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Phylogeographic Patterns in a Semi-lithophilous Burrowing Scorpion, *Opistophthalmus pallipes*, from South Africa

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Southern Africa has a diverse endemic scorpion fauna, but a paucity of information currently confounds conservation of the group. Phylogeographic approaches represent a useful tool to identify the patterns and processes which underpin scorpion diversity, but these studies are lacking for southern African species. Among southern African scorpions, the semi-lithophilous *Opistophthalmus pallipes* has strict habitat requirements, and a distribution historically subjected to profound environmental turnover. As such, the species offers a model system to investigate the interplay between intrinsic and extrinsic factors as drivers of diversity and endemism. To investigate spatial genetic patterns within *O. pallipes* and the possible drivers thereof, the current study combines mitochondrial DNA and ecological information under a phylogeographic approach. The species is characterized by several genetically discrete and divergent populations. The factors which shape these genetic patterns appear to be both intrinsically (ecological specificity) and extrinsically (landscape structure and ecogeographic conditions) influenced, with major divergences corresponding to periods of profound environmental changes. Taken together, the findings of this study provide evidence of spatial genetic isolation and genetic diversity within a stenotopic southern African scorpion species. These findings partly explain the staggering diversity and endemism in southern African scorpions, but further phylogeographic studies are necessary to propose conservation scenarios for this group.

Key words: ecogeographic areas, Knersvlakte Region, Namaqua burrowing scorpion, *Opistophthalmus pallipes*, phylogeography, South Africa

INTRODUCTION

Southern Africa hosts a diverse and endemic scorpion fauna (140 species, of which nearly 96% are endemic; Prendini, 2001, 2005). Even so, a paucity of data regarding biological aspects of southern African scorpion species characterizes the literature. This data deficiency currently impedes the conservation of the group, with no species that has been assessed for the IUCN or benefits from formal protection outside of existing nature reserves (IUCN, 2019). As such, this lack of conservation focus may have far-reaching consequences in the face of modern threats to biodiversity (e.g., Tilman et al., 2017), especially given the vulnerability of scorpion species to climate change (e.g., Ureta et al., 2020).

Conservation of biodiversity relies predominantly upon assessments performed by the IUCN (see e.g., Child et al., 2017), with these assessments considering taxonomy (defined as species, species complexes, newly described/undescribed species, subspecies or subpopulations) as units when identifying and protecting unique and narrow-endemic lineages. In this regard, molecular data and phylogeographic approaches have been employed successfully in identifying cryptic scorpion diversity in other regions of the world (e.g., Gantenbein et al., 2001; Santibáñez-López et al., 2014; Gonzalez-Santillan and Prendini, 2015; Sharma et al., 2015; Esposito et al., 2017; Graham et al., 2017). Coupled to this, phylogeographic approaches have also enabled the identification of the possible processes which underpin and maintain this diversity (e.g., Gantenbein and Keightley, 2004; Habel et al., 2012; Graham et al., 2012, 2013a, b, 2017; Bryson et al., 2013a, b, 2014; Shi et al., 2013; Ceccarelli et al., 2016).

Taken together, the drivers of scorpion diversity appear centered on the long-term interplay between their sedentary nature and often narrow ecological requirements (intrinsic characteristics), coupled to the spatiotemporal evolution of the landscape (extrinsic factors such as geomorphological and climatic changes [Graham, 2012, 2017; Bryson et al., 2013a, b]). Although largely speculative, species richness and endemism among southern Africa scorpions have been suggested to be underpinned by similar processes (e.g., Prendini, 2001, 2005; Prendini and Bird, 2008; Foord et al., 2015). Species across the subregion are frequently ecologically specialized (stenotopic), exhibiting stringent ecological requirements and high habitat fidelity (Fet et al., 1998; Prendini, 2001, 2005 and references therein; Druce et al., 2007; Visser and Geerts, 2020). Furthermore, these stenotopes exhibit a poor dispersal ability (as little as a few meters per year; Polis

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et al., 1985), thereby increasing the propensity for diversification across spatiotemporally heterogeneous landscapes (Prendini, 2001, 2005; Foord et al., 2015).

