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Limited dispersal and local adaptation promote allopatric speciation in a biodiversity hotspot

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Abstract

Recently diverged or diverging populations can offer unobstructed insights into early barriers to gene flow during the initial stages of speciation. The current study utilised a novel insect system (order Mantophasmatodea) to shed light on the early drivers of speciation. The members of this group have limited dispersal abilities, small allopatric distributions and strong habitat associations in the Cape Floristic Region biodiversity hotspot in South Africa. Sister taxa from the diverse family Austrophasmatidae were chosen as focal species (*Karoophasma biedouwense*, *K. botterkloofense*). Population genetics and Generalized Dissimilarity Modelling (GDM) were used to characterise spatial patterns of genetic variation and evaluate the contribution of environmental factors to population divergence and speciation. Extensive sampling confirmed the suspected allopatry of these taxa. However, hybrids were identified in a narrow region occurring between the species' distributions. Strong population structure was found over short geographic distances; particularly in *K. biedouwense* in which geographic distance accounted for 32% of genetic variation over a scale of 50 km ($r = .56$, $p < .001$). GDM explained 42%–78% of the deviance in observed genetic dissimilarities. Geographic distance was consistently indicated to be important for between species and within population differentiation, suggesting that limited dispersal ability may be an important neutral driver of divergence. Temperature, altitude, precipitation and vegetation were also indicated as important factors, suggesting the possible role of adaptation to local environmental conditions for species divergence. The discovery of the hybrid-zone, and the multiple allopatric species pairs in Austrophasmatidae support the idea that this could be a promising group to further our understanding of speciation modes.

KEYWORDS
biome, Cape Floristic Region, diversification, general dissimilarity modelling, hybridisation, Mantophasmatodea, speciation

1 | INTRODUCTION

Processes giving rise to new species have long been the subject of intense study and lively debate (e.g., Brown & O'Neill, 2010; Butlin et al., 2008, 2012; Butlin & Smadja, 2018; Crespi & Nosil, 2013; Czekanski-Moir & Rundell, 2019; Foote, 2018; Hausdorf, 2011; Nei & Nozawa, 2011; Nosil, 2012; Seehausen et al., 2008; Smadja & Butlin, 2011). The speciation process is a continuum starting from a single species, which diverges into at least two lineages via the accumulation of genetic mutations and changes in allelic frequencies, and ends in full reproductive isolation. Speciation is typically studied retrospectively once "good biological species" have evolved (Harvey et al., 2019; Via, 2009). However, studying the causes of partial reproductive isolation between diverging populations can shed light on the early barriers to gene flow. Using a population-based approach can be very valuable as it can illuminate patterns which are later obscured by the accumulation of further interspecific differences (Feder et al., 2012; Mullen & Shaw, 2014; Seehausen et al., 2014; Via, 2009). Many of the differences which arise subsequent to speciation may be unrelated to reproductive isolation.

Reproductive isolation is central to the speciation process and can arise as a byproduct of genetic divergence (Dobzhansky, 1937). It may be caused by pre- or post-mating isolation mechanisms, though typically premating isolation mechanisms (including mate recognition) achieve initial reproductive isolation (Coyne & Orr, 2004; Nei & Nozawa, 2011). Premating isolation can occur due to geographic, behavioural or ecological isolation, whilst post-mating isolation can arise due to mechanical or gametic isolation mechanisms (reviewed in Feder et al., 2017). The modes of establishing reproductive isolation can be defined based on geography (i.e., sympatric, parapatric or allopatric), or based on the key factors and mechanisms involved, such as natural selection, sexual selection, hybrid speciation and genetic drift (Feder et al., 2017). Examples exist in the literature for all of these mechanisms, and it is now understood that speciation may frequently involve many modes and mechanisms which vary through time and space (Coyne & Orr, 2004; Nosil et al., 2012).

The relative importance of each of these mechanisms for the generation of biodiversity remains to be elucidated. Likewise, determining the order in which barriers to reproductive isolation evolve, and which of these most strongly drive initial divergence, remains a significant goal in speciation research (Christie & Strauss, 2018).

By studying multiple species pairs with a range of divergence times, the drivers of reproductive isolation can be studied throughout the continuum, from initial divergence to the maintenance of species barriers, and their relative order of appearance, and importance, can be evaluated. Studying multiple-species pairs can also allow insights in the geographic modes of speciation once species distributions are known and a dated phylogeny is available, illuminating how geography shapes the origin of species. With the aim of taking an initial step to contribute to these questions, recently diverged sister taxa from the insect order Mantophasmatodea (Heelwalkers) (Klass et al., 2002) were used to infer early drivers of differentiation, and to examine whether environmental factors, geographic distance or vibrational communication are implicated in differentiation in this group. All Mantophasmatodea are secondarily wingless with limited dispersal capacity, and occur in semi-arid regions on specific vegetation types, typically in allopatry. These generalist carnivore taxa are cryptically coloured to match their plant habitat, with within-species colour polymorphisms described in several taxa (Klass et al., 2003; Roth et al., 2014). The most speciose family, Austrophasmatidae, currently comprises approximately 14 taxa (described or awaiting formal description) with many genera that are monospecific or which contain allopatric sister taxa (Eberhard et al., 2018). This family experienced a rapid radiation during the Late Miocene (Dool et al., 2018) coincident with the establishment and diversification of the Succulent Karoo and younger elements of the Fynbos Biomes (Verboom, Archibald, et al., 2009; Verboom et al., 2015), with which it is associated (Picker et al., 2002). Within Austrophasmatidae, the sister taxa *Karoophasma biedouwense* and *K. botterkloofense* (Kbi and Kbo henceforth in the text) were selected for this exploratory study for several reasons: previous studies revealed that mtDNA divergence within Kbi was almost as high as interspecific divergence with Kbo (5.6% vs. 6.5% based on 819 bp COI, Damgaard et al., 2008); the two species have very similar morphology (Klass et al., 2003) and biotremulation signals used in mate location (Eberhard & Eberhard, 2013; Eberhard & Picker, 2008); and they occur in the same part of the Cape Floristic Region (CFR), a biodiversity hotspot. These close similarities could reflect recent divergence or incomplete speciation, making these taxa ideal for investigating early speciation processes (i.e., the order of appearance and relative importance of mechanisms causing reproductive isolation). Additionally, diversity hotspots are excellent locations to study the mechanisms driving diversification (Rymer et al., 2010; Verboom, Dreyer, et al., 2009). The drivers of faunal radiations in the CFR have been generally poorly-studied compared to those of the flora (Colville et al., 2014). Therefore, a further aim of the current study was to evaluate whether heelwalkers could be a promising group for investigating drivers of speciation in the animal kingdom.

The overall aim of the study was thus to elucidate the forces promoting the earliest divergence in *Karoophasma*, and assess the relative order and importance of factors leading to reproductive isolation, with a view to contributing to understanding the mechanisms giving rise to speciation within a recent radiation (Austrophasmatidae). An extensive sampling was conducted for both species in South Africa. Thereafter, an integrative approach including population genetic (microsatellite and mtDNA), morphological, and behavioural data in addition to site-based habitat and climatic variables were used to evaluate potential factors (e.g., geographic, behavioural, ecological) and mechanisms (e.g., selection, drift) affecting divergence. Specifically, the genetic data set was used to quantify the extent of population and species divergence and diversity, as geographic-based genetic variation is a prerequisite for many scenarios of speciation (Gavrilets et al., 2000) and reproductive isolation is known to correlate with genetic distance (Christie & Strauss, 2018; Coyne & Orr, 1989). Although microsatellites are presumed to be neutrally evolving loci and thus cannot

be used to inform on specific adaptations, they nevertheless have utility in providing an initial assessment of the relative importance of neutral or selective forces driving divergence (Geue et al., 2016; Orsini et al., 2013). Correlations between neutral loci and environmental variables that cannot be accounted for by geographic distance alone may suggest divergent selection (Geue et al., 2016; Nosil, 2009). The morphological data set was used to quantify interspecific differentiation and to examine associations with habitat or environmental traits which could indicate a role for local adaptation. Behavioural data in the form of biotremulations (via drumming) were examined due to their role in mate recognition and potentially choice (Cocroft et al., 2008; Rodríguez, 2019). Climate, altitude and habitat (e.g., vegetation type) data were included to examine ecological associations between either population or species-level divergences with such parameters.

