female chickens are the main determinant of their vulnerability to gastrointestinal parasites infections. The results of this study revealed that female chickens had more *A. galli* infections compared to males in the South region. Asumang et al. (2019) and El-Dakhly et al. (2019) recorded a high incidence of gastrointestinal parasites in females than males in their investigation on indigenous and exotic breeds (Asumang et al., 2019), which is comparable with our findings. Abdelqader et al. (2008) also reported contrary to our findings observed in the south coast region that *A. galli* infections were encountered more in males than in females among local chickens. However, in agreement with those obtained in the north coast region, where infections were generally higher in males compared to females. In this study there was an observed mixed infection by Ascarididae and Heterakidae species which accounted for 23.8%. The highest rate of mixed infections was observed in Ozwathini (70%), followed by Gingindlovu (50%), and Shongweni (33.3%). The overall mixed infection rate of the current study is comparable with the findings of Ogbaje et al. (2012) who recorded 23.9% of mixed infections in domestic fowls. In contrast, Uhuo et al. (2013) reported 86.6% of mixed infections in local chickens. This study revealed that females harbored more mixed infection compared to males. In contrast, Wuthijaree et al. (2019) reported more mixed infections in males (72.4%) than in females (61.1%). Mixed infection with multiple species might be attributed by the food selected and consumed by the chicken at a particular time which may be contaminated and as a result lead to infection (Smyth, 1976).

Although *Ascaridia* infection in indigenous chickens is well known to exist in sub-Saharan Africa, application of molecular techniques in the identification is still scarce and as a result there is insufficient reference sequence for comparison. Results from this study confirmed the identification of *Ascaridia* specimens in this study as *A. galli*. These isolates showed a minimum percentage identity of 99%, and further formed a weekly supported clade with other South African (KT388440.1, KT388438.1) isolates documented by Malatji et al. (2016) and China (KT613902.1). The results further showed that *Ascaridia* isolates used in the analysis formed 9 haplotypes, of which 4 haplotypes are from South African isolates. Although the isolates from this study did not show uniqueness with location, the isolates were however unique to country (South Africa) and with the mutations occurring in 6 sites.

Heterakis gallinarum isolates, based on the *cox1* gene, from this study formed a strong supported clade by neighbor-joining. The results also showed that although majority of our specimens were closely related to Chinese isolates, some of the isolates from this study were closely related to the isolate from the United States. Similar relationship was depicted by the haplotype analysis, where isolates from this study either formed haplogroup (H_2) with the Chinese isolates, or they showed few mutational divergences from the Chinese isolates. This was the first molecular confirmation of *H. gallinarum* in South Africa.

The ITS marker identified specimens from this study as *H. beramporia* and *H. gallinarum*. According to Biswas et al. (2021), *Heterakis beramporia* is one of the

most common nematodes in gallinaceous poultry in Asian countries. Phylogenetic analysis showed that *H. beramporia* specimens formed a strongly supported monophyletic clade with *H. gallinarum* specimens. Similar phylogenetic relationship was illustrated by Biswas et al. (2021), who further showed that *H. indica* and *H. beramporia* formed sister clade, which was also observed in this study. Haplotype analysis showed that *H. beramporia* isolates from this study formed one haplogroup with GenBank isolates from Bangladesh (LC592731.1) and China (KU529974.1). The remaining isolate MP5H formed its own haplotype, which showed mutations on 7 sites from H_1 which contained other isolates from this study. This is the first study identifying *Heterakis* species based on the ITS marker, and the first confirmation and report of *H. beramporia* in South Africa.

CONCLUSIONS

The prevalence of gastrointestinal nematodes of family Ascarididae and Heterakidae in free-range chickens is high in 3 of the 4 sampled rural communities of KwaZulu-Natal province of South Africa. This study reported for the first time, the occurrence of *H. beramporia* in free range chickens using molecular techniques in KwaZulu-Natal Province of South Africa. Future research should focus on more to provide fundamental evidence on the geographical limits of the presence of *H. beramporia* in southern Africa. Furthermore, identifying and barcoding these species will provide a clear insight over the infection dynamics and the epidemiology of gastrointestinal nematodes in free-range chickens in South Africa.

ACKNOWLEDGMENTS

The authors acknowledge Biomedical Resource Centre (BRU) staff for looking after the chickens.

Ethics statement: This study was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/049/019M) in using experimental animals and was carried out in compliance with the relevant ethical standards according to the South African national guidelines on animal care, handling and use in biomedical research.

The project was funded by SM's Research Productivity funds (UKZN) and National Research Foundation of South Africa provided bursaries for SlM.

DISCLOSURES

Authors declare no conflict of interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101979.

REFERENCES

Abdelqader, A., M. Gauly, C. B. A. Wollny, and M. N. Abo-Shehada. 2008. Prevalence and burden of gastrointestinal helminths among local chickens, in northern Jordan. Prev. Vet. Med. 85:17–22.

