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Substrate specialisation drives an unexpectedly diverse radiation in barking geckos (*Ptenopus*: Gekkonidae)

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A B S T R A C T

Barking geckos (genus *Ptenopus*) are terrestrial, burrowing lizards endemic to southern Africa, currently with three recognised species. Two species are range-restricted (*P. kochi* and *P. carpi*) and display clear differences in substrate preference (soft sand vs. hard gravel). The third and most widespread species, *P. garrulus,* occurs on a variety of substrates of differing hardness, across potential geographic barriers, and over a steep climatic gradient. Variations in morphology and advertisement calls indicates that *P. garrulus* may be a species complex. Two subspecies of *P. garrulus* are currently recognised: *P. g. maculatus* and *P. g. garrulus*. To investigate species boundaries, we produced the first comprehensive phylogeny for the genus. We used a novel application of multiple regression on matrices models to assess multiple environmental drivers of diversification, as contrasted to isolation by distance. We show that *P. kochi, P. carpi,* and *P. g. garrulus* are valid species, but that *P. g. maculatus* is a paraphyletic complex of five previously unrecognised taxa. Specialisation onto different substrates was likely the main driver of divergence, with parapatric occurrence of two to four clades occurring at each of the three substrate transition zones identified *a priori*. The region encompasses diverse bioclimatic regions and potential geographic barriers, and these likely played a role in some divergence events.

1. Introduction

Ptenopus (barking geckos) is a genus of terrestrial, burrowing geckos, endemic to the more xeric parts of southern Africa. Members of the genus are uniquely vocal amongst non-avian reptiles, the males producing loud advertisement calls in large choruses around sunset (Haacke, 1969; Smith, 1849). Only three species are currently recognised: *P. garrulus* Smith, 1849, *P. carpi* Brain, 1962, and *P. kochi* Haacke, 1964. Both *P. kochi* and *P. carpi* are range-restricted (<30,000 km^2) and are endemic to the Namib Desert in western Namibia (Bauer and Becker, 2020; Becker and Bauer, 2020; Haacke, 1975). *Ptenopus garrulus* is much more widespread (1,350,000 km^2) and consists of two subspecies, with *P. g. maculatus* Gray, 1865, inhabiting the most arid southwestern and southern portion of the range, while *P. g. garrulus* is distributed in the semi-arid savanna further east and northeast (Bates et al., 2020; Haacke, 1975; Oelofsen and Vorster, 1976).

The species *P. kochi* and *P. carpi* inhabit relatively specialised and localised habitats, while the widespread *P. garrulus* occurs across a substantial bioclimatic gradient, on different substrates, and across several potential geographic barriers. These factors (bioclimate, substrate, geographic barriers) have been implicated in various lizard radiations across southern Africa (Bauer and Lamb, 2005; Makokha et al., 2007; Medina et al., 2016; Tolley et al., 2008, 2004). It is therefore possible that the widespread *P. garrulus* is a species complex. Haacke (1975) described variation in mid-body and inter-orbital scale-counts, and in advertisement calls, among geographically disparate hypothesised populations of *P. garrulus*. Haacke (1975) interpreted these variations as subspecific and merely revised the geographic boundary between the subspecies. However, he noted that the *P. g. maculatus* (western form) occurring in the Namib erg (a large area of aeolian sand) had scale-counts similar to *P. g. garrulus* (eastern form) from the Kalahari erg, in contrast to the *P. g. maculatus* populations occurring on the surrounding gravel plains. There had been no systematic revision of the genus since Haacke's, 1975 publication, and no comprehensive phylogenetic analysis has been conducted on *Ptenopus*. Only *P. carpi* and *P. garrulus* sequences were included in previous phylogenetic studies of gekkonids, but were not the study focus (e.g. Cai et al., 2021; Han et al., 2004). The phylogenetic placement of *Ptenopus* within Gekkonidae has been investigated, showing it is not closely related to other gecko genera in southern Africa. Its closest known relative on the subcontinent is *Narudasia festiva* (Gamble et al., 2012; Pyron et al., 2013) from which it diverged more than 90 Mya (Zheng and Wiens, 2016: Appendix S3).

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Barking geckos dig elaborate burrow systems within which they live, mate, and lay eggs (Haacke, 1975; Hibbitts et al., 2012, 2005; Polakow, 1997), making substrate type an important component of their environment. They have specialised hand and foot morphologies for their burrowing lifestyle: strong nails on the fingers are for excavation, while rows of fringed scales along the lateral margins of all toes aid in scooping loose soil backwards (Brain, 1962; Haacke, 1975; Polakow, 1997). Toe fringes have evolved multiple times in different species of lizards including geckos, and are usually associated with burrowing or moving in aeolian sands (Bauer and Russell, 1991; Luke, 1986). As in other lizard groups, the extent of these fringes in *Ptenopus* is linked to substrate hardness (Haacke, 1975, 1964). The two extremes are evident in the two substrate specialists: *P. kochi,* occurs on soft sand in the Namib erg, and has the most extensive fringing, whereas *P. carpi*, occurs on hard gravel plains and has the least extensive fringing. *Ptenopus garrulus* appears to be a substrate generalist and has intermediate toe fringing (Haacke, 1975). This species inhabits hard gravels, silty soils, and soft sandy plains in the Namib and Kalahari ergs. Given the importance of substrate in gecko morphology and their distribution, it is possible that speciation events in *Ptenopus* are associated with their occurrence on a diversity of substrates.

In addition to substrate, there are several climatic and geographic factors which may also have driven or facilitated divergence in *Ptenopus,* particularly within the range of *P. garrulus*. We focused on two climatic patterns which affect ecoregion-level changes in fauna and flora in southern Africa (Dinerstein et al., 2017; Olson et al., 2001; Sayre et al., 2013). The first is the divide between primarily summer and winter rainfall zones (Neukom et al., 2014), with only the south-westernmost *Ptenopus g. maculatus* populations occurring in the latter zone. The second is a steep gradient in bioclimate, thermotype and ombrotype, eastwards from the subcontinental west coast (see Sayre et al., 2013). This gradient is caused by the Atlantic Benguela cold water current which causes regular foggy, cold, windy, and generally hyper-arid conditions in the coastal Namib Desert and a steep climatic gradient eastward from this zone (Atlas of Namibia Team, 2022a). Finally, several potential barriers to dispersal dissect the range of *Ptenopus,* including mountain ranges or broad rocky formations which are devoid of *Ptenopus*. Three of these potential barriers have been linked to cladogenesis in other lizard taxa on the subcontinent (Lamb and Bauer, 2000, Scott et al., 2004, Bauer et al., 2006, Makokha et al., 2007, Childers et al., 2021). These include the Great Escarpment, an area of ancient uplift along the southern and eastern margin of the continent, and two major westward-flowing rivers: the Orange river, marking the southern border of Namibia with South Africa, and Swakop River in the central Namib Desert, western Namibia. These various bioclimatic and geographic factors may have contributed to divergence among *Ptenopus* clades including cryptic species not yet identified.