A number of hypotheses were examined in the current study. Distinct vegetation types have been mapped in great detail across South Africa (Dayaram et al., 2019). If habitat (vegetation) or climatic conditions impose strong selection, then we expect to find that vegetation type or climatic variable(s) would influence the pattern of genetic divergence between sites (H1). Equally, it is known that mate choice based on vibrational communication in the *Enchenopa binotata* species complex of treehoppers was associated with divergence and speciation (Cocroft et al., 2008; Fowler-Finn & Rodríguez, 2013). If vibrational communication plays a role in diversification in Austrophasmatidae, we would predict that population and species divergence will be correlated with divergence in call parameters (H2). Given the complex topography in the study area, elevation may play a role in driving divergence through the promotion of geographical isolation, as found for plant lineages in the fynbos biome in South Africa (Verboom et al., 2015). We hypothesise that we will find evidence for strong associations between elevation and genetic differentiation in *Karoophasma* if elevation resulted in isolation of lineages (H3). Similarly, given the limited dispersal capacity of heelwalkers, their small distributional ranges and multiple allopatric species pairs, we predict that geographic distance has played a substantial role in divergence through neutral drift (H4). Finally, as it is now known that multiple drivers of divergence can occur simultaneously or sequentially with varying importance throughout the speciation process (e.g., Villoutreix et al., 2020), we predict that several of the above processes would prove important to some degree in speciation in this group (H5).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Sampling was conducted during the Southern Hemisphere late winter/early spring (August–September, 2015–2017) in the Succulent Karoo and Fynbos Biomes of the Western and Northern Cape provinces of South Africa. Nymphs and some adults were collected by bush beating and were taken back to the lab for rearing and recording (see Table 1 for site based sample details). The vegetation type at sampling sites was assigned using the National Vegetation Map of South Africa 2018 (Dayaram et al., 2019).

Animals were housed individually in lidded plastic pots (60 mm high, 80 mm diameter) at room temperature with a stick to climb to facilitate moulting until adulthood when vibrational signals could be recorded. Specimens were then preserved in absolute EtOH for later genetic and morphological analyses. Additional individuals were available from previously published studies (Eberhard & Eberhard, 2013; Eberhard & Picker, 2008) or from previous field collections and were used in the current study to augment the sampling for the morphological (n = 5), vibrational (n = 23) and population genetic (n = 18) analyses for sites which were poorly represented (see Tables S1–S3 for further details).

2.2 | DNA extraction, genotyping and sequencing

Whole genomic DNA was extracted from one leg using the DNeasy blood and tissue kit (Qiagen) after mechanical lysis. Twenty-three microsatellite loci which were designed specifically for Kbi were genotyped in three multiplex reactions as described in Dool et al. (2018), with one modification: locus KB_4 was labelled with a yellow dye (Atto-550) instead of green (HEX). The potential presence of scoring errors was examined in MICROCHECKER v. 2.2.3 (Van Oosterhout et al., 2004). The frequency of null alleles was estimated in GENEPOP v.4.6.9 (Rousset, 2008). Linkage and departures from Hardy–Weinberg Equilibrium (HWE) were assessed using FSTAT v. 2.9.3.2 (Goudet 2001). Of the 23 loci amplified, 17 were retained for Kbi (n = 277), 11 for Kbo (n = 62) and 12 in common to both species (n = 339; see Table S1 for genotypes). No linkage or deviations from HWE were detected. Due to low per-site sample sizes for Kbo, nearby sites were pooled for some analyses (see Table 2). Both species had sites represented by a single individual which were excluded from analysis where appropriate (i.e., for site-based estimates such as F_{ST}). Pooling and exclusion of singleton sites are indicated in the legends of presented results.

Partial COI was amplified for 11 individuals (Table S4) using the primers C1-J-2183 and TL2-N-3014 (Simon et al., 1994) to complement available COI data for these taxa (Damgaard et al., 2008; Klass et al., 2003). PCRs were conducted in 10 μl volumes containing 1 μl DNA, 1× PCR buffer -Mg (Invitrogen), 1.5 mM MgCl2, 0.3 μM of each primer, 0.3 mM dNTPs and 2 U Platinum Taq Green Hot Start DNA Polymerase (Invitrogen) on an Applied Biosystems Veriti thermocycler. Amplification conditions were: 94°C for 2 min; two cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by two cycles each at annealing temperature in 2°C decrements from 55°C (53–45°C); 72°C for 5 min. Products were purified using ExoSAP-IT (Thermo Fisher) and sequenced on an ABI 3130xl Genetic Analyser (Applied Biosystems). Sequences were edited using CODONCODE ALIGNER v. 4.2.7 (CodonCode Corporation).

TABLE 1 Sampling information for *K. biedouwense* and *K. botterkloofense* in South Africa

	Site code	Field site	Lat S	Long E	Alt (m)	Micro	Morph	Biotrem
Karoophasma biedouwense (Kbi)	BV	Biedouw Valley	32.143	19.309	345	6	4	1
	BVS	Biedouw Valley Sandy Field	32.141	19.265	308	44	65	29
	CL	Clanwilliam Dam	32.212	18.868	140	54	75	59
	DO	Doringbos	31.973	19.223	183	15	9	10
	DR	Driefontein Farm	32.000	19.228	224	34	38	16
	HO	Hoopvol	32.116	18.856	83	1	1	1
	NNWO	North North Wolfdrif farm	31.952	18.975	177	5	3	2
	NWO	North of Wolfdrif farm	32.013	19.024	251	2	2	1
	SBO	South of Botterkloof Pass	31.938	19.238	233	10	6	7
	SKL	South Klaver	31.955	18.819	298	14	6	4
	TR	Traveller's Rest	32.071	19.076	311	26	33	26
	WCL	West Clanwillaim	32.170	18.846	233	5	3	4
	WF	Waterfall Farm	31.791	18.911	208	15	7	15
	WF2	Waterfall2	31.761	18.915	210	2	2	1
	WO	Wolfdrif Farm	32.018	19.058	278	35	47	32
	NA	Nardouw	31.949	18.912	259	9	5	5
		n sites = 16				277	306	213
Karoophasma botterkloofense (Kbo)	BO	Botterkloof farm	31.790	19.280	724	1	1	1
	CA	Calvinia	31.371	19.532	863	5	4	1
	KD	Klein Doring	31.198	19.452	595	1	1	1
	LO	Loriesfontein	30.944	19.417	827	1	1	1
	LO1	Loriesfontein 1	30.980	19.470	939	4	3	3
	LO2	Loriesfontein 2	31.070	19.290	625	1	0	3
	LO4	Loriesfontein 4	31.016	19.488	845	5	2	6
	NBO	North of Botterkloof Pass	31.806	19.270	703	7	4	1
	NBO1	North of Botterkloof Pass 1	31.744	19.308	687	5	3	0
	NF	Nieuwoudtville Falls	31.320	19.118	661	15	6	8
	OG	Oorlogskloof Glacial Fields	31.421	19.125	732	13	6	3
	OR	Oorlogskloof River	31.486	19.353	751	1	1	1
	OR1	Oorlogskloof River 1	31.537	19.382	802	2	2	0
	OR2	Oorlogskloof River 2	31.577	19.359	781	1	1	0
		n sites = 14				62	35	29
		***n* total sites = 30**				**339**	**341**	**242**

Note: Numbers of individuals used in each data set are indicated: Micro (microsatellite); Morph (morphology); Biotrem (biotremulation).

2.3 | Population genetics

To obtain base-line information on within- and between-species genetic diversity, differentiation and structure, a suite of methods were employed using the three microsatellite data sets (17 loci Kbi, 11 loci Kbo and 12 loci in common to both species).

