Widespread anthropogenic translocation of the African Clawed Frog across its native range is revealed by its co-introduced monogenean parasite in a co-phylogeographic approach

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ABSTRACT

1. The management of biological invasions relies upon the development of methods to trace their origin and expansion. Co-introduced parasites, especially monogenean flatworms, are ideal tags for the movement of their invasive hosts due to their short generations, direct life cycles and host specificity. However, they are yet to be applied to trace the intraspecific movement of species in their native ranges.

2. As proof of this concept, we conducted a co-phylogeographic analysis based upon two mitochondrial markers of a globally distributed frog *Xenopus laevis* and its monogenean flatworm parasite *Protopolystoma xenopodis* in both its native range in southern Africa and its invasive range in Europe.

3. Translocation of lineages was largely masked in the frog's phylogeography. However, incongruent links between host and parasite phylogeography indicated host switches from one host lineage to the other after these were brought into contact due to human-mediated translocation in the native range. Thus, past translocation of host lineages is revealed by the invasion success of its co-introduced parasite lineage.

4. This study demonstrates the concept that parasite data can serve as an independent line of evidence in invasion biology, also on the intraspecific level, shedding light on previously undetected invasion dynamics. Based upon the distribution of these invasive parasite lineages, we infer that the widespread translocation of hosts is mainly facilitated by the frog's use as live bait by the local angling communities and not via official export routes.

5. *Synthesis and applications.* Data from co-introduced, host-specific parasites can add value to investigations in invasion biology and conservation. A better understanding of the translocation history and resulting genetic mixing of animals in their native ranges prior to introduction into new environments can inform management strategies in the invasive range. Knowledge of the intraspecific movement of different lineages of animals in their native ranges also has conservation implications, since contact between divergent lineages of hosts and parasites can facilitate host switches and altered parasite dynamics in both native and invasive populations. Therefore, we recommend the inclusion of parasite data as a more holistic approach to the invasion ecology of animals on the intraspecific level.

Keywords: biological invasion, co-invasion, holistic specimen, intraspecific cryptic invasion, introduction pathway, *Protopolystoma xenopodis*, *Xenopus laevis*

1 INTRODUCTION

The Anthropocene is characterised by the unparalleled human-mediated passage of organisms from their natural ranges to ecosystems across the world (Capinha *et al.*, 2015; Ricciardi, 2007). If these non-native organisms manage to disperse from their initial introduction point and seed new populations in their non-native range, they are regarded as invasive (Blackburn

et al., 2011; Richardson *et al.*, 2011). This reshuffling of the world's biota is a decisive agent of global change, as evidenced by the fact that invasive species are implicated as the cause of a third of recent animal extinctions (Blackburn *et al.*, 2019).

Clearly, the development of methods to trace both the origin and expansion of biological invaders is an urgent, but not always straightforward, task. For one, organisms can be both native and invasive within the borders of their country of origin, especially in large countries with high climatic and habitat diversity (van Wilgen *et al.*, 2020). These so-called domestic exotics occur outside their native range, yet remain within one administrative region, a feature which hinders their detection and management (Guo & Ricklefs, 2010). Along the same lines is what is known as intraspecific cryptic invasion. This describes the process where one lineage of a species spreads into parts of the species' native range where another lineage is already present, often with human help (Morais & Reichard, 2018; Saltonstall, 2002).

Genomic tools can be highly informative for the reconstruction of invasion histories, the prediction of potential range expansion and invasion success and the development of control strategies (e.g. de Busschere *et al.*, 2016; Dufresnes *et al.*, 2017; Dlugosch & Parker, 2008; Hiller & Lessios, 2017; Lee & Gelembiuk, 2008; Prentis *et al.*, 2009; Stouthamer *et al.*, 2017; Zahiri *et al.*, 2019). Nonetheless, in many cases the available molecular data or chosen molecular markers are of insufficient resolution by themselves to trace the movement of species (e.g. Ahyong *et al.*, 2017; Criscione *et al.*, 2006). In fact, the use of molecular population genetics at the short timescales and coarse-grained genomic resolution of most biological invasions seems to be applicable only in rare instances (Fitzpatrick *et al.*, 2012).

A more holistic approach to solving the origin and range shifts of invasive species involves molecular data from co-introduced parasites. Such an integrative approach has been proposed to investigate invasions that are difficult to trace (Clavero *et al.*, 2016). For example, the long-debated cryptogenic history of the European marine snail *Littorina littorea* was solved with the help of an associated digenean parasite (Blakeslee *et al.*, 2008). Likewise, intraspecific morphometric variation of monogenean flatworm parasites identified both the origin and introduced life stage of invasive clupeid fish in Africa (Kmentová *et al.*, 2019), shed light on the history of an invasive population of odontobutid fish in Europe (Ondračková *et al.*, 2012) and provided information on the introduction route of an invasive goby in Europe (Huyse *et al.*, 2015). Therefore, the use of parasites as tags for host connectivity is not new (Gagne *et al.*, 2021). Yet, the co-phylogeographic analysis of host and parasite data has never been applied to trace intraspecific cryptic invasions within the native ranges of species. In a proof of concept investigation, we employed a globally distributed frog-parasite system to uncover within-species movement.

The African Clawed Frog *Xenopus laevis* (Daudin, 1802) (Anura: Pipidae) is arguably the world's most widely distributed and well-studied amphibian (Measey *et al.*, 2012; van Sittert & Measey, 2016). The ability to trace the native origins of the

invasive populations of *X. laevis* is brought about by the high levels of phylogeographic structuring of this frog in its native range of southern Africa (de Busschere *et al.*, 2016; Furman *et al.*, 2015). Previous studies on the morphology and genetics of *X. laevis* have identified several differentiated populations in South Africa that are segregated by large mountain ranges and different climatic regions (de Busschere *et al.*, 2016; du Preez *et al.*, 2009; Furman *et al.*, 2015; Grohovaz *et al.*, 1996; Measey & Channing, 2003).

Based upon the widespread occurrence of both natural and human-mediated translocation of *X. laevis* in the region, a consequence of the domestic export of the frog for teaching and learning and the widely adopted practice of translocating the frog for use as live bait in recreational angling, some authors advocate for its recognition as a domestic exotic (Measey & Davies, 2011; Measey *et al.*, 2017; van Sittert & Measey, 2016; Weldon *et al.*, 2007). These translocations should leave a genetic trace (van Sittert & Measey, 2016). Nonetheless, the cryptic invasion of *X. laevis* over long distances and across major geographic barriers in the native range has not been confirmed in previous phylogeographic studies of this frog (de Busschere *et al.*, 2016; du Preez *et al.*, 2009; Furman *et al.*, 2015; Measey & Channing, 2003).

Thus, we posit that the bladder polystomatid flatworm *Protopolystoma xenopodis* (Price, 1943) Bychowsky, 1957 (Monogenea: Polystomatidae) is the ideal tool to trace the hitherto unconfirmed contact between different lineages of its host *X. laevis* across its native range of southern Africa. In natural populations across sub-Saharan Africa, *P. xenopodis*, the most widely distributed of the species of *Protopolystoma*, is restricted to a single host species complex, *X. laevis sensu lato*, even in sympatry with other *Xenopus* species (Tinsley & Jackson, 1998a,b). Patterns of host-specificity and speciation within *Protopolystoma* reflect the phylogenetic relationships among their hosts, hinting at a very close co-evolutionary association (Tinsley & Jackson, 1998a). Furthermore, in *P. xenopodis*, notable intraspecific morphological variation exists between populations of the polystomatid in southern Africa and those infecting more northerly host subspecies (Tinsley & Jackson, 1998b). These factors suggest that the phylogeny of *P. xenopodis* might mirror that of *X. laevis* also on an intraspecific level, meaning that this host-parasite system is a good candidate for a co-phylogeographic study.