To investigate the hypothesis that environmental factors may have contributed to diversification in *Ptenopus*, we produced the first comprehensive molecular phylogeny for the genus. We included samples from all three currently recognised species and both subspecies of *P. garrulus,* from across a broad geographic range and across potential geographic barriers, different substrates, and bioclimatic zones. We assessed the species diversity of the genus using two operative criteria from the general lineage species concept (de Queiroz, 2007, 1998): phylogenetic and ecological. We used species delimitation methods to test species boundaries. Then, we used distance-based models (multiple regression on distance matrices models) to test the hypothesised drivers of divergence against each other and, simultaneously, against the null-hypothesis of isolation-by-distance (IBD), thereby providing a secondary test of species-boundaries. Based on variation in scale-counts and corresponding habitat differences (Haacke, 1975), we hypothesised that *P. garrulus* is a complex of two to three species, while *P. carpi* and *P. kochi* each represent a single species.

2. Methods

2.1. Study species, study area, and sampling design

Four currently recognised taxa were sampled (*P. carpi, P. kochi, P. g. garrulus,* and *P. g. maculatus*), all of which show differences in their substrate preference specificity. Specialists tend to have smaller distributions (*P. carpi* and *P. kochi*). Extent of toe fringing generally reflects the softness and specialisation of preferred substrate and is most extensive in the soft sand specialist but least extensive in the hard gravel specialist. Toes also tend to be broader in the sand-living *Ptenopus* (Table 1).

Barking geckos were sampled from across the range of each *Ptenopus* species and subspecies in Namibia and South Africa, covering a range of ecoregions, climatic gradients, rainfall zones, and a variety of substrate types. Geckos were located using a T16 LED Lenser torch to spot eyeshine at night, and/or by their vocalisations at dusk. Tail tips were taken as tissue samples and preserved in 99 % ethanol, NAP buffer (EDTA, Sodium citrate, Ammonium sulphate buffer) or in solution of 20 % dimethyl sulfoxide saturated with NaCl.

"Soil texture" refers to the properties of moist soils (USDA, 2016), but *Ptenopus* dig their burrows in dry soils. Therefore, soil surface "hardness", while dry, is a more appropriate classification of *Ptenopus* substrate. No formal classification exists for assessing this, so careful notes in the field were compared with soil type data according to the Harmonised World Soil Database (Fischer et al., 2008) and the Atlas of Namibia (Atlas of Namibia Team, 2022b). Soil type, which is related to soil texture class, appeared to be a relatively good indicator of soil hardness, but this may vary on a single soil type. Soil hardness was classified into three categories, based on how easily the soil surface could be disturbed or excavated (Table 2). On a landscape scale, we used data from the Harmonized World Soil Database (Fischer et al., 2008) and the Atlas of Namibia (Atlas of Namibia Team, 2022b), with certain soils classified under one of the three categories (Table 2).

Three substrate transition zones were identified in Namibia prior to sampling, at the edges of the Namib and Kalahari ergs (Fig. 1). At these places, the substrate transitions from the soft sand on the ergs to hard substrates, over tens of metres. *Ptenopus* were captured from the ergs and on the adjacent hard substrates to test for parapatric occurrence of genetically divergent clades. The three transition zones were:

A) Northern edge of the Namib erg: in the central Namib Desert near Gobabeb (approx. –23.5, 15.4), where the soft arenosols of the Namib erg occur on the south-western side of the Kuiseb River, while hard gravel plains (gypsisols) occur on the northeastern side. On the southern side of the river, inter-dune plains are mostly inhabited by *P. g. maculatus* while the dunes themselves are usually inhabited by *P. kochi*.
B) Southwestern edge of the Kalahari erg: the area around Koës (approx. −25.8, 19.3) was sampled, with the soft Kalahari erg (arenosols) to the northeast and the hard calcisols to the southwest.
C) Southern edge of the Namib erg: the area east of Lüderitz, with soft arenosols in and at the edges of the Namib erg (the tall dunes are uninhabited by *Ptenopus*) that connect with coastal sand southwards (including compacted arenosols and regosols), while hard (including durisols and compacted regosols) plains occur further east (inland), south of the erg.

2.2. DNA extraction, amplification and sequencing

A total of 79 *Ptenopus* samples from a broad geographic range and across all three recognised species were sequenced for three genes (Appendix A). Two *Narudasia festiva* samples were included as the closest outgroup taxon. Two mitochondrial markers (*ND2* and *16S*) were used for all phylogenetic analyses, while one nuclear marker (*c-mos*) was

Table 1
Substrate preference, distribution and foot morphology of *Ptenopus* species/subspecies.

Taxon	Substrate preference	Range (km^2)	Foot morphology (typical)
P. kochi	soft sand (specialist)	28,000	
P. g. garrulus	sand (semi-specialist)	830,000	
P. g. maculatus	sand + gravel + other (generalist?)	520,000	
P. carpi	hard gravel (specialist)	20,000	

Table 2
Substrate classification for sampled locations for Ptenopus.

Classification	Soil types included	Observational criteria	General description
'soft'	Arenosols	A person leaves clear, deep (>5 mm) tracks	Wind-blown or finer-textured river sand, with no or very little cementation or compaction of any kind
'intermediate'	Regosols; Cambisols; compacted Arenosols	No deep tracks, but easy to excavate with bare hand	Soils containing mostly sand, but some level of cementation or compaction is present, often coarser-grained sands or mixed soils, and often having some vegetation cover
'hard'	Calcisols; Durisols; Gypsisols; compacted Regosols; deeper/ softer pockets within Leptosols	Not easy or impossible to excavate with bare hand	Gravelly soils and/ or soils with a hard crust, i.e. considerable surface cementation or compaction present

sequenced for selected individuals only, to resolve the relationships of deeper phylogenetic divergences.