For all three data sets, the partitioning of genetic variation was assessed by means of F_{ST} (in FSTAT), and a principal component analysis (PCA), neighbour joining tree (NJ) and ancestry proportions analysis in R 3.4.2. (R Core Team, 2017). The packages ADEGENET v.2.1.1 (Dray & Dufour, 2007; Jombart, 2008) and APE v.5.3 (Paradis & Schliep, 2018) were used to generate the PCA and NJ, whilst ancestry proportions were estimated in the package LEA v. 2.0.0 based on sparse nonnegative matrix factorization and least-squares optimisation (Frichot & François, 2015; Frichot et al., 2014) after subsampling to generate an even data set (Puechmaille, 2016). Parameters for LEA were *K* 1–10, with 20 replicates at each *K* and alpha 20. Cross entropy was used to select *K* and the best run.

Parameters were estimated to better understand the ecological and sex-based determinants of dispersal, due to its key role in population connectivity. The potential for sex-biased dispersal was evaluated using the largest data set only (Kbi, 17 loci) in HIERFSTAT v. 0.04–22 (Goudet, 2005; Goudet et al., 2002), using the mAI_c (mean assignment index) test, and 10,000 permutations using the "two

TABLE 2 Molecular diversity indices for *K. biedouwense* and *K. botterkloofense*

	Site code	*N*	No. of alleles	Ho	He	Gene Div.	R	Fis
K. biedouwense (Kbi)	BV	6	5.81	0.74	0.79	0.74	2.90	0.064
	BVS	44	12.71	0.70	0.78	0.78	2.98	0.097
	CL	54	9.56	0.59	0.59	0.56	2.36	0.01
	DO	15	7.87	0.68	0.72	0.63	2.55	0.056
	DR	34	11.56	0.68	0.70	0.66	2.68	0.03
	HO	1	2.00	-	-	0.59	-	-
	NNWO	5	5.07	0.73	0.76	0.67	2.66	0.052
	NWO	2	2.71	0.61	0.74	0.61	-	0.22
	SBO	10	7.07	0.60	0.69	0.61	*	0.092
	SKL	14	6.71	0.54	0.63	0.63	2.58	0.135
	TR	26	7.53	0.57	0.62	0.62	2.45	0.064
	WCL	5	4.33	0.69	0.71	0.62	2.50	−0.01
	WF	15	5.63	0.53	0.55	0.52	2.15	−0.021
	WF2	2	2.50	0.50	0.72	0.25	-	0
	WO	35	10.65	0.63	0.71	0.71	2.78	0.101
	NA	9	6.63	0.70	0.74	0.69	2.72	0.047
K. botterkloofense (Kbo)	BO/NBO	8	5.00	0.56	0.62	0.62	2.44	0.073
	CA	5	4.25	0.75	0.72	0.52	2.25	−0.048
	KD	1	2.00	NA	NA	0.82	NA	NA
	LO/LO1	5	4.70	0.64	0.73	0.66	2.60	0.088
	LO2	1	2.00	NA	NA	0.64	NA	NA
	LO4	5	3.91	0.49	0.64	0.64	2.43	0.188
	NBO1	5	4.44	0.53	0.70	0.58	2.31	0.19
	NF	15	6.70	0.60	0.64	0.58	2.35	0.007
	OG	13	4.89	0.56	0.62	0.51	2.14	0.075
	OR/OR1/OR2	4	4.00	0.64	0.71	0.58	2.40	0.115

Note: Neighbouring sites in *K. botterkloofense* were pooled in three cases to allow site-based statistics. Pooled sites take the name of the underlined site. R: allelic richness based on a minimum sample size of two. Increasing this minimum (incurring loss of sites) does not change the trend observed. -:could not be calculated due to low sample size; * could not be calculated due to failure at two loci (KB23 10/10 failed, KB5 9/10 failed).

sided" alternative method (Helfer et al., 2012). Only sites with more than five individuals and at least two males and two females were used. The noneffective dispersal rate (m^{ij}) between sites was estimated using the Bayesian method in BIMR v. 1.0 (Faubet & Gaggiotti, 2008) under default settings. Three independent runs were conducted. The lowest loglikelihood was used to select the best run.

For the data set of 12 loci in common only (i.e., assessing between species divergence), analysis of molecular variance (AMOVA) was examined in ARLEQUIN v. 3.5.2.2 (Excoffier & Lischer, 2010), allelic frequencies were calculated and plotted in R using ADEGENET and ADE4 v. 1.7–13 (Dray & Dufour, 2007), and the potential for hybrid taxa was assessed. Statistical evidence for hybrid individuals was examined using parallelnewhybrids (Wringe et al., 2017) which calls the NEWHYBRIDS software v.1.1b (Anderson & Thompson, 2002). Three independent runs of 100,000 sweeps with Jeffrey's priors and a burnin of 50,000 were used. The results were validated using a simulated data set of parental, F1, F2 and backcross individuals made using HYBRIDLAB v.1.0 (Nielsen et al., 2006) and by jackknifing over loci.

Using the two individual species' data sets (i.e., assessing within species population divergence), standard measures of molecular diversity were calculated in FSTAT and ARLEQUIN and the relationship between genetic and geographic distances were examined (i.e., isolation by distance, IBD) was assessed using 10,000 permutations in ECODIST v. 2.0.1 (Goslee and Urban 2007).

In addition to the microsatellite data, mtDNA was analysed to uncover phylogenetic and phylogeographic structure and to date divergences. Therefore, a dated Bayesian gene tree was constructed for the newly generated mtDNA sequences in combination with previously published data, using BEAST v.1.8.4 (Heled & Drummond, 2010), three MCMC chains of 10 million generations sampled every 1000 generations and a substitution rate as in (Dool et al., 2018).

Yule speciation, a strict clock and a UPGMA starting tree were used as tree priors. Sequences were also used to generate a haplotype network using NETWORK v. 5.0.1.1 (Bandelt et al., 1999).

2.4 | Biotremulation

Biotremulation (substrate born vibrational signals) was investigated to examine the association between this mode of communication and population and species divergence. Vibrational signals (via abdominal drumming) of nonmated adults (n = 242; Table S2) were recorded at room temperature (mean 24°C) by placing drumming individuals on the membrane of a loudspeaker (SC13, 13 cm diameter impedance 8 Ohm, Visaton GmbH & co. KG) connected to a Tascam Linear PCM recorder (DR-05, sampling frequency 44.1 kHz, WAV 16-bit format, TEAC corporation). Males were encouraged to drum if necessary by tapping next to or on the loudspeaker, by placing a drumming female in a pot adjacent to the loudspeaker, or by playing back a female recording. Females rarely spontaneously drummed and were played recordings of males to elicit a response. A minimum of three recording attempts were made, each typically lasting 5–10 min, separated by several hours to a few days, before terminating recording attempts for that individual. Recordings were analysed using BATSOUNDPRO 3.31a (Pettersson Elektronik AB). Parameters measured were: number of pulses per pulse train (NP), duration of pulse train (DPT), interpulse interval (IPI), pulse repetition time (PRT), pulse train repetition (PTR) and duty cycle (DC); see Figure 1 in Eberhard and Picker (2008) for an illustration of these features. Parameter means were taken from multiple pulses (females) or pulse trains (males) per individual (mean number of pulses per individual Kbi females: n = 15.8, males: n = 54.5; Kbo females: n = 71.5, males: n = 56). Sexual dimorphism in call structure necessitated the separate analysis of male and female calls. Female calls have a very simple structure generated from regular tapping of the abdomen, resulting in a single measurable parameter (pulse repetition time). Call parameters were analysed in R using the baseline packages STATS and GRAPHICS (R Core Team) to generate PCAs and box plots, which were visualised using GGPLOT2 v. 2.2.1 (Wickham, 2016), GGFORTIFY v. 0.4.5 (Tang et al., 2016), and RESHAPE v. 0.8.7 (Wickham, 2007). Tests for significant differences in call parameters (dependent variable) between sites (independent variable) were conducted using analysis of variance (ANOVA) with Tukey's honest significant differences (TukeysHSD) and p-values adjusted for multiple comparisons.