In general, monogenean parasites (Platyhelminthes: Monogenea) exhibit sufficient levels of phylogeographic structuring to assess intraspecific spatial patterns in their hosts (Baldwin *et al.*, 2012; Hahn *et al.*, 2015; Huyse *et al.*, 2017; Kmentová *et al.*, 2018). At the very least, the phylogeographic structuring of monogenean lineages can support evidence revealed by the host data set or historic records, such as the well-documented anthropogenic introduction of salmonid fish host stocks across Europe that is reflected in the phylogeography of their gyrodactylid monogenean lineages (Hahn *et al.*, 2015). In particular, monogenean parasites are regarded as ideal tags of host genealogy due to their short generations, direct life cycles

and generally high host-specificity (Nieberding & Olivieri, 2007; Pariselle *et al.*, 2011).

All things considered, the well-studied, widely distributed and intimately associated *X. laevis* and *P. xenopodis* system may act as a proof of concept that intraspecific parasite phylogeography can provide information on the cryptic introductions of their host lineages. Therefore, in the present study, we will assess the performance of this integrative approach in the detection of recent translocation of *X. laevis* lineages in the native range. We hypothesise that the phylogeography and morphological divergence of *P. xenopodis* will mirror the documented intraspecific divergence of its host *X. laevis*. Thus, we propose that a co-phylogeographic analysis of this host and parasite can identify recent human-mediated contact between distinct lineages of *X. laevis* and provide new insights on invasion pathways in the native range and beyond.

2 MATERIALS AND METHODS

2.1 Host and parasite sampling

In the native range of southern Africa, 46 adult *X. laevis* were captured from March 2017 to April 2019 in liver baited funnel traps from 30 localities (Fig. 1; Appendix S1 for locality details). The localities were representative of the phylogeography of the frog, based upon the lineage assignment of de Busschere *et al.* (2016) (Appendix S2 for locality classification). In the invasive range, 13 adult *X. laevis* were collected from 12 localities in western France and one in Portugal during the summers of 2017, 2018 and 2019 (Appendix S1 for locality details). In France, frogs were captured in baited traps by members of the EU LIFE CROAA team as part of the regional eradication programme for invasive amphibians. Frogs in Portugal were obtained from the locally captured laboratory stock of the University of Lisbon.

All frogs underwent double euthanasia with anaesthesia in 6% ethyl-3-aminobenzoate methanesulfonate (MS222) (Sigma-Aldrich Co., USA) followed by pithing as required by the institutional ethics committee. During subsequent dissection, 59 *P. xenopodis* (one per frog) were retrieved from the bladders for molecular analyses. Polystomatids and liver tissue from their corresponding frog hosts were preserved in 70% ethanol. A further 23 polystomatids were retrieved from the same frogs hosts at eight of the 30 sampled localities in South Africa for the morphometrical comparison of polystomatids from the southwest of South Africa versus the northeast of South Africa (see Appendix S3 for morphometrical methods and findings).

2.2 Molecular data collection

The DNA was extracted from host and parasite tissue with the PCRBIO Rapid Extract PCR kit (PCR Biosystems Ltd., UK) or the *Quick*-DNA™ Miniprep Plus kit (Zymo Research, USA). Subsequently, amplification reactions for the mitochondrial *COX1* and *rrnS* genes of both frogs and polystomatids were prepared with 2 to 5 μL genomic DNA, 1.25 μL forward primer

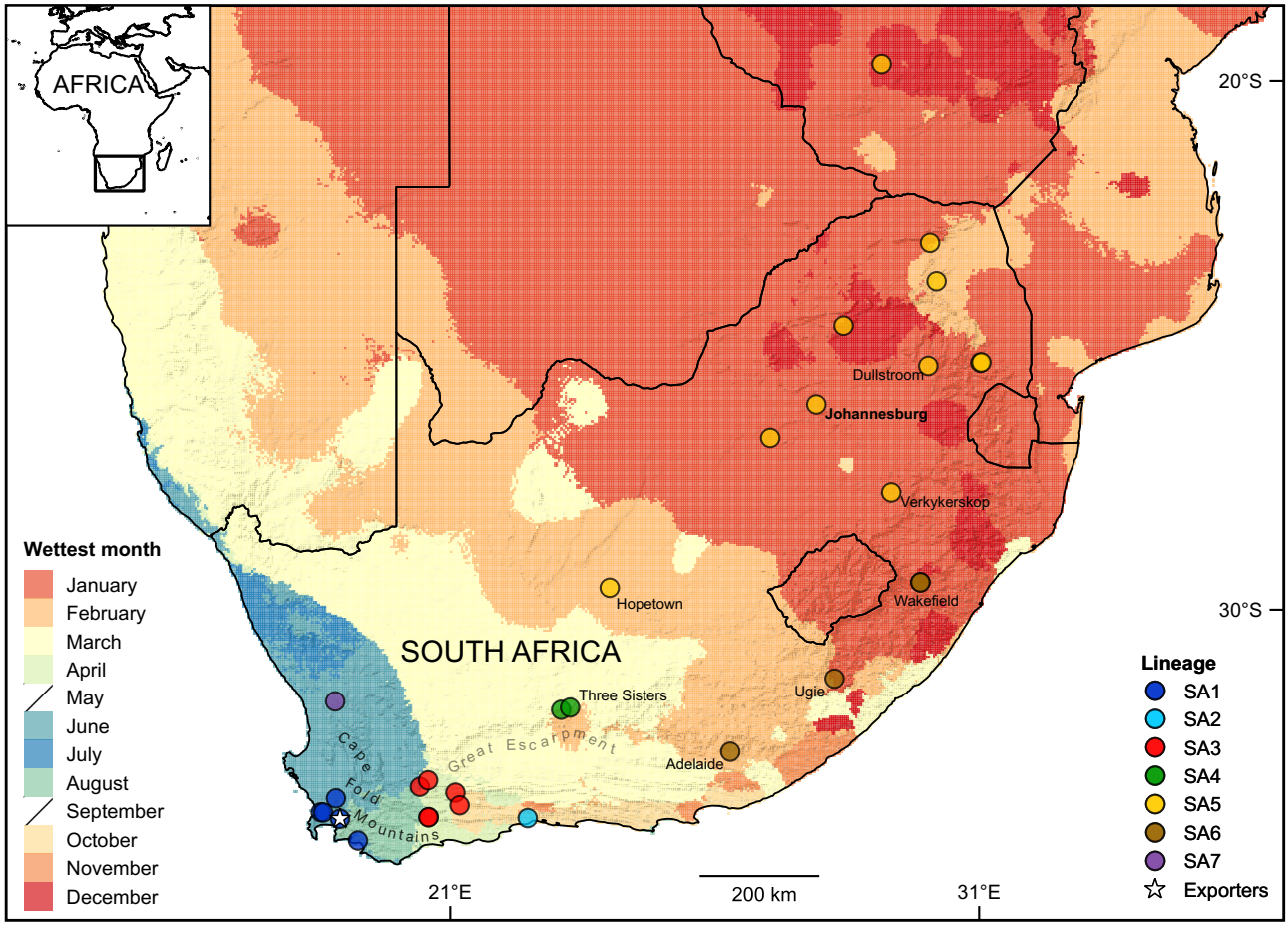

FIGURE 1 Localities in southern Africa where 46 adult *Xenopus laevis* were collected. Localities are classified according to the geographic extent of the native phylogeographic lineages of *X. laevis* (de Busschere *et al.*, 2016). Key localities are named according to the closest town. Jonkershoek (☆) is the source locality of the majority of historic *X. laevis* exports. Mountain ranges are indicated with shaded relief and rainfall seasonality is represented by the wettest month of each 25 km^2 grid cell. Monthly mean precipitation data, obtained from the WorldClim 2.1 database at a spatial resolution of 2.5 arc-minutes (Fick & Hijmans, 2017), were used to identify the months with the highest historical mean precipitation from 1970 to 2000 for each grid cell. Elevation data were obtained from the Natural Earth database. The map was constructed in QGIS *version* 3.10.2-A Coruña (QGIS Development Team, 2018) with the Mercator projection.