With limited dispersal ability and patchy populations, *Opistophthalmus* is the most species-rich genus, containing 59 recognized species (Prendini, 2000, 2001; Harington, 2002). Distributions of *Opistophthalmus* species closely track soil hardness and texture (Eastwood, 1978; Prendini, 2001), with the genus harboring a multitude of stenotopic species. Among stenotopes, the semi-lithophilous Namaqua burrowing scorpion *Opistophthalmus pallipes* is associated with rocky areas across western South Africa, where it occupies burrows under separate rocks (Eastwood, 1978; Prendini, 2001; Leeming, 2003). Because rocky habitat across the region is not continuous, *O. pallipes* populations display a heterogeneous distribution across the species' range (J.H. Visser, personal observation). Furthermore, population sizes across the arid northern part of the distribution (especially in the Namaqualand Region) appear relatively small (< 10 individuals per population, J.H. Visser, personal observation) and geographically patchy (populations occur several kilometers apart), and are therefore possibly in need of conservation focus.

The distribution of *O. pallipes* across western South Africa has been subjected to major historical climatic and geomorphological upheavals. For instance, a major uplift event during the early Pliocene, (uplift of 150–300 m; Partridge and Maud, 1987, 2000) initiated the Post-African II erosion cycle (erosion of the newly uplifted geological surface and incision of major valleys), driving the development of soil types across the region (Campbell, 1983; Deacon et al., 1992). During this period, a major marine ingression (sea-level of > 300 m above present) also inundated large portions of the low-lying inland coastal areas (Siesser and Dingle, 1981), with climatic conditions during the later Pleistocene further alternating between mesic (during interglacial periods), and arid (during glacial periods) periods, thereby altering the spatiotemporal extent of climatic regimes and vegetation types (Van Zinderen Bakker and Mercer, 1986 and references therein). In addition to these upheavals, western South Africa hosts the Knersvlakte Region – an arid plain (~18 Myr in age, Moon and Dardis, 1988; 40–100 kilometres in breadth, Kounov et al., 2008) implicated as a major phylogeographic disruptor (barrier to gene-flow) in a multitude of vagile rock-dwelling vertebrate taxa (e.g., Matthee and Robinson, 1996; Lamb and Bauer, 2000; Matthee and Flemming, 2002; Smit et al., 2007; Daniels et al., 2010; Portik et al., 2011; Visser et al., 2020).

Through investigating phylogeographic patterns in the lithophilous scorpion *Smeringurus vachoni* across the Sonoran and Mojave deserts, a recent investigation indicated that profound historical landscape changes since the Miocene (climatic changes, lake formation and drainage evolution) drive vicariant events, and consequently genetic structures and genetic diversity in this stenotopic species (see Graham et al., 2017). Consequently, *O. pallipes* represents a suitable model to evaluate these biogeographic hypotheses surrounding the drivers of diversity and endemism in an African context (e.g., Prendini, 2001, 2005; Prendini and Bird, 2008; Foord et al., 2015). Therefore, this study combines molecular (mitochondrial DNA) data and ecological information under a phylogeographic approach to evaluate spatial genetic patterns in *O. pallipes*, and highlights possible key processes which underpin these patterns. Following major historical climatic and geomorphological upheavals across the species' range, it is expected that genetic structure will follow the fragmentation and isolation of populations in disparate areas of suitable habitat. As such, spatially separate populations should show evidence of genetic exclusiveness. Furthermore, it is expected that the Knersvlakte Region may form a dominant phylogeographic disruptor to *O. pallipes*, similar to other taxa that are rupicolous or habitat specialists. Finally, given the establishment of ecogeographically distinct areas across the studied region since the Pliocene, it is expected that spatially disparate populations may be isolated in areas with vastly different ecological compositions. This information is a critical first step to investigate patterns of diversity and endemism in understudied southern African scorpion species, and ultimately guide conservation of this group.

MATERIALS AND METHODS

Specimen collection

To assess phylogeographic hypotheses, *O. pallipes* specimens (n = 44) were collected (CapeNature Permit Number: CN44-59-9347) from seven localities across a large portion of the species' range (Table 1, Fig. 1A). The selected localities represent geographically disparate populations, thereby allowing for a repre-

Table 1. List of *Opistophthalmus* specimens included in this study. For each, the species' name, number of specimens included, sampling locality, sampling coordinates and DDBJ/ENA/GenBank accession number(s) for the COI sequence(s) are indicated.

