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Intraspecific morphological and genetic variation in South African populations of a polystomatid flatworm parasite

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Abstract. The African Clawed Frog *Xenopus laevis*, a global invader, exhibits a marked phylogeographic divergence among native populations in southern Africa, which seems to enhance its invasive potential. The polystomatid flatworm, *Protopolystoma xenopodis*, is the frog's most frequently co-introduced metazoan parasite. In an integrative approach, we utilised morphometrics and molecular markers to assess variation in *P. xenopodis* in its native range. We measured twelve key morphological characters from 23 flatworms and compared these statistically between flatworms collected from the northern- and southernmost distribution in South Africa. Phylogenetic analyses were based on three concatenated markers, namely *28S* and *12S rDNA* and *COX1*, from six flatworms. The combination of five morphological characters, which involve egg size, gut morphology and size of the attachment hooks, differentiated northern and southern populations of *P. xenopodis*. The multilocus phylogenetic analyses showed a cluster of northern *P. xenopodis* and two southern lineages with more basal positioning. These findings demonstrate a relatively high level of intraspecific variation in *P. xenopodis* in its native range. The presented intraspecific variation of *P. xenopodis* could be potentially informative to trace geographic origin in its non-native range.

Keywords. Integrative taxonomy, phylogeography, *Protopolystoma xenopodis*, *Xenopus laevis*.

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Introduction

Worldwide, the African Clawed Frog *Xenopus laevis* (Daudin 1802) (Anura: Pipidae) is one of the most widespread amphibians. Its native range covers much of southern Africa (Furman *et al*. 2015). In the

native range, the frog harbours a diverse parasite fauna (TINSLEY 1996). One of the most prevalent parasites of *X. laevis* is the flatworm *Protopolystoma xenopodis* (Price, 1943) (Monogenea: Polystomatidae), a sanguinivorous parasite infecting the frog's bladder in its adult form. In the native range of southern Africa, *P. xenopodis* has been recovered from more than 90% of *X. laevis* populations and more than 50% of all sampled hosts in a recent survey (SCHOEMAN *et al*. 2019). Moreover, *P. xenopodis* is its host's most frequently co-introduced metazoan parasite (TINSLEY & JACKSON 1998b; KUPERMAN *et al*. 2004; RODRIGUES 2014; SCHOEMAN *et al*. 2019).

In general, *P. xenopodis* is differentiated from its congeners, which infect other species of *Xenopus* in Africa, based upon the morphology of the gut, large posterior attachment hooks and spines on the male reproductive organs (TINSLEY & JACKSON 1998b). The number of gut diverticula and anastomoses varies between but also within species (TINSLEY & JACKSON 1998b). All species of *Protopolystoma* possess a pair of large hooks, or hamuli, with two roots and a sharpened terminal hook, used to attach to the wall of the host's bladder (TINSLEY & JACKSON 1998b). Finally, species of *Protopolystoma* have a muscular, bulb-shaped male reproductive organ armed with sixteen spines that are arranged in two concentric rings of eight spines each (TINSLEY & JACKSON 1998b). In their redescription of *P. xenopodis*, TINSLEY & JACKSON (1998b) noted geographical variation in the size of the genital spines between southern and more northerly populations of *P. xenopodis* from *X. laevis* across South Africa and Zimbabwe. The authors also noted intraspecific variation in the morphology of the large hamulus and the caecal branches, although this was not correlated with geographic distance (TINSLEY & JACKSON 1998b).

So far, only a single study of TINSLEY & JACKSON (1998b) provides information on morphological variation of *P. xenopodis* from *X. laevis* from southern and northern South Africa and Zimbabwe. Given the current wide distribution of *P. xenopodis* in many different parts of the world, such information is potentially valuable to trace the geographic origin of this parasite in its non-native range. The present study offers an exploratory investigation of the morphological and genetic variation in *P. xenopodis* collected from *X. laevis* from localities on the northern and southern sides of the Great Escarpment in its native range in South Africa, belonging to two distinct phylogeographic lineages according to DE BUSSCHERE *et al*. (2016). In an integrative approach, we will rely on a combination of evidence from one nuclear and two mitochondrial genes and 12 key morphological characters to assess the extent of intraspecific variation in *P. xenopodis* between two geographically distanced populations.

Material and methods

Specimen collection

From March to July 2017, 20 adult *X. laevis* were captured in funnel traps baited with chicken liver at eight sites in South Africa (Table 1). These localities were chosen to reflect extremities of geographic distribution of *P. xenopodis* (SCHOEMAN *et al*. 2022) and its host frog (DE BUSSCHERE *et al*. 2016) in South Africa (Fig. 1). These sampling sites lie on either side the Great Escarpment, a geological feature that influenced the population structure of *X. laevis* (FURMAN *et al*. 2015).

The frogs underwent double euthanasia according to institutional ethics guidelines under ethics approval number NWU-00380-16-A5-01: first anaesthesia in 6% ethyl-3-aminobenzoate methanesulfonate (MS222) (Sigma-Aldrich Co., USA) and then euthanasia through pithing. Frogs were dissected and adult specimens of *P. xenopodis* were obtained from the urinary bladder. The 29 retrieved polystomatids were processed for either morphological or molecular analyses (Table 1).

TABLE 1

Information on the geographic origin of *Xenopus laevis* and their associated *Protopolystoma xenopodis* specimens. Localities are assigned according to the expected phylogeographic lineage of *X. laevis* in South Africa (DE BUSSCHERE *et al*. 2016). Locality names refer to the nearest town and are given along with the collection date, geographic coordinates of the sampled water bodies, number of adult *X. laevis* hosts captured (N_X) and number of *P. xenopodis* parasites collected for morphometry ($N_{P[m]}$) and DNA sequencing ($N_{P[s]}$).

