Intraspecific Patterns of Mitochondrial Variation in Natural Population Fragments of a Localized Desert Dung Beetle Species, Pachysoma gariepinum (Coleoptera: Scarabaeidae)

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Phylogenetic, population, and coalescent methods were used to examine the genetic structuring of *Pachysoma gariepinum*, a flightless dung beetle species endemic to the arid west coast of southern Africa that exhibits interrupted south to north morphological clinal variation along a distributional gradient. Mitochondrial cytochrome oxidase I sequence data of 67 individuals from 5 localities revealed the presence of 3 geographically distinct evolutionary lineages (with an overall nucleotide divergence of 5.7% and a per-locality divergence of 1.9–3.8%) which display significant levels of genetic structuring. The separation of the lineages was estimated to have occurred between 2.2 and 5.7 million years ago—which is the late Miocene, early Plio-Pleistocene era—possibly in response to the ebb and flow of the Orange and Holgat River systems as well as the interactions between the moving and stable sand dune systems. Moreover the species' current range appears to have been influenced by the formation of advective fog resulting in a constant source of water in an area with low precipitation thereby allowing for the beetles to radiate to areas that were previously inhospitable. Fu's *F*-statistics and population parameters based on recent mutations indicated that little to no recent population growth has occurred. This together with changing anthropogenic factors and the recovery of 3 geographically discrete management units, points to a need for census data in order to monitor and conserve the genetic diversity of this species.

Species more often do not consist of geographically structured populations, many of which have experienced little or no genetic contact for long periods due to limited dispersal abilities of the individuals, or as a result of habitat discontinuities or due to the extinction of intermediate haplotypes (Avise et al. 1987; Carisio et al. 2004). In addition to selective forces, factors that contribute to these associations are past events such as colonization history and current demography (Juan et al. 1998). By examining the variation among these populations and their historical associations, the processes underpinning the present genetic structure can often be revealed (Wright 1931). Species complexes among these geographically isolated populations have great potential for historic inferences, in that they represent the extreme of spatial patterning (Kirchman et

al. 2000). By linking genetic differentiation, ecological adaptation, and environmental change, speciation, historical demography, and geographic movement of the taxa of interest can be investigated (Zheng et al. 2003).
Dung beetles have a worldwide fauna of at least 5000 species belonging to 234 genera (Hanski and Cambefort 1991; Villalba et al. 2002; Ocampo and Hawks 2006). They are known for their distinctive and diverse morphology, coloration, behavior, and ecology (Ocampo and Hawks 2006). Dung beetles feed mainly on dung, which represents a patchy, ephemeral, and limited food source. The main climatic factors affecting the spatial and temporal distribution of dung beetles are temperature and precipitation (Halffter and Edmonds 1982). They are most diverse in moist tropical regions and poorly represented in colder climates or arid regions with most species being restricted to areas where annual precipitation is above 250 mm per year and the annual daily temperature is higher than 15 °C (Halffter and Edmonds 1982). *Pachysoma* species are quite different from other Scarabaeines in their habitat preference, food relocation behavior, and unique morphology (see Scholtz et al. 2004).

The genus *Pachysoma* MacLeay (1821) represents a group of 13 atypical flightless dung beetle species belonging to the ball-rolling tribe Scarabaeini that are distributed along the west coast of southern Africa from Cape Town (33°56'S–18°28'E) in South Africa to the Kuiseb River (22°58'S–14°30'E) in Namibia (Figure 1), with *Pachysoma gariepinum* occupying the central portion of the entire *Pachysoma* distribution, occurring from the Buffels River (29°33'S–17°24'E) in South Africa to the Agub Mountains (26°59'S–15°58'E) in Namibia (Figure 1). *Pachysoma gariepinum* occurs in pockets of discontinuous populations on coastal sands, covering two distinct biomes, the Succulent Karoo Biome, south of the Orange River, and the Namib Desert, north of the Orange River (Figure 1).

