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Persistent effects of historical sea levels on the population structure of a temporary wetland copepod

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Abstract

1. Temporary wetland ecosystems are common in arid and semi-arid environments, and are inhabited by diverse invertebrate communities. Little is known about the dynamics of genetic connectivity in the geographically scattered populations of these wetland specialists.
2. The current study investigated the spatial genetic structure and dispersal history of a recently described calanoid copepod, *Lovenula raynerae*, reported from temporary wetlands in the Eastern Cape province of South Africa. We tested whether the species represents a single, well-connected population or comprises different regional genetic groups, some of which may be rare or endangered.
3. Mitochondrial COI sequences were generated for 365 specimens from 46 temporary wetlands spread across the species' known distribution range. Isolation-by-distance and isolation-by-environment patterns of partitioning genetic variations across the landscape were evaluated. In addition, the presence of historical impediments to gene flow between contemporary populations was investigated using a combination of Monmonier's algorithm and Bayesian reconstruction of phylogeographical diffusion in continuous space.
4. The wetland populations were highly structured across the landscape and could be assigned to six distinct evolutionary lineages, potentially representing some level of cryptic speciation. Two distinct phases were identified in the dispersal history of these lineages. Initially, dispersal only occurred inland of a postulated barrier, but eventually the barrier disappeared and the species extended its range by spreading into regions close to the coastline. Molecular dating shows that the barrier represents the upper limit of the coastline during the Pliocene, and that its crossing was facilitated by Pliocene sea regression in southern Africa.
5. Our finding shows that complex demographic histories can be preserved in the mitochondrial DNA of temporary wetland crustaceans because of limited effective

Arsalan Emami-Khoyi, Candice M. Jooste and Ryan J. Wasserman contributed equally to this study.

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gene flow after initial colonisation events. This makes them an interesting study system to explore the long-term effects of climate change on arid ecosystem communities.

KEYWORDS
calanoid copepod, dispersal barrier, historical demographic change, *Lovenula raynerae*, sea-level changes

1 | INTRODUCTION

Temporary endorheic wetlands are dynamic waterbodies that dominate the hydrology of arid and semi-arid regions around the world (Dalu & Wasserman, 2022; Vanschoenwinkel et al., 2011a). These ecologically productive ecosystems are typically isolated from larger permanent waterbodies and from each other (Fugate, 1998; Ripley & Simovich, 2009; Sacerdote & King, 2009), and undergo cyclic wet and dry phases (Dalu et al., 2016). A diverse array of aquatic crustaceans with a high degree of endemicity inhabit these distinctive ecosystems, and have evolved to exploit temporary wetlands as feeding grounds or as stepping-stones for dispersal (Williams, 2006). These species survive dry phases through dormant propagule production, capable of withstanding prolonged desiccation periods that are followed by successful hatching under favourable environmental conditions (Bird et al., 2019; Keeley & Zedler, 1998; Zedler, 2003).

Studies on temporary wetland species have focussed mainly on taxonomy and biodiversity (Bird et al., 2019), meta-community processes (Vanschoenwinkel et al., 2007, 2011b) and, more recently, on trophic interactions (Cuthbert et al., 2020; Dalu et al., 2017; Wasserman et al., 2018). To date, only a handful of studies have investigated the phylogeography of branchiopod crustaceans in temporary wetlands, mostly in Europe, North Africa and Australia (Kappas et al., 2017; Lukić et al., 2019; Pinceel et al., 2013; Reniers et al., 2013; Vanschoenwinkel et al., 2012). As a result, the dynamics of population structure, the extent of connectivity between populations, and the presence of historical impediments to gene flow are poorly understood (Pinceel et al., 2013).

Copepods are a diverse group of crustaceans that constitute more than 13,000 species (Humes, 1994). They inhabit various marine, brackish and freshwater environments (Boxshall & Defaye, 2008), and form integral food-web conduits between primary producers and higher trophic levels. Most copepod species occur in permanent waterbodies and have not developed the ability to produce propagules that can withstand desiccation (Hansen, 2019), but members of the subfamily Paradiaptominae are freshwater specialists that produce large quantities of desiccation-resistant propagules under dry environmental phases in their life cycle (Suárez-Morales et al., 2015). In this way, these copepods have evolved similar adaptations to those of large branchiopod crustaceans (Brendonck et al., 2008), and it has been suggested that mechanisms which govern dispersal and demographic dynamics in both groups are likely to be similar (Brendonck et al., 2017; Vanschoenwinkel et al., 2008).