Locality	Date	Coordinates	N_X	$N_{P[m]}$	$N_{P[s]}$
SA1 (southern South Africa)					
Cape Town, Western Cape	June 2017	33.8355º S, 18.5528º E	2	2	1
Durbanville, Western Cape	July 2017	33.8392º S, 18.6003º E	2	4	0
Hermanus, Western Cape	June 2017	34.3702º S, 19.2571º E	3	4	1
SA5 (northern South Africa)					
Dullstroom, Mpumalanga Province	April 2017	25.3981º S, 30.0380º E	4	3	1
Modimolle, Limpopo Province	April 2017	24.6384º S, 28.4369º E	2	2	1
Potchefstroom, North-West Province	March 2017	26.7555º S, 27.0506º E	3	3	1
Tzaneen, Limpopo Province	May 2017	23.7988º S, 30.1951º E	2	2	1
White River, Mpumalanga Province	April 2017	25.3391º S, 31.0226º E	1	1	0
White River, Mpumalanga Province	April 2017	25.3320º S, 31.0433º E	2	2	0

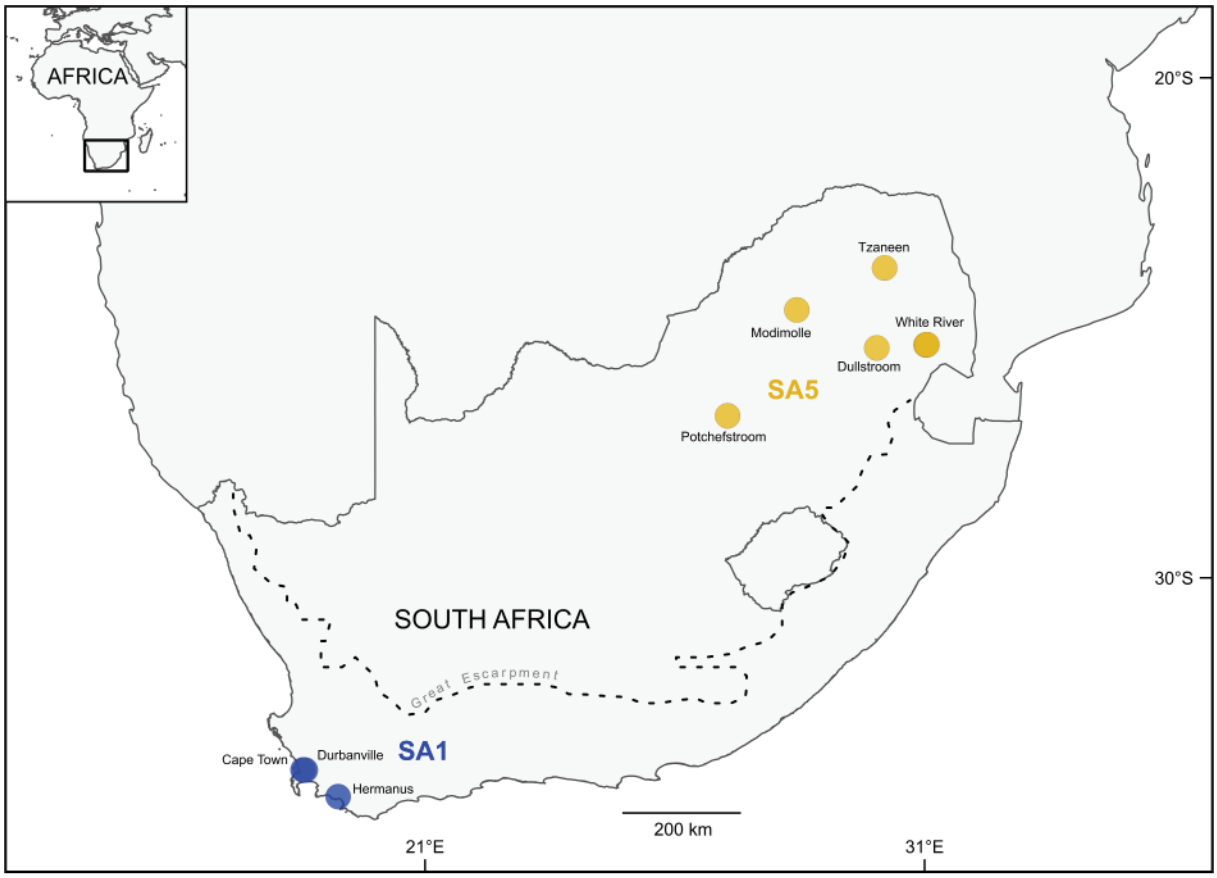

Figure 1 – The eight localities where *Protopolystoma xenopodis* was obtained from *Xenopus laevis*, coloured according to the frogs' expected phylogeographic lineage following DE BUSSCHERE *et al*. (2016), namely SA1 (in blue) to the south of the Great Escarpment Mountain Range (dotted line) or SA5 (in yellow) to the north. The locality names, derived from the nearest town, are indicated. The map was constructed in QGIS *version* 3.10.2-A Coruña (QGIS Development Team 2018) with the Mercator projection.

Morphometrical analyses

In total, 23 of the retrieved polystomatids from the eight localities were processed for morphological analyses. The live polystomatids were placed in a drop of tap water on a microscope slide and gently heated from underneath until they relaxed, following SNYDER & CLOPTON (2005). They were then fixed in 10% neutral buffered formalin or 70% ethanol under coverslip pressure. Polystomatids preserved in both 10% neutral buffered formalin and 70% ethanol were hydrated through a decreasing ethanol series to the tap water, with 10 minutes spent on each step. The specimens were stained overnight in acetocarmine. Thereafter, the specimens were dehydrated in an increasing ethanol series to absolute ethanol, 10 minutes per step, with colour corrections by hydrochloric acid incorporated whilst the specimens were in the 70% ethanol. The specimens were cleared in xylene and mounted in Canada balsam (Sigma-Aldrich Co., Steinheim, Germany). The mounts were dried at 50ºC for approximately 48 hours.