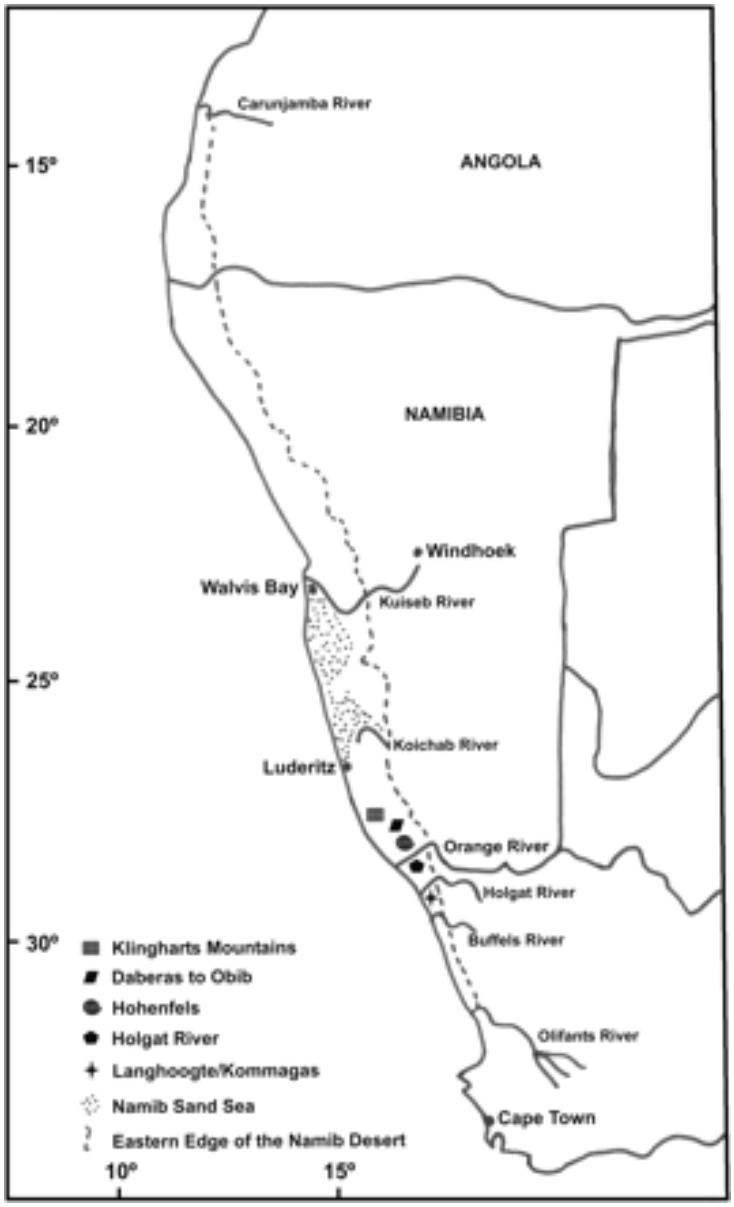

Figure 1. Map indicating the entire *Pachysoma* distribution, from the Cape, in South Africa, to Walvis Bay, in Namibia, as well as schematically representing collecting localities of *Pachysoma gariepinum*.

Individuals of *P. gariepinum* that occupy the northern part of their range are adapted to live in one of the driest areas on the African continent, the southern Namib Desert (south of Lüderitz and north of the Orange River (Figure 1)), which has a mean annual temperature below 18 °C and is classified as true desert in which deficient rainfall occurs in winter but has a high incidence of fog (Pickford and Senut 1999). The southern part of

P. gariepinum distribution, the Succulent Karoo, has annual winter rainfall of 20 mm, supplemented by fog and dewfall, and temperatures as low as –4 °C in the coldest months.

Their food preference is unusual in that the beetles feed on dry herbivore dung pellets and detritus that they drag forward, whereas their *Scarabaeus* relatives form balls from wet herbivore dung, which they roll backwards. Their dung burial activity also differs from other ball-rolling dung beetles. *Pachysoma* first locate food, dig a burrow (Scholtz 1989), then forage repeatedly using polarized light for orientation (Dacke et al. 2002), until they have collected sufficient dung fragments or bits of detritus. Related rollers locate dung form a ball at the source and roll it away to be buried in a suitable place. Flightlessness has resulted in atypical morphology in these species such as the absence of humeral calli, semi-contiguous mesocoxae, and short mesosterna (Harrison and Philips 2003). Added to which, Harrison (1999) showed that body size, elytral sculpture, indument, and size of the mesepisternal protuberance varied within and between localities, with distinct south to north morphological clinal variation. *Pachysoma* has been shown to be monophyletic and sister to the main *Scarabaeus* sensu stricto lineage that radiated in the Namib Desert after the onset of hyperaridity (Harrison and Philips 2003; Sole et al. 2005).