[0.2 μM], 1.25 μL reverse primer [0.2 μM], 12.5 μL master mix and PCR-grade water to the final volume of 25 μL. The 2× PCRBIO HS Taq Mix Red (PCR Biosystems Ltd.) or the OneTaq® 2× Master Mix with Standard Buffer (New England Biolabs Inc., USA) were used with the temperature of the elongation steps as per supplier instructions (68 °C or 72 °C).

For the frog *COX1*, the 'LCO1490'/'HCO2198' (Folmer *et al.*, 1994) and 'VF1'/'VR1' (Ivanova *et al.*, 2006; Ward *et al.*, 2005) primer pairs were implemented with the thermocycling profile: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 50 °C for the first primer pair and 54 °C for the second and elongation for 2 min, terminated by a final elongation step of 7 min. In the case of the '12SA-F'/'12SB-R' primer pair (Kocher *et al.*, 1989) to obtain the frog *rrnS*, the profile of Amaro *et al.* (2013) was followed. For the amplification of the

polystomatid *COX1* and *rrnS*, the profile of Héritier *et al.* (2015) was implemented for the primer pairs 'L-CO1p'/'H-Cox1R', 'L-CO1p'/'H-Cox1p2', '12SpolF1'/'12SpolR1' and '12SpolF1'/'12SpolR9' (Héritier *et al.*, 2015; Littlewood *et al.*, 1997).

For purification and sequencing, PCR products were sent to a commercial company (Inqaba Biotec, RSA) that used the ExoSAP protocol (New England Biolabs Ltd.) 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 Prime *version* 2021.0.3 (www.geneious.com). The 236 obtained sequences were deposited in GenBank (accession numbers to be added after article acceptance).

2.3 Phylogeographic analysis

The phylogeography of the 59 *X. laevis* and the 59 *P. xenopodis* was visualised in two haplotype networks. All genes were aligned using the Clustal Omega algorithm under default settings (Sievers & Higgins, 2017) implemented in Geneious Prime. For the protein-coding *COX1* gene, translation alignment was implemented under the vertebrate mitochondrial genetic code (table 2) for the frog and the echinoderm and flatworm mitochondrial genetic code (table 9) for the polystomatid. The gene alignments were trimmed to the length of the shortest sequence since gaps are masked during the subsequent analysis (Leigh & Bryant, 2015). The two aligned genes of each species were concatenated by individual in Geneious Prime. Minimum spanning networks (MSN) of the frog and polystomatid data sets were inferred in PopART *version* 1.7.2 (Bandelt *et al.*, 1999; Leigh & Bryant, 2015).

The genetic diversity of the two host and the two parasite genes was studied based upon these trimmed alignments prior to concatenation. Number of haplotypes and polymorphic sites, haplotype diversity, nucleotide diversity and mean percentage of pairwise differences were calculated in in Arlequin *version* 3.5 (Excoffier & Lischer, 2010) for the alignments as a whole and for predefined groups. Three groups were defined based upon collection locality (see Appendix S2 for full details), namely specimens collected in South Africa to the southwest of the Great Escarpment (SA1, SA2, SA3, SA4 and SA7), to the northeast of the Great Escarpment (SA5 and SA6), or in France. Specimens from Portugal were not included in the analyses because of small sample size.

2.4 Phylogenetic analyses

For the phylogenetic analyses, duplicate sequences were identified with the help of CD-HIT (Fu *et al.*, 2012; Li & Godzik, 2006) implemented online (Huang *et al.*, 2010). Specimens with unique *COX1* or *rrnS* gene haplotypes were retained for the analysis. The *COX1* and *rrnS* gene sequences of the congeneric Müller's clawed frog *X. muelleri* (Peters, 1844) (GenBank

accession numbers EU599031, AY581657; Evans *et al.*, 2004, Evans *et al.*, 2008) and its polystomatid *P. occidentalis* Tinsley *et* Jackson, 1998 (GenBank accession numbers KR856179, KR856121; Héritier *et al.*, 2015) were added as outgroups. This polystomatid only infects *X. muelleri*-like species, even in sympatry with *X. laevis* (Tinsley & Jackson, 1998b). Furthermore, its host *X. muelleri* reportedly reproduces sterile offspring when hybridisation occurs between this species and its sympatric congener *X. laevis* (Fischer *et al.*, 2000). Therefore, the selected outgroups are applicable to an intraspecific analysis of the *X. laevis* and *P. xenopodis* host-parasite system. The resulting data sets, after removal of duplicates and addition of the outgroups, were aligned and concatenated as for the haplotype networks, but not trimmed. Descriptive statistics of the two alignments were derived in DnaSP *version* 6.12.03 (Rozas *et al.*, 2017).

Initially, the concatenated alignments comprised four partitions each, namely the three codon positions of the partial *COX1* gene and the *rrnS* gene. The ModelFinder selection routine (Kalyaanamoorthy *et al.*, 2017), in conjunction with the FreeRate heterogeneity model (Soubrier *et al.*, 2012), implemented in IQ-TREE *version* 2.1.2 (Minh *et al.*, 2020), was employed to select the optimal model of molecular evolution for each partition. To reveal the optimal partitioning scheme and most suitable evolutionary models for each alignment, the initial partitions were sequentially merged (Chernomor *et al.*, 2016) until model fit, as evaluated by the Bayesian Information Criterion (BIC), ceased to improve. For both the data sets, the optimal partitioning scheme was a single partition of all genes and codon positions.

The maximum likelihood (ML) phylogeny of the host was inferred under the TIM2 model (Posada, 2003) with edge-unlinked FreeRate heterogeneity models (Soubrier *et al.*, 2012), proportions of invariant sites (Gu *et al.*, 1995) and discrete gamma models (Yang, 1994). Similarly, the ML tree of the polystomatid was inferred under the TPM3 model (Kimura, 1981) with a FreeRate heterogeneity model (Soubrier *et al.*, 2012). The analyses were performed in IQ-TREE *version* 2.1.2 (Minh *et al.*, 2020), with the assessment of branch support through ultrafast bootstrapping (UFboot2; Hoang *et al.*, 2018) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon *et al.*, 2010), each with 10 000 replicates. The two phylogenies were rooted with respect to the outgroups in TreeGraph2 *version* 2.15.0-877 *beta* (Stöver & Müller, 2010). Clades with UFboot2 support lower than 95% or SH-aLRT lower than 80% were collapsed to polytomies in the same programme.

2.5 Co-phylogenetic analyses

The resulting phylogenies formed the basis of a global-fit approach to the co-phylogenetic analysis of *X. laevis* and *P. xenopodis*. The global congruence of the two phylogenies was assessed by the implementation of both Procrustean Approach to Co-

phylogeny (PACo) (Balbuena *et al.*, 2013) and the fourth-corner statistical approach of ParaFit (Legendre *et al.*, 2002). Both these methods also consider the contribution of individual species interactions to phylogenetic congruence (Balbuena *et al.*, 2013; Legendre *et al.*, 2002; Poisot & Stouffer, 2015). Moreover, both methods can accommodate trees that are not fully resolved and support instances of uneven numbers of terminal nodes of hosts and parasites.