Lovenula raynerae Suárez-Morales, Wasserman & Dalu, 2015 is a recently described species of Paradiaptominae that has been exclusively reported from temporary wetlands in parts of the Eastern Cape province on the south-east coast of South Africa (Jooste et al., 2019). This area is known for its exceptionally high biodiversity, as it hosts seven of South Africa's nine biomes (Lubke et al., 1986) and forms a geographical and climatic transition zone between southern Africa's different rainfall areas (Cloete & Lubke, 1999; Lubke et al., 1986). Compared to most other freshwater copepods, *L. raynerae* is exceptionally large (4–5 mm), making it the largest freshwater copepod recorded to date (Suárez-Morales et al., 2015).

Like other temporary wetland specialist crustaceans, *L. raynerae* is believed to rely on strong winds, occasional floodings and vector species such as birds, mammals and amphibians, to passively disperse its desiccation-resistant propagules across the landscape (Brendonck & Riddoch, 1999; Figuerola & Green, 2002; Vanschoenwinkel et al., 2008; Vanschoenwinkel, Mergeay, et al., 2011; Vanschoenwinkel, Waterkeyn, et al., 2011). However, the significance of this unique mode of dispersal on the species' evolutionary history, and the timescale at which it acts on populations, is currently unknown.

The spatial partitioning of genetic variation across a landscape depends not only on a species' dispersibility, but also on its reproductive success and the extent of density-dependent competition it faces once it has arrived in a new habitat (Waters et al., 2013). Hence, the order of arrivals in founder populations that colonise an unoccupied habitat can affect the spatial structuring of genetic diversity in future generations (De Meester et al., 2002). The founder populations readily establish themselves to reach high densities. By contrast, secondary dispersers may face intense competition from the thriving founder populations and, as a result, the competitive exclusion of late dispersers can counteract the homogenising effects of the movement of individuals between populations, eventually creating distinct lineages (Waters, 2011; Waters et al., 2013). Evidence for such density-dependent sorting of genetic diversity is widespread and includes taxa from bacterial and yeast colonies, to the migration of hominids out of Africa (Emerson et al., 2000; Fraser et al., 2009; Hallatschek et al., 2007; Juan et al., 1995; Oppenheimer, 2006, 2012; Seddon et al., 2001; Sequeira et al., 2000).

In the current study, we reconstructed the dispersal history of *L. raynerae* using samples collected from temporary wetlands throughout its known contemporary range, and investigated its genetic structure by testing whether (a) the species represents a single, well-connected population or (b) it comprises different regional genetic

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groups, some of which may be rare or endangered. The significance of historical sea-level changes on the species' phylogeography and the signature of historical events in the contemporary genetic makeup of this arid ecosystem species are discussed. This study serves as a baseline investigation for more comprehensive analyses of aquatic temporary wetland fauna in southern Africa.

2 | METHODS

2.1 | Sample collection and extraction

Samples of *L. raynerae* were collected from 46 distinct temporary wetlands across parts of the Eastern Cape province, South Africa (Table S1; Figure S1). Samples were collected with zooplankton nets with a mesh size of 200 μm and a diameter of 57 cm. Individual specimens were immediately transferred into Eppendorf tubes containing 80 μl of CTAB buffer (Doyle & Doyle, 1987) and 10 μl of Proteinase K (Qiagen, Hilden, Germany). Individuals were identified following Suárez-Morales et al. (2015), and the extraction of DNA commenced upon returning to the laboratory (within 3–4 days of collection) using the CTAB method (Doyle & Doyle, 1987).