Hyperaridity of the Namib Desert is closely associated with, and maintained by, the upwelling of cold subsurface oceanic water commonly known as the Benguela current (Tankard and Rogers 1978; de Wit 1993; Pickford and Senut 1999). This cold water causes the condensation of moist sea air forming coastal fog banks which in turn are windblown inland, by the prevailing south westerly winds, resulting in a constant source of water for the desert-dwelling organisms (Pickford and Senut 1999). Total annual precipitation from the fog may reach as high as 40–50 mm (Pickford and Senut 1999). The Namib Desert is characterized by barren sandy landscapes and is considered an evolutionary hot spot with many endemic groups of fauna and flora exhibiting unusual adaptations for survival in the harsh climatic conditions (Pickford and Senut 1999). The hyperaridity of the Namib Desert is no older than the middle Miocene (ca. 15 million years ago [mya]) indicating that rates of evolution of the flora and fauna associated with the post-Miocene Namib Desert Phase (Ward and Corbett 1990; de Wit 1993) were much faster than previously anticipated (for an example of rapid radiation, see Sole et al. 2005). Rapid radiations would therefore imply more severe selection pressures and enhanced generation of genetic variability or a combination of both (Pickford and Senut 1999; Sole et al. 2005).

Pachysoma gariepinum represents a rare endemic of conservation concern due to its commercial value to collectors, the current habitat threat posed by the removal of natural vegetation for farming in the western Cape, and the mining for diamonds and other minerals in Namibia and South Africa. Added to this, it is an organism that has undergone recent intraspecific geographic isolation. Life-history traits and time since fragmentation will influence the effect that habitat fragmentation has on a species. Genealogies, in a coalescent framework, are useful for deducing population demographics (Moya et al. 2004). Cytochrome oxidase I (COI) data were generated in order to assess the

phylogeographic and demographic patterns of *P. gariepinum*. We investigated the relationships between the COI haplotypes, time to most recent common ancestor (TMRCA), time of population divergence, and lastly we used the influence of landscape modifications, major climatic events, or anthropogenic actions to explain the overall genetic patterns observed.

Materials and Methods

Sampling, Amplification, and Sequencing

Sixty-seven individuals from 5 localities, covering the entire *P. gariepinum* distribution range (Figure 1), were collected. Total genomic DNA was extracted from thoracic muscle tissue and the 5' end of the COI gene, which was previously shown to be the most parsimony informative (Sole et al. 2005) for 46 genetically characterized individuals representative of the 13 morphological species of *Pachysoma*, was targeted for characterization in this study. Sequence data corresponding to 960 bp of the 5' end of the COI gene were generated with the TL2–N-3014 PCR primer and the internal C1-J-2183 primer (Simon et al. 1994) as described previously (Sole et al. 2005). Sequence chromatograms were visualized and edited in Sequence Navigator (Perkin-Elmer, Foster City, CA) and aligned in CLUSTAL X (Thompson et al. 1997). All sequences were deposited in the Genbank database under accession numbers AY9655087–AY965153.

Data Analysis

A genealogy was constructed using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). MrBayes uses the Metropolis-coupled Markov chain Monte Carlo (MCMC) technique to estimate the posterior probability distribution of trees. The Bayesian analysis utilized the GTR + I (0.763) + G (1.005) model with base composition of A = 32.89%, C = 16.08%, G = 11.97%, and T = 39.06% (as favored by the Akaike Information Criterion implemented in MrModel Test 2.2 (Nylander 2004)). Analyses were initiated from random starting trees using 1 cold and 3 incrementally heated metropolis-coupled chains (0.01) run for 10 million iterations with trees being sampled every 100th iteration, of which 20% were discarded during the burn in. Three independently repeated MCMC runs were performed. Phylogenetic analyses were rooted with *Pachysoma denticollis*. As haplotype networks are a better representation of the connections between haplotypes than tree-based criteria (Excoffier et al. 1992), SPLITSTREE4 was used to construct an unrooted network with the uncorrected *P*, NeighborNet distance (Husen and Bryant 2006).

Nucleotide (π) and haplotype diversity (h) within each collecting locality was calculated using Arlequin 2.000 (Schneider et al. 2000) (see network estimation and genealogy from above). Characteristic patterns such as high values of h and π indicate sustained large population sizes, whereas a high value of h and a low value of π are consistent with recent population expansion (Russell et al. 2005).

Hierarchical structuring of genetic variation was determined using analysis of molecular variance (AMOVA) (Excoffier et al. 1992, new version: Schneider et al. 2000), which

produces Φ-statistics similar to the F-statistics of Wright (1951, 1965). Levels of significance of Φ_{ST}-statistics were determined through 10 000 random permutation replicates (Schneider et al. 2000). A Mantel (1967) test was used to determine significant associations between genetic distances, calculated in MEGA version 4 (Tamura et al. 2007) and geographical distances between the 5 localities. One thousand randomized permutations were performed using Mantel version 2.0 (Liedloff 1999).