Measurements and photomicrographs were taken using a Nikon ECLIPSE E800 compound microscope in conjunction with the software NIS-Elements Documentation *version* 3.22.09 (Nikon Instruments Inc., Tokyo, Japan). The following nine characters were measured: body length from the tip of the haptor to tip of the false oral sucker, body width at the widest point, length and width of the haptor, length of the ventral roots of the two large hamuli, length of the dorsal roots of the two large hamuli, length of the terminal hooks of the two large hamuli and the length and width of the egg (if present) at the longest and widest points, respectively (Fig. 2). The following three structures were counted: number of post-ovarian inter-caecal anastomoses, number of medial diverticula of the caecum and number of

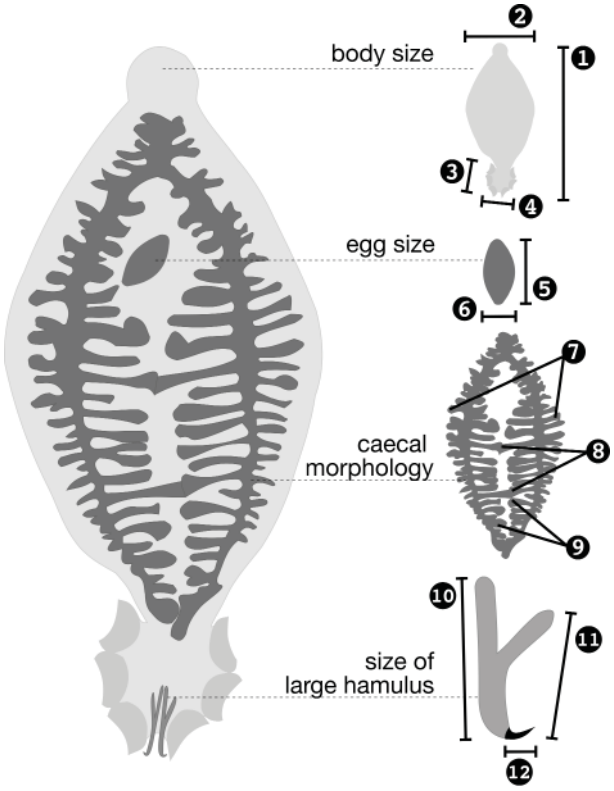

Figure 2 – Measured morphological characters of adult *Protopolystoma xenopodis*: (1) body length, (2) body width, (3) haptor length, (4) haptor width, (5) egg length, (6) egg width, (7) number of lateral diverticula, (8) number of post-ovarian inter-caecal anastomoses, (9) number of medial diverticula, (10) length of the dorsal root of the large hamulus, (11) length of the ventral root of the large hamulus, (12) length of the terminal hook of the large hamulus.

lateral diverticula (Fig. 2). The hamuli and the medial and lateral diverticula from the two sides of the polystomatids were measured or counted separately and then averaged for each specimen to give a single value for each character for subsequent analyses. Of these 12 characters, only egg length and width have not been compared within and among *Protopolystoma* spp. by TINSLEY & JACKSON (1998b).

The 12 characters were compared statistically based upon geographic origin (SA1 or SA5) in the software R *version* 4.1.2 (R CORE TEAM 2021). Unless otherwise mentioned, data treatment and visualisation were performed with the help of the R packages *broom* (ROBINSON *et al*. 2022), *factoextra* (KASSAMBARA & MUNDT 2020), *ggdist* (KAY 2021), *ggtext* (WILKE 2020), *patchwork* (LIN PEDERSEN 2020), *png* (URBANEK 2013), *skimr* (WARING *et al*. 2021) and *tidyverse* (WICKHAM *et al*. 2019). Missing data points were imputed by the random forest method in the R package *missForest* (STEKHOVEN 2013) using a random seed of 666 as starting point. This method was preferred since it has a non-parametric approach suitable to the small sample size and because it can handle mixed variable types (STEKHOVEN & BÜHLMANN 2012). Since certain characters can vary with parasite age, or its proxy, body size, the median body length and width and haptor length and width were compared between the two groups with the non-parametric Wilcoxon-Mann-Whitney (WMW) test to ensure that the groups contained polystomatids of similar size distributions. The WMW test was further employed to test whether there was a significant difference in the median number of post ovarian inter-caecal anastomoses and lateral and medial diverticula, the median length of the terminal hook and dorsal and ventral roots of the large hamuli and egg length and width between *P. xenopodis* from the two phylogeographic lineages of the host. The WMW tests were performed and visualised via the R package *ggsignif* (CONSTANTIN & PATIL 2021).

A principal components analysis (PCA), which is commonly employed in numerical taxonomy, also that of monogeneans (e.g., HAHN *et al*. 2011 or VANHOVE *et al.* 2021), was employed to evaluate the correlation among polystomatids from different localities based upon the variance in the characters that were shown to be significantly different between the two groups. The PCA visualised whether the combination of significantly different morphological characters could discriminate between polystomatids from SA1 and SA5 hosts, despite overlap in the measurements of all these characters between the two groups, without considering geographical origin *a priori*. The visualisation further identified the characters that contributed most to the variation between groups. Since the Euclidean distances utilised in a PCA are sensitive to different units of measurement, the data were column-standardised beforehand in the R package *vegan* (OKSANEN *et al*. 2020) as recommended by THORPE (1981). The PCA itself was performed in base R, utilising the singular value decomposition method.