2.5 | Morphology

Morphology data were collected from individuals at all sites to uncover site and species based differences which could be implicated in population divergence. Thirty-five body length measurements with discriminatory power in interspecific relationships (Klass et al., 2003) were measured on a subset of individuals. The eight most informative measures were retained for the remainder of the samples (n = 341; Table S3), following preliminary analyses (ANOVA) with

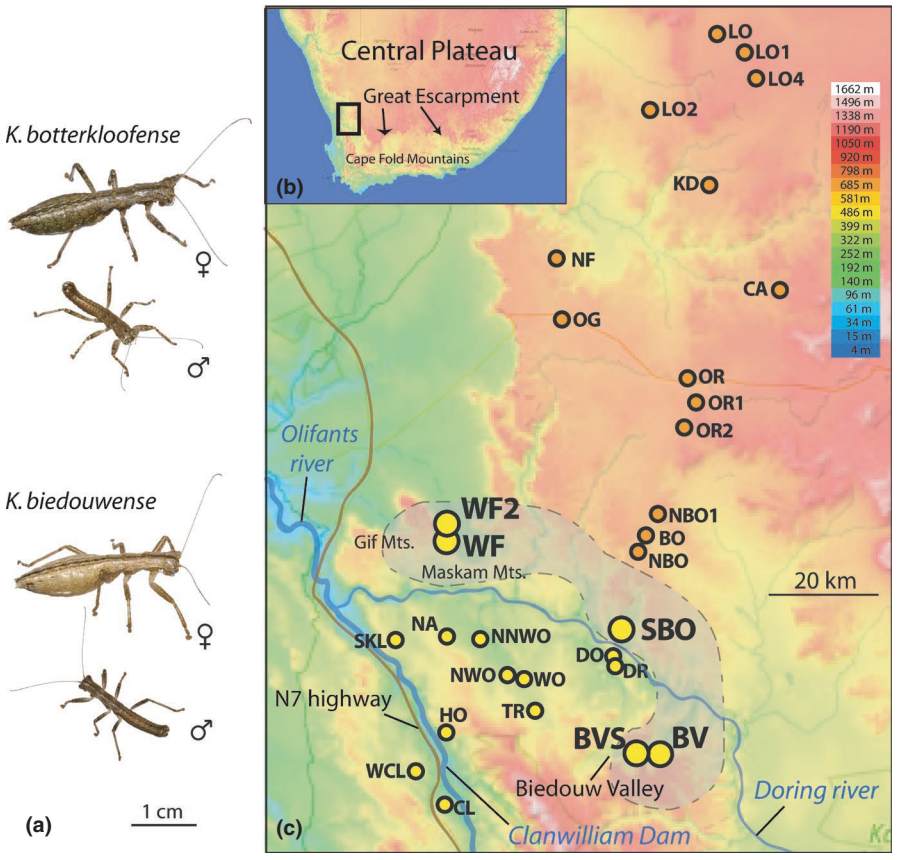

FIGURE 1 (a) Representatives of each species. (b) Topography of South Africa with sampling area marked by a black rectangle. (c) Sampling sites used in this study for *K. biedouwense* and *K. botterkloofense*. Potential hybrid sites are indicated in bold within a grey shaded area (WF, WF2, SBO, BV, BVS). Map credit: topographic-map.com

a subset of individuals. These were: pronotum length (prnl), mesonotum length (msnl), protibia length (prtl), profemur length (prfl), eye length (eyel), eye height (eyeh), gena height (genh), and the distance between the anteroventral corner of the eye and the posterior articulation of the mandible (emad); illustrated in Figure 3 of Klass et al. (2003). Measurements were made using a SteREO Discovery. V20 microscope (Carl Zeiss Microscopy GmbH) and the associated AxioVision v. 4.9.1 SE64 software. To account for body size differences, measurement ratios were calculated as described in Klass et al. (2003). Species, sex and population differences were assessed using R packages as in the biotremulation analysis in addition to the packages MASS v. 7.3-50 (Venables & Ripley, 2002), FLIPMULTIVARIATES v. 0.1 (R Core Team, 2017) and NORMALR v. 1.0.0 (Courtney & Chang, 2018). Species and population differences were explored using PCA, linear discriminant analysis (LDA, in DisplayR, www.displayr.com) and ANOVA. The data sets for Kbi were subsampled to match the sample sizes of Kbo for the LDA. 70% of the data set was used for training and 30% for testing. The model was validated by testing the prediction accuracy on the test data. Pronounced sexual dimorphism necessitated the separate treatment of male and female data sets.

2.6 | Drivers of divergence

The potential processes driving divergence were investigated using a simple correlative approach and landscape genetics. Associations between morphological, biotremulation, genetic and geographic distance matrices were assessed using a mantel-based analyses using Moran spectral randomization (Crabot et al., 2019; Wagner & Dray, 2015). This approach was found to efficiently correct for spurious correlations whilst conserving the ability to detect true correlations when present, thus avoiding spatial autocorrelation and inflated type 1 error rates (Crabot et al., 2019). Reynolds genetic distance between sites was calculated in adegenet before using the function msr.mantelrtest in ADESPATIAL (Dray et al., 2012).

Factors which may be facilitating or restricting gene flow were further investigated using general dissimilarity modelling (GDM) using the R package GDM v.1.4.2.1 (Fitzpatrick et al., 2021). This nonlinear matrix regression technique fits relationships between environmental variables and biological variation through I-spline functions (Ferrier et al., 2007; Fitzpatrick & Keller, 2015) offering great insights into population and species divergence (Gibson & Moyle, 2020; Oliveira et al., 2018). The default of three I-spline basis functions per predictor was used. I-splines are partial regression fits that can indicate the importance of predictor variables and how these change along the gradient concerned while holding all other variables constant. The best predictors were selected using a stepwise matrix permutation and backward elimination approach (500 permutations), in which the least important predictor variable is removed at each step. This procedure is repeated until all variables remaining in the final model make a significant contribution to explained deviance ($p < .05$). All spatial layers were prepared in R and converted to grids using RASTER v. 3.4–5 (Hijmans, 2020). The spatial data sets included: the 19 climatic variables available from WorldClim version 2.1 climate data for 1970–2000 (Fick & Hijmans, 2017), insect diversity and endemism (Succulent Karoo Ecosystem Programme Expert Maps Insects 2002; (SANBI Biodiversity GIS, 2002), altitude (SRTM30, http://srtm.csi.cgiar.org/), and South African Vegetation (Dayaram et al., 2019; SANBI Biodiversity GIS, 2018). A separate analysis was performed using genetic distance (Kbi and Kbo individually, and both species together) as the response variable. This method was also used to assess the factors influencing variation in the biotremulation and morphology data sets (i.e., morphology and call were analysed in turn as the response variables for the data sets Kbi and both species together).

3 | RESULTS

3.1 | Sample collection

Due to the morphological similarity of Kbi and Kbo, microsatellite genotyping was used to confirm species identification. Kbi were thus identified at 16 sites and Kbo at 14 sites (Figure 1, Table 1, Figure S1). The two species were never found together at the same site and were separated by a minimum of 15 km (the distance between the closest sites: NBO and SBO; Figure 1). Kbo was found at higher elevations (595–939 m), whereas Kbi was found at lower elevations (83–345 m; Table 1).

3.2 | Population genetics

Levels of missing data across genotypes were low for both species: Kbi, $n = 277$, 1.5%; Kbo, $n = 62$, 1.9% (Table S1). All analyses of the two-species data set supported two distinct species. Differentiation of populations between the two species were generally higher than differentiation between population within either species (average between species F_{ST}: 0.377; within Kbi 0.194; within Kbo 0.137; Table S5). Genetic variance between species accounted for 23% of the total variance whereas within species population variation accounted for 13.5% (Table S6). Species-level differentiation was also apparent in the ancestry analysis, PCA and NJ tree (Figure 2). However, the model-based method for hybrid detection indicated that all individuals at five Kbi sites were assigned with high posterior probability to a hybrid class (sites BV, BVS, SBO, WF, WF2; Table S7). The simulations demonstrated the limited power to assign specific hybrid categories using the current data set: simulated F1 hybrids were assigned correctly only 83% of the time (they were otherwise mistaken for F2); F2 hybrids were assigned correctly 94%; and backcross individuals 47%, otherwise being mistaken for F2 hybrids (40%; Table S7). Thus, distinguishing with certainty between hybrid classes was not possible. The results remained unchanged when any individual loci were removed (data not shown). Nevertheless, the existence of mixed ancestry at these sites was reflected in their ancestry proportions (Figure 2a) and supported by their intermediate position