Tissue samples were rinsed three times with deionised water and dried in a vacuum centrifuge prior to DNA extraction. Total genomic DNA was extracted using a salt extraction protocol (Aljanabi and Martinez, 1997) or with the Qiagen DNeasy Blood & Tissue Kit following the manufactures instructions. PCR amplification was carried out using the following forward and reverse primer sets: for *c-mos*: FU-F and FU-R (Gamble et al., 2008); for *16S*: 16Sa and 16Sb (Palumbi, 1996); for *ND2*: vMet2 and vTrp (Cunningham and Cherry, 2004). PCR cycling conditions were as follows: *16S*: denaturation (95 °C, 4 min), 30 cycles of denaturation (94 °C, 45 s), annealing (51 °C, 45 s), extension (72 °C, 1 min), and final extension (72 °C, 10 min). For *ND2*: denaturation (95 °C, 4 min), 38 cycles of denaturation (95 °C, 35 s), annealing (55 °C, 45 s), extension (72 °C, 1 min), and final extension (72 °C, 10 min). For the *c-mos*: denaturation (95 °C, 4 min), 30 cycles of denaturation (94 °C, 45 s), annealing (50 – 60 °C varied by sample, 45 s), extension (72 °C, 40 s), and final extension (72 °C, 10 min). For some samples, annealing temperatures and denaturation times were adjusted to achieve amplification. PCR amplicons were visualised by electrophoresis on a 0.8 % agarose gel with ethidium bromide. Sanger sequencing was carried out at Macrogen (Amsterdam, The Netherlands) using the forward primers for each gene.

2.3. Phylogenetic analyses

The concatenated matrix consisted of 2,016 base pairs (bp), with 14.5 % missing data (see Appendix A) including the missing sequences for some individuals: *16S* = 550 bp (6.6 % missing data plus 4 missing sequences); *ND2* = 1,050 bp (9.3 % missing data); *c-mos* = 416 bp (5.5 % missing data plus 24 missing sequences). Sequences were checked for quality, edited, and aligned in Geneious Prime 2022.1.1 (https://www.geneious.com). Alignments were inspected manually noting protein translation, to confirm the absence of stop codons for coding regions (in *ND2* and *c-mos*). For the nuclear gene sequences where the trace file showed a possible heterozygote, the base was recorded as an ambiguity

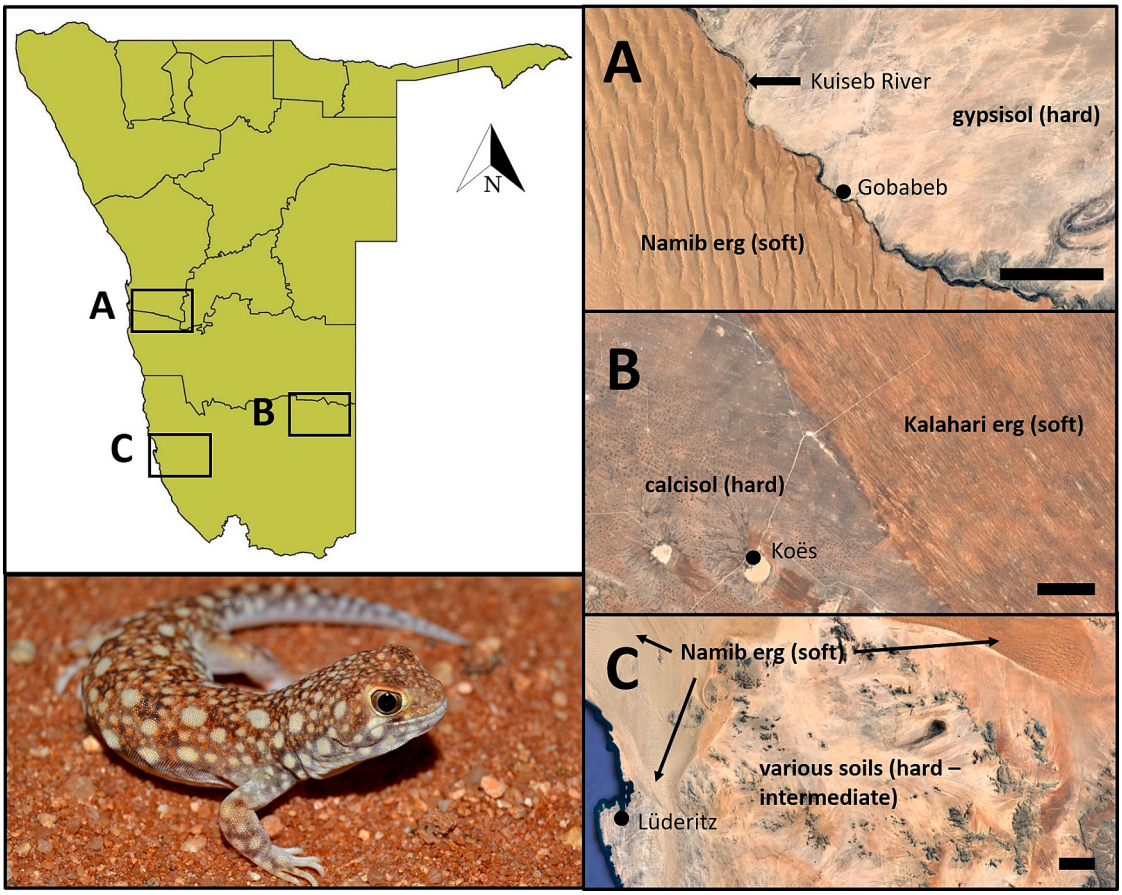

Fig. 1. Substrate transition zones (A – C described above) at the edges of the Namib and Kalahari ergs in Namibia, with hard vs. soft erg substrates (boldface labels) clearly visible on the satellite images. Scale bars indicate 10 km. Study animal (*Ptenopus garrulus*) beneath map insert.

according to the relevant IUPAC codes. A total of 19 alignment gaps were removed from the hypervariable region of the mitochondrial *16S* ribosomal RNA gene.

Bayesian inference (BI) and maximum likelihood (ML) analyses were run on the combined dataset of 2,016 characters, partitioned into the three genes. The partition homogeneity test in PAUP4 (Swofford, 2001) was used to test for topological congruence between the nuclear and mitochondrial trees. The ML analysis was carried out with the command line local version of RAxML-VI-HPC v7.0.4 (Stamatakis, 2006), GTR model, and 1,000 rapid bootstraps were used to assess branch support. The Bayesian analysis was run in MrBayes 3.2.7 (Huelsenbeck and Ronquist, 2001) through the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v3.3 (https://www.phylo.org/portal). The program jModelTest 2.1.10 (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to assess the evolutionary model that best fitted each of the partitions using the Akaike information criterion (AIC) test and confirming similar results with the Bayesian Inference Criterion (BIC) test. This was incorporated into the Bayesian phylogenetic analyses (parameters for *16S* and *ND2*: GTR model, nst = 6, rates = gamma, ncat = 4; for *c-mos*: HKY model, nst = 2, rates = equal).