Molecular and phylogenetic analyses

One nuclear marker, namely *28S rDNA*, and two mitochondrial markers, namely *12S rDNA* and *COX1*, were chosen for the phylogenetic analyses. These markers have been used previously for both taxonomic and phylogenetic studies of Polystomatidae, leading to the availability of family-specific primers for these genes (VERNEAU *et al*. 2009; HÉRITIER *et al*. 2015, 2018).

Extracts of DNA were obtained from six additional polystomatid specimens from six of the eight localities (Table 1) with the PCRBIO Rapid Extract PCR Kit (PCR Biosystems Ltd., London, United Kingdom). Subsequent amplification reactions were performed with 2 to 5 mL extracted DNA, 1.25 mL [0.2 mM] forward primer and 1.25 mL [0.2 mM] reverse primer, 12.5 mL [1 ×] master mix from the PCRBIO HS *Taq* Mix Red (PCR Biosystems Ltd., London, United Kingdom) and PCR grade water to the final volume of 25 mL. The fragment of nuclear *28S rDNA* of the six specimens of *P. xenopodis* was amplified using the method of VERNEAU *et al*. (2009) with the primer pair ’LSU5’

(5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') (Littlewood *et al*. 1997) and 'LSU1500R' (5'-GCTATCCTGAGGGAAACTTCG-3') (Tkach *et al*. 1999).

For purification and sequencing, all PCR products were sent to a commercial company (Inqaba Biotec, Pretoria, South Africa) that used the ExoSAP protocol (New England Biolabs Ltd., United States) for purification and obtained the sequences with BigDye® Terminator *version* 3.1 Cycle Sequencing, utilising the corresponding primer pairs used in the PCR reaction, on an ABI3500XL analyser. Sequences were assembled and manually edited in Geneious *version* 9.0 (Saint Joseph, Missouri, United States). The *28S rDNA* sequences were uploaded to GenBank and can be found under accession numbers PP263027–PP263032. The *12S rDNA* and *COX1* sequences, derived from the same polystomatid specimens, were obtained as part of a previous study – available under Genbank accession numbers OP046050–OP046051, OP046054–OP046056, OP046059, OP038566–OP038567, OP038670–OP038572 and OP038575 (Schoeman *et al*. 2022).

The sequences from the six specimens of *P. xenopodis* were aligned separately for each gene in Seaview *version* 4.7 (Gouy *et al*. 2010) with the MUSCLE algorithm *version* 3 at default settings (Edgar 2004). For the protein-coding *COX1*, alignment was performed on the amino acid sequences, translated by the echinoderm and flatworm mitochondrial genetic code. The percentage of differing bases between the sequence pairs in each alignment was calculated in Geneious. Model-corrected pairwise genetic distances were calculated through maximum likelihood (ML) analysis in IQ-TREE *version* 2.1.2 (Minh *et al*. 2020), which first selected the optimal model of molecular evolution for each gene with the ModelFinder selection routine (Kalyaanamoorthy *et al*. 2017) with the FreeRate heterogeneity model (Soubrier *et al*. 2012) based on the Bayesian Information Criterion (BIC). The substitution models were TPM2u+F (Kimura 1981; Soubrier *et al*. 2012) for the partial *28S rDNA*, HKY+F+I (Gu *et al*. 1995; Posada 2003; Soubrier *et al*. 2012) for the partial *12S rDNA* and TIM2+F+G (Yang 1994; Posada 2003; Soubrier *et al*. 2012) for the partial *COX1* gene. The same analyses calculated the number of invariant and parsimony informative sites for each sequence alignment.

For the subsequent phylogenetic analyses, previously published *COX1*, *28S* and *12S* sequences of the closely related *P. occidentalis* (accession numbers KR856179.1, KR856121.1 and KR856160.1, respectively) were included as outgroup (Héritier *et al*. 2015) and the sequence sets were realigned as detailed above. The aligned sequences were concatenated in SequenceMatrix *version* 1.8 (Vaidya *et al*. 2011). The optimal models of molecular evolution for the *12S* and *28S rDNA* genes and the three *COX1* codon positions (Chernomor *et al*. 2016) were selected based on the BIC with the ModelFinder selection routine (Kalyaanamoorthy *et al*. 2017) implemented in W-IQ-TREE *version* 1.6.7 (Trifinopoulos *et al*. 2016). The five partitions were initially analysed separately (Chernomor *et al*. 2016) and then sequentially merged with the implementation of a greedy strategy until model fit no longer improved (Kalyaanamoorthy *et al*. 2017). The new selection procedure was implemented which included the FreeRate heterogeneity model (Soubrier *et al*. 2012). The selection routine identified three partitions in the alignment, namely the *12S rDNA* and *COX1* first codon position with best-fit model the HKY+G (Hasegawa *et al*. 1985; Yang 1994), the *28S rDNA* and *COX1* second codon position with TIM2+I (Gu *et al*. 1995; Posada 2003) and the *COX1* third codon position with TIM2 (Posada 2003).

For tree reconstruction, both ML analysis and Bayesian inference of phylogeny (BI) were performed to increase confidence in the resulting topology. The ML tree was inferred under the three partitions suggested by the selection routine. The parameter estimates were edge-unlinked for all partitions. The analysis was performed in IQ-TREE *version* 1.6.7 (Nguyen *et al*. 2015), with the assessment of branch support through ultrafast bootstrapping (UFboot2; Hoang *et al*. 2018) and the Shimodaira-Hasegawa-like (SH-like) approximate likelihood ratio test (SH-aLRT; Guindon *et al*. 2010), each with 10 000 replicates.