Species	# specimens	Locality	Coordinates	Accession #
Opistophthalmus macer	1	Stilbaai	34°21′S, 21°24′E	MT582271
Opistophthalmus capensis	1	Trekoskraal	32°53′S, 17°52′E	MT582273
Opistophthalmus capensis	1	Dwarskersbos	32°41′S, 18°14′E	MT582272
Opistophthalmus pallipes	10	Sterkfontein	32°47′S, 18°34′E	MT582287–MT582296
Opistophthalmus pallipes	21	Redelinghuys	32°28′S, 18°29′E	MT582297–MT582317
Opistophthalmus pallipes	1	Koningskop	32°29′S, 18°50′E	MT582286
Opistophthalmus pallipes	4	Klawer	31°44′S, 18°36′E	MT582280–MT582283
Opistophthalmus pallipes	2	Rooiberg	31°12′S, 18°28′E	MT582284–MT582285
Opistophthalmus pallipes	3	Garies	30°33′S, 17°56′E	MT582277–MT582279
Opistophthalmus pallipes	3	Kamieskroon	30°05′S, 17°45′E	MT582274–MT582276

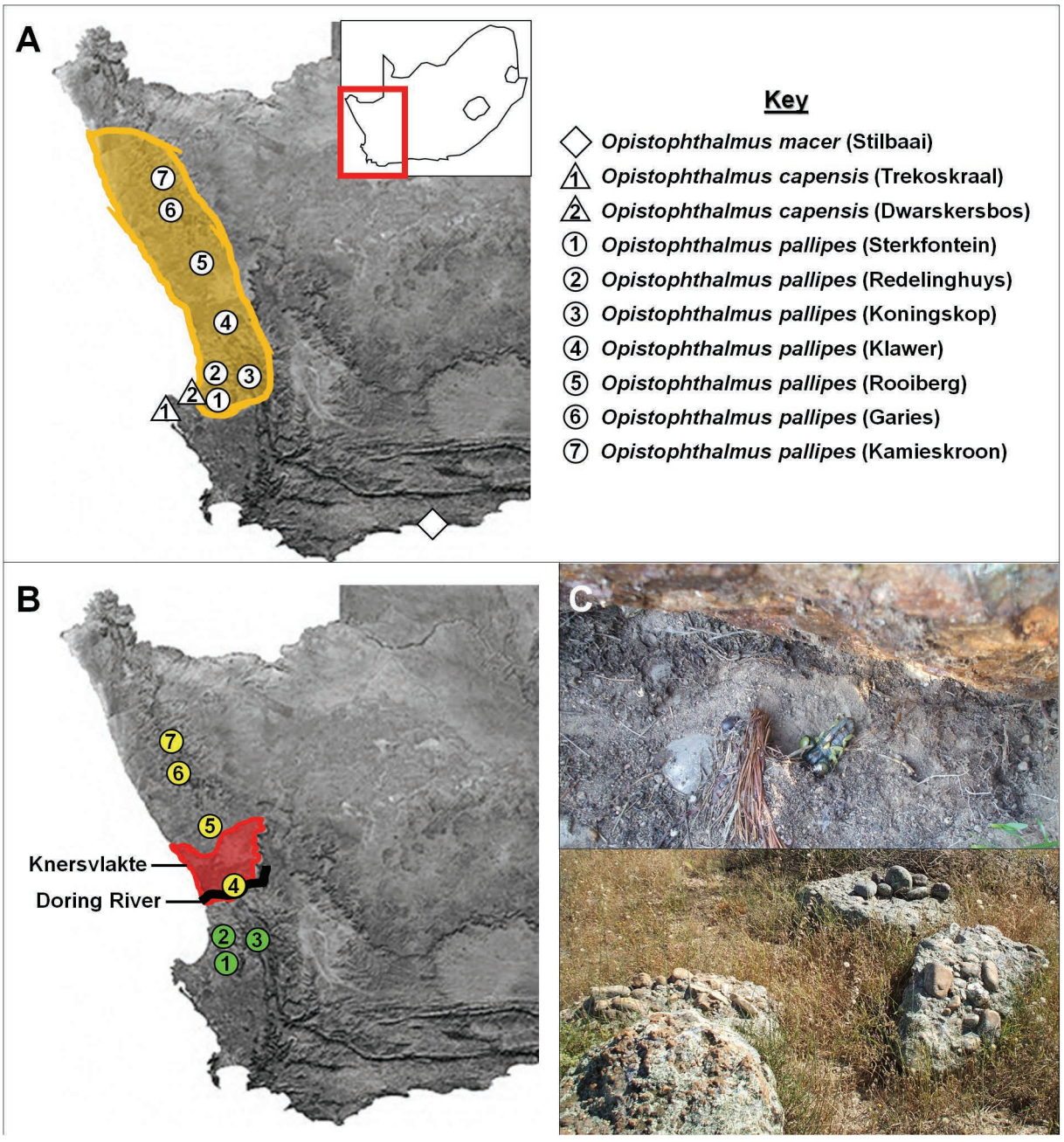


Fig. 1. Sampling localities for the *Opistophthalmus* specimens and the habitat of *O. pallipes*. **(A)** Sampling localities, with an indication of the geographic distribution of *O. pallipes* (shaded area) based on the point-locality data available from the Atlas of African Scorpions (http://vmus.adu.org.za/?vm=ScorpionMAP). **(B)** Populations which constitute the two major clades (Cape [1–3] and Namaqualand [4–7] lineages; Figs. 2, 3), along with the geographic location of the major geographic features (Knersvlakte and Doring River). **(C)** An *O. pallipes* specimen in its burrow system under a rock, along with a representation of the rocky habitat occupied by the species.