The entire data set and the individual locality data were tested for selective neutrality using Tajima's (1989) *D* (estimated in DNASP 4.0; Rozas et al. 2003) and Fu's (1997) *F*s (estimated in Arlequin). If Tajima's *D* and Fu's *F*s are found to be significantly negative, it would suggest the presence of selection or the occurrence of population growth. Population expansion was estimated in Arlequin by plotting the frequency of pairwise differences between the haplotypes of each sampling locality (the mismatch distribution). This evaluates the hypothesis of recent population growth (Rogers and Harpending 1992) with the underlying assumption that population growth or decline leaves distinctive signatures on the DNA sequences compared with constant population size. Recent growth should generate a unimodal distribution of pairwise differences, but the exact mode of growth (exponential, stepwise, or logistic) cannot be distinguished (Rogers and Harpending 1992).

In most instances, historical population demographics are estimated. However, in certain cases, recent population trends are needed for conservation or management purposes. With this in mind, different estimators of θ place different weights on mutations occurring in different time periods. Fu's UPBLUE estimate puts emphasis on recent mutations, revealing recent population processes, whereas Tajima's estimate puts more weight on ancient mutations, reflecting past population trends (Su et al. 2001). Comparing Tajima's with Fu's UPBLUE estimate will give an idea of population size change in recent time. Tajima's (1983) estimate of θ was estimated in Arlequin, whereas Fu's (1994a, 1994b) UPBLUE estimate of θ was estimated by Fu's phylogenetic estimator of θ online (http://hgc.sph.uth.tmc.edu/cgi-bin/upblue.pl). Tajima's estimate is based on the calculation of the mean number of pairwise differences of the sequences, whereas Fu's UPBLUE estimate is calculated by incorporating the genealogical information of the sequences (Su et al. 2001).

Mutation rate was estimated by following the procedure in Rooney et al. (2001). Firstly, the number of nucleotide substitutions per site was estimated by comparing the in-group *P. gariepinum* with *P. Pachysoma bennigseni* (Sole et al. 2005) using the formula $d = (\mathrm{Tv} + \mathrm{Tv}R)/m_T$, where Tv is the number of transversions between the focal and sister-species, *R* is the ratio of transitions to transversions within the focal species, and m_T is the length of the investigated DNA sequence. Secondly, the rate of nucleotide substitution (γ) per site, per lineage, per year was estimated by $\gamma = d/2T$, where *T* stands for the divergence time of the 2 compared species (this was estimated in MEGA using Brower 1994; 2.3% pairwise divergence per million years). Thirdly, it was possible to estimate mutation rate per nucleotide site, per generation (μ) by solving the equation $\mu = \gamma t_g$, where t_g is generation time in years, which in this case was taken to be a single generation per year.

The program Beast v.1.4.7 (Drummond and Rambaut 2007), a Bayesian coalescent analysis with the MCMC procedure, was used to estimate lineage ages (TMRCA) (Rutchman 2006). Lineage age was estimated under the lognormal uncorrelated model (relaxed molecular clock), assuming the Yule speciation model with all estimates utilizing the GTR + I + G model of substitution. Two independent Markov chains were run for 20 million iterations using a random starting tree. The program TRACER v. 1.3. (Rambaut and Drummond 2007) was used to assess the convergence between runs and posterior probabilities of the estimates. The number of independent samples from which estimates are drawn can be monitored through the effective sample size values as being 100 or more, this is an indication that the samples are a good estimator of the posterior distribution. As no fossil evidence is available with which to estimate the time of origin of the lineages, a standard mutation rate of 0.014 mutational changes per million years (see calculation below in the Results) was used.

Results

Genetic Diversity

The COI sequence data of *P. gariepinum* were characterized by high levels of diversity. An overall high haplotype diversity (h = 0.997 ± 0.004) was obtained with 62 unique haplotypes of a total 67 individuals being recognized. No haplotypes were shared between localities. The overall nucleotide diversity was (π) 0.057 ± 0.001, ranging from 0.022 ± 0.012 for Hohenfels samples to 0.042 ± 0.022 for the Klingharts Mountains (Table 1). The average number of nucleotide differences (k) was 54.195 for the entire data set. Overall sequence divergence was 5.7%, ranging from 1.9% in the Holgat River locality to 3.8% in Namibia.

Table 1. Molecular diversity indices for *Pachysoma gariepinum* by sampling locality

Locality	n	n_h	H	k	π(standard deviation)
Langhoogte/Kommagas	12	8	0.909	19.924	0.026 (0.014)
Holgat River	17	16	0.993	18.022	0.023 (0.012)
Hohenfels	13	13	1.000	17.154	0.022 (0.012)
Daberas/Obib Dunes	11	11	1.000	29.218	0.038 (0.020)
Klingharts Mountains	14	14	1.000	32.637	0.042 (0.022)
Overall	67	62	0.997	54.195	0.057 (0.001)

n, number of individuals sequenced; n_h, number of haplotypes; H, haplotype diversity; k, nucleotide differences; π, nucleotide diversity.