A multi-species coalescent analysis was conducted with the supported putative species clades (see 3.4 and 3.5, below). This Bayesian analysis was run in BEAST v. 2.6.6 (Bouckaert et al., 2019), through CIPRES, using the StarBeast2 template, with the site and clock models unlinked among all three genes and the tree models unlinked between the nuclear and mitochondrial datasets, at 100 million replicates. The jModelTest results were used to set priors for the HKY site models. Tracer (Rambaut et al., 2018) was used to confirm that model parameters have converged, and the consensus phylogeny was compiled using BEAST TreeAnnotator, with 10 % burn-in, 0.5 posterior probability limit, and mean node heights settings.

Divergences between the clades retrieved from the phylogenetic analyses were estimated in MEGA-X (Kumar et al., 2016) using uncorrected net p-distances for each of the three genes on all available ingroup individuals.

2.4. Phylogenetic species delimitation analyses

The automatic barcode gap detection (ABGD Puillandre et al., 2012) was run on the combined mitochondrial data and separately on the nuclear *c-mos* (ABGD is designed for single-locus data), via the ABGD web interface (web version '11/18/2022'). Simple p-distance metric was selected, with priors for minimum relative barcode gap width = 1 and (default) intraspecific divergence minima (0.001) and maxima (0.1).

A Bayesian general mixed Yule-coalescent model (bGMYC) was implemented using the package bGMYC v. 1.0 (Carstens and Reid, 2012) in R (v 3.2.4, R Core Team 2016), on the concatenated matrix and the single-locus (*ND2* + *16S*) mitochondrial dataset, separately. We used BEAST 2.6.6 (Bouckaert et al., 2019) through the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v3.3 (https://www.phylo.org/portal) to sample a posterior distribution of ultrametric trees. The genes were partitioned separately, the HKY site model was used for each partition, and the relaxed log-normal clock model with default primers were implemented. The MCMC were run for 100 million generations, sampling every 5,000 generations. The bGMYC analysis was applied to 1,000 posterior trees, randomly sampled from the distribution of trees after 50 % burn-in from the BEAST run. The bGMYC was run for 1 million generations with a 10 % burnin, sampling every 100 generations. Because bGMYC tends to over-split species (e.g., Miralles and Vences, 2013), the most conservative estimates from this

analysis (lower probability thresholds in bGMYC output) were presented in the results.

The candidate species that were identified by bGMYC and ABGD were interpreted in a spatial context to distinguish between true species-splits and isolation-by-distance (Wright, 1943). Sequence divergence between the putative species was estimated in MEGA-X (Kumar et al., 2016) using uncorrected net mean p-distances for each of the genes separately (mitochondrial *ND2* and *16S;* nuclear *c-mos*).

2.5. Spatial regression analyses

We developed a robust approach for testing various hypotheses for drivers of genetic divergence against each other to evaluate the most likely scenario to explain potential species-boundaries. We used multiple regression on distance matrices models (MRMs) to test for a positive relationship between sequence divergence (p-distance) and various explanatory variables (as distance matrices) or hypothesised drivers of divergence, against each other. This approach maximises the strengths of the geographic and taxonomic sampling design applied in this study. Moreover, MRMs are more versatile and have better power for detecting linear relationships, than the commonly used partial Mantel test (Legendre and Fortin, 2010; Lichstein, 2007). Only the mitochondrial dataset was used for these analyses, as the nuclear *c-mos* dataset contained insufficient mutation differences and/or samples to assess spatial patterns.

IBD was chosen as the "null-hypothesis" of genetic divergence (i.e., a significant positive relationship between genetic and geographic distance). The alternative hypotheses considered as drivers of diversification were: difference in soil hardness (between sample pairs), difference in climatic gradient, difference in summer/winter rainfall zone, and vicariance caused by different potential barriers. These models were conducted on the whole genus, on groups of clades (e.g., *P. garrulus*), and on each of the putative species.

Distance table matrices (among relevant samples) for all explanatory variables were generated in R (v. 4.1.0, R Core Team, 2021) using packages *ape* (Paradis and Schliep, 2019), *seqinr* (Charif and Lobry, 2007), *raster* (Hijmans, 2022), and using *base-R*. Full models including all explanatory variables were fitted in using the function 'glm' in R. The function 'step' was then used to select the AIC-best model in terms of complexity. The R package *relaimpo* (Grömping, 2006) was used to calculate the total percentage explained by the AIC-best model and by each explanatory variable, as an average of two alternative but similar R^2 metrics developed for GLMs (Genizi, 1993; Zuber and Strimmer, 2011). A strong positive relationship with p-distance thus indicates support for the relevant explanatory variable or hypothesis.

The explanatory variable soil hardness was categorised as 1 = soft, 2 = intermediate, and 3 = hard (see Table 2), and the difference in these values between all samples pairs was calculated, with 0 = same soil hardness, 1 = slightly different hardness, 2 = most different hardness. Difference in distance from the west coast (km) was used as a proxy for (difference in) position along the climatic gradient. Difference in primarily summer/winter rainfall zone was scaled as a binomial factor, with 0 = same zone, 1 = different zones. The final variable was substrate-driven vicariance, scaled as a binomial factor for which samples across the hypothesised barrier were scaled as 1 = separated, vs. 0 = in contact (on the same side of the barrier). If more than one potential barrier was relevant for a particular clade or group of clades, alternative barrier models were included in alternative full models (e.g., including all barriers, only one barrier, or a combination of some barriers). The AIC-best barrier model (if any) was then retained. For a detailed list and map of all barriers tested (including those not validated and not included in the results) and the clades for which they were included, see Appendix D. Once the AIC-best model was chosen, the R^2 for each variable was estimated to quantify relative variable importance, as variation in p-distance explained.

3. Results

3.1. Molecular phylogeny

The concatenated Bayesian (Fig. 2) and maximum likelihood (Appendix B: Fig. B.1) topologies differed only slightly (Appendix B: Fig. B.2), but the differing nodes in the maximum likelihood phylogeny were unsupported. Both analyses recovered the described species *P. kochi* and *P. carpi* and the subspecies *P. g. garrulus,* as well-supported monophyletic clades, with *P. carpi* being at the basal node within the genus (Fig. 2). By contrast, the subspecies *P. g. maculatus* was paraphyletic, with one clade (*P. g. m.* North) placed as sister to *P. kochi* and four other clades being more closely related to *P. g. garrulus*: *P. g. m.* Central, South, East, and Southeast (Fig. 2). The mitochondrial (Appendix B: Fig. B.2) and nuclear (Appendix B: Fig. B.3) topologies were congruent, with the partition homogeneity tests showing no evidence for significant differences between the topologies (partition homogeneity test p = 1.000).

3.2. Species delimitation and spatial regression analyses

The species delimitation analysis using ABGD identified six putative taxa in the initial partitions, resulting in a barcode gap distance (inter vs. intra p-distance) of 9.1 %, based on the concatenated mitochondrial dataset (Fig. 2; see Appendix C). In the recursive partition of the ABGD analysis, up to 28 taxa were identified, but this was largely incongruent with the concatenated matrix phylogeny topology (see Appendix C). The ABGD analysis on the nuclear *c-mos* gene resulted in only one taxon, despite several parameter adjustments, being incongruent with currently recognised species.