Amor, N., S. Farjallah, O. B. Mohammed, A. Alagaili, and L. Bahri. 2018. Molecular characterization of the nematode *Heterakis gallinarum* (Ascaridida: Heterakidae) infecting domestic chickens (*Gallus gallus domesticus*) in Tunisia. Tunisia J. Vet. Anim. Sci. 42:388–394.

Anderson, R. C., A. Chabaud, and S. Willmott. 1974. *CIH Keys to the Nematode Parasites of Vertebrates* (No. QL 391. N4. C54). Commonwealth Institute of Helminthology, St Albans, Herts, UK.

Asumang, P., J. Akoto Delali, F. Wiafe, Z. Kamil, G. Iddrisu Balali, V. Afua Dela Gobe, W. Nketiah Siaw, and G. Pinamang. 2019. Prevalence of gastrointestinal parasites in local and exotic breeds of chickens in Pankrono−Kumasi, Ghana. J. Parasitol. Res. 2019:1–7.

Bandelt, H. J., P. Forster, and A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Bio. Evol. 16:37–48.

Basit, A., A. A. Ali, M. S. Malik, A. N. Malik, M. Iftikhar, H. M. A. Haq, and S. M. Nadeem. 2014. A study of gastrointestinal helminths in native peafowl and comparative efficacy of Albendazol and a Pyrantel pamoate against the helminth parasites. J. Infect. Mol. Biol. 2:22–25.

Bazh, E. K. 2013. Molecular identification and phylogenetic analysis of *Heterakis gallinae* from native chickens in Egypt. Parasitol. Res. 112:3557–3560.

Biswas, P. G., Y. Ohari, U. K. Mohanta, and T. Itagaki. 2021. Development of a multiplex PCR method for discriminating between *Heterakis gallinarum, H. beramporia*, and *H. indica* parasites of poultry. Vet. Parasitol. 295:109463.

Bobrek, K., J. Hildebrand, J. Urbanowicz, and A. Gaweł. 2019. Molecular identification and phylogenetic analysis of *Heterakis dispar* isolated from geese. Acta Parasitol 64:753–760.

Chabaud A.G., *Key to genera of the surperfamilies Cosmocercoidea, Seuratoidea, Heterakoidea and Subuluroidea. CIH Keys to the Nematode Parasites of Vertebrates*, 1978, Commonwealth Agricultural Bureaux; Farnham Royal, England.

Davidson, R. K., A. Mermer, and Ø. Øines. 2012. *Toxocara cati* larva migrans in domestic pigs-detected at slaughterhouse control in Norway. Acta Vet. Scan. 54:1–3.

El-Dakhly, K. M., M. A. El-Seify, E. S. Mohammed, I. S. Elshahawy, S. A. M. Fawy, and M. A. Omar. 2019. Prevalence and distribution pattern of intestinal helminths in chicken and pigeons in Aswan, Upper Egypt. Trop. Anim. Health Prod 51:713–718.

Eshetu, Y., E. Mulualem, H. Ibrahim, A. Berhanu, and K. Aberra. 2001. Study of gastro-intestinal helminths of scavenging chickens in four rural districts of Amhara region, Ethiopia. Rev. Sci. Tech. *(International Office of Epizootics)* 20:791–796.

FAO. 2004. Small-scale Poultry Production: Technical Guide. Food and Agriculture Organization of the United Nations, Rome, Italy. Retrieved from http://www.fao.org/3/a-y5169e.pdf.

Gasser, R. B. 2006. Molecular tools—advances, opportunities, and prospects. Vet. Parasitol. 136:69–89.

Guéye, E. 2003. Gender issues in family poultry production systems in low-income food-deficit countries. *Am. J. Altern. Agric* 18:185–195, doi:10.1079/AJAA200350.

Gupta, S. P. 1960. Nematode parasites of vertebrates of east pakistan: iv. ascaroid nematodes from amphibia, birds, and mammals. Can. J. Zool. 38:315–329.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98.

Katakam, K. K., P. Nejsum, N. C. Kyvsgaard, C. B. Jørgensen, and S. M. Thamsborg. 2010. Molecular and parasitological tools for the study of *Ascaridia galli* population dynamics in chickens. Avian Pathol 39:81–85.

Katoch, R., A. Yadav, R. Godara, J. K. Khajuria, S. Borkataki, and S. S. Sodhi. 2012. Prevalence and impact of gastrointestinal helminths on body weight gain in backyard chickens in subtropical and humid zone of Jammu. India. J. Parasit. Dis. 36:49–52.

Kaufmann, F., G. Daş, B. Sohnrey, and M. Gauly. 2011. Helminth infections in laying hens kept in organic free-range systems in Germany. Livest. Sci. 141:182–187.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. boil. evol. 33:1870–1874.