Genealogical Relationships among Haplotypes

A neighbor-joining phylogram is presented with posterior probabilities obtained from the Bayesian analysis of the 62 haplotypes identified in our study, rooted with *P. bennigseni* (Figure 2). Two main monophyletic groups of haplotypes can be identified: A South Africa group (75 posterior probability) of haplotypes which includes all the haplotypes collected in South Africa and the second group is a Namibia group (99 posterior probability) which includes all the haplotypes found in Namibia. Within the South African group, 2 subgroups of haplotypes could be identified and they correspond to the 2 collecting localities, the Holgat River and Langhoogte/Kommagas localities (100 and 74 posterior probability, respectively). Even though 2 subgroups of haplotypes (92 and 100 posterior probability) could be identified within the Namibian group they were not geographically discrete and comprised a mixture of haplotypes from the 3 localities in Namibia. They were therefore treated as a single, Namibian group. The unrooted SPLITSTREE NeighbourNet network in Figure 3 provides a graphical representation of haplotypes which is not purely dichotomous. Reticulation indicates alternative mutational pathways (i.e., homoplasy) that appear to occur within groups. The 3 main groups of the phylogram are identified by labeled circles.

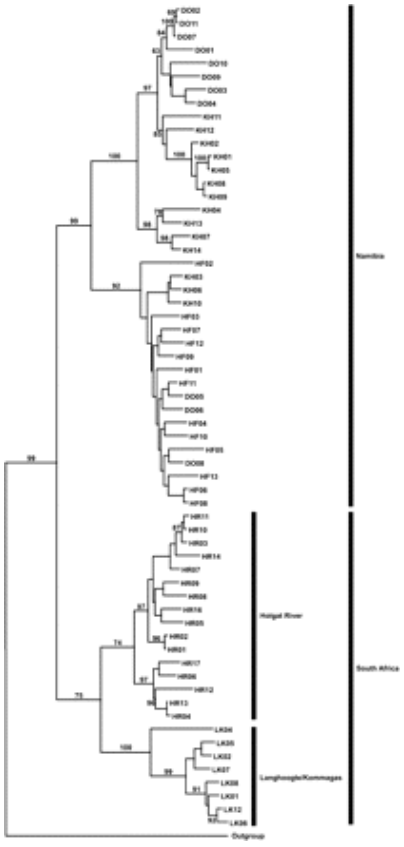

Figure 2. Neighbor-joining phylogram of haplotype relationships with support represented by Bayesian posterior probabilities. (see Table 1 one for abbreviations, numbers represent individuals).

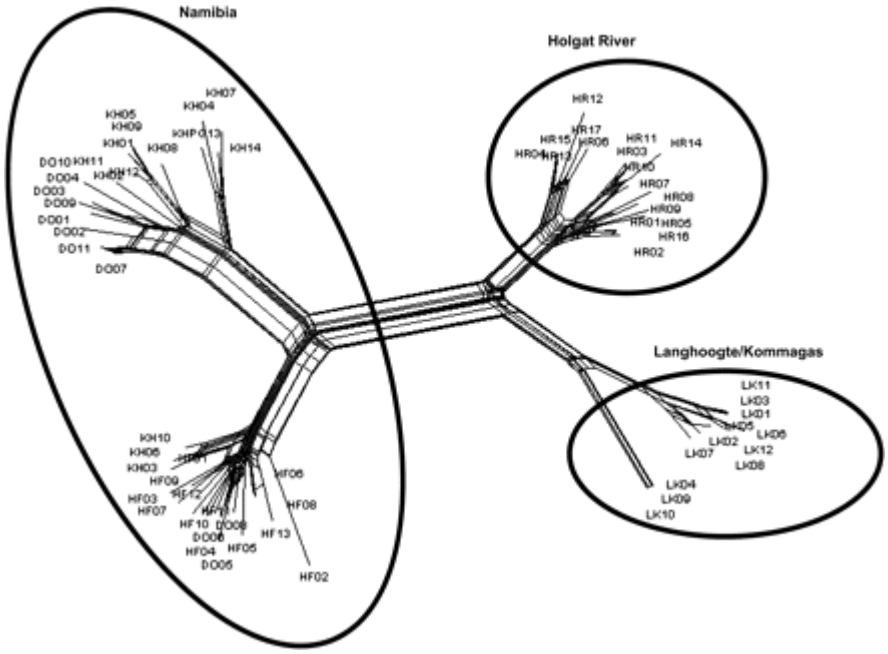

Figure 3. NeighbourNet network for 62 mtDNA haplotypes of *Pachysoma gariepinum*. Groups have been circled and labeled to correspond with the 3 main genetically and geographically distinct groups indicated in the phylogenetic tree (Figure 2).