The bGMYC analysis on the mitochondrial dataset identified 24 taxa at a threshold probability of $P > 0.95$ (Fig. 2), 22 taxa at $P > 0.9$, and 12 taxa at $P > 0.5$ (Fig. 2; Appendix C). The concatenated matrix dataset (including nuclear *c-mos*) resulted in 32 taxa. Overall, the bGMYC has provided what is likely a considerable over-estimation of species diversity. Only the most conservative of these estimates (12 taxa) was included as a comparable putative number of species to ABGD clades (Fig. 2).

The multiple regression on (distance) matrix models (MRMs) supports the existence of ten putative species, including *P. kochi*, five species in *P. g. maculatus* (North, South, Central, East and Southeast), two species within *P. g. garrulus* (North and South), and two species within *P. carpi* (North and South) (Fig. 2; Table 3). When all the *P. garrulus*-group clades were combined into a single dataset, geographic distance explained only a small portion of the variation in genetic divergence, while substrate-based barriers, rainy season, distance from the west coast, and the escarpment, were more important explanatory variables (Table 3). When each putative species (named) clade was analysed separately, geographic distance was the most important explanatory variable for some, but not all, of these putative species (Table 3). Overall, substrate barriers (i.e., large tracts of unsuitable substrate) explained most of the variation in genetic divergence among the *P. garrulus*-group clades (Table 3). The *P. g. garrulus* North and South clades are not separated by any obvious barrier, although the Orange River was the best explanatory variable (Table 3). The divergence between *P. g. garrulus* North + South and *P. g. maculatus* Southeast, was best explained by the presence of the escarpment (Table 3). The main explanatory variables for genetic divergence between *P. g. garrulus* (North + South) + *P. g. maculatus* Southeast and *P. g. maculatus* South, were difference in rainy season and distance from the west coast (Table 3). The Swakop River as a barrier was an important explanatory variable for genetic divergence between *P. carpi* North and South, and within the clade *P. g. maculatus* North (Table 3). Substrate hardness was the only supported explanatory variable for divergence among *P. kochi* samples, with samples from the edge of the gravel plains being highly divergent from those within the Namib erg. Distance from the west coast

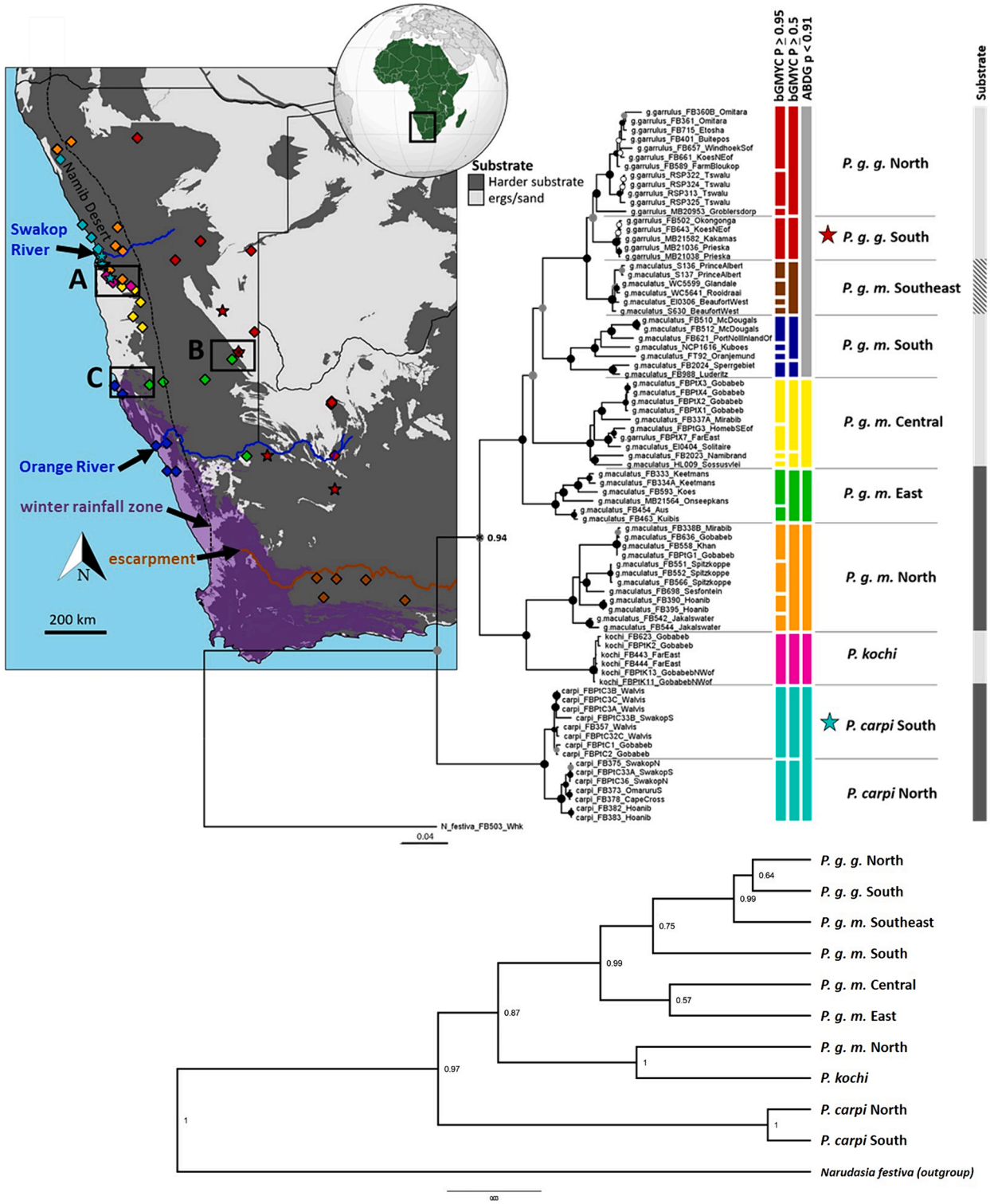

Fig. 2. Bayesian concatenated topology of *Ptenopus* (right), map of sample locations (left), and coalescent species tree (bottom). On the concatenated tree, nodes supported by both maximum likelihood (≥70 % bootstrap) and Bayesian (≥0.95 posterior probability) analyses denoted by black circles; Bayesian only, with grey circles; maximum Likelihood only, with white circles; one node with 0.94 posterior probability is indicated by '*'. On the species tree, posterior probability or support is indicated for each node. Bars to the right of the phylogeny indicate putative species based on two delimitation analyses, with clade names indicated for putative species supported by phylogenetic and spatial analyses (see MRMs); bar colours/symbols correspond to localities on the map, with unique colours assigned to putative species that are ecologically divergent from their sister clades – otherwise only distinguished by a different symbol. The white asterisk indicates co-occurrence of *P. g. g.* North and South. Preferred substrate of each clade corresponds to that on the map (clade *P. g. macultus* Southeast occurs on sandy hummocks, on hard substrate). Relevant features and barriers are indicated by arrows and text.