sentative rendering of the phylogeographic patterns across this region. Outgroup taxa included two other *Opistophthalmus* species (*Opistophthalmus capensis* and *Opistophthalmus macer*; Fig. 1A). The sampled species are all readily identifiable based on external morphology, as well as from species' distributional data (e.g., Atlas of African Scorpions, FitzPatrick Institute of African Ornithology, Department of Biological Sciences, University of Cape Town). Sampling of *O. pallipes* and *O. capensis* followed active searching along patches of suitable rocky habitat (i.e., the turning of stones), with *O. macer* collected using a UV light during nocturnal hours. Specimens were collected with a pair of long forceps, and were euthanized by immersion in 70% ethanol (as outlined in Cooper, 2011), with storage in this form prior to DNA extraction.

DNA extraction and sequencing

Total genomic DNA was extracted from a back leg of specimens using a commercial DNA extraction kit (DNeasy Tissue and Blood kit; Qiagen) following the manufacturer's protocol. The mitochondrial DNA fragment cytochrome *c* oxidase subunit I (COI) was amplified using universal primers (LCO1490 and HCO2198, Folmer et al., 1994). PCR amplifications followed standard protocols and were performed in a MultiGene Optimax system (Labnet

International, Inc.) at a region-specific annealing temperature of 48°C. Successful amplifications were verified on a 1% agarose gel and amplicons were sequenced using BigDye chemistry and analysed on an ABI 3730 (Life Technologies). Electropherograms of the raw data were aligned and checked manually (Geneious Pro™ 7.0 software; Biomatters Ltd).

Data analyses

To visually represent genetic diversity and structure within *O. pallipes* populations, a haplotype network was constructed in Network version 10.1.0.0 (Fluxus Technology Ltd) using the Median Joining algorithm. Phylogenetic relationships among populations were assessed through Bayesian Inference (BI) methods and Maximum Likelihood (ML) with nodal support determined through posterior probabilities and bootstrapping, respectively. Prior to genealogical analyses, the best-fit substitution model (GTR + I + G) was determined through Modeltest version 2.1 (Guindon and Gascuel, 2003; Darriba et al., 2012) using the Akaike Information Criterion (AIC, Akaike, 1973).

BI trees were constructed in BEAST version 1.10.4 (Drummond et al., 2012) and MrBayes version 3.2.7 (Ronquist et al., 2011) by running 50 × 10^6 generations and 5 × 10^6 generations respectively,