2.2 | DNA amplification and sequencing

A fragment of the cytochrome oxidase c subunit I gene (COI) was amplified in 365 individuals using universal primers LCO1490 (5′–GGT CAA CAA ATC ATA AAG ATA TTG G–3′) and HCO2198 (5′–TAA ACT TCA GGG TGA CCA AAA AAT CA–3′) (Folmer et al., 1994). Each 20-μL reaction comprised 3 μL of DNA template, 1.5 mM of each primer (Integrated DNA Technologies), 2.5 mM of dNTPs (Sigma-Aldrich), 2.5 μl of 10 × PCR Buffer (Promega), 2.2 mM of $MgCl_2$ (Separation Scientific), 7.14 μl of double-distilled water, 1 μl of BSA (20 mg/ml) (New England Biolabs), and 0.16 μl of Super-Therm Taq polymerase (5 units/μl; Separation Scientific). Amplification was performed on a MultiGene™ OptiMax thermal cycler (Labnet International). The PCR protocol consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 49°C for 45 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The amplicons were visualised on a 2% agarose gel containing GelRed® Nucleic Acid Stain (Biotium) to verify successful amplification. Sequencing of amplified fragments was performed using BigDye Terminator version 3.1 cycle sequencing kit (ThermoFisher Scientific) on an ABI 3730 DNA analyser (Applied Biosystems).

2.3 | Sequence analyses

Low-quality sequences were manually removed using MEGA version 6 (Tamura et al., 2013), and quality-filtered DNA sequences were aligned using MAFFT version 7 (Katoh & Standley, 2013; Kuraku et al., 2013). To investigate the partitioning of the genetic diversity between wetlands, an analysis of the molecular variance (AMOVA) (Excoffier et al., 1992) in Arlequin version 3.5b (Excoffier & Lischer, 2010) was performed by using the program's default settings, and the estimated pairwise Φ_{ST} distance matrix was visualised in R version 4 (www.r-project.org). The population size parameters $\hat{\theta}_s$ and $\hat{\theta}_\pi$ were estimated for each wetland using the same software.

2.4 | Testing for isolation-by-distance and isolation-by-environment

In order to test for relationships between genetic distance, geographical distance and environmental conditions, 10 soil variables for each pond were obtained from the SoilGrids database (Hengl et al., 2017) at a 250-m spatial resolution and a soil depth of 0–5 cm (Table S2). The raster values of these soil variables were then extracted in ArcMap GIS version 10.8.1 (ESRI, 2019). In addition, two climatic variables, the precipitation of the wettest quarter and the precipitation of the driest quarter, were downloaded from the WorldClim database (Fick & Hijmans, 2017) at a 30 arc-second (i.e., 1 × 1 km) resolution.

Collinearity between environmental variables was investigated based on the variance inflation factors (VIF) calculated in the R package *olsrr* version0.5 (Hebbali & Hebbali, 2017), and predictors with VIF > 10 were considered significantly collinear and removed from the analysis.

A principal components analysis (PCA) was then performed on the centred environmental variables using the *prcomp* function in R. The distance between two locations on the resulting PCA axes, that accounts for >65% of the total variation, was used to approximate the environmental distance that separates ponds. Nei's genetic distance (Avise, 1994; Nei, 1972, 1978) between populations was estimated in adegenet version 2 (Jombart, 2008).

The effects of isolation-by-distance and isolation-by-environment on the evolutionary history of the species were firstly investigated by performing a series of Mantel tests (Mantel, 1967) in the R package *ecodist* version 2.09 (Goslee & Urban, 2007). Mantel tests only show the correlation between linear components of variations; thus, it is necessary to first investigate such a pattern in the data. For this purpose, a piecewise correlogram between genetic distance, geographical distance and environmental distance was created using the "pmgram" function in the same package, and the linear correlation between matrices was visually inspected. Then, the correlation between genetic, geographical and ecological dissimilarity matrices, as well as the partial correlation between genetic and environmental distance, once the effect of geographical distance is taken into account, was estimated.

Next, the pairwise matrices of genetic, geographical and PCA-transformed environmental distances were used in a multiple matrix regression with randomization (MMRR) analysis (Wang, 2013), and

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the statistical significance of the correlation between matrices was evaluated using 9,999 permutations.

2.5 | Investigating the presence of dispersal barriers

More detailed spatial genetic structure of the populations was investigated using two different methods. In the first method, Monmonier's maximum difference algorithm (Manni et al., 2004; Monmonier, 1973), implemented in adegenet, was used to identify barriers to gene flow between wetlands. The method places a barrier where the genetic differences between pairs of populations are highest, which are then plotted on a map. The number of independent runs was set to 4, and a Gabriel graph (Matula & Sokal, 2018) was selected for visualisation, with default settings for all other threshold values.