The BI was performed in MrBayes *version* 3.2.6 (RONQUIST *et al*. 2012) implemented through the CIPRES Science Gateway *version* 3.3 on XSEDE (MILLER *et al*. 2010). Posterior probabilities were calculated with four different Metropolis-coupled Markov chains over 10^6 generations, with sampling of the Markov chain every 10^3 generations. The first quarter of the samples was discarded as burn-in. Stationarity of the Markov chains was reached, as indicated by a deviation of split frequencies of 0.001, by a potential scale reduction factor converging to 1 and by the absence of a trend in the plot of log-probabilities as function of generations. The substitution models implemented in MrBayes were adapted from the selection of ModelFinder as the next more complex model under the BIC in terms of substitution rates available in MrBayes. Thus, the HKY model (HASEGAWA *et al*. 1985) was implemented for the first partition, allowing for a discrete gamma model and the GTR (TAVARÉ 1986) model was implemented with and without a proportion of invariant sites for the second and third partitions, respectively. All parameter estimates were edge-unlinked.

Results

Morphological variation

None of the four indicators of body size, namely body length and width and haptor length and width, were significantly different between the polystomatids from the northern (SA5, n = 13) and southern (SA1, n = 10) frog hosts (Fig. 3a–d). Therefore, because of the isometric growth of body structures in *P. xenopodis* (TINSLEY & JACKSON 1998b), no adjustment for size was needed in the subsequent analyses.

Polystomatids from the southern region had significantly longer and wider eggs than those from northern hosts (Fig. 3e–f). There were marked differences in the gut morphology between the polystomatids from the two regions. Polystomatids retrieved from the southern region had significantly more medial and lateral diverticula of the caeca than those from the northern one (Fig. 3g–h). On the other hand, even though there were some northern polystomatids with up to four post-ovarian intercaecal anastomoses, as opposed to their southern counterparts where no specimen had more than two, there was no significant difference in this character between polystomatids from these two regions (Fig. 3i). In terms of large hamulus shape and size, there was no overall difference in the length of the dorsal and ventral roots of the large hamuli between polystomatids from the two regions (Fig. 3j–k). However, southern polystomatids had significantly longer terminal hooks than the northern polystomatids (Fig. 3l).

Thus, *P. xenopodis* from the south displayed less variation in the number of anastomoses ($SA1_{min:max}$ = 1–2; $SA5_{min:max}$ = 1–4), possessed more diverticula, both laterally ($SA1_{median}$ = 27; $SA5_{median}$ = 23) and medially ($SA1_{median}$ = 28; $SA5_{median}$ = 20), had longer terminal hooks of the hamuli ($SA1_{median}$ = 46.75mm; $SA5_{median}$ = 36.50mm) and had longer ($SA1_{median}$ = 250.0 mm; $SA5_{median}$ = 220.5 mm) and wider eggs ($SA1_{median}$ = 84 mm; $SA5_{median}$ = 78 mm) than their northern counterparts. Moreover, egg length displayed no overlap in measurements between the two groups of polystomatids ($SA1_{min:max}$ = 236–271 mm; $SA5_{min:max}$ = 191–228 mm).

Notably, for eight of the characters, including body length, width and haptor length, the polystomatids from the southern region displayed a greater range of measurements than individuals from the northern sampling sites (Fig. 3).