Lalchhandama, K. 2010. On the structure of *Ascaridia galli*, the roundworm of domestic fowl. Sci.Vis 10:20–30.

Lalchhandama, K. 2011. Vermicidal activity of *Millettia pachycarpa* on the nematode, *Heterakis gallinarum*. Int. J. Pharm. Sci. Res. 2:2430.

Madsen, H. 1950. Studies on species of *Heterakis* (nematodes) in birds. Dan. Rev. Game Biol. 1:1–43.

Mafu, J. V., and P. J. Masika. 2003. Small scale broiler production by rural farmers in the central Eastern Cape Province of South Africa: *12*Fort Hare Papers, 25–34.

Malatji, D.P. 2017. A genomic insight into the diversity and pathogenesis of village chickens in a Ascaridia galli infested environment Doc. diss., University of Pretoria, South Africa.

Malatji, D. P., A. M. Tsotetsi, E. Van Marle-Koster, and F. C. Muchadeyi. 2016. Population genetic structure of *Ascaridia galli* of extensively raised chickens of South Africa. Vet. Parasitol. 216:89–92.

Maxwell, O. N., O. E. Roseline, O. O. Daniel, and J. O. Cornelius. 2016. Haemoparasitism of local and exotic chickens reared in the tropical rainforest zone of Owerri Nigeria. Alex. J. Vet. Sci. 51:1–7.

McAinsh, C. V., J. Kusina, J. Madsen, and O. Nyoni. 2004. Traditional chicken production in Zimbabwe. Worlds Poult. Sci. J 60:233–246.

Melewas, J. N. 1989. The contribution of poultry industry to the National economy. Proceedings of the 7th Tanzania Veterinary Association Scientific ConferenceTVA 13–35.

Minga, U. M., A. M. Katule, T. Maeda, and J. Musasa. 1989. Potential and problems of the traditional chicken industry in Tanzania. Proceedings of the 7th Tanzania Veterinary Scientific ConferenceArusha 207–215.

Mtileni, B. J., F. C. Muchadeyi, A. Maiwashe, M. Chimonyo, and K. Dzama. 2012. Conservation and utilisation of indigenous chicken genetic resources in South Africa. Worlds Poult. Sci. J. 68:727–748.

Mukaratirwa, S., T. Hove, J. B. Esmann, and C. J. Hoj. 2001. A survey of parasitic nematode infections of chickens in rural Zimbabwe. Onderstepoor J. Vet.Res. 68:183–186.

Mukaratirwa, S., and M. P. Khumalo. 2010. Prevalence of helminth parasites in free-range chickens from selected rural communities in KwaZulu-Natal province of South Africa. J. S. Afr. Vet. Assoc. 81:97–101.

Mungube, E. O., S. M. Bauni, B. A. Tenhagen, L. W. Wamae, S. M. Nzioka, L. Muhammed, and J. M. Nginyi. 2008. Prevalence of parasites of the local scavenging chickens in a selected semi-arid zone of Eastern Kenya. Trop. Anim. Health Prod. 40:101–109.

Mushi, E. Z., M. G. Binta, R. G. Chabo, R. Ndebele, and T. Thibanyane. 2000. Helminth parasites of indigenous chickens in Oodi, Kgatleng District, Botswana. J. S. Afr. Vet. Assoc. 71:247–248.

Mwale, M., and P. J. Masika. 2011. Point prevalence study of gastrointestinal parasites in village chickens of Centane district, South Africa. Afr. J. Agric. Res. 6:2033–2038.

Newton, S. E., P. R. Boag, and R. B Gasser. 2002. Opportunities and prospects for investigating developmentally regulated and sex-specific genes and their expression in intestinal nematodes of humans. Pages 235−268 in the Geohelminths: Ascaris, Trichuris and Hookworm. Springer, Boston, MA.

Ogbaje, C. I., E. O. Agbo, and O. J. Ajanusi. 2012. Prevalence of *Ascaridia galli, Heterakis gallinarum* and Tapeworm infections in birds slaughtered in Makurdi township. Int. J. Poult. Sci. 11:103–107.

Oliveira, C. M. G. D., A. R. Monteiro, and V. C. Blok. 2011. Morphological and molecular diagnostics for plant-parasitic nematodes: working together to get the identification done. Trop. Plant Pathol. 36:65–73.

Oniye, S. J., P. A. Audu, D. A. Adebote, B. B. Kwaghe, O. J. Ajanusi, and M. B. Nfor. 2001. Survey of helminth parasites of laughing dove (Streptopelia senegalensis) in Zaria Nigeria. Afr. J. of Nat. Sci. 4:65–66.