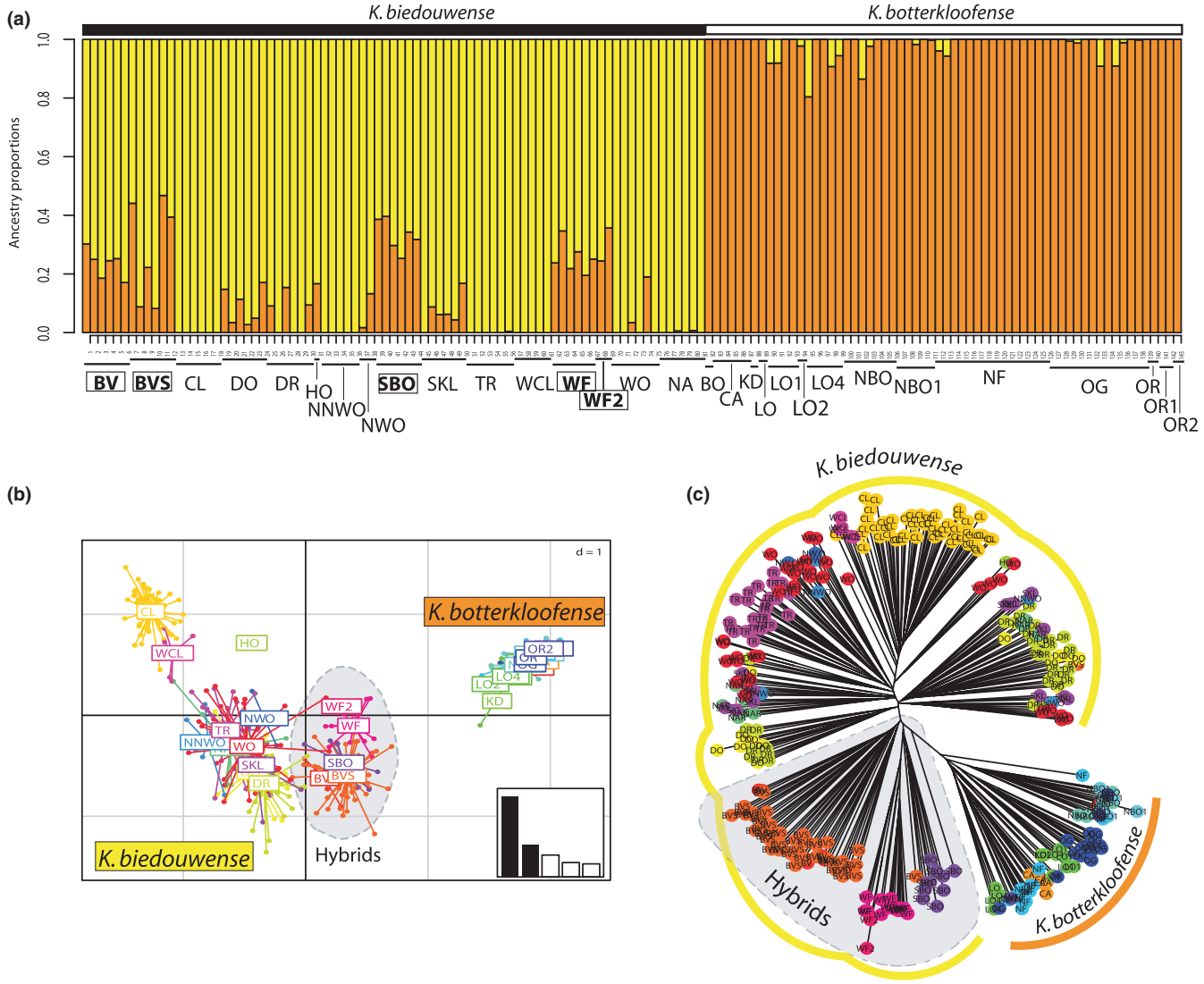

FIGURE 2 Interspecific genetic structure between *K. biedouwense* and *K. botterkloofense*, based on (a) Individual ancestry coefficients; (b) PCA (eigenvalues corresponding to the represented components are filled in black, PC 1, 18%, PC2 7%) and (c) NJ tree. Sites containing putative hybrid individuals are highlighted in bold and by a box (a), or by grey shading (b, c). All individuals were used with no pooling; data set: 12 loci in common

in the PCA and NJ analyses (Figure 2b,c). The individual-based ancestry proportions analysis indicated that the genetic exchange is asymmetric, from Kbo into Kbi (Figure 2a), with no obvious sex-bias in the hybrid individuals ($n = 34$ female; $n = 43$ male). Allelic frequencies at four loci displayed similar frequency patterns between the putative hybrid sites and Kbo localities (KB20 Figure 3; and loci KB4, KB7, KB14 Figure S2).

Measures of diversity were approximately similar in both species (H_e 0.55–0.79 Kbi, 0.62–0.73 Kbo), with some sites in both species having very low genetic diversity (e.g., Kbi, WF, WF2, CL; Kbo, OG; Table 2). Population differentiation was moderate to high (0–0.52 in Kbi and 0.02–0.28 in Kbo), with the highest differentiation between the sites with the lowest genetic diversity versus all others (Tables S8 and S9). The PCA analyses also supported geographic-based genetic structure in both species, particularly in Kbi (Figure S3). In Kbi the first axis differentiated sampling sites along an East-to-West gradient (28.7%), whilst the second axis was driven by variance caused by the low diversity, highly differentiated sites (17.3%). In Kbo the first axis differentiated sampling sites along a North-to-South gradient (27.5%), with the second axis driven by the low diversity site (OG) (21.9%).

Strong geographic, almost site-based structuring was also evident in both species in the Bayesian assignment and NJ tree analyses (Figure 4, Figure S4). Particularly for Kbi, sampling sites were either assigned to their own genetic cluster, or when such clusters were shared between sites, it was between geographically very closely located sites. A strong signature of IBD over a short geographic distance (~50 km), was evident in Kbi (Figure S5, $r = .563$, $p < .001$). We detected no signature of IBD in Kbo, though it's distribution spanned twice the distance (~100 km; Figure S5, $r = .107$, NS). However, having few sites ($n = 8$) for this test strongly limits statistical power (Jenkins et al., 2010). Reducing Kbi sampling to match that of Kbo

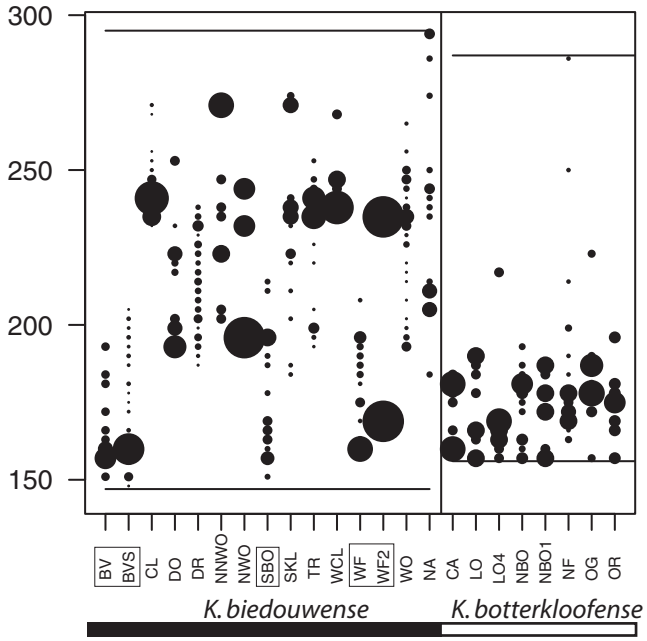

FIGURE 3 Allelic frequencies for locus KB20, illustrating that the frequency patterns of the *K. biedouwense* sites containing hybrids (BV, BVS, SBO, WF, and WF2) are similar to that found in *K. botterkloofense*. Site on *x*-axis, allele size on *y*. Bubble diameters reflect allele frequencies which sum to one in each column. Plots of all allelic frequencies are presented in Figure S2. Singleton sites were removed, pooled sites were used in *K. botterkloofense*; data set: 12 loci in common

(11 loci of equivalent variability, eight sites and matching numbers of individuals per site) also resulted in a nonsignificant association ($p = .077$). The same analysis repeated for 12 sites resulted in greater statistical power ($p = .008$).