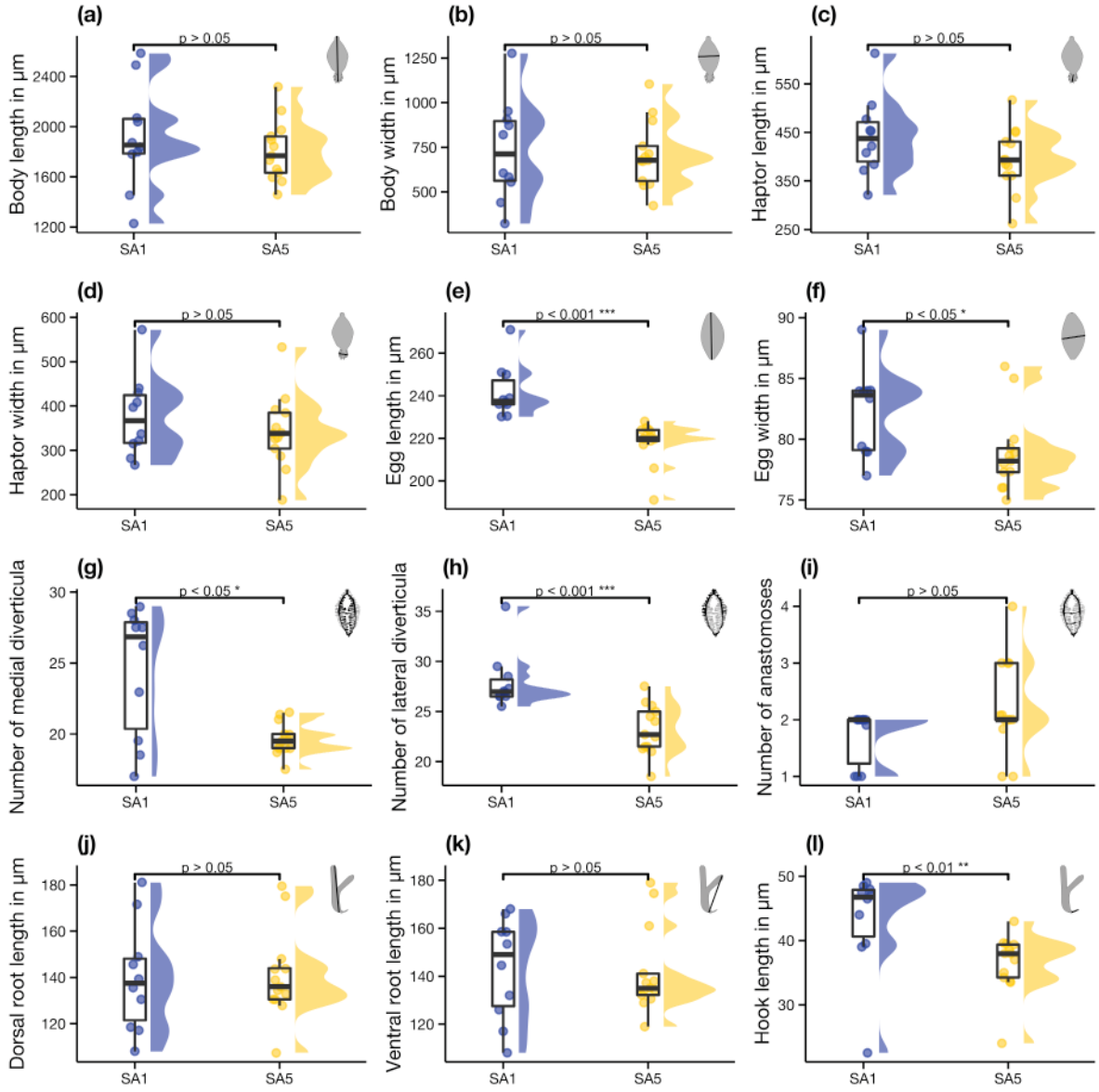

Figure 3 – Raincloud plots of 12 morphometric characters of the parasite *Protopolystoma xenopodis*, compared based on the geographic origin of their host *Xenopus laevis*, namely SA1 and SA5. The characters are (**a**) body length, (**b**) width, (**c**) haptor length, (**d**) width, (**e**) egg length, (**f**) width, (**g**) number of medial, (**h**) lateral diverticula and (**i**) post-ovarian intercaecal anastomoses, (**j**) length of the dorsal root, (**k**) ventral root and (**l**) terminal hook of the large hamuli. Points indicate raw data along with their distributions to the right and summary statistics, the first, second and third quartiles, are given in boxplots to the left. The brackets above the plots indicate the significance levels calculated by Wilcoxon-Mann-Whitney tests that compared the characters between the two groups.

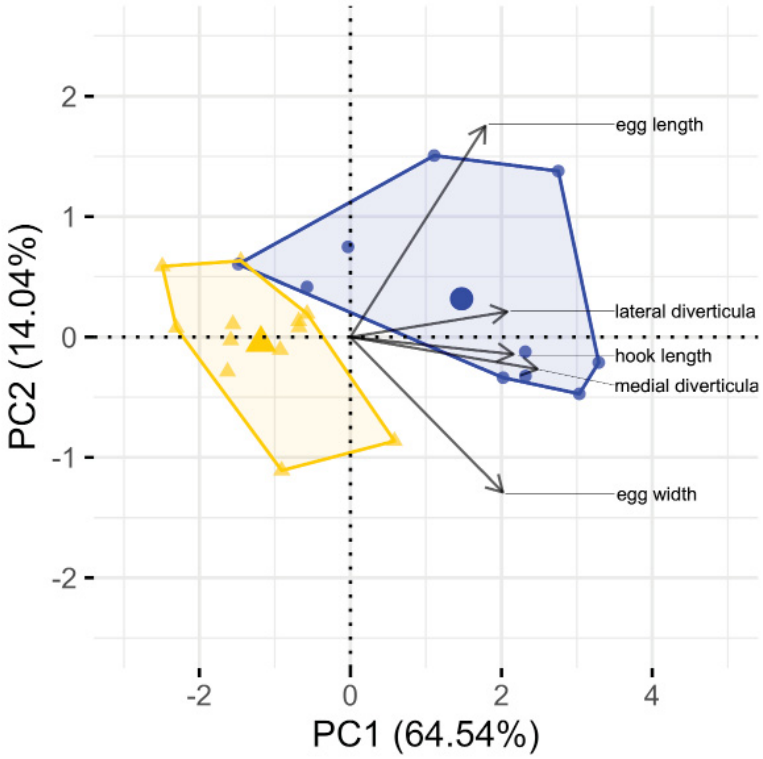

Figure 4 – The first two principal components derived from five morphometric variables—length and width of the egg, length of the terminal hooks of the hamuli (in μm) and the number of lateral and medial diverticula—of 23 *Protopolystoma xenopodis* associated with the southern (SA1, circles in blue) and northern (SA5, triangles in yellow) phylogeographic lineages of their frog host *Xenopus laevis*.

According to the results of the PCA, the combination of the five significantly different morphological characters, namely egg length and width, terminal hook length and number of lateral and medial diverticula, allowed reasonable discrimination between the polystomatids from the two regions (Fig. 4). The first two principal components (PCs) accounted for 64.54% and 14.04% of the observed variance, together explaining 78.58% of the variance in the data. The loadings of PC1 and PC2 were both positive and negative.

Phylogenetic structure

In the case of the partial *28S rDNA* alignment without the outgroup sequence, a total of 1721 bases contained 18 variable and 7 parsimony informative sites. Model-corrected genetic distances in the *28S rDNA* sequences among the six specimens ranged from 0 to 1.6% (Table 2). The partial *12S rDNA* data set without the outgroup sequence was represented by an alignment of 505 base pairs. Of the 505 sites, 67 were variable and 23 were parsimony informative. Model-corrected genetic distances in the *12S rDNA* sequences among the six *P. xenopodis* specimens ranged from 0.12 to 3.77% (Table 2). For the *COX1* gene alignment, the data set without the outgroup amounted to 418 base pairs, where 25 of the 74 variable sites were parsimony informative. Model-corrected genetic distances in the *COX1* sequences among the six specimens ranged from 0.14 to 9.00% (Table 2). The concatenation of the three alignments with the outgroup sequences, which was used for the subsequent phylogenetic analyses, yielded a total of 2667 base pairs. There were 250 variable sites, of which 93 were parsimony informative.