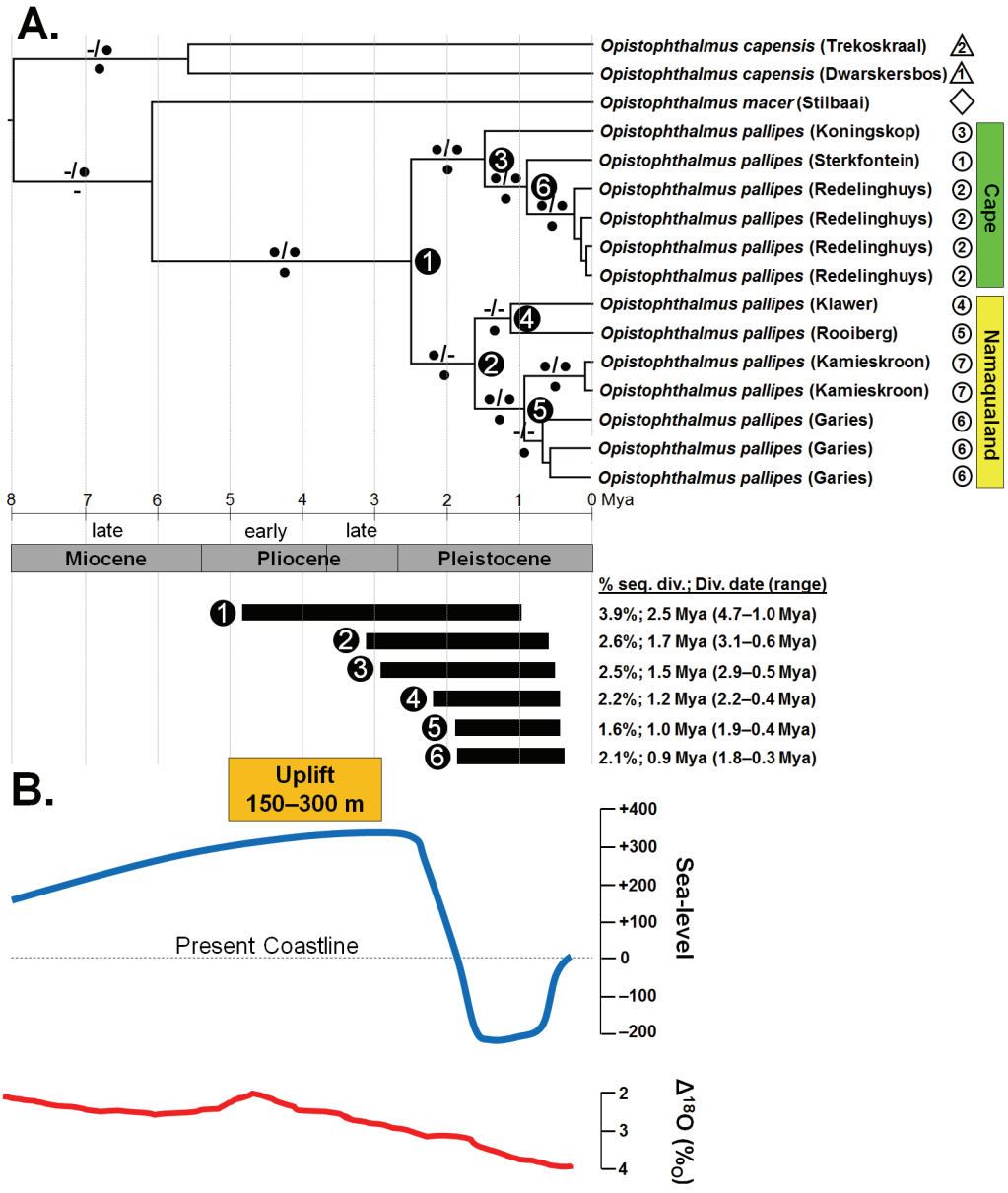


Fig. 2. A time-calibrated phylogeny of *Opistophthalmus pallipes* with paleogeographic and paleoclimatic changes. **(A)** A phylogeny generated in BEAST based on the COI data. Symbols above nodes represent posterior probability values derived from the BI in BEAST and MrBayes, respectively, while symbols below nodes indicate bootstrap values derived after ML. Support values of a posterior probability > 0.95 and bootstraps of 100% are indicated by black circles, while values below these thresholds are designated with a "-". Symbols at each terminal node correspond to those in Fig. 1. The temporal extent of divergence date ranges (black bars) for each of the major divergences (nodes 1–6), and with the uncorrected sequence divergence (%) and divergence date estimates (confidence interval for this estimate in parentheses) are shown as an inset. **(B)** The temporal scale of a major uplift event (which initiated the Post-African II erosion cycle), as well major sea-level changes (blue line; adapted from Siesser and Dingle, 1981) and climatic trends (red line, deduced from δ18O of marine sediments with higher values indicating colder temperatures; adapted from Cowling et al., 2009) across the identical timeframe are indicated.