In PACo, we assessed the dependency of the parasite phylogeny upon that of the host through asymmetrical Procrustean superimposition of host and parasite distance matrices, whilst ParaFit tested the dependence of both phylogenies upon one another (Balbuena *et al.*, 2013; Legendre *et al.*, 2002). Prior to analyses, the outgroups were removed from the ML phylogenies. The phylogenies were converted to matrices of patristic distances by the package *ape* (Paradis & Schliep, 2019) in R *version* 4.0.2 (R Core Team, 2020). These matrices served as input for both analyses, along with the corresponding host-parasite association matrix, where the polystomatids were assigned to their original host individuals, or to a host with an identical gene sequence to the original host individual.

The principal coordinates for PACo were derived with the R package *paco* (Hutchinson *et al.*, 2017) *version* o.4.2 for the Procrustes superimposition in conjunction with the Cailliez correction (Cailliez, 1983) to avoid the production of negative eigenvalues of non-Euclidean phylogenetic distances. The PACo analysis was performed, using the asymmetric Procrustes statistic to test for phylogenetic tracking by the parasite of its host, in *paco* with 10 000 permutations, with conserved row sums and number of interactions. The dynamics of the interacting clades were further explored by identifying those interactions that most contribute to the possible concordance between the two phylogenies through a jackknife procedure in *paco*. Those interactions with the smallest residual distances were interpreted as stronger supporters of overall congruence.

The ParaFit analysis (Legendre *et al.*, 2002) was carried out with *ape* based upon the patristic distance matrices, using 10 000 permutations with the Cailliez correction (Cailliez, 1983). Both the ParaFitLink tests were employed to assess the significance of the individual links (Legendre *et al.*, 2002).

A tanglegram of the host and parasite ML phylogenies with a weighted interaction network, based upon the residuals yielded by the PACo jackknife procedure, was constructed with the R package *phytools* (Revell, 2012) with the function `cophylo()`, which has an internal algorithm that rotates nodes to optimally match tips. Due to the removal of duplicate sequences, certain host and parasite terminal nodes were involved in multiple interactions.

3 RESULTS

3.1 Sequence diversity

The trimmed and concatenated alignment of the two host genes used for the network analysis was 1010 bp in length. In the case of the two parasite genes, a concatenated alignment of length 900 bp included seven sites with gaps that were not included for inference. The genetic diversity of the two parasite genes were generally higher than the two orthologous host genes, with greater genetic diversity among the samples collected in South Africa than in France in both host and parasite (Table 1). Genetic diversity was generally higher in the hosts collected to the southwest of the Great Escarpment than in those collected from the northeast, which was not the case for the parasites (Table 1).

In terms of the alignments used for the phylogenetic analyses, analysis of redundancy identified 30 unique sequence combinations in the concatenated *X. laevis* data set with 23 unique haplotypes of *COX1* and 10 of *rrnS*. Including the outgroup *X. muelleri*, 31 haplotypes were aligned for the ML analysis to a total of 1153 bp of 717 bp *COX1* and 436 bp *rrnS* containing 189 sites with missing data and 183 variable sites, of which 88 were parsimony informative. Similarly, after removal of duplicates, the concatenated parasite alignment contained 44 unique *P. xenopodis* sequences, a combination of 34 unique

TABLE 1 Genetic diversity of the bladder flatworm *Protopolystoma xenopodis* and its frog host *Xenopus laevis*, grouped according to sampling locality – collected in South Africa to the southwest of the Great Escarpment or to the northeast of the Great Escarpment, or in France. Indices were inferred separately by gene for each group and for all samples combined from the aligned and trimmed partial mitochondrial *COX1* and *rrnS* genes used in the haplotype network analyses.

	n	H	L	S	Hd	π	d (%)
X. laevis COX1							
Southwestern SA	30	11	641	78	0.823 ± 0.061	0.026 ± 0.013	2.6
Northeastern SA	16	6	641	74	0.742 ± 0.084	0.025 ± 0.013	2.5
France	12	3	641	50	0.591 ± 0.108	0.014 ± 0.008	1.4
All samples	58	16	641	86	0.880 ± 0.023	0.048 ± 0.024	4.8
X. laevis rrnS							
Southwestern SA	30	7	369	13	0.662 ± 0.088	0.008 ± 0.005	0.8
Northeastern SA	16	3	369	2	0.342 ± 0.140	0.001 ± 0.001	0.1
France	12	2	369	5	0.303 ± 0.148	0.004 ± 0.003	0.4
All samples	58	9	369	14	0.681 ± 0.044	0.009 ± 0.005	0.9
P. xenopodis COX1							
Southwestern SA	30	20	416	94	0.959 ± 0.022	0.070 ± 0.035	7.0
Northeastern SA	16	15	416	89	0.992 ± 0.025	0.072 ± 0.037	7.2
France	12	3	416	3	0.591 ± 0.108	0.004 ± 0.003	0.4
All samples	58	34	416	110	0.958 ± 0.014	0.072 ± 0.035	7.2
P. xenopodis rrnS							
Southwestern SA	30	14	484	78	0.903 ± 0.036	0.050 ± 0.025	5.0
Northeastern SA	16	14	484	87	0.983 ± 0.028	0.055 ± 0.029	5.5
France	12	2	484	1	0.485 ± 0.106	0.001 ± 0.001	0.1
All samples	58	28	484	98	0.930 ± 0.020	0.048 ± 0.024	4.8

Abbreviations: SA, South Africa; *n*, sample size; H, number of haplotypes; L, length of sequence; S, number of polymorphic sites; Hd, haplotype diversity; π, nucleotide diversity; d, mean percentage of pairwise differences.

haplotypes of *COX1* and 35 of *rrnS*. Along with the outgroup *P. occidentalis*, the 45 aligned *COX1* sequences of 423 bp and the *rrnS* sequences of 511 bp resulted in a concatenated alignment of 934 bp in length that was used to obtain the ML phylogeny of the parasite. Of these sites, the 799 complete sites contained 225 sites that were polymorphic with a subset of 174 parsimony informative sites.

3.2 Host and parasite phylogeographic clustering

The MSN of the frog host showed three major haplotype clusters (Fig. 2a) that clearly corresponds to the three major lineages revealed by the phylogenetic analysis of *X. lavis* (Fig. 3). The northeastern frog cluster, which corresponded to lineage NE in the frog phylogeny, included frog specimens from SA4, SA5 and SA6 localities and France. This cluster exhibited a star-like topology with two dominant native haplotypes and four satellite haplotypes that were separated from the dominant haplotypes by one to seven mutations. The two southwestern frog clusters, which corresponds to SW_{coast} and SW_{inland} from the frog phylogeny, included frogs from SA1, SA2, SA3, SA4, SA6 and SA7 localities and France and Portugal. One central dominant haplotype in the southwestern clusters represented specimens from SA1 and SA3, as well as France and Portugal. Satellite haplotypes from SA1, SA2, SA3, SA4 and SA7 fanned out from the dominant southwestern haplotype with a star-like topology and, with the exception of a cluster of four highly divergent haplotypes that differed from the most frequent haplotype by 36 to 50 mutations, the rest were separated from the central haplotype by one to seven mutations. The specimens in the divergent southwestern cluster, which corresponds to the SW_{inland} lineage identified by the phylogenetic analysis, also included a specimen collected at one of the SA6 northeastern localities (Wakefield). Furthernore, frog specimens from SA6 localities (Wakefield and Ugie) had similar haplotypes that fell between the northeastern and southeastern clusters, separated from the nearest northeastern haplotype by 29 mutations and the nearest southwestern haplotype by 45 mutations. Frog specimens from the SA4 localities were present in all three the clusters.