Table 3
AIC-best MRM results, based on distance tables of p-distance vs. explanatory variables, of various putative species clades in *Ptenopus*. 'Group/clade' refers to the clades in Fig. 1 and 'Var. explained' is the total percentage variation in p-distance or divergence explained by the model. 'Variables' are the explanatory variables in the current model, which include a combination of geographic distance (km), one or more barrier to dispersal (binomial variable), distance from the west coast (km), and/or substrate hardness category (1 – 3). For each explanatory variable the p-value ('P') and R^2 (proportion of p-distance variation explained) is indicated.

Group/clade	Variables	M coef.	P	% explained
P. garrulus group	barrier: substrate	0.040	≤ 0.001	22
clades (excluding paraphyletic	log (geo distance)	0.007	≤ 0.001	9
P. g. maculatus North)	distance W coast	0.000	0.030	8
	barrier: scarp	0.014	≤ 0.001	5
	rainy season	0.005	0.035	4
	substrate	0.009	≤ 0.001	1
	TOTAL			50
P. g. g. North + South +	rainy season	0.082	≤ 0.001	51
P. g. m. Southeast +	distance W coast	0.000	0.002	18
South	barrier: scarp	0.008	≤ 0.001	7
	log (geo distance)	0.005	≤ 0.001	5
	barrier: Orange R	0.013	≤ 0.001	4
	TOTAL			84
P. g. g. North + South +	barrier: scarp	0.022	≤ 0.001	26
P. g. m. Southeast	barrier: Orange R	0.015	≤ 0.001	14
	log (geo distance)	0.003	≤ 0.001	9
	substrate	−0.006	0.007	1
	TOTAL			50
P. g. g. North + South	barrier: Orange R	0.020	≤ 0.001	16
	log (geo distance)	0.003	0.006	7
	TOTAL			23
P. g. m. South	geo distance	0.000	≤ 0.001	70
	barrier: Orange R	0.007	0.166	16
	substrate	0.009	0.048	4
	distance W coast	0.000	0.042	1
	TOTAL			91
P. g. m. Central	log (geo distance)	0.008	<0.001	73
Central	barrier: Kuiseb R	0.013	0.001	8
	TOTAL			81
P. g. m. East	geo distance	0.000	<0.001	60
	substrate	0.017	0.001	28
	TOTAL			88
P. g. m. North	barrier: Swakop R	0.022	<0.001	23
	log(geo distance)	0.004	0.003	23
	distance W coast	0.000	0.130	5
	TOTAL			51
P. kochi	substrate	0.004	<0.001	69
P. carpi	barrier: Swakop R	0.021	0.000	42
	log(geo distance)	0.001	0.153	7
	distance W coast	0.000	0.052	4
	TOTAL			53

was also of minor to intermediate importance within most of the coastal or near-coastal clades (Table 3).

The Bayesian multi-species coalescent tree had a near-identical topology to the concatenated tree (Fig. 2). However, not all nodes were supported, including the sister-relationship between *P. g. maculatus* East and Central, which differed from the concatenated topology (Fig. 2). By contrast, in the concatenated tree, *P. g. maculatus* East was recovered as basally divergent among the *P. garrulus*-group clades (i.e., excluding *P. g. maculatus* North), with good support from both Bayesian and maximum likelihood analyses (Fig. 2).

There was a notable gap between intra- and inter-clade p-distances for most putative species identified by the delimitation analyses. The intraspecific p-distance was unusually high in *P. g. maculatus* South and North (Tables 4 – 5) compared to most interspecific differences. There was no intraspecific variation in nuclear *c-mos,* except for 1 – 2 bp variations in *P. g. maculatus* Central and *P. g. garrulus* North (Table 6). Interspecific pairs with lower-than-intraspecific mitochondrial p-distances included *P. carpi* South & North, *P. g. garrulus* South & North, and the latter pair with *P. g. maculatus* Southeast (Tables 4 – 5). There was no difference in *c-mos* sequences between *P. g. garrulus* South and *P. g. maculatus* South (Table 6).

4. Discussion

The phylogenetic analyses uncovered a previously undetected radiation within the genus *Ptenopus*, that is far more diverse than current taxonomy or previous morphological analyses suggested. While three species (*P. garrulus, P. kochi* and *P. carpi*) including two subspecies (*P. g. garrulus* and *P. g. maculatus*) are currently recognised, species delimitation analyses suggested between six and 24 putative species (Fig. 2; Appendix D). Ten of these putative species are supported by spatial analyses of genetic divergence, showing evidence of ecological divergence and/or vicariance scenarios between the divergent groups, and these putative species all form well-supported clades (Fig. 2; Table 3). While several other putative species-level partitions also formed well-supported clades, most of these divergences appear to represent populations isolated by distance (Table 3), rather than truly separate species. The species *P. kochi* and *P. carpi* and the subspecies *P. g. garrulus* (within the species *P. garrulus*) are each monophyletic, but the subspecies *P. g. maculatus* is paraphyletic, with one clade being sister to *P. kochi,* and several others forming a clade along with *P. g. garrulus* (Fig. 2). Overall, substrate specialisation and vicariance due to separation by tracts of unsuitable substrate, explain most of the variation in genetic divergence between the most divergent clades (Table 3).

Patterns showing the effect of ergs was clearest at the three substrate transition zones at the edges of the Namib and Kalahari ergs. Parapatric occurrence of four putative species occurred at the northern border of the Namib erg: *P. carpi* and *P. g. maculatus* North to the gravel plains north of the (ephemeral) Kuiseb river, and *P. kochi* and *P. g. maculatus* Central on the Namib erg to the south. Both the latter (erg) species also had representatives on the northern side of the river, on the gravel plans (although *P. kochi* never occur more than 100 m from the river). In *P. kochi*, this difference in substrate hardness accounted for 69 % of variation in genetic divergence (Table 3). In *P. g. maculatus* Central, the populations north of the Kuiseb River (on gravel) were highly divergent from those immediately to the south (on sand), which resulted in the Kuiseb River explaining 8 % of genetic divergence in this clade, while the remaining samples were generally isolated by distance (Table 3). Some of the southern samples also occurred on harder substrates. Thus substrate hardness was not a good predictor of genetic divergence in the clade as a whole, only at the northern border of the Namib erg. This indicates that speciation due to divergent substrate specialisation may have occurred several times at the northern edge of the Namib erg (four deep divergence events), while similar speciation events are also currently in progress here (two shallower divergence events). At the southern border of the Namib erg, two putative species occur

Table 4
Inter- and intra-clade uncorrected pairwise net sequence divergences (%) for the putative species of *Ptenopus,* based on the *ND2* mitochondrial gene; intraclade distances on the diagonal. Outlier values (≤6% interclade or > 6 % intraclade p-distance) are shown in boldface.