In the second method, the phylogeographical history of the species across the Eastern Cape landscape was reconstructed using BEAST version 1.10 (Suchard et al., 2018). To this end, a single representative of all the unique COI haplotypes present in each wetland was selected using FAbox version 1.41 (Villesen, 2007). The optimal sequence substitution model was estimated using the Bayesian phylogenetic site model averaging package bModelTest (Bouckaert & Drummond, 2017) implemented in BEAST version 2 (Bouckaert et al., 2019), and the substitution rate matrix for the phylogenetic reconstruction was manually modified to reflect the rates that were calculated during this step.

Then, the marginal likelihood of two phylogeographical models implemented in the BEAST package, diffusion in continuous space (Lemey et al., 2009) and diffusion in discrete space (Lemey et al., 2009), were estimated using stepping-stone and path sampling methods, and the best phylogeographical model was selected for further analyses. In the discrete model, each pond location was assigned a unique discrete identifier, and in the continuous model, the latitude and longitude for each wetland were added to the analysis as a bivariate trait. For the trait (location) evolution in the discrete model, a symmetrical substitution model with social network inference was specified, and for the continuous model, the marginal likelihoods of the Brownian random walk, Gamma relaxed random walk (RRW), Lognormal RRW and the Cauchy RRW model were calculated. To optimise the performance of the diffusion in continuous space analysis when all collected samples cannot be associated with unique geographical coordinates, a random jitter with a window size equal to 0.01 (Dellicour et al., 2021) was added to each tip. The remaining parameters were set to their default values.

The marginal likelihood for each model was estimated using 200 steps of path sampling, each with 20 million iterations. The model with the highest marginal likelihood was selected based on the Bayes Factor and was subsequently run for an additional 10 independent Markov chain Monte Carlo (MCMC) chains, each one billion iterations in length and with an initial burnin of 40%.

The trace files from the 10 independent chains were combined using LogCombiner version 2.1.3 (Rambaut & Drummond, 2014), and their convergence was checked using Tracer version 1.7.1 (Rambaut et al., 2018). A maximum clade credibility tree using mean heights and a 30% burnin was constructed in TreeAnnotator (Bouckaert et al., 2019), and the resulting tree was visualised in Figtree version 1.4.3 (Rambaut, 2016). The sequence of dispersal events between wetlands was visually summarised in SpreaD3 (Bielejec et al., 2011).

2.6 | Calibrating the Bayesian phylogenetic tree

In order to test whether sequences in the Bayesian phylogenetic tree evolve in a strict clock-like manner, a least-squares (LS) statistical test with 1,000 bootstraps and a composite matrix of GTR distances between sequences (Xia, 2009) was performed in DAMBE version 6 (Xia, 2017). Then, a likelihood ratio test was done to estimate the probability of error if the null hypothesis of a constant evolutionary rate among different lineages is falsely rejected. Finally, the presence of saturation in the sequence substitutions was tested using the same software. The reconstructed Bayesian phylogenetic tree was calibrated with a consensus mutation rate for the COI gene in crustaceans (Winkler et al., 2008).

2.7 | Estimation of the genetic distances between pairs of evolutionary lineages

In order to determine whether genetic distance among evolutionary lineages exceeded the threshold that may indicate the presence of distinct species (Bezeng & van der Bank, 2019; Dowton et al., 2014; Hebert et al., 2004; Klimov et al., 2019; Liu et al., 2017; Rossini et al., 2016; Smith et al., 2005), mean Kimura two-parameter (Kimura, 1980) (K2P) distances between pairs of evolutionary lineages were estimated in MEGA version 6. In addition, a generalised mixed Yule coalescent method (Fujisawa & Barraclough, 2013) implemented in the web server https://species.h-its.org/gmyc/ was used to statistically test the presence of different putative species across the landscape.

3 | RESULTS

3.1 | Sequence analysis

A total of 365 COI sequences, each 480 bp in length, were generated, and 58 distinct haplotypes were identified. The AMOVA analysis indicated that the major component of genetic variations resides between populations (93%, $p < 0.00001$). Ninety-four percent of all pairwise Φ_{ST} values between populations were statistically significant ($p < 0.05$), and the mean value of Φ_{ST} between pairs of populations was estimated at 0.93, confirming very high levels of population differentiation (Figure S2). The values of $(\hat{\theta}_s)$ and $(\hat{\theta}_\pi)$

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for total populations were 23.78 (*SD* 4.98) and 50.69 (*SD* 24.31), respectively.