In the polystomatid MSN (Fig. 2b), a cluster of parasite specimens from northeastern hosts, that corresponds to **ne** in the polystomatid phylogeny, represented nine of the sixteen specimens collected from the northeastern SA5 and SA6 localities. The seven remaining parasite specimens from SA5 and SA6 localities (Adelaide, Dullstroom, Hopetown, Johannesburg, Ugie, Verkykerskop and Wakefield) were spread across the remainder of the network, therefore clustering with polystomatids from southwestern hosts. Moreover, parasites collected at the SA4 localities were spread across the entire network.

The polystomatids from France and Portugal clustered together as a peripheral dominant haplotype that included frogs from the southwestern SA1 and SA3 localities with two satellite haplotypes that differed by two or four mutations, respectively.

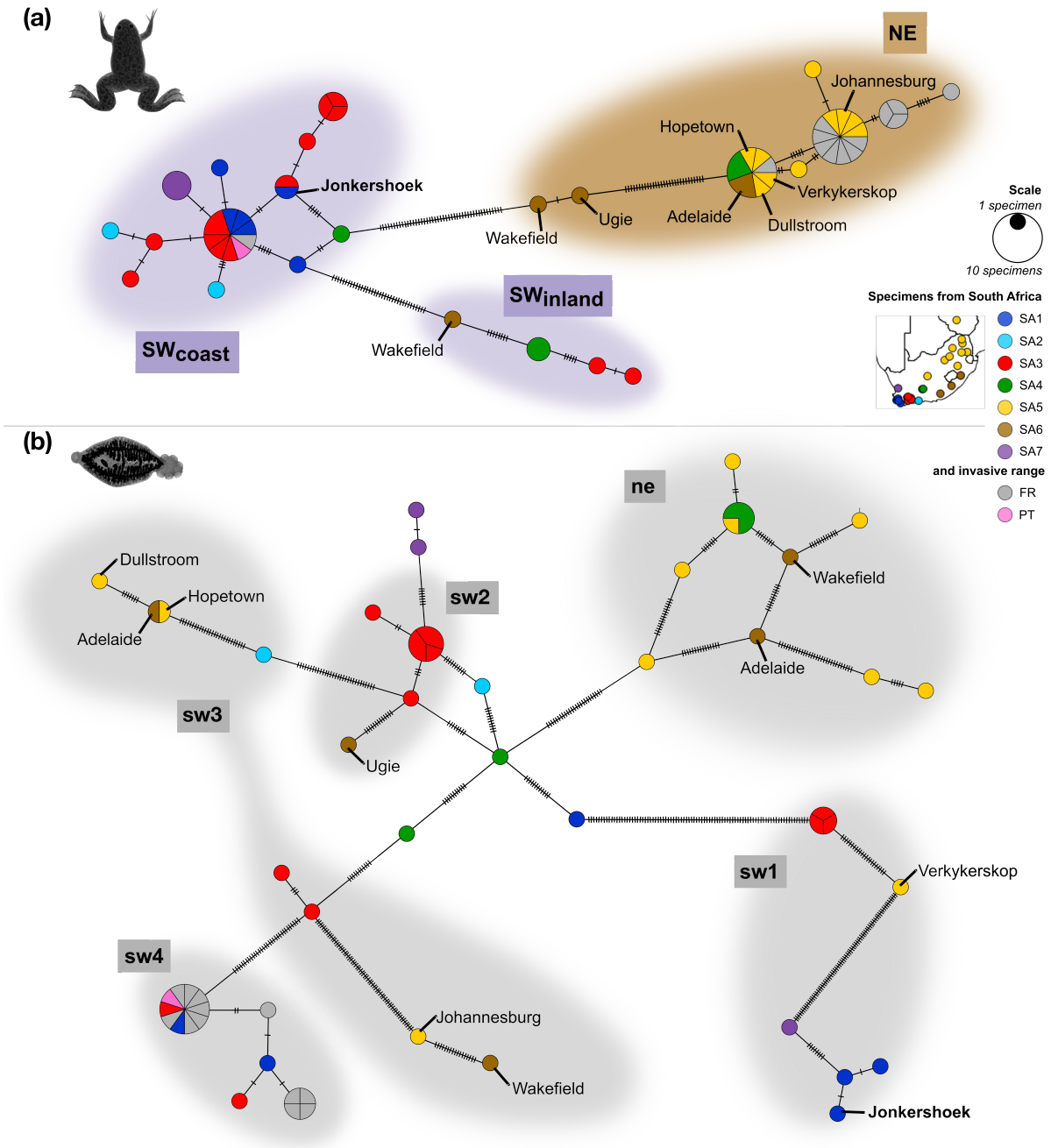

FIGURE 2 Median spanning haplotype networks of the partial *COX1* and *rrnS* genes of **(a)** 59 *Xenopus laevis* specimens and **(b)** their corresponding 59 *Protopolystoma xenopodis* parasites. For frogs and their polystomatids collected in the native southern Africa (see inset map), colours refer to the phylogeographic lineages of the host (de Busschere *et al.*, 2016). Co-introduced *X. laevis* and polystomatids are from France (grey) and Portugal (pink). Highlighted haplotypes correspond to the clades revealed by the phylogenetic analyses (Fig. 3): northeastern host (NE) and parasite (ne) and southwestern host (SW) and parasite (sw) clades. Key localities with host lineage switches are named, as well as Jonkershoek, the source locality of most of the *X. laevis* exports. Sizes of circles are proportional to haplotype frequencies (see scale). The number of mutations is marked on the connecting branches.

This haplogroup of polystomatid specimens from the invasive localities, which corresponds to **sw4** of the polystomatid phylogeny, was separated from the specimen from Jonkershoek (Fig. 1), the source of the majority of *X. laevis* exports, by

255 to 260 mutations.

3.3 Host and parasite phylogeny and congruence

Tree reconstruction showed that frogs from the northeastern SA5 and SA6 localities in the native range form a well-supported clade (clade NE in Fig. 3 with UFboot2 = 95% / SH-aLRT = 95.8%). In addition, two clades with unresolved relationships to one another and to NE could be identified among sampled frogs from the southwestern localities in the native range of *X. laevis* (clades SW_{coast} with UFboot2 = 81% / SH-aLRT = 86.1% and SW_{inland} with UFboot2 = 98% / SH-aLRT = 99.7% in Fig. 3). Members of the first clade originate from localities closer to the coastline (clade SW_{coast} in Fig. 3 from SA1, SA2, SA3 and SA7 localities) than the second one (clade SW_{inland} in Fig. 3 from SA3, SA4 and SA6 localities).

The two SA4 localities (near Three Sisters in the Karoo) are suggested as a contact zone between NE, SW_{coast} and SW_{inland}, with sampled individuals from this area present in all three host lineages. Likewise, frogs from an SA6 locality (Wakefield near Lesotho) were also present in both NE and SW_{inland}.

The non-native frog from the invasive population in Portugal, along with one of the non-native frogs from western France, grouped together with the native frogs in SW_{coast} in the host phylogeny, thus placing these invasive frogs in the same clade as the frog from Jonkershoek (Fig. 1), the source of the majority of *X. laevis* exports. Nonetheless, the invasive population of *X. laevis* in France also harboured several frogs that clustered with frogs from the native northeastern localities in the NE clade.

In the same manner as the host phylogeny, the ML inference of the *P. xenopodis* tree revealed a single, well-supported clade of polystomatids from hosts from the northeastern SA5 and SA6 localities and the SA4 contact zone (clade **ne** in Fig. 3 with UFboot2 = 97% / SH-aLRT = 87.6%). Several clades basal to the **ne** group emerged among the polystomatids from hosts collected at the southwestern SA1, SA2, SA3 and SA7 localities and the SA4 contact zone (clades **sw1**, **sw2**, **sw3** and **sw4** in Fig. 3). The marked phylogenetic divergence of the polystomatid was supported by significant morphological differences between polystomatids from the southwest of South Africa and the northeast of South Africa (see Appendix S3 for morphometrical methods and findings).