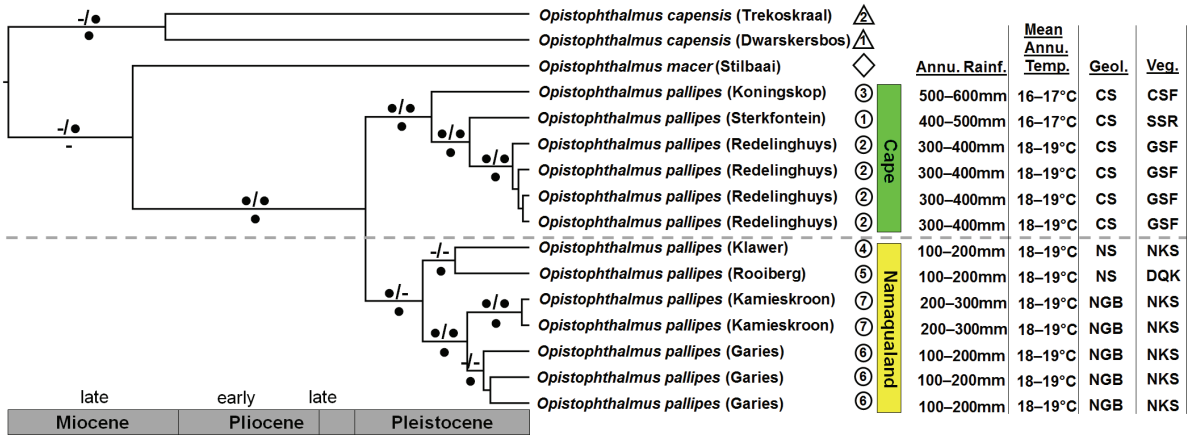
with sampling every 1000 generations. To assess adequate convergence of the MCMC chain, the Effective Sample Size (ESS) of parameters was verified in Tracer version 1.5 (Rambaut and Drummond, 2003). After discarding the first 25% of the trees as burnin, a majority rule consensus tree with posterior probabilities was assembled and visualized in Figtree version 1.4.2 (available at: http://tree.bio.ed.ac.uk/software/figtree/). To obtain estimates of divergence times for the various clades, a strict molecular clock model with a Yule tree prior and an UPGMA starting tree was applied to the COI data in BEAST. A mean mutation rate of 0.00925 substitutions/site/million years was used based on available COI mutation rate data in *Brachistosternus* scorpions (Ceccarelli et al., 2016), and a mean standard deviation of 0.004 was specified to include mutation rate estimates from other investigations (0.007, Gantenbein et al., 2005; 0.0133, Ceccarelli et al., 2017; 0.00393–0.00734, Graham et al., 2017). Samples from the posterior distribution of trees were summarized in TreeAnnotator version 1.6.1 (Drummond et al., 2012) with burnin set to 25%. ML analyses were performed in RAxML version 7.0.4 (Stamakis, 2014), which comprised 1000 bootstrap replicates. A majority rule consensus tree was constructed and visualized using Dendroscope version 3.5.8 (Huson and Scornavacca, 2012). To determine the amount of genetic divergence among groups identified in the phylogenetic analyses, sequence divergences (uncorrected p-distances) were calculated in DnaSP version 5.10.01 (Librado and Rozas, 2009).

Finally, environmental attributes of each *O. pallipes* population were mapped onto the phylogeny. These include the annual rainfall, mean annual temperature (sourced from the online GIS platform, https://gis.elsenburg.com), the vegetation type (sourced from Mucina and Rutherford, 2006) and geological system (sourced from Keyser, 1997) of the sampled areas. These environmental attributes may be a proxy for the ecological specificity of populations given that the habitat requirements of scorpions rarely change over evolutionary time (Prendini, 2005).