3.2 | Isolation-by-distance and isolation-by-environment

A piecewise Mantel correlogram showed a clear monotonic linear relationship between geographical distance and both genetic and PCA-transformed environmental distances (Figure S3); therefore, the Mantel tests provide adequate estimates of the correlation between dissimilarity matrices. Specifically, the Mantel test analysis shows a statistically significant correlation between genetic distance versus geographical distance ($r=0.40$, $p=0.001$), and genetic distance versus enviromental distance ($r=0.09$, $p=0.03$). However, when the partial effect of geographical distance was considered, the correlation between genetic and environmental distance was no longer statistically significant ($r=-0.1$, $p=0.9$).

The MMRR analysis showed a similar pattern. In the model that did not include geographical distance as a predictor, the correlation between genetic and PCA-transformed environmental distances was marginally non-significant ($R^2=0.01$, $\beta=1$, $p=0.05$) and the model did not adequately explain variation in the data. However, when the geographical distance was considered, the geographical distance between pairs of ponds became the most significant predictor of genetic variation ($R^2=0.21$, $\beta=5.58$, $p=0.001$) and the model performance was considerably improved. This pattern highlights the major role of isolation-by-distance in the spatial genetic structure of the study species.

3.3 | Presence of dispersal barriers to geneflow

Monmonier's algorithm identified a barrier to gene flow that divides the wetlands into those associated with the coastal belt and those further inland (Table S3).

The bModelTest analysis identified a model with four rate changes ($r_{ac}=r_{cg}$; $r_{at}=r_{gt}$; r_{ag}; and r_{ct}) and a Gamma-shaped site heterogeneity as the optimal sequence evolution model, and this model was selected for the Bayesian phylogeographical reconstruction. Among the competing phylogeographical models, a phylogeographical model in continuous space with Cauchy RRW as the optimal model of trait evolution had the highest marginal likelihood in the log space and was selected to reconstruct the species' dispersal history (Table S4). However, this reconstruction remained unchanged when suboptimal models were run (not shown), pointing to the strong genetic signature of the dispersal history in the contemporary populations.

The Bayesian phylogenetic reconstruction assigned sequences to six distinct evolutionary lineages (Figure 1a). Reconstruction of the dispersal history of *L. raynerae* identified a geographical barrier in the species' contemporary distribution whose location matched