The earliest diverging clade is a well-supported grouping of polystomatids collected from SA1, SA3 and SA7 (clade **sw1** in Fig. 3 with UFboot2 = 94%/SH-alrt = 88.1%). Two other native southwestern clades included a clade of polystomatids from the SA3 localities (clade **sw2** in Fig. 3 with UFboot2 = 53%/SH-alrt = 90.7%) and another clade from a combination of SA2 and SA3 localities (clade **sw3** in Fig. 3 with UFboot2 = 61% / SH-aLRT = 86.6%). Another well-supported clade,

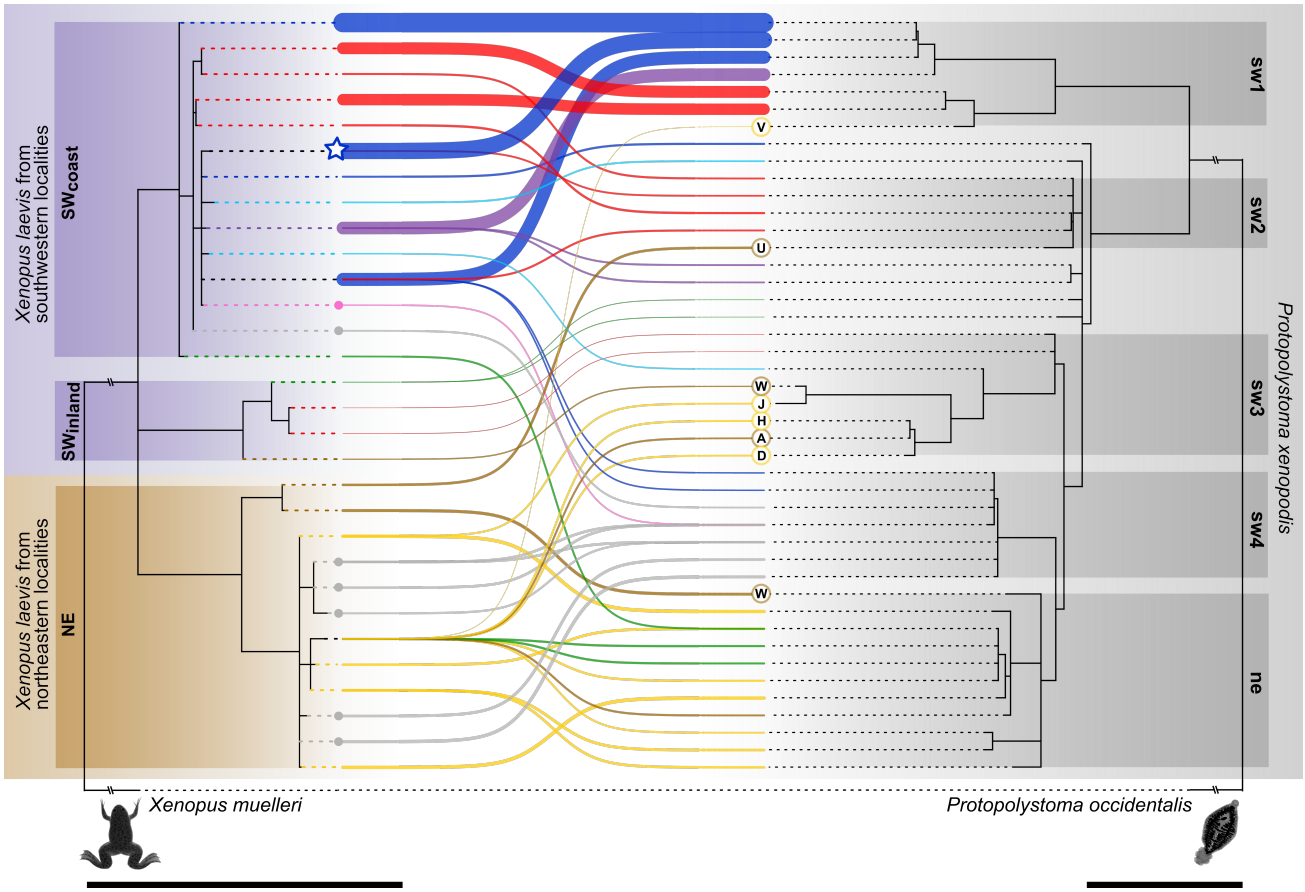

FIGURE 3 The interaction network and phylogenies of the frog *Xenopus laevis* (left) and its polystomatid *Protopolystoma xenopodis* (right) with closely related *X. muelleri* and *P. occidentalis* as outgroups. Topologies are based on the maximum likelihood analyses of the concatenated *COX1* and *rrnS* gene alignments of 31 hosts and 45 parasites with low support clades (UFboot2 $<$ 95% or SH-alrt $<$ 80%) collapsed. Links indicate the observed host-parasite associations, weighted according to the contribution of each interaction to the overall phylogenetic congruence with thicker lines corresponding to greater support. Colours refer to geographic origin, following the host phylogeography of de Busschere *et al.* (2016): SA1 (dark blue), SA2 (light blue), SA3 (red), SA4 (green), SA5 (yellow), SA6 (brown) and SA7 (purple) (see Fig. 1) with main lineages named and highlighted. Invasive *X. laevis* and co-introduced *P. xenopodis* from France (grey) and Portugal (pink) are also included. Key localities are Adelaide (A), Dullstroom (D), Hopetown (H), Johannesburg (J), Ugie (U), Verkykerskop (V) and Wakefield (W). Jonkershoek (☆) is the origin of the majority of *X. laevis* exports. Scale bars indicate 0.1 nucleotide substitutions per site for the two phylogenies.

the sister clade to **ne**, contained polystomatids from SA1, France and Portugal (clade **sw4** in Fig. 3 with UFboot2 = 100% / SH-aLRT = 100%). However, overall low support values of many internal nodes in the parasite ML tree hampered the interpretation of phylogenetic clustering among some of the polystomatids collected from the southwestern SA1, SA2, SA3, SA4 and SA7 localities.

A few links represented mismatches between the host and parasite phylogenies that could be explained by long distance translocation of frogs from the SW host lineages to the northeastern localities followed by spill-over of **sw1**, **sw2** and **sw3** polystomatids to NE hosts. These indications of host switching were identified in frog-polystomatid pairs from SA5 and SA6

localities (Dullstroom, Hopetown, Johannesburg, Verkykerskop, Adelaide, Ugie and Wakefield).

The phylogeny of *X. laevis* exhibited significant overall congruence with that of *P. xenopodis* (PACo m^2_{xy} = 0.1034, p = 0.0018, n = 10000; ParaFitGlobal = 0.0202, $p < 0.0001$, n = 10000), with the PACo analysis suggesting that the phylogeny of *P. xenopodis* tracked that of *X. laevis*. According to PACo, links between frogs from SW_{coast} and their corresponding polystomatids that fall within **sw1** in the parasite phylogeny, generally contributed the most to the overall phylogenetic congruence. The PACo jackknife procedure further identified the interactions between frog–polystomatid pairs from the invasive range in France and Portugal as links conveying low support to congruence. The ParaFitLink tests reported 30 out of 48 links as significant at a level of 0.05. Out of the 18 links that did not significantly support congruence, 11 signified host switches by parasites to another host lineage that occurred in the invasive range of France and Portugal and at SA5 and SA6 localities in the native range (Johannesburg, Three Sisters, Ugie and Wakefield).