TABLE 2

Pairwise genetic distances (%) of three partial gene sequences from six specimens of *Protopolystoma xenopodis* from *Xenopus laevis* collected at six localities, here named according to the nearest town. Model-corrected distances are given above the diagonal and percentage of non-identical bases are given below.

	CPT	HRM	MDM	DLS	TZN	PTC
Nuclear *28S rDNA*						
Cape Town (CPT)	–	0.05	0.42	0.40	0.50	0.56
Hermanus (HRM)	0.09	–	0.37	0.36	0.61	0.48
Modimolle (MDM)	0.53	0.59	–	0.74	1.60	0.85
Dullstroom (DLS)	0.47	0.53	0.88	–	0	0.07
Tzaneen (TZN)	0.47	0.53	0.88	0	–	0.05
Potchefstroom (PTS)	0.59	0.65	0.10	0.12	0.06	–
Mitochondrial *12S rDNA*						
CPT	–	2.17	2.02	2.15	2.15	2.11
HRM	9.76	–	1.39	3.77	1.37	1.37
MDM	10.18	4.50	–	2.45	0.41	0.40
DLS	10.14	6.52	6.94	–	2.87	2.70
TZN	9.96	4.08	1.02	6.52	–	0.12
PTS	10.37	4.08	1.02	6.92	0.41	–
Mitochondrial *COX1* gene						
CPT	–	9.00	9.00	9.00	9.00	9.00
HRM	11.99	–	0.24	9.00	0.29	0.32
MDM	12.98	4.09	–	9.00	0.20	0.23
DLS	12.23	7.43	8.89	–	9.00	9.00
TZN	13.19	3.84	2.40	9.11	–	0.14
PTS	12.47	4.32	2.64	8.39	1.68	–

In agreement with the morphometric analyses and genetic distances, phylogenetic tree reconstruction based on the concatenated *12S* and *28S rDNA* and *COX1* gene alignments revealed existence of several lineages in *P. xenopodis* related to geographic origin. Polystomatids from the northern localities (SA5) formed a well-supported clade (Fig. 5). *Protopolystoma xenopodis* from Hermanus, Durbanville and Cape Town (southern localities, SA1) were earlier diverging than those from the northern localities and were rendered paraphyletic by the northern clade in both the BI and ML analyses. Additionally, the BI could not resolve the relationships between *P. xenopodis* from Dullstroom, Tzaneen and Potchefstroom (northern localities, SA5), even though the sister relationship of *P. xenopodis* from Potchefstroom and Tzaneen to *P. xenopodis* from Dullstroom had high support in the ML phylogeny.

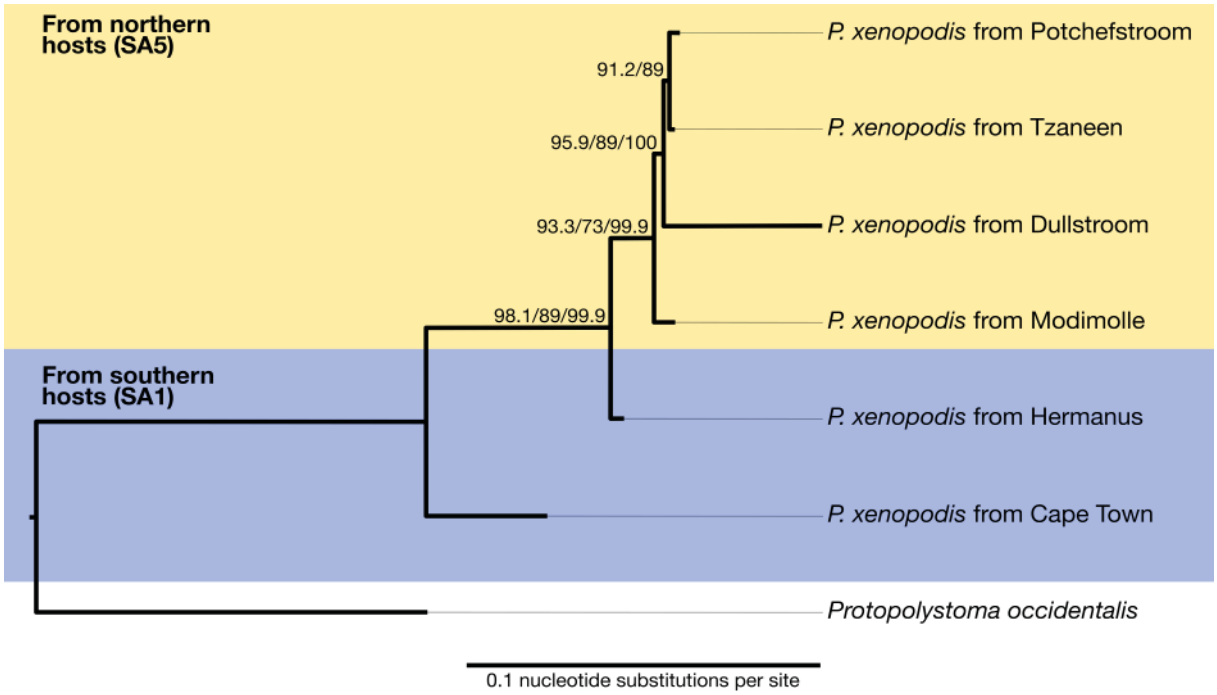

Figure 5 – Maximum likelihood consensus phylogram of six *Protopolystoma xenopodis* specimens, inferred from the *COX1* gene and *12S* and *28S rDNA* sequences. The polystomatids were recovered at six localities, indicated by the name of the nearest town, from *Xenopus laevis* frog hosts from two phylogeographic lineages (SA1 in blue, SA5 in yellow). The closely related *P. occidentalis* from *X. muelleri* from Togo was used to root the phylogram. Values at the nodes indicate support, where available, as calculated by ultrafast bootstrapping (first value), SH-like approximate likelihood ratio test (second value) and posterior probabilities (third value).