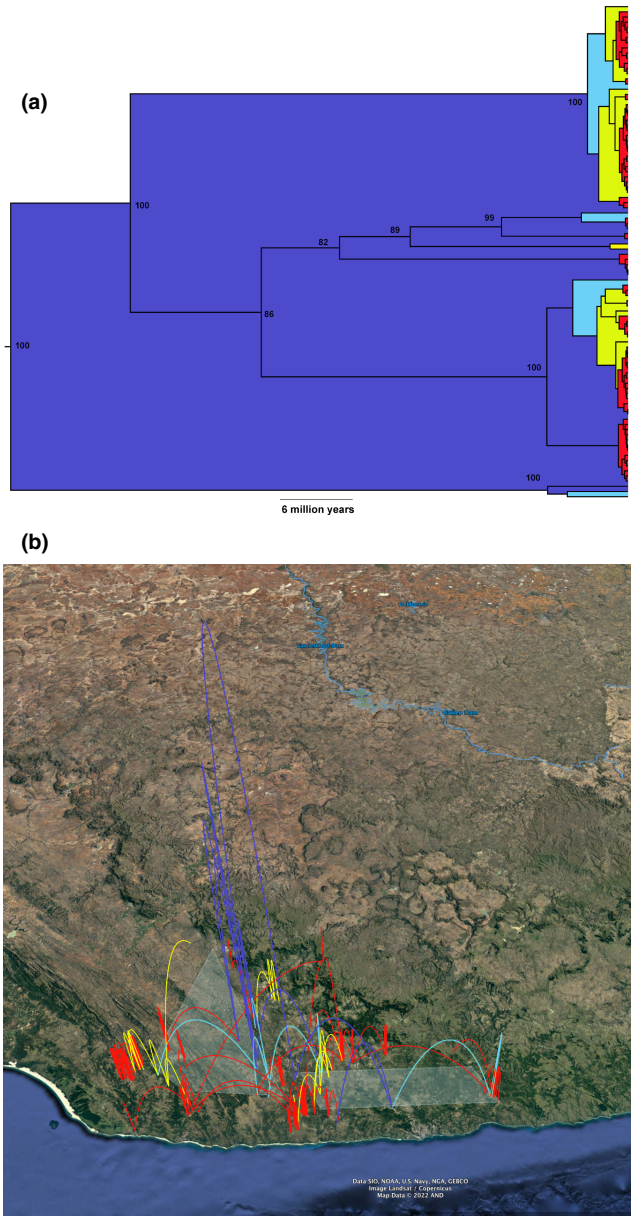

FIGURE 1 Phylogenetic tree reconstructed from COI sequences of *Lovenula raynerae* recovered across the species' known distribution range in South Africa's Eastern Cape province (a). The background colours on the phylogenetic tree represent the node age (the red represents divergence events that occurred <1 million years ago, yellow divergence 1–3 million years ago, light blue divergence 3–5.8 million years ago, and purple divergence >5.8 million years ago); (b) the sequence of events in the dispersal of *L. raynerae*, as visualised by SpreaD3. Branches on the map are coloured according to the age of their corresponding parent nodes in the phylogenetic tree above. The polygons show the approximate location of the reconstructed barrier. The purple and yellow branches show two major ancient dispersals extending species' distribution into the coastal zone. For a detailed animation of the dispersal events depicted in this figure, please see Video S1.

that inferred using the Monmonier's algorithm. Initially, dispersal only occurred between wetlands situated inland to the north-west of the barrier, until it effectively disappeared, and wetlands close to

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the coastline were colonised for the first time. Following this event, dispersal occurred in all directions (Figure 1b; Video S1).

Population size statistics $\hat{\theta}_s$ and $\hat{\theta}_\pi$ were higher for the populations located to the north-west of the reconstructed barrier compared to those located along the coastal belt (25.8 and 51.36 versus 23.16 and 44.68, for Θ_s and Θ_π, respectively). This pattern places the most likely source of the current populations into the geographical region containing the inland ponds. Likewise, the Bayesian phylogeographical reconstruction placed the most likely location of the root of the Bayesian phylogenetic tree in areas associated with inland ponds (−33.0969°S, 27.1638° E) (Video S2).

The null hypothesis that sequences did not evolve in a strict clock-like manner was rejected at a significance level of 5%, justifying the use of a uniform strict molecular clock across the Bayesian phylogenetic tree. No evidence for saturation in sequence substitution was observed in the dataset. Assuming a crustacean COI mutation rate of 0.007 (or 0.7%) per million years per lineage (Winkler et al., 2008), the earliest evidence for the crossing of the barrier by a haplotype dates back to 6.6 million years (95% highest posterior distribution [HPD]: 4.1–9.2). Soon after this event, another haplotype crossed the barrier approximately 1 million years ago (95% HPD: 0.22–1.98) (Figure 1; Video S1). The remaining haplotypes then dispersed into coastal areas within the past 1 million years.

3.4 | Genetic distances between evolutionary lineages

Mean K2P distances between lineages exceeded the 2%–4% thresholds that potentially define distinct species (Bezeng & van der Bank, 2019; Dowton et al., 2014; Hebert et al., 2004; Klimov et al., 2019; Liu et al., 2017; Rossini et al., 2016; Smith et al., 2005). In most cases, K2P values ranged from 16% to 26%. The likelihood ratio test in GMYC species delimitation analysis also confirmed that the model which split populations into a minimum of six putative species fitted the data significantly better (LR = 11.353, $p = 0.003$) than the null model that assumes a single species.

4 | DISCUSSION

Ephemeral wetlands, despite their small size and temporary nature, are important features of the landscape in arid and semi-arid southern Africa, as they support a wide diversity of aquatic and terrestrial species in what is otherwise inhospitable habitat (Goudie & Wells, 1995). Several invertebrate species, including the world's largest freshwater calanoid, *L. raynerae*, evolved to exploit these seasonal habitats (Bird et al., 2019). The present study investigated the spatial genetic structure and dispersal history of this recently discovered species in temporary wetland systems in the Eastern Cape province of South Africa.