	Putative species	1	2	3	4	5	6	7	8	9	10
1	*P. carpi* South	1.3 %									
2	*P. carpi* North	**4.6 %**	1.6 %								
3	*P. g. m.* Southeast	22.6 %	22.4 %	1.3 %							
4	*P. g. m.* Central	20.7 %	20.0 %	12.9 %	6.0 %						
5	*P. g. m.* East	19.7 %	19.6 %	11.8 %	9.1 %	6.4 %					
6	*P. g. garrulus* North	22.1 %	21.6 %	6.5 %	11.8 %	10.4 %	3.2 %				
7	*P. g. garrulus* South	22.2 %	21.3 %	7.6 %	12.5 %	11.3 %	**5.2 %**	0.7 %			
8	*P. g. m.* South	19.1 %	18.7 %	10.1 %	8.5 %	8.0 %	9.9 %	10.9 %	**8.8 %**		
9	*P. g. m.* North	18.2 %	17.6 %	18.0 %	14.9 %	14.2 %	17.4 %	18.2 %	13.9 %	6.0 %	
10	*P. kochi*	22.6 %	22.3 %	21.7 %	19.5 %	18.8 %	22.2 %	22.7 %	17.6 %	13.7 %	0.5 %

Table 5
Inter- and intra-clade uncorrected pairwise net sequence divergence (%) for the supported putative species of *Ptenopus,* for *16S* mitochondrial gene; intraclade distances on the diagonal. Outlier values (≤3.5 % interclade or > 3.5 % intraclade p-distance) are shown in boldface.

	Putative species	1	2	3	4	5	6	7	8	9	10
1	*P. carpi* South	0.3 %									
2	*P. carpi* North	**0.1 %**	0.6 %								
3	*P. g. m.* Southeast	11.2 %	10.9 %	0.8 %							
4	*P. g. m.* Central	10.0 %	9.6 %	7.2 %	2.8 %						
5	*P. g. m.* East	9.5 %	9.1 %	6.2 %	5.9 %	2.9 %					
6	*P. g. garrulus* North	10.7 %	10.2 %	**1.7 %**	6.8 %	5.4 %	1.8 %				
7	*P. g. garrulus* South	10.4 %	10.0 %	**2.1 %**	6.8 %	5.9 %	**2.1 %**	0.2 %			
8	*P. g. m.* South	8.4 %	7.9 %	6.3 %	4.8 %	3.5 %	5.1 %	5.9 %	**4.3 %**		
9	*P. g. m.* North	10.2 %	9.9 %	10.7 %	8.8 %	7.4 %	10.0 %	9.9 %	7.2 %	**3.5 %**	
10	*P. kochi*	13.3 %	13.1 %	12.6 %	10.4 %	9.1 %	12.1 %	12.0 %	9.0 %	4.3 %	0.6 %

Table 6
Inter- and intra-clade uncorrected pairwise net sequence divergence (%) for the supported putative species of *Ptenopus,* for *c-mos* nuclear gene; intraclade distances on the diagonal. Outlier values (0 % interclade, >0% intraclade p-distance, or unable to calculate = NA) are shown in boldface.

	Putative species	1	2	3	4	5	6	7	8	9	10
1	*P. carpi* South	0.00 %									
2	*P. carpi* North	**0.00 %**	0.00 %								
3	*P. g. m.* Central	0.67 %	0.70 %	**0.18 %**							
4	*P. g. garrulus* North	0.68 %	0.78 %	0.21 %	**0.14 %**						
5	*P. g. garrulus* South	0.68 %	0.77 %	0.07 %	**NA**	0.00 %					
6	*P. g. m.* Southeast	0.95 %	1.04 %	0.54 %	0.16 %	0.20 %	0.00 %				
7	*P. g. m.* East	0.93 %	1.02 %	**NA**	0.22 %	0.06 %	0.51 %	0.00 %			
8	*P. g. m.* South	0.70 %	0.79 %	0.27 %	**NA**	**0.00 %**	0.26 %	0.26 %	0.00 %		
9	*P. g. m.* North	0.94 %	1.02 %	0.55 %	0.27 %	0.26 %	0.53 %	0.52 %	0.27 %	0.00 %	
10	*P. kochi*	1.20 %	1.29 %	0.62 %	0.52 %	0.51 %	0.78 %	0.77 %	0.53 %	0.26 %	0.00 %

parapatrically, with *P. g. maculatus* South occurring on the southern edges of the erg and on coastal dunes, while *P. g. maculatus* East occurs on harder substrates southeast of the erg (Fig. 2). An east–west divide between summer and winter rainfall zones is evident here, which may be an additional ecological divide between these putative species (although they are not sister clades). Finally, at the southwestern border of the Kalahari erg, two putative species occur parapatrically, with *P. garrulus* North on the erg and *P. g. maculatus* East on the harder fluvial substrates to the southwest (Fig. 2). The most obvious contrast between all parapatric putative species was their occurrence either on ergs, or not. These patterns suggest that speciation events in *Ptenopus* may be primarily driven by the colonisation of a novel substrate (either sand or a harder substrate).

Climatic zonation also likely played a role in some of the divergence events. The putative species *P. g. maculatus* South is endemic to, and allopatric in the winter rainfall zone of the southern Namib Desert, while all other clades occurr in the predominantly summer rainfall zone. Rainfall seasonality was the most important (51 % variation) explanatory variable of divergence between *P. g. maculatus* South and its sister clades. Thus, it would be of interest to know if populations in different rainfall zones have diverged with regards to the timing of breeding since this could act as an isolation mechanism. Distance from the west coast, which was used as a proxy for a climatic gradient, was also an important explanatory variable of divergence (Table 3). Although distance from the west coast was not the primary divergence driver identified on other clades, there is a clear distributional pattern evident: most putative species were endemic to the greater Namib Desert (hyper-arid west-coastal regions), while only the clades *P. g. maculatus* East, *P. g. maculatus* Southeast, and *P. g. garrulus* North and South, occurred inland.