4 DISCUSSION

In this study, the widespread, yet previously hidden, domestic expansion of *X. laevis* lineages is brought to light by the intraspecific phylogeography of its co-invasive parasite, *P. xenopodis*, a participant in the invasion history that is often forgotten. The use of parasite data to reveal host connectivity is hardly novel (reviewed in Gagne *et al.*, 2021). Yet, the present study is the first application of host-parasite co-phylogeographic analyses to the detection of intraspecific cryptic invasions. The study is not only a proof of concept that parasite data can provide additional information to trace biological invaders on the intraspecific level but it also demonstrates how parasite data can illuminate invasion pathways, thus informing management strategies.

4.1 Parasite clustering mirrors the intraspecific phylogeography of the host

The phylogeographic analyses of the parasite *P. xenopodis*, together with morphometric comparisons, reveal marked intraspecific geographic structuring across the native range of the species. The observed structuring in the parasite corroborates and expounds upon the phylogeography of the host *X. laevis* which also shows notable phylogeographic structure across its native range (de Busschere *et al.*, 2016; du Preez *et al.*, 2009; Furman *et al.*, 2015; Grohovaz *et al.*, 1996; Measey & Channing, 2003). Yet, the parasite data set provides an independent line of evidence and not a mere duplication of the phylogeographic structure of the frog, since each parasite represents a separate infection event from a pool of free-swimming larvae. Furthermore, with the exception of a brief free-swimming period that allows host switches to occur, *P. xenopodis* completes the whole of its life cycle in the excretory system of *X. laevis* (Theunissen *et al.*, 2014), which translates to a very

specific and intimate host-parasite association that can faithfully portray the phylogeographic structure of the frog. Therefore, based upon the phylogenetic congruence between the host and parasite, the phylogeography of the parasite can provide insight on the translocation of the host.

The genetically distinct host-parasite clusters display distributions that roughly follow the two climatic regimes in the region, namely the summer rainfall region to the northeast and the winter rainfall region to the southwest, with the plateau edge of the Great Escarpment and the many ridges of the Cape Fold Mountains as assumed geographical barriers to natural dispersal (Furman *et al.*, 2015). This divergence is further echoed by the significant morphological differences between polystomatids from the northernmost and the southernmost localities. The more pronounced clustering among hosts and parasites and the higher haplotype diversity in hosts that hail from the southwest of the Great Escarpment is likely brought about by the presence of the many ridges of the Cape Fold Mountains (de Busschere *et al.*, 2016; du Preez *et al.*, 2009; Furman *et al.*, 2015; Grohovaz *et al.*, 1996; Measey & Channing, 2003). On the other hand, well-documented human-mediated translocation of *X. laevis* to the southwest of the Great Escarpment (van Sittert & Measey, 2016; Weldon *et al.*, 2007), in conjunction with widespread overland migration of the frog with the help of the many interconnected artificial water bodies in the region (de Villiers & Measey, 2017; Measey, 2016), ensure some gene flow – and exchange of parasites – between the divergent frog populations in this area.

4.2 Parasite spillover is an indication of contact between divergent host lineages

Parasite phylogeography offers a tool to recover historical insights of host dispersal pathways, since host lineage switches reflect past interactions between host organisms at various time scales (e.g. Barbosa *et al.*, 2012; Criscione *et al.*, 2006; Galbreath & Hoberg, 2015; Galbreath *et al.*, 2020; Nieberding *et al.*, 2004). The usefulness of parasite species in reflecting the introduction pathways of their hosts has been demonstrated for fish hosts and their monogenean parasites on a few occasions (Huyse *et al.*, 2015; Kmentová *et al.*, 2019; Ondračková *et al.*, 2012; Reyda *et al.*, 2020). However, these studies focused on species-level invasions from the native to the non-native range, rather than intraspecific invasions of different lineages within the native range. Our study, on the other hand, demonstrates the use of parasites as tags to trace intraspecific cryptic invasions. Multiple instances of host lineage switches revealed by the phylogeographic analyses of *P. xenopodis* act as an indicator for contact between phylogeographically distinct *X. laevis* lineages. This provides us with the means of recognising contact between *X. laevis* lineages even in cases where the locally non-native host lineage may have gone extinct after the introduction event or where they are not detected at a certain sampling effort.

In fact, thus far, genetic evidence for the admixture of the lineages of *X. laevis* with native ranges on opposite sides of the Great Escarpment has not been found, despite historical records pointing towards large scale exports from the southwest to the rest of southern Africa (van Sittert & Measey, 2016; Weldon *et al.*, 2007). In our study, the host phylogeographic analyses suggest the presence of contact zones between the northeastern (NE) and southwestern lineages (SW_{inland} & SW_{coast}) at two localities to the northeast of the Great Escarpment (Fig. 2). Since these zones are situated to the north of a predicted geographical barrier, namely the plateau edge of the Great Escarpment (Furman *et al.*, 2015), one could surmise that anthropogenic translocation had a hand in bringing about this sympatry of multiple clades of *X. laevis*, pointing towards more recent translocation events.

Moreover, it is this sympatry of multiple host clades at localities that makes possible host lineage switching by parasite lineages. In turn, host switches provides more evidence for the translocation of *X. laevis* across southern Africa. Indeed, our study confirmed the presence of southwestern parasite lineages (**sw1**, **sw2** & **sw3**) at seven localities to the northeast of the Great Escarpment, where one would expect the northeastern parasite lineage (**ne**) to occur (Fig. 3). In these instances, southwestern parasite lineages that are translocated along with their respective host lineages, thus locally non-native at these new localities, spill over to the locally native northwestern host lineage when these divergent, previously allopatric host lineages come into contact (Galbreath & Hoberg, 2015).

Differential survival between locally native and non-native *X. laevis* lineages in southern Africa can partially explain the fewer observed instances of establishment by non-native *X. laevis* lineages when compared to its co-introduced polystomatid. In translocation experiments of different lineages in the native range, *X. laevis* tadpoles and metamorphs exhibited remarkable phenotypic plasticity when translocated between rainfall regimes at the cost of longterm survival (Kruger, 2020). Under these circumstances, the locally native *X. laevis* lineage would outcompete the non-native lineage, preventing successful local invasion. Thus, the employment of host phylogeography alone to trace long range translocation of *X. laevis* in southern Africa would mask a number of introduction events.

Upon arrival in the new area, the co-introduced *P. xenopodis* lineage may rapidly spread in a population of locally native and therefore naive *X. laevis*. It has been shown that primary infections by *P. xenopodis* in previously uninfected *X. laevis* hosts elicit strong long term immunological responses against secondary infections by the same parasite species (Jackson & Tinsley, 2001). Correspondingly, a cross-infectivity experiment between sympatric and allopatric *X. laevis* and *P. xenopodis* lineages further suggested that *X. laevis* lineages in their native ranges might be more susceptible to infection by the locally non-native *P. xenopodis* lineage than to their corresponding locally native parasite lineage (Jackson & Tinsley, 2005). In turn,

this effect will boost the nascent spread of non-native *P. xenopodis* lineages in the locally native lineage of *X. laevis*.

The rural locations of the parasite spillover events revealed by our analyses of polystomatid phylogeography suggest that smaller scale human-mediated dispersal events, as opposed to large official consignments from the southwestern region to centers of higher learning across South Africa, have far-reaching effects. The role of recreational fishing across South Africa in the dispersal of *X. laevis*, and by extension, *P. xenopodis*, has been recognised before (Weldon *et al.*, 2007). Previously documented interviews with local anglers and suppliers exposed the use of *X. laevis*, frequently bought from pet shops and local breeders, as live bait for predominantly catfish angling (Weldon *et al.*, 2007). Our own informal discussions with local anglers at the localities where we collected *X. laevis* affirm the widespread use of *X. laevis* as live bait for the capture of both black bass (*Micropterus* spp.) and catfish (*Clarias gariepinus*).