RESULTS

Genetic diversity and the uniqueness of *O. pallipes* populations

Herein studied *O. pallipes* populations were retrieved as



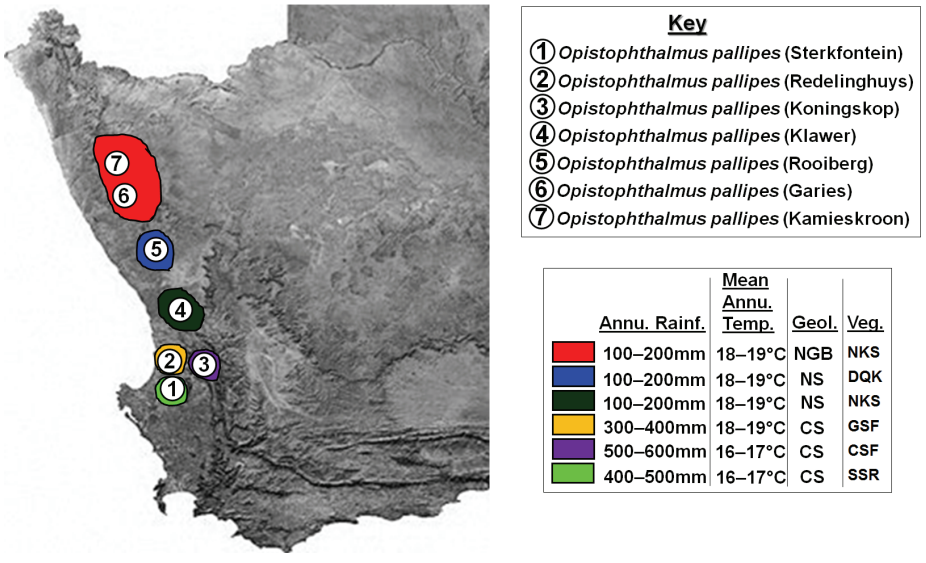


Fig. 3. Ecological characteristics of each population of *Opistophthalmus pallipe* and locations of the ecogeographic areas. Phylogeny is identical with that in Fig. 2A. Symbols at each terminal node correspond to those in Fig. 1. Ecological characteristics of each population are pertaining to the annual rainfall (Annu. Rainf.), mean annual temperature (Mean Annu. Temp.), and the geological system (Geol.) and vegetation type (Veg.) prevalent within each sampled area. Abbreviations: CS = Cape System; NS = Nama System; NGB = Namaqualand Granitized Belt, Vegetation: CSF = Cederberg Sandstone Fynbos; SSR = Swartland Shale Renosterveld; GSF = Graafwater Sandstone Fynbos; NKS = Namaqualand Klipkoppe Shrubland; DQK = Doringrivier Quartzite Karoo.

divergent (separated by six to 40 mutational steps) and genetically distinct, with no shared haplotypes present (see Supplementary Figure S1). Individuals generally appear genetically fixed (in instances where multiple specimens were included), with the exception of the Redelinghuys population, where four haplotypes were present, but with a common haplotype that characterized most specimens.

Phylogenetic structure of *O. pallipes* populations

Two major geographically discrete clades were recovered and are designated here as the Cape and Namaqualand lineages, corresponding to their respective ecogeographic regions (Figs. 1B, 2A, 3A). Within these clades, individual populations appear as divergent and geographically discrete (Figs. 2A, 3A, and see Supplementary Figure S1). The divergence between the two major *O. pallipes* lineages dates to the Pliocene/Pleistocene boundary (with the divergence initiated in the early Pliocene and extending into the Pleistocene; node 1, Fig. 2B), with the divergences among the separate populations occurring during the Pleistocene (nodes 2–6, Fig. 2B), but with two divergences initiated in the late Pliocene (nodes 2 and 3, Fig. 2B).

Ecogeographic differences exist between the ranges occupied by the Cape and Namaqualand lineages with respect to annual rainfall, geological system and vegetation type of the area (Fig. 3B, C). To some extent, mean annual temperature also differs between these clades; however, the Redelinghuys population (within the Cape lineage) occurs in a region with a similar temperature regime to that of the Namaqualand lineage. Ecogeographical differences are also apparent among the individual populations (Fig. 3B, C), mostly pertaining to mean annual temperature, annual rainfall and vegetation type (between the Koningskop, Sterkfontein and Redelinghuys populations in the Cape lineage; between the Klawer/Rooiberg and Garies/Kamieskroon populations in the Namaqualand lineage).

DISCUSSION

Populations in *O. pallipes* are characterised by genetic exclusiveness, substantial sequence divergence and relatively ancient divergence date estimates (Fig. 2A, B, and see Supplementary Figure S1). These patterns resemble those recovered for a North American lithophilous scorpion species, *Smeringurus vachoni*, with a temporal overlap in the timeframe of divergences (see Graham et al., 2017). Protracted isolation of separate *O. pallipes* populations is therefore likely, and may follow intrinsic attributes (ecological specificity) in combination with extrinsic factors (spatial connectivity among suitable habitat areas). *Opistophthalmus pallipes* populations closely track suitable rocky habitat across the landscape and the distribution of the species is patchy, with populations separated by sometimes vast areas devoid of rocky habitat. As a result, gene-flow among spatially disparate populations is likely limited, resulting in inbreeding (fixation of common haplotypes) and genetic drift (divergence of haplotypes), as is suggested by the data presented here (see Supplementary Figure S1). The majority of divergences temporally follow the Post-African II erosion cycle (nodes 2–6 on Fig. 2B) – a period when erosion sculpted the newly uplifted landscape. Erosion of rocky areas and the establishment of soils therefore likely covered and diminished regions of rocky habitat, thereby increasing the spatial isolation among ancestral populations of the rock-dwelling *O. pallipes*.

The isolation of populations in different ecogeographic areas (Fig. 3B, C) may further contribute to their genetic exclusiveness. For instance, within the Cape lineage, the observed genetic structure coincides with differences in rainfall and temperature regimes, as well as in phytogeography (Fig. 3B). This pattern is also observed within the Namaqualand lineage, but here genetic structure coincides with geological and phytogeographic patterns only. Divergences among separate populations (Fig. 2B) follow a period characterized by pronounced environmental turnover produced by the Post-African II erosion cycle (Partridge and Maud, 1987, 2000) as well as the continued development of climatic regimes and vegetation changes produced by a variable climate (alternating glacial and interglacial cycles; Tyson and Partridge, 2000). These environmental changes therefore likely isolated ancestral *O. pallipes* populations in novel ecogeographic areas, thereby contributing to their isolation and divergence.