The Swakop River and Orange River appear to have been geographic barriers, causing cladogenesis in *Ptenopus*. Substantial divergence in the *ND2* mitochondrial gene occurred between *P. carpi* North and South across the Swakop River (Fig. 2: phylogeny; Table 3; Appendix D). In clade *P. g. maculatus* North, samples from across the Swakop River and the Khan River (tributary of the Swakop) were also highly divergent, despite the small geographic distance separating them. The Swakop River is not currently a boundary to dispersal: it is ephemeral and both *P. carpi* and *P. g. m.* North were found within the dry riverbed. However, the recovery of *Hippopotamus amphibius* skulls from the lower Swakop River suggests it was likely a perennial river in recent millennia (Irish, 2012). For example, during the late Pliocene, uplift in central Namibia caused an increase in fluvial activity in west-flowing rivers, including

the incision of the Swakop River canyon from ~ 2.8 Mya (Van der Wateren and Dunai, 2001). This has also been implicated in speciation between the lacertid species *Pedioplanis inornata* and *P. undata* (Childers et al., 2021). Similarly, the location of the Orange River explained some genetic divergence within the clade *P. g. maculatus* South and between *P. g. garrulus* North and South. However, this pattern may be an artefact of the overall north–south occurrence pattern. These and other potential geographic barriers identified in this manuscript, could be further assessed as tenable hypotheses using a dated phylogeny.

The levels of mitochondrial divergence among most of the putative species clades (see Tables 4 – 6) are comparable to species-level divergences in other squamates, including some gecko clades, (e.g., Agarwal et al., 2014; Branch et al., 2021; Busschau et al., 2019; Castiglia and Annesi, 2011; Conradie et al., 2022). These divergence estimates likely indicate divergence times of millions of years, when compared to time-calibrated gekkonid phylogenies using the same loci (Gamble et al., 2015, 2011; Heinicke et al., 2011). Additionally, the genetic distance between geographically closest samples of parapatric putative species is large compared to the net mean p-distances calculated. For instance, while the mean *ND2* p-distance between clades *P. g. maculatus* East and *P. g. garrulus* North is 10 %, two nearby individuals (40 km apart) from these clades are 14 % divergent. Within *P. g. maculatus* East, by comparison, two samples 290 km apart are only 8 % divergent. The two geographically closest samples between *P. g. maculatus* South and East (100 km), are 15 % divergent for *ND2*. Between *P. g. maculatus* North and Central, two samples that are < 2 km apart, are 22 % divergent in *ND2*. Intraspecific mitochondrial divergence within *P. g. maculatus* South and *P. g. maculatus* East are deeper than several of the other putative species divergences and more comprehensive sampling of these clades is required to clarify species boundaries. However, the divergence pattern among currently included samples suggest that both these clades represent metapopulations isolated by distance, rather than truly separate species (see Table 3).

Interclade mitochondrial p-distances between putative species *P. carpi* North and South, between *P. g. garrulus* North and South, and between *P. g. garrulus* (North + South) and *P. g. m.* Southeast, are lower than intraclade p-distance within several other putative *Ptenopus* species (Tables 4 – 5) and did not show obvious ecological divergence (substrate or bioclimate) between clades. However, the spatial pattern of divergence indicates clear splits in these cases, and the divergences are comparable to interspecific divergences in some gecko genera (e.g., Cyriac, 2020; Sayyed et al., 2020). In the case of *P. g. garrulus* North vs. South, there is a possibility of mitochondrial introgression having occurred between two previously isolated populations. The nuclear *c-mos* gene was useful for resolving relationships between the deepest-divergent clades, with a few consistent base-pair differences evident among most putative species-clades (Table 6). However, there were no allelic differences between *P. g. maculatus* East and Central, between the clades *P. g. maculatus* South + *P. g. maculatus* Southeast + *P. g. garrulus* South + North, or between *P. carpi* North and South. Hence, difference in the nuclear genome supports for only six of the ten putative *Ptenopus* species indicated by the species delimitation analyses. A faster evolving nuclear gene should be used to investigate whether the proposed species show congruent differences in the nuclear genome.

The results of our study have taxonomic implications in light of the general lineage species concept (de Queiroz, 1998,2007). There is evidence for evolutionary independence between *P. carpi* North and South, and between *P. g. garrulus* North and South, from the phylogenetic operative criterion only. Following a conservative approach, we do not recommend any taxonomic changes for these four putative species, pending additional lines of evidence. Our results provide evidence from two operative criteria, phylogenetic and ecological divergence, to support eight species within the genus *Ptenopus*: *P. carpi, P. kochi, P. g. maculatus* North, *P. g. maculatus* East, *P. g. maculatus* Central, *P. g. maculatus* South, *P. g. maculatus* Southeast, and *P. g. garrulus* (Fig. 2). The sister-relationships among these taxa were well-supported in the concatenated phylogenies, but not all were supported in the multi-species coalescent analysis (Fig. 2). Additional gene-sampling may help to resolve these relationships with greater confidence. According to our results, the genus is in need of a taxonomic revision, including the description of four new species and the elevation of the subspecies *P. g. maculatus*, to species-level, pending an investigation into which clades are represented by the respective type specimens.

Despite the deep genetic divergences evident within the "*Ptenopus garrulus*" species complex, previous studies uncovered very few morphological differences among geographically genetically divergent *P. garrulus* populations (only some variation in mid-body and inter-orbital scale counts: see Haacke, 1975). The morphological conservatism evident in this species-complex has likely led to the underestimation of species diversity in the past. However, differences recorded in advertisement calls show promise for species delineation and identification among the morphologically similar lineages identified here (Becker, 2023). In addition, the advertisement calls provide an ideal model for testing mate recognition hypotheses in this genus and are likely to play a role in driving speciation.

5. Conclusions

Our results reveal a previously undocumented radiation within the genus *Ptenopus,* with multiple lineage divergences at various stages of progress. We provide evidence for the existence of at least eight species of *Ptenopus,* including the four recognised taxonomic subunits (*P. kochi, P. carpi, P. g. garrulus*, and *P. g. maculatus sensu stricto*), and four additional species within the paraphyletic species complex "*P. g. maculatus*". Divergent substrate specialisation appears to be the main driver of speciation in the genus, with some species occurring on ergs while others are adapted to harder substrates. Adaptation to different bioclimatic zones and isolation due to geographic barriers probably played a less prominent role in this radiation. The central Namib Desert in Namibia is the centre of diversity for the genus. This region contains four of the eight proposed species, including the most deeply divergent lineages. A further two proposed species also occur within the southern Namib Desert.

CRediT authorship contribution statement

Francois S. Becker: Conceptualization, Writing – original draft, Investigation, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Visualization. **Graham J. Alexander:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Krystal A. Tolley:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108104.

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