In terms of sheer numbers, the impact of these translocation events on the domestic dispersal of *X. laevis* has been deemed negligible when compared to exports from Jonkershoek, the most prolific exporter, and other official suppliers to the domestic market (van Sittert & Measey, 2016; Weldon *et al.*, 2007). Nonetheless, previous studies on other species have pointed out that the use of live bait in angling is an important pathway for introductions, even if the market might be small in economic terms (Kalous *et al.*, 2013; Kilian *et al.*, 2012). Therefore, the impact of anglers on the spread of locally non-native *X. laevis* lineages to more remote areas, as opposed to the bigger centra that received the documented official consignments, should not be underestimated. Our informal interviews identified a myriad of ways for *X. laevis* to be released in new environments by anglers, from escapees *en route*, to the deliberate release of surplus bait, to the intentional stocking of water bodies for future use.

4.3 Parasite phylogeography as an underutilised tool in invasion biology

As shown above, the present study acts as a proof of concept for the potential of the co-phylogeographic approach to the clarification of patterns in intraspecific host divergence and dispersal. In turn, this independent line of evidence can provide additional insight on invasion pathways and inform management strategies in profound ways. Here, we highlight some of the potential implications to demonstrate the broader applicability of this integrative approach.

Our approach was able to provide a new perspective on the origin of the relatively high haplotype diversity of the invasive population of *X. laevis* in France, recovered in our analysis and others, when compared to other invasive populations in Portugal, Sicily and Chile (de Busschere *et al.*, 2016; Lillo *et al.*, 2013; Lobos *et al.*, 2014). The idea of multiple introduction events from various source populations in South Africa has been offered before as the most parsimonious explanation for

this genetic diversity in the invasive population of *X. laevis* in western France (de Busschere *et al.*, 2016). Since we found evidence for host switches by *P. xenopodis* in the native range, an event that necessarily involves the sympatry of divergent host lineages, it is a certainty that multiple host lineages co-occur at certain localities in the native range. In this, our results build upon the evidence provided by historical export records (van Sittert & Measey, 2016; Weldon *et al.*, 2007), dispersal patterns (de Villiers & Measey, 2017; Measey, 2016) and anecdotal information (Weldon *et al.*, 2007) to show that the mixing of lineages most likely already happened in the source populations in South Africa prior to the co-introduction of *X. laevis* and *P. xenopodis* to western France (van Sittert & Measey, 2016; Weldon *et al.*, 2007).

The novel insights provided by the addition of the parasite data set has implications for both the conservation of *X. laevis* and its host-specific parasites. The widespread anthropogenic translocation of this host-parasite system disrupts interactions between *X. laevis* lineages and the *P. xenopodis* lineages that tracked their phylogenetic divergence, ancient interactions that deserve to be targets of conservation (Gómez & Nichols, 2013). Subsequent host switches between closely related lineages set the stage for new diversity to arise and for novel ecological interactions to be established (Galbreath *et al.*, 2020; Sures *et al.*, 2017). In the case of host-specific parasites, translocation of lineages within the native range of a species may actually be of more concern than translocation to far-flung non-native ecosystems, since these ecosystems will probably not harbour close relatives of the invasive host to facilitate host switches (Blackburn & Ewen, 2017). Host-parasite interactions are also something to keep in mind when conducting conservation re-introduction programmes, where captive-bred or locally non-native individuals of a species are used to re-stock locally extinct populations of endangered species (Britt *et al.*, 2004). Our results highlight that the evaluation of the genetic and parasitological suitability of a source population for translocation should also encompass the potential history of local introductions that may have influenced the intraspecific parasitism of the host population (Jørgensen, 2015; Northover *et al.*, 2018).

This approach further holds great promise in the management of invasive species. Prior to invasion, anthropogenic translocations in the native range may have already altered the source populations of invasive species, as is the case of *X. laevis*. We need to take altered phylogeographic structure into account to adequately monitor the course of an invasion or the novel interactions that may arise. Parasites as passengers or invaders themselves easily slip under the radar in invasion biology (Blackburn & Ewen, 2017; Solarz & Najberek, 2017). Yet, the role of parasites on the move in emerging infectious diseases and habitat transformation via indirect density-mediated effects cannot be overemphasised (Amundsen *et al.*, 2013; Bobadilla-Suarez *et al.*, 2017; Daszak, 2000; Dunn *et al.*, 2012; Goedknegt *et al.*, 2016; Lymbery *et al.*, 2014; Roy & Lawson Handley, 2012; Sures *et al.*, 2017). Furthermore, the identification of the local angling community as a major player

in the translocation of *X. laevis* in the native range allows policy makers to focus their intervention efforts in the management of *X. laevis* as a domestic exotic.

4.4 Recommendations to make parasite data sets work for invasion biologists

All in all, our findings agree with the necessity of a more holistic approach to data collection in invasion biology (Clavero *et al.*, 2016), especially with regard to parasites. The idea of the ”holistic specimen” has recently gained some traction (Cook *et al.*, 2017; Galbreath *et al.*, 2019). This approach to the collection and study of organisms advocates for the integrated specimen-based studies of animals and their associated parasites and pathogens. The optimisation of sampling effort is crucial to improve the allocation of resources, both in terms of funding and specimen use, especially when working with endangered species or specimens that require considerable effort to obtain, or in view of other ethical concerns.

The inclusion of parasite data during the data collection phase also ensures that the host and parasite data sets are comprised of directly comparable individuals which greatly improves the feasibility and value of comparative phylogeographic analyses. For example, the non-overlap between host and parasite data sets was cited as a limitation in the co-phylogeographic analyses conducted by both Barbosa *et al.* (2012) and Huyse *et al.* (2017). Even in cases where parasites are not the targets of the current project, the deposition of voucher material of both host and parasite into curated institutional collections, in other words overlapping data sets, can be vital for later studies (Galbreath *et al.*, 2019; Thompson *et al.*, 2021).

Another aspect to keep in mind is the choice of markers for co-phylogeographic analyses. In general, it is regarded as best practice to employ orthologous molecular markers in co-phylogenetic analyses (de Vienne *et al.*, 2013). Specifically, the use of orthologous markers for host and parasite in co-phylogeographic analyses to answer questions in invasion biology may have the benefit of making the findings from the two lines of evidence more directly comparable. The independent yet comparable DNA signature provided by the parasite data set may ameliorate inference based upon the phylogeographic structure of the host data set that is often blurred by the recent mixing of previously isolated genotypes in invasive populations (Rius & Turon, 2020).

Ultimately, this study serves as a proof of concept that the use of parasite data sets will add value to investigations in invasion biology, especially in the case of intraspecific cryptic invasions. In fact, we show that the utility of parasite data sets can go beyond the provision of supporting evidence and provide greater resolution that may lead to elucidation of patterns that went undetected in the host data set. Our study echoes the sentiments of Hoberg *et al.* (2015) who argued for an integrated approach to parasitology as a discipline that holds the keys to understanding and managing global change in the

Anthropocene.

DATA AVAILABILITY STATEMENT

All DNA sequences used for co-phylogeographic analyses will be made available on the public database GenBank, along with relevant metadata and accession numbers will be added upon article acceptance. Detailed locality data and data for additional morphometrical analyses are contained in the supplementary information that can be made available upon request.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

ALS conceived the initial idea, collected and analysed the data and led the writing of the manuscript. All authors refined the initial concept, designed the methodology, contributed to interpretation of data, contributed critically to the drafts and gave final approval for publication.

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