There was no evidence for sex biases in dispersal in *Kbi* (mAIc −0.76, $p = .44$). These results were unchanged when only the five largest sites were used (minimum sample size 26; mAIC −1.71, $p = .08$), suggesting that the result is not impacted by low sample sizes. Estimated migration rates between sites within and between species showed that some low diversity sites had low numbers of immigrants and emigrants (e.g., BVS, NBO1) but there was no evidence for increased migration from Kbo to presumed Kbi hybrid sites (Tables S10–S12).

Analysis of mtDNA supported the monophyly of the two species and demonstrated strong geographic structure in Kbi (Figure 4, Figure S6). No haplotypes were shared between species.

3.3 | Biotremulation

Kbi were recorded from all sites ($n = 16$), whilst fewer Kbo individuals were recorded from 10 sites (Table S2). Males and females of both species were always analysed separately due to their distinctive biotremulation signals.

Using the full data set of parameters for males, and the pulse repetition time of females, substantial overlap was evident between the two species for signals of both sexes (Figure 5). Between species differentiation in the male signals was driven by the longer intervals between pulse trains in Kbo males (longer PTR & IPI) and by the faster signalling rate in Kbo females (lower PRT). Signals of hybrid individuals were not intermediate between the species. Male hybrids had a typical call type for males of Kbi (Figure 5a); female Kbi hybrids had a higher PRT than Kbo, with almost no overlap in values (Figure 5b).

Substantial site variation in signal parameters occurred in both sexes and species (Figure S7). Kbi males from the site WF had a higher mean number of pulses per pulse train (mean 6.99, range 5–11) compared to all other sites (mean 5.17 range 2–7), but otherwise parameter variation did not produce an easily discernible pattern. If all male signal parameters were considered, there were no significant differences between sites (ANOVA, honest significant differences, *p*-values adjusted for multiple comparisons, $p = .82–1.00$); when individual parameters were considered there were significant differences between sites, but these varied with the parameter under consideration and did not yield a consistent pattern (Table S13). The pulse repetition time (PRT) for Kbi females was extremely variable both between and within sites and did not appear to be related to geographic proximity of sites (Figure S7C). Ten site pairwise comparisons were significant, six of which involved the site CL (ANOVA, honest significant differences, $p = 0–.02$, Table S14).

3.4 | Morphology

The variance in morphology overlapped partially between the two sexes and two species (Figure 5c,d, Figure S8). Nevertheless, the LDA suggested that good species predictions can be made using few measures (i.e., profemur and protibia length for both sexes, in addition to genal height for males, Table S15). Sites with hybrid individuals were not morphologically intermediate between the species, but were completely nested within the variation found in Kbo (Figure 5c,d). The ratios of greatest importance for characterising the variation were consistent irrespective of the data set (e.g., between species, within species between sexes, or between sites in a particular sex; Figure S8, Figure 5c,d): prfl_genh (i.e., the ratio profemur length:gena height), prfl_eyeh, prnl_genh, and eyel_genh. Within species, there was little site based differentiation in either species or sex. The variation found at sites with the highest sampling effort (e.g., BVS, WO, LO) often encompassed the variation found within the species (Figures S8–S12). No significant differences were found between sites in any sex or species (Tables S16 and S17).

3.5 | Drivers of divergence

Geographic distance was supported as an important factor for interspecific divergence in both call ($p = .02$) and morphological ($p = .009$) parameters for males according to the mantel-based and GDM analyses. Geographic distance was found to be important for interspecific divergence in calls only for females in the gdm analysis

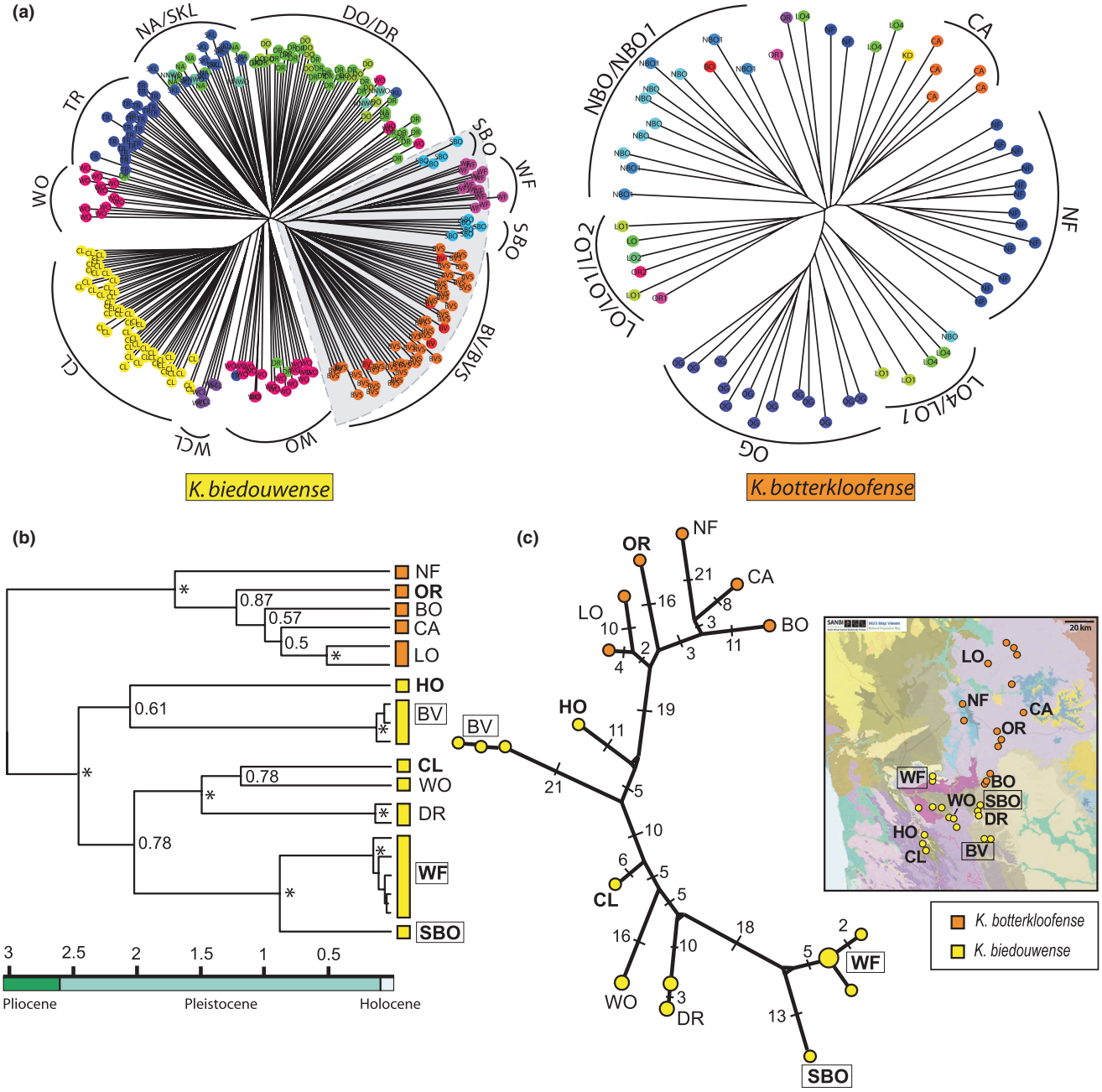

FIGURE 4 (a) NJ tree for *K. biedouwense* and *K. botterkloofense*. All individuals were used in these analyses; data sets 17 loci and 11 loci, respectively. Hybrid populations marked with grey shading. (b) Bayesian mtDNA gene-tree (COI) illustrating the relationships and divergence times between *K. biedouwense* and *K. botterkloofense* (outgroups not shown). Posterior probabilities are indicated at nodes (* = BPP 0.99/1). Timescale in millions of years. See Figure S4 for estimates of node ages. (c) Median joining haplotype network illustrating the relationships between the two species. Branch lengths represent a single mutation unless otherwise indicated. A sampling map indicating the subset of sites used in the mtDNA analyses is shown in inset. Site names in boxes indicate putative hybrid localities. Site names in bold (OR, HO, CL, WF, SBO) indicate that there are both mtDNA sequences and microsatellite genotypes for these individuals

($p = 0$), which can accommodate nonlinearity, but not when using the mantel-based approach. There was no influence of vegetation type or altitude, nor any consistently important climatic variables on variation in calls or morphology.