Opara, M. N., D. K. Osowa, and J. A. Maxwell. 2014. Blood and gastrointestinal parasites of chickens and turkeys reared in the tropical rainforest zone of south-eastern Nigeria. Open J. Vet. Med. 4:308.

Pandey, V. S., and J. Jiang. 1992. Observation of helminth parasites of domestic fowl in Zimbabwe. Zimb. Vet. J. 20:15–17.

Permin, A., M. Bisgaard, F. Frandsen, M. Pearman, J. Kold, and P. Nansen. 1999. Prevalence of gastrointestinal helminths in different poultry production systems. Br. Poult. Sci. 40:439–443.

Permin, A., J. B. Esmann, C. H. Hoj, T. Hove, and S. Mukaratirwa. 2002. Ecto-, endo-and haemoparasites in free-range chickens in the Goromonzi District in Zimbabwe. Prev. Vet. Med. 54:213–224.

Permin, A., and J. W. Hansen. 1998. The epidemiology, diagnosis, and control of parasites in poultry. FAO Rome 20:233–240.

Permin, A., and G. Pedersen. 2002. The need for a holistic view on disease problems in free-range chickens. Network for Smallholder Poultry Development. The Royal Veterinary and Agricultural University Frederiksberg, Denmark.

Phiri, I. K., A. M. Phiri, M. Ziela, A. Chota, M. Masuku, and J. Monrad. 2007. Prevalence and distribution of gastrointestinal helminths and their effects on weight gain in free-range chickens in Central Zambia. Trop. Anim Health Prod 39:309–315.

Ramadan, H. H., and N. Y. Abouznada. 1992. Morphology and life history of Ascaridia galli in the domestic fowl that are raised in Jeddah. Sci. 4:87–99.

Raza, A., A. G. Qamar, K. Hayat, S. Ashraf, and A. R. Williams. 2019. Anthelmintic resistance and novel control options in equine gastrointestinal nematodes. Parasitol 146:425–437.

Rozas, J., J. C. Sánchez-DelBarrio, X. Messeguer, and R. Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinform 19:2496–2497.

Saha, D., 2003. Status of rural poultry production in North 24 Parganas district of West Bengal. Doctoral, Indian Veterinary Research Institute. India.

Smyth, J. D. 1976. Introduction to Animal Parasites. 2nd ed Hazzel Watson and Viney Limited, Aylesbury, Bucks, UK.

Soulsby, E. J. L. 1982. Helminths. *Arthropods and Protozoa of Domesticated Animals*. 7th ed. Bailliere Tindall, East Sussex, UK.

Ssenyonga, G. S. Z. 1982. Prevalence of helminth parasites of domestic fowl (*Gallus domesticus*) in Uganda. Trop. Anim. Health Prod. 14:201–204.

Swatson, H. K., R. Gous, P. A. Iji, and R. Zarrinkalam. 2002. Effect of dietary protein level, amino acid balance and feeding level on growth, gastrointestinal tract, and mucosal structure of the small intestine in broiler chickens. Anim. Res 51:501–515.

Tarbiat, B., D. S. Jansson, and J. Höglund. 2015. Environmental tolerance of free-living stages of the poultry roundworm *Ascaridia galli*. Vet. Parasitol. 209:101–107.

Taylor, M. A., R. L. Coop, and R. L. Wall. 2007. Parasites of poultry and gamebirds. Vet. Parasitol. 3:459–557.

Thekisoe, M. M. O., P. A. Mbati, and S. P. R. Bisschop. 2003. Diseases of free-ranging chickens in the Qwa-Qwa District of the northeastern Free State province of South Africa. J. S. Afr. Vet. Assoc. 74:14–16.

Tran, N. H. B., T. N Bich, and N. P Khanh. 2015. Situation of parasitic helminth infections in the gastrointestinal tract and some blood physiological parameters in caged chickens in Binh Thuy district, Can Tho city. J. Sci. Can Tho Univ. 37:6–10.

Uhuo, A. C., F. C. Okafor, O. O. Odikamnoro, C. S. Onwe, M. C. Abarike, and J. N. Elom. 2013. Common gastrointestinal parasites of local chicken (*Gallus domesticus*) slaughtered in some selected eatery centres in Abakaliki, Ebonyi State: implication for meat quality. Int. J. Dev. Sustain 2:1416–1422.

Wuthijaree, K., C. Lambertz, T. Vearasilp, V. Anusatsananun, and M. Gauly. 2019. Prevalence of gastrointestinal helminths in Thai indigenous chickens raised under backyard conditions in Northern Thailand. J. Appl. Poult. Res. 28:221–229.

Zhou, H., and S. J. Lamont. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim. Genetics 30:256–264.

Zuk, M., T. Kim, S. I. Robinson, and T. S. Johnsen. 1998. Parasites influence social rank and morphology, but not mate choice, in female red junglefowl, *Gallus gallus*. Anim. Behav 56:493–499.