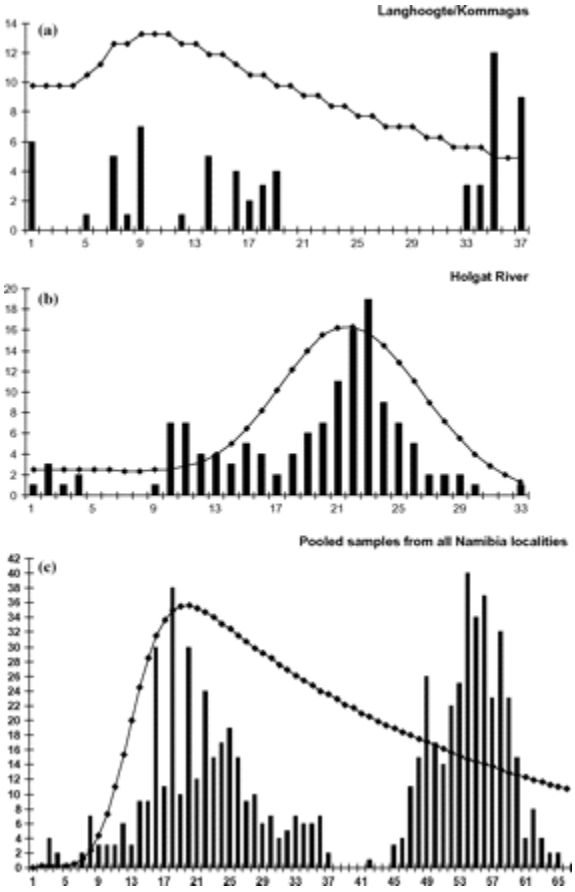

Figure 4. Mismatch frequency distributions of pairwise nucleotide differences for the 3 designated groups of *Pachysoma gariepinum*. (**a**) Represents the Langhoogte/Kommagas group, (**b**) the Holgat River group, and (**c**) all the individuals collected in Namibia.

Demographic History

Based on both the phylogram and unrooted SPLITSTREE NeighbourNet network, 3 distinct groupings could reliably be identified, namely, 1) a Namibian group, in which all the individuals from Namibia were pooled, 2) a Langhoogte/Kommagas group, and 3) a Holgat River group, were defined for the AMOVA. The results of AMOVA (Table 2) revealed that differences among these 3 defined groups accounted for 49.6% of the variance (Φ_{CT} = 0.496; P = 0.001). A high and significant Φ_{ST} value of 0.704 (P = 0.001) indicated strong genetic structure between the 3 designated groups. Pairwise comparisons between the Φ_{ST} therefore clearly support the distinctiveness of the 3 groups. The remaining variation could be attributed to Φ_{SC} = 0.415 (among group within locality variation), which was significant (P = 0.001), accounting for 20.9% of the overall variation. A Mantel test revealed a significant association between genetic and geographical distances (g = 2.055, r = 0.6206, P < 0.025), indicating that the level of resemblance between the genetic groupings is dependent on distance.

Table 2. Summary of F_{ST} statistics calculated by AMOVA (Excoffier et al. 1992) for the 3 *Pachysoma gariepinum* groups

	Φ_{ST}	%	*P*
Among groups	Φ_{CT} 0.496	49.6	<0.001
Among groups within populations	Φ_{SC} 0.415	20.9	<0.001
Within populations	Φ_{ST} 0.705	29.5	<0.001

P values were determined from 10 000 random permutations.

The frequency distribution for the pairwise nucleotide differences was investigated for the 2 groups identified in South Africa and the single group in Namibia. The mismatch distributions for the Holgat River locality (Figure 4b) show similar unimodel curves as expected with a historically expanding population. Both the variance (sum of the squared deviation [SSD]) and Harpending's Raggedness Index (HRI) suggest that the simulated and expected curves do not differ significantly under a model of expansion. For the individuals collected in Namibia and the Langhoogte/Kommagas locality (Figures 4a, c), the SSD and HRI are significant, indicating that the expected and simulated curves differ. Because they are multimodel/ragged curves, this is an indication that these lineages are approaching mutation–drift equilibrium.