Considering genetic divergence, the minimum temperature of the coldest month, precipitation of the driest quarter, altitude and geographic distance were indicated as important factors implicated in species divergence ($p < .05$, Table 3, Figure 6). However, within species, geographic distance tended to be the most important and significant factor (Table 3, Figure 6). Based on just a few variables, a high percentage of deviance was explained (42%–78%, Table 3). Although vegetation type was indicated as important for divergence

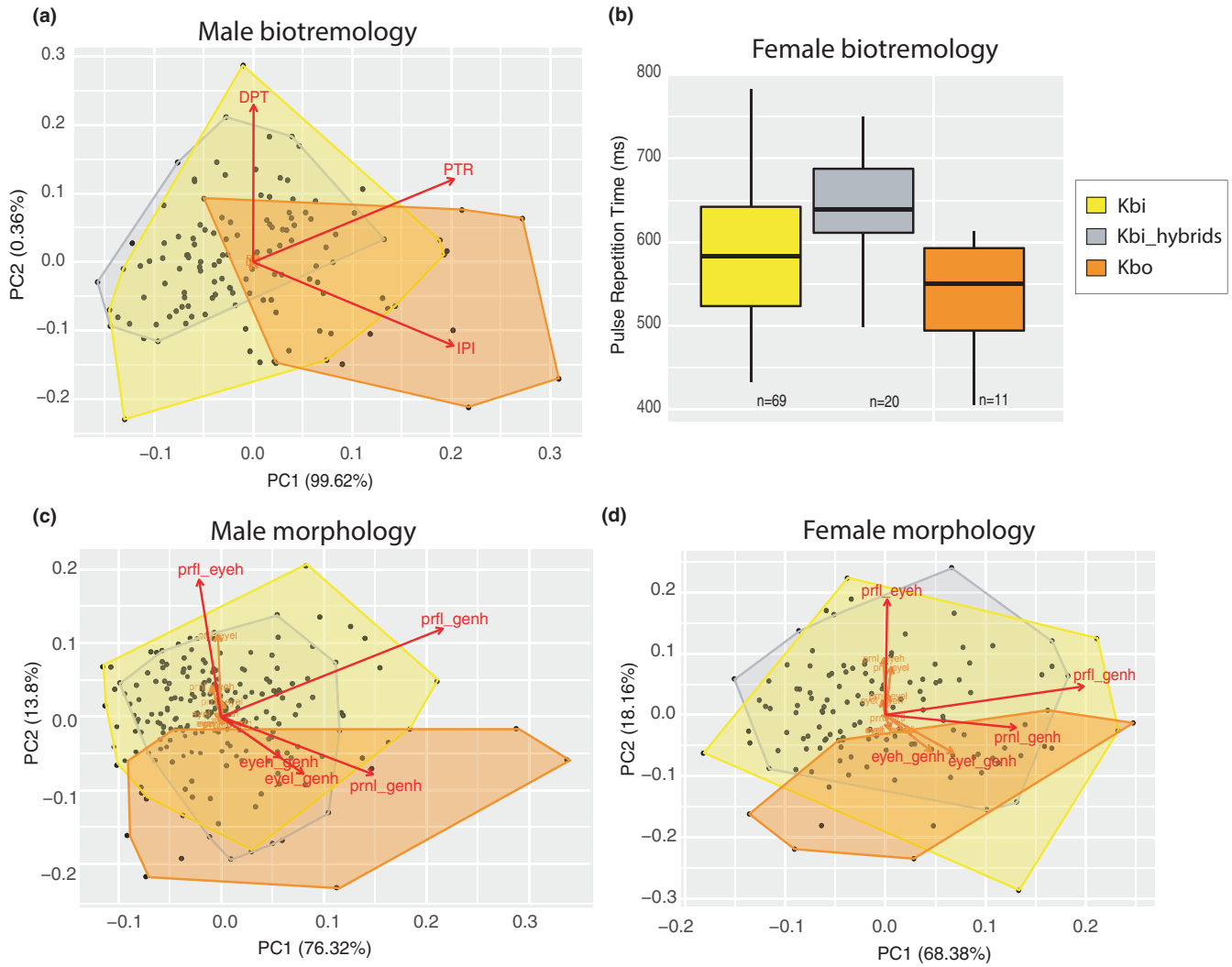

FIGURE 5 Variation in call parameters (a, b) and morphology (c, d) between *K. biedouwense*, *K. botterkloofense* and hybrid sites, for males (a, c) and females (b, d). (a) is based on five call parameters for males, (b) on a single call parameter (PRT) for females, and (c and d) on 13 ratios between eight morphological measurements (see Table S3)

in Kbo, with only eight sites, five of which occur in the same vegetation type, this result should be interpreted with caution.

Overall, our results do not demonstrate associations which would indicate selective pressures on morphological or call traits. It is possible that variation in these traits could be due to ecological drift, though local adaptation was not specifically tested for in this study and should be verified experimentally. It appears that geographic distance has played a role in population genetic divergence, before adaptation to local conditions probably occurred and grew in importance.

4 | DISCUSSION

Current results suggest that the genus *Karoophasma* diverged into two species 2.96 Ma (95% HPD 2.33–3.67), rapidly followed by strong geographic-based divergence in Kbi during the Pleistocene, in agreement with Dool et al., 2018. This early divergence and maintenance of distinct Kbi clades resulted in the current pattern of mtDNA differentiation being almost as high within Kbi as between the two species. Within the order Mantophasmatodea, allopatry is the norm and most genera contain few species or are monotypic. Divergence followed by differentiation in allopatry may be a typical pattern in the order, enhanced by the limited dispersal abilities of these taxa.

In the current study, no evidence was found for interspecific mtDNA introgression in *Karoophasma*. However, site-based sample sizes were low for this data set, including the sites containing potential hybrid populations (BV $n = 3$, WF $n = 5$, SBO $n = 1$). Rates of mitochondrial and nuclear introgression often differ and numerous hypotheses have been proposed to explain this, for example, sex-specific hybrid mating behaviour, or sex biases in dispersal (Patten et al., 2015; Petit & Excoffier, 2009; Toews & Brelsford, 2012). Using spatially explicit, individual-based simulations, Bonnet et al. (2017) demonstrated that under a scenario of high dispersal, a spatial invasion can result in substantial and easily detectable introgression of

TABLE 3 Relative importance of predictor variables for genetic divergence between and within species determined by summing the coefficients of the three GDM I-splines

Predictor variable	KbiKbo_MF	KbiKbo_F	KbiKbo_M	Kbi_MF	Kbi_F	Kbi_M	Kbo_MF
Geographical distance	0.02	0	0.12	0.04	0.04	0.14	0.02
Altitude	0.15	0.23					
Min. temperature of coldest month (BIO_6)		0.29	0.29				
Precipitation of driest month (BIO_14)				0.01	0.01		
Precipitation of driest quarter (BIO_17)	0.28						
Vegetation type							0.09
Percentage of deviance explained	58.40	78.26	76.25	42.96	42.36	73.36	49.12

Note: Only predictors found to be statistically significant are shown ($p < .05$, 500 permutations). Due to sexual dimorphism in morphology and call traits, these data sets were only included in the model when treating sexes separately. KbiKbo_MF (both species, both sexes), KbiKbo_F (both species, females only), etc.