The significant level of genetic differentiation between wetlands indicates that potential vector-mediated movement of diapausing propagules, and intermittent connectivity of adjacent habitats during extreme climatic events, was insufficient to evenly distribute genetic diversity among populations. The effective role of vector species in the long-distance dispersal of crustacean propagules has been discussed elsewhere (Muñoz et al., 2013). However, the evidence for the significant population structure in the populations of *L. raynerae* confirms that vector-mediated gene flow may primarily contribute to the expansion of the species' distribution range into adjacent vacant habitats, as the magnitude of this dispersal does not counteract other evolutionary forces that promote genetic differentiation between populations.

Statistically significant isolation-by-distance partitioning of genetic variation across the landscape shows that effective gene flow between wetlands decreases significantly as the geographical distance separating populations increases. By contrast, when the partial effects of geographical distance were considered, the environmental dissimilarity between different ponds did not explain the structured genetic diversity across the landscape.

The dormant propagules produced by *L. raynerae* are exceptionally large (Suárez-Morales et al., 2015), and this is likely to have implications for both wind and zoochorous transport between wetlands that are presently not well understood. The observed pattern is consistent with an evolutionary scenario in which different regional genetic groups with limited effective gene flow coexist across the landscape.

In passively dispersed crustaceans, the dispersal of a small number of desiccation-resistant propagules can result in the foundation of new populations in suitable habitats (Frisch et al., 2021), and these populations then grow exponentially in size and form a large number of diapausing egg banks (Muñoz et al., 2013). Secondary dispersers are confronted by intense competition from populations established by the original founder individuals, which may have become locally adapted and developed a competitive edge over time, reducing the contribution of late dispersers to the genetic diversity in future generations (De Gelas & De Meester, 2005; De Meester et al., 2002; Gómez et al., 2000; Muñoz et al., 2008, 2013). Consequently, even substantial vector-mediated transport of propagules will not necessarily manifest in an appreciable level of effective gene flow. Founder effects during periods of range expansion will thus promote the formation of local "lineage patches" with long-lasting genetic signatures of the original founder events in the contemporary populations.

The genetic distances between these lineages were all considerably greater than the 4% threshold beyond which interbreedings between crustaceans have rarely been reported (Lagrue et al., 2014), and all of the haplotypes within each lineage descended from a common ancestor to form a monophyletic group (Figure 2). The observed pattern could indicate an early stage of cryptic speciation in *L. raynerae* across the Eastern Cape (Nixon & Wheeler, 1990).

The reconstruction of the dispersal history of *L. raynerae* in the Eastern Cape identified an invisible barrier to gene flow across the species' distribution range. This barrier divides the wetland sites into those near the coast versus those situated inland. The dispersal of the species was initially limited to inland sites until the barrier

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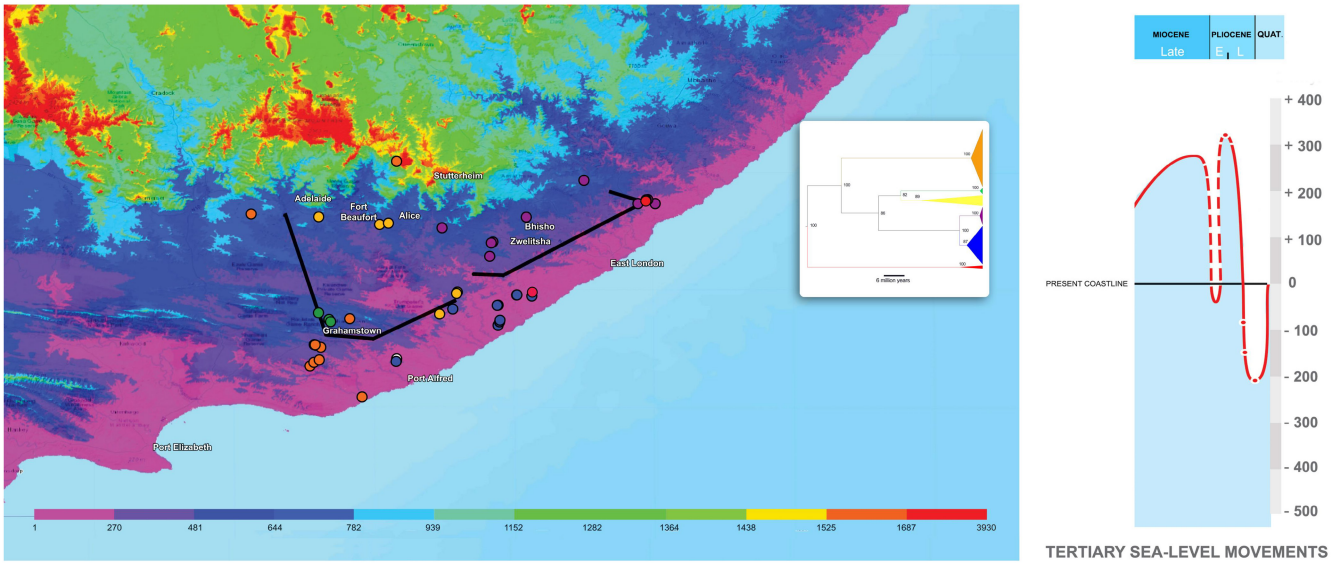