The results for the neutrality tests are summarized in Table 3. The overall general trend for recent expansion indicates that most lineages have yet to reach mutation–drift equilibrium. This is reflected in Fu's *F* values which, albeit highly variable among groupings, tend to be significantly negative. However, Tajima's *D* does not support this. When comparing Fu's UPBLUE estimate with Tajima's estimate of θ, the UPBLUE estimate is approximately one and zero for all estimates indicating little to no recent growth within the identified groups.

Table 3. Results of the neutrality tests calculated for the 3 geographically distinct lineages (as identified in the NeighbourNet Network)

	Fu's *F*	Tajima's *D*	Tajima's estimate	Fu's UPBLUE	UPBLUE/Tajima
Langhoogte/Kommagas	2.65 (ns)	0.53 (ns)	17.55	20.89	1.17
Holgat River	–3.77 (*)	–0.14 (ns)	18.16	1.25	0.04
Namibia	–14.86 (***)	0.42 (ns)	31.18	1.17	0.07
Overall	–16.45 (**)	0.92 (ns)	42.94	217.13	5.06

Statistical significance as follows: ns, nonsignificant, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Coalescent Time Estimates

Using the 960 bp of the COI sequence, the average number of nucleotide substitutions per site (*d*) was calculated and a value of 0.08 obtained. The divergence time between *P. gariepinum* and *P. bennigseni* (Sole et al. 2005) was estimated to have occurred 2.8 mya. This gives the estimate of nucleotide substitutions per site, per lineage, per year (π) to be $0.08/(2 \times 2\,800\,000) = 1.4 \times 10^{-8}$. The mutation rate per nucleotide site, per generation (μ) was therefore 1.4×10^{-8} or 0.014 mutational changes per million years.

Lineage age estimates in million years, upper and lower bounds at the 95% confidence interval as well as standard deviation, based on the Bayesian approach using a relaxed molecular clock, are listed in Table 4. The chronogram depicting confidence intervals estimated during the MCMC runs is presented in Figure 5.

Table 4. Estimated parameters for coalescence time of lineages of *Pachysoma gariepinum*

	Mean	Upper 95%	Lower 95%	Standard deviation
Langhoogte/Kommagas	2.21	3.76	1.09	0.08
Holgat River	2.28	4.20	1.27	0.12
South Africa	4.69	6.82	2.40	0.15
Namibia	5.77	6.62	2.64	0.12

Lineage age estimates are in mya using the uncorrelated lognormal substitution model with an estimated substitution rate of 0.014. Mean, upper, and lower lineage age estimates are presented as calculated from 20 million MCMC iterations under the GTR + G + I model.

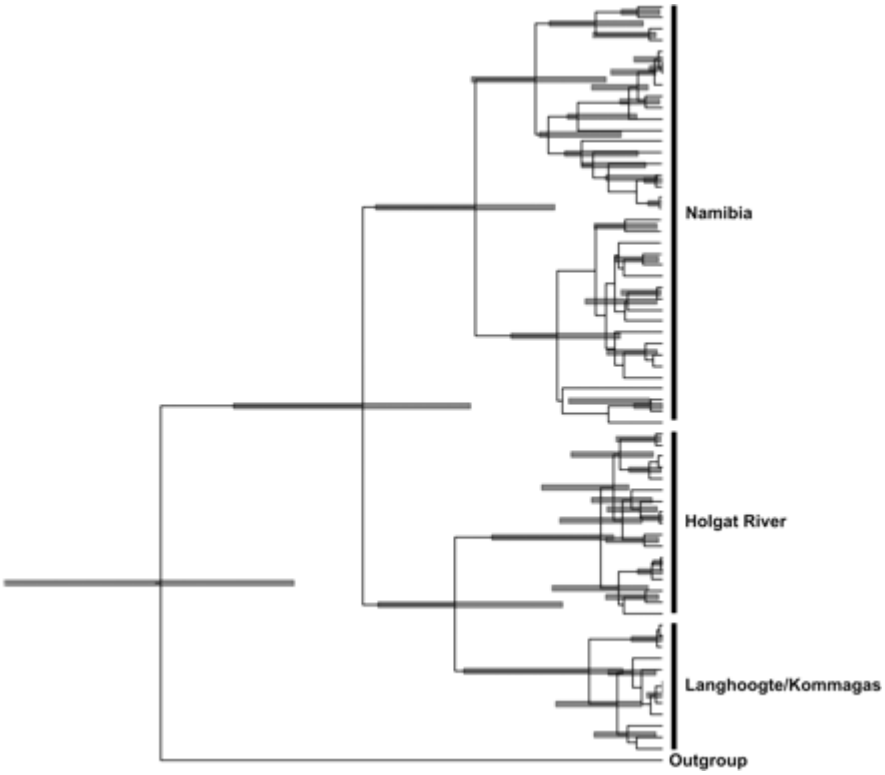

Figure 5. One of the sampled trees by the MCMC run in the Bayesian-based estimate of lineage ages. The bars on the lineages correspond to confidence intervals estimated during the MCMC runs.