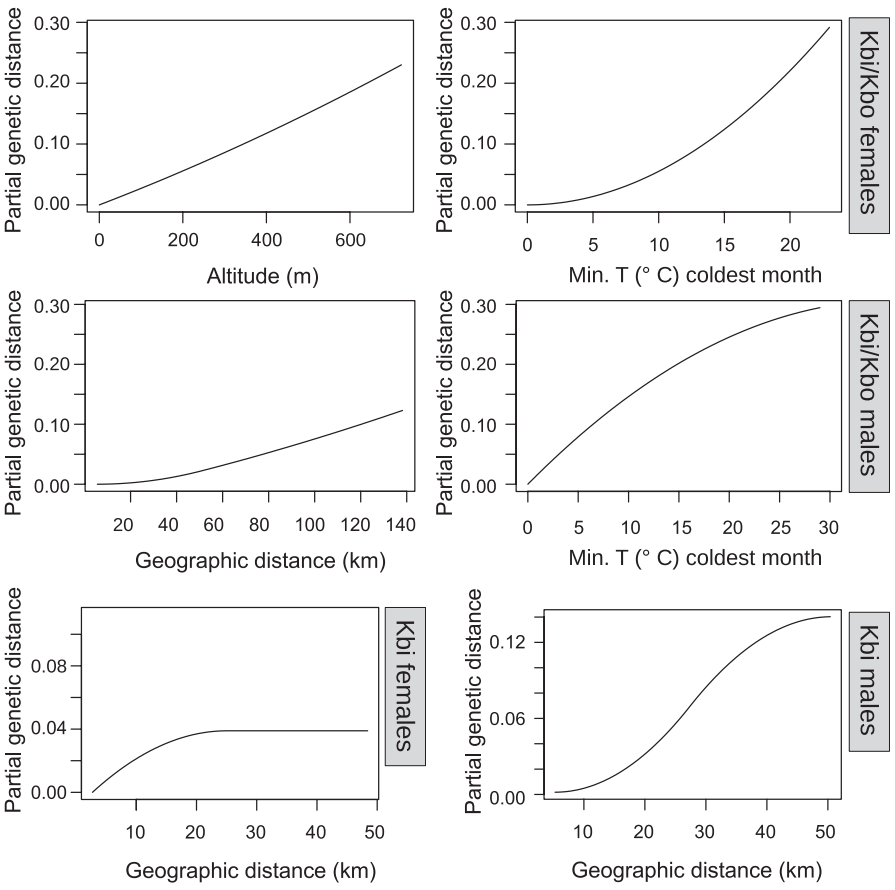

FIGURE 6 Generalised dissimilarity model-fitted I-splines (partial regression fits) for variables found to be important and significant for interspecific (top and middle panel) and intraspecific divergence in Kbi (bottom panels). See also Table 3

the nuclear genome. The microsatellite results suggest asymmetric nuclear introgression from Kbo to Kbi, at three localities (WF/WF2, BV/BVS, and SBO). Currently there is no evidence for sympatry between these taxa at any site, though their distributional ranges are contiguous. The absence of Kbo individuals at the hybrid sites and assignment of all individuals at these sites to hybrid categories suggests extensive past introgression and subsequent limited gene flow. This situation can arise when hybrids can mate successfully with other hybrids and with both parental species (Allendorf et al., 2010; Dufresnes & Dubey, 2020; Ficetola et al., 2019; Pacheco-Sierra, 2016; Phifer-Rixey & Nachman, 2015). The hybrid sites occur between the two species distributions and at the edge of high elevations occupied by Kbo (Figure 1). Mountains, and particularly rivers may have acted as effective geographic barriers constraining further spread of introgressed alleles. Currently the two species are separated by the substantial Doring river, whose peak flow coincides with the emergence period of *Karoophasma*. Climatic fluctuations during the Plio–Pleistocene (Linder & Verboom, 2015) may have caused fluctuations in the distributions of these species, thereby facilitating dispersal and interspecific introgression.

Adaptive introgression is a possible explanation for the unidirectional introgression of alleles but is not required to account for a phenomenon which may be entirely nonadaptive. However, it is also known that introgressed adaptive alleles may act as barrier loci and thus contribute to divergence only during later phases of in situ population divergence (Ravinet et al., 2017). Asymmetric introgression can be found during species formation when closely related taxa are incompletely isolated for millions of years (Mallet, 2005) and experience alternating periods of isolation and gene flow (Harrison & Larson, 2014). This may have been the case during unusually dry periods when the Doring river could be crossed by the wingless *Karoophasma*. Prezygotic mating barriers may be lost in relatively short time-frames if they are costly to maintain or simply due to drift, greatly facilitating interspecific hybridisation upon secondary contact (Myers & Frankino, 2012; Sánchez-Guillén et al., 2016). Given the recent divergence of these taxa and their high similarity in morphology and call structure it is possible that reproductive isolation remains incomplete. As the two species are not known to naturally occur at the same site, this would need to be tested experimentally. The detection of hybrids was unaffected by jackknifing across loci, suggesting widespread introgression across the genome. This does not corroborate a scenario of adaptive introgression wherein selectively advantageous genes only are introgressed.

Vegetation type was not found to play an important role in species divergence, nor in accounting for variation in call or morphological characteristics. Neither vibrational communication signals nor morphological parameters were implicated in either within, or between species divergence. The strong relationship between geographic and genetic distance was evident from the strong signal of IBD and geographic-based structure within species. Based on modelling, geographic distance was found to be an important and significant factor implicated in early population divergence, suggesting that the strongly limited dispersal capacity of these animals could be an important early driver of divergence in these taxa. Examining factors influencing between species divergence suggested that climatic variables, followed by altitude were the most important, whilst geographic distance remained a significant variable (thus, aspects of H1, H3 and H4 were partially supported). Given the higher altitudes that Kbo occupies (viz the Niewoudtville and Calvinia plateaus) it is possible that there are physiological adaptations that restrict it from occupying the lower and warmer habitats of Kbi. Together these results illustrate that mechanisms driving divergence may be multiple and vary in their importance over space and time (i.e., H5 is the best supported), with however, a strong role for geographic distance throughout the process, particularly during early divergence.

The plant communities of the CFR, including those occupied by heelwalkers, are well studied. These communities are composed of plant species with an exceptionally low migration rate (due to short seed-dispersal distance, McDonald et al., 1995) and a fast rate of speciation. In fact, migration rates were found to be two orders of magnitude lower than in tropical rain forest systems and speciation rates were found to be higher than in any known plant system (Klak et al., 2004; Latimer et al., 2005). This low migration system historically generated a spatially structured suite of species-rich communities isolated by drier lowlands. Ecological and genetic drift in the isolated communities is thought to have driven divergence and species formation over short spatial scales (1–100 km; Latimer et al., 2005). Our results suggest that a similar situation may have occurred in Mantophasmatodea. Similarly, Verboom et al. (2015) studied the radiations of six prominent fynbos lineages and found support for the hypothesis that niche conservatism in the context of Miocene-Pliocene climatic changes and topographic heterogeneity drove vicariant speciation for these high-elevation flora. The CFR may be particularly favourable to geographic modes of speciation due its archipelagic nature in the form of "sky islands" (Verboom et al., 2015) and extensive geological heterogeneity and soil types. Within these climatically and geologically distinct high-elevation zones, drift may act strongly on the isolated populations. Many mammal and reptile species in this region also have distinct genetic lineages confined to specific mountain ranges (see Linder et al., 2010 and references therein).

5 | CONCLUSION

The current study sought to investigate the early drivers of speciation using sister taxa in Austrophasmatidae and to explore whether this group could be a promising target for studying modes of speciation. Recently diverged taxa have the advantage that fewer confounding variables obscure the signal of the factors promoting early differentiation (Wagner & Mandeville, 2017). For our study taxa, geographic distance was an important driver of divergence, especially for initial divergence, whereas local selection in response to climate and habitat conditions appeared to be more important later in divergence. The *Karoophasma* hybrid-zone opens the possibility to identify introgressed genomic regions and assess what impact these have on reproductive isolation. If further hybrid zones are found in Austrophasmatidae, the patterns of hybridisation in species-pairs can be assessed with respect to genomic and phenotypic divergence. This will allow us to distinguish between factors shaping differentiation across the taxonomic group generally, and those which are specific to taxon-pairs, contributing to a better understanding of how biodiversity is generated and maintained.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors planned the field collection and contributed reagents or analytical tools. Serena E. Dool collected the field data with help from Mike D. Picker. Serena E. Dool generated the data sets, analysed the data and wrote the paper. All authors revised the manuscript. The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The mtDNA (COI) sequences generated in the current study and metadata are available on GenBank MN612053–MN612063. The individual-based genotype, call and morphological data sets are available in Tables S1–S3 and include site, latitude, longitude, date of collection and sex for each individual.

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