FIGURE 2 Partial reconstruction of the Eastern Cape coastline showing the approximate geographical location of the dispersal barrier in *Lovenula raynerae* (black lines). The ponds are coloured based on the evolutionary lineage that was numerically dominant in each pond, with the phylogenetic tree (insert) showing the lineages represented by each colour (this tree is a simplified version Figure 1). This map shows that the area where today's coastal belt wetlands are found was inundated during most of the Pliocene (light purple areas). The scale bar on the map shows the altitude relative to the present-day sea level. Changes in the sea level across southern Africa based on the highest altitude at which fossilised remains of marine invertebrates were found (*y*-axis) are shown on the right (modified from Siesser & Dingle, 1981).

eventually disappeared, and coastal areas were colonised for the first time during the Pliocene. This epoch is chronologically linked to marine regressions (Siesser & Dingle, 1981) following marine flooding of the Eastern Cape during the Tertiary, which created the Alexandria Formation that is currently found tens of kilometres inland in various locations (Oliver, 1971; Ruddock, 1947). Fossilised remains of Pliocene marine invertebrate species, including the foraminiferans *Florilus victoriense*, *Ammonia ammoniformis* and *A. italica*, were found in the calcified sand deposits at altitudes up to 330 m (King, 1972), and this information can be used to estimate the contemporary boundaries of ancient marine beds.

Partial reconstruction of the Pliocene coastline in the Eastern Cape, based on the highest altitude at which fossilised remains of Pliocene marine fauna were discovered (King, 1972), suggests that the geographical locations where the contemporary coastal belt wetlands are located were underwater during most of the Miocene and early Pliocene, and gradually emerged later in the Pliocene when the sea regressed (Figure 2). By contrast, sea-level high stands during the Tertiary never reached the area to the north-west of the historical dispersal barrier where today's inland wetlands are located (Daniel et al., 1974) (Figure 2).

We suggest that the historical barrier evident in the reconstructed dispersal history of *L. raynerae* reflects the upper limit of the Eastern Cape coastline during the Pliocene. When the sea regressed later in the Pliocene, a subset of founder lineages from inland wetlands dispersed into habitats previously inundated by seawater. Then, a combination of low effective gene flow, genetic drift, potential monopolisation of available resources by founder populations (De Meester et al., 2002; Frisch et al., 2021), and environmental filtering (Frisch et al., 2021) may have influenced the species' evolutionary history to create the contemporary lineage patches.

The fact that *L. raynerae* diversified into multiple evolutionary lineages during the Pliocene has potential taxonomic and conservation implications for southern African ephemeral pond fauna in general. The finding that ancient phylogeographical patterns can persist long after the processes that created them are no longer present, and deep divergence that suggests the existence of distinct cryptic species, indicates that this landscape may harbour many more unique regional populations or species that may require protection. As such, the present study thus represents a starting point for further research into overlooked biodiversity in southern Africa that could include exploring morphological differences, reproductive isolation, and adaptation to regionally unique environmental conditions between evolutionary lineages.

AUTHOR CONTRIBUTIONS

Conceptualisation: PT, RW, TD, BJ. Developing methods: PT, RW, TD, AE. Data analysis: AE, CJ, PT, MR. Preparation of figures and tables: AE, CJ, MR. Conducting the research, data interpretation, writing: all authors contributed.

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CONFLICT OF INTEREST STATEMENT

None of the authors declare any conflict of interest.

DATA AVAILABILITY STATEMENT

The DNA sequences generated in this study are available from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/) under accession numbers OL376366 - OL376423.

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