Discussion

The present investigation is the first integrative approach to the intraspecific diversity of *P. xenopodis* in South Africa, the widespread bladder parasite of the globally invasive frog *X. laevis*. Both morphological and molecular data reveal a notable intraspecific variation in *P. xenopodis* collected from two regions in South Africa. The combination of egg length and width, the number of diverticula of the gut and the length of the terminal hook of the large hamulus provides a set of key characters that differs considerably between geographically distanced populations of *P. xenopodis* in South Africa. The pattern of morphological variation matches the genetic diversity in mitochondrial and nuclear genes.

Intraspecific variation in morphological characters has been reported before in many species of Polystomatidae and herein, *P. xenopodis* is no exception. Especially the number of intercaecal anastomoses and medial and lateral diverticula are suggested as highly variable characters in polystomatid monogeneans, including *P. xenopodis* (e.g., TINSLEY 1974, 1978; TINSLEY & JACKSON 1998b; DU PREEZ *et al*. 2002; AISIEN & DU PREEZ 2009). Likewise, the high variability in the length of the terminal hook of the large hamulus and the genital spine length in *P. xenopodis* has been pointed out previously (TINSLEY & JACKSON 1998b). Yet, the genital spine length in *P. xenopodis* is the only character for which the link between morphological variation and geographic distance has been explored in the previous study (TINSLEY & JACKSON 1998b). Unfortunately, the genital spine length could not be measured in the present study. The mounting procedure that we applied to the available specimens, whilst it is

ideal for measurements of the soft structures and large hamuli, does not allow for sufficient flattening of the specimens to ensure that the smaller sclerites, such as the genital spines and marginal hooklets, are mounted horizontally. Nonetheless, given the overlap in measurements between the specimens of *P. xenopodis* from different geographic regions, we assume that the observed variation is still within the bounds of the intraspecific level. The examined parasites originate from regions inhabited by the two most geographically distanced lineages of the host (Furman *et al*. 2015; de Busschere *et al*. 2016; Premachandra *et al.* 2023).

In accordance with the variation in morphometrical characters, the mitochondrial *COX1* gene and *12S rDNA* show a remarkable intraspecific variation both within and between the southern and northern lineages of *P. xenopodis*. In fact, the diversity in the *COX1* gene of *P. xenopodis* far exceeds that of species of *Madapolystoma* from frog hosts across Madagascar (proportion on non-identical bases up to 13.2% for *P. xenopodis* and 1.8% in species of *Madapolystoma*), the only other polystomatid genus for which an intraspecific genetic variation has been assessed to date (Berthier *et al*. 2014). Also, a higher intraspecific variation was observed for the *28S rDNA* (proportion on non-identical bases up to 1.6% for *P. xenopodis* and 0.2% in species of *Madapolystoma*) (Berthier *et al*. 2014).

Given the continuous genetic divergence of the host species reflecting a geographical gradient (de Busschere *et al*. 2016; Premachandra *et al.* 2023), the reported morphological and genetic variation of *P. xenopodis* most likely represent the extent of the natural diversity as opposed to a complete north-south division (Schoeman *et al*. 2022). This conclusion is supported by the "significant, but continuous" variation in genital spine length that has been observed in *P. xenopodis* from various host species belonging to *Xenopus* across Africa in the study by Tinsley & Jackson (1998b). Likewise, similar investigations of monogenean flatworms (Kmentová *et al.* 2020; Thys *et al.* 2022) or fishes and reptiles (e.g., Manier 2004; Risch & Snoeks 2008; Ennen *et al*. 2014; Van Steenberge *et al*. 2011, 2015) revealed that phenotypic variation represents geographical variation along a cline.

The Great Escarpment is a continuous mountain range that seems to have shaped the diversification or restricted the expansion of many species in South Africa, including representatives of insects, frogs, snakes, lizards and small mammals (e.g., Makokha *et al*. 2007; Predel *et al*. 2012; Barlow *et al*. 2013; Mynhardt *et al*. 2015; Nielsen *et al*. 2018). This geological feature likely also had an impact on the population structure of *X. laevis* (Furman *et al*. 2015) and, by extension *P. xenopodis*, given the ancient association of species of *Xenopus* and *Protopolystoma* (Tinsley & Jackson 1998a). Nonetheless, the Escarpment is no barrier to the well-documented human-mediated domestic translocation of *X. laevis* from the southernmost part of its range to other localities in southern Africa (Measey & Davies 2011; van Sittert & Measey 2016). Therefore, more detailed investigations that consider the phylogeography of corresponding host-parasite pairs can shed light on the evolutionary and ecological consequences of the anthropogenic movement of *X. laevis* in its native and non-native range.

In summary, we report both morphological and genetic variation in *P. xenopodis* in South Africa. Our findings suggest a possible link between the evolutionary histories of both frog host and flatworm parasite. The relatively high morphological and molecular variation of *P. xenopodis* is a factor to assess in terms of their ability to colonise and adapt to new environments, as was noted for the frog host in France (de Busschere *et al*. 2016). In addition, the phylogeographic analysis of *P. xenopodis* could act as additional evidence in the reconstruction of the invasion histories of this parasite species, as has been demonstrated in a handful of other studies on the monogenean parasites of invasive fish (Ondračková *et al*. 2012; Huyse *et al*. 2015; Kmentová *et al*. 2019).

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