Following from the patterns retrieved for individual populations, it is not surprising that the major divergence in *O. pallipes* (divergence between the Cape and Namaqualand lineages, Figs. 1B, 2A) coincides with an expansive area of low landscape connectivity, as well as high ecogeographic turnover. The Knersvlakte Region displays a distinct lack of suitable rocky habitat and therefore represents an enduring barrier to gene-flow to *O. pallipes* populations straddling this region. The initial separation between the Cape and Namaqualand lineages (node 1 on Fig. 2A), however, also corresponds to a major marine ingression at the Pliocene/Pleistocene boundary (Fig. 2B; Siesser and Dingle, 1981). These elevated sea-levels (> 300 m above present) would have inundated the low-lying Knersvlakte, thereby further isolating a much more widely distributed ancestral *O. pallipes* stock to the north (the Namaqualand lineage) and south (the Cape lineage), respectively. Indeed, the flooding of low-lying areas by a major sea-level ingression has been demonstrated to drive divergences among scorpion species in the *Tityus bolivianus* complex across South America (Ojanguren-Affilastro et al., 2017).

The Knersvlakte Region furthermore represents an abrupt change in landscape composition, geology, climate, and phytogeography relative to adjacent areas. Moreover, the adjacent areas (occupied by the Cape and Namaqualand lineages) represent vastly different ecogeographic regions (with respect to geological systems, rainfall patterns and vegetation type; Fig. 3B, C). As with individual populations, the divergence between the Cape and Namaqualand lineages also corresponds to a period of major environmental turnover (spatiotemporal variation of climatic regimes and vegetation types; Van Zinderen Bakker and Mercer, 1986 and references therein), which was further accompanied by hydrological and edaphic evolution, (subsequent to the major uplift early Pliocene event and Post-African II erosion cycle; Fig. 2B; Campbell, 1983; Deacon et al., 1992). As a result, ancestral *O. pallipes* populations likely became established in separate ecogeographic environments spanning the Knersvlakte Region, with the region further acting as an enduring ecogeographic barrier.

The genetic affinity of the Klawer population (directly south of the Knersvlakte) to the northern Namaqualand lineage (this population is related to the Rooiberg population directly north of this region; Figs. 2A, 3A, Supplementary Figure S1) may represent a subsequent expansion by members of this clade across the Knersvlakte – a situation similarly retrieved in the rock-dwelling rock hyrax, *Procavia capensis*, across this region (Visser et al., 2020). Conversely, it is possible that historical drainage evolution of the Doring River (following uplift in the early Pliocene; Campbell, 1983; Deacon et al., 1992) directly south of the Klawer population may have acted as a phylogeographic disruptor, enforcing long-term isolation between the two major clades. Indeed, the uplift of the Andes and subsequent drainage evolution of major rivers during the Pliocene have been similarly demonstrated as major factors in the divergence between two scorpion species (*Brachistosternus paposo* and *Brachistosternus roigalsinai*) along the Chilean coastal desert (Ceccarelli et al., 2017). Similarly, drainage evolution of major river systems and the development of lakes drive genetic structure among populations of the lithophilous scorpion *S. vachoni* across the Mojave and Sonoran deserts (Graham et al., 2017).

Taken together, the factors which shape genetic patterns in *O. pallipes* appear both intrinsically (ecological specificity of the species) and extrinsically (landscape structure and ecogeographic condition) influenced, with major divergences corresponding to a period of profound environmental changes. However, the factors which drive diversity in this stenotopic species are not mutually exclusive, and may have had a synergistic effect in isolating ancestral populations. Even so, this habitat specialist species is characterized by divergent and genetically discrete populations, which are probably further isolated by adherence to separate ecogeographic areas. Indeed, similar patterns may characterize other southern African endemic scorpion species which, in part, explains the staggering diversity and endemism in scorpions observed across the region. Further phylogeographic and reproductive studies may therefore be necessary to gain a better understanding of scorpion diversity (and the drivers thereof) across southern Africa, ultimately informing the conservation of this group.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

JHV contributed to the experimental design, conducting of experiments, interpretation of data, and writing of the manuscript. SG contributed to the experimental design and writing of the manuscript. BJvV contributed to the conducting of experiments, interpretation of data, and writing of the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs200094)

Supplementary Figure S1. Haplotype network of the sampled *O. pallipes* populations based on the mitochondrial (COI) dataset.

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