Discussion

Demographic Patterns and Biogeographic Inferences

The breakup of Gondwanaland would have resulted in the west coast landmass being exposed to the South Atlantic Ocean and the factors responsible for influencing its climate. The area occupied by Namib Desert today was probably previously part of the Gondwana Desert, which had a much milder climate (lower temperatures, more regular precipitation, and aseasonality) with its own fauna. These organisms being preadapted to an arid environment would therefore have had the monopoly on the unfilled niches, which became available as the Namib climate started evolving. The present Namib fauna has also been shown to be descended from the Gondwanan fauna in that sandy pockets at river mouths, sand accumulations in the lower Orange River area, and coastal/littoral dunes in the western Cape have been shown to be possible areas for the origin of psammophilous (sand dwelling) taxa (Endrödy-Younga 1978, 1982a, 1982b; Irish 1990). Being confined to sand, their invasion of the Namib would have followed sand movements. Dune dispersal in a unidirectional wind regime has resulted in south to north evolutionary trends seen in many of the Namib's psammophilous taxa (Endrödy-Younga 1982b; Irish 1990) in that those with the more plesiomorphic characters are found in the southern Namib or along the Namaqualand coast with their apomorphic counterparts occurring in the northern Namib. Examples of this are seen in the clinal morphs of Lepismatidae, Scarabaeidae, and Tenebrionidae (Irish 1990). In these ways, the Namib endemics would have come into being.

Although the Orange River currently forms the boundary between the Nama-Karoo Biome and the Namib Desert, it appears to be of minor or sporadic importance as a gene barrier with many southern Namib species occurring on either side of it (Irish 1990). The beetles could have crossed the river by falling in and being swept across, by being wind blown or by simply walking across the silt rivulets, which are known to have formed across or near the mouth area (Moore J, personal communication). The Holgat River, on the other hand, was a major watercourse during wet phases of the Pleistocene (Pickford and Senut 1999) and could have caused the separation between the localities in South Africa. This implies that the barrier between related arid-adapted species appears to lie more south than the Orange River, around the Holgat or Buffels Rivers (see Figure 1). The northern distribution of *P. gariepinum* is represented by the northern most limits of the Klingharts Dunes.

No shared haplotypes were noted between any of the collecting localities or groupings. This could be due to inadequate sampling, an effect of limited genetic diversity (which does not appear to be the case in this study, see explanation below) or as the result of the dynamics of the natural history of *P. gariepinum* (because the beetles are flightless, the chances of individuals finding each other for mating purposes are reduced leading to the extinction of intermediate haplotypes). The NeighbourNet network and phylogenetic tree shows historical intermixing of individuals from different localities within Namibia (Figures 2 and 3). Despite the lack of shared haplotypes, traces of historical movement

have not completely vanished, in that samples can be meaningfully arranged according to their genetic proximity (as seen in the phylogenetic tree). Fragmentation of a population over geological time, a process that results in genetic divergence, can be distinguished from anthropogenic fragmentation, which acts over a short period of time and leads to the overall loss of genetic diversity (Moya et al. 2004). *Pachysoma gariepinum* has maintained its genetic variation over time indicating that the most probable cause for the structure seen today would be geological fragmentation.

Diamond mining takes place along some 130 km of Namibian coastline north of the Orange River and has been ongoing for just over 100 years. This occurs in the Orange River palaeobeach deposits along the coast as well as from offshore marine deposits. Two of the 3 collecting localities (Daberas to Obib and the Klingharts Mountains) occur within the restricted diamond mining area. Any form of tourism or human movement through the area is prohibited except under strict conditions. Diamond mining in Namibia often results in the complete destruction of a limited area, due to the extensive open cast mining practices used. However, in this case much of the remaining area appears to be undamaged, thus resulting in large areas of relatively natural habitat being undisturbed. South of Lüderitz, the dune fields comprise two types, the permanent dune fields—Hohenfels, Daberas to Obib, and Klingharts dune fields—and the windblown barchan or shifting sand dunes. The barchan dunes which move between one and 100 m per year (Prendini 2001) may historically have linked the permanent dunes which would have allowed for movement of the beetles between systems. This could have resulted in the single lineage in Namibia.

South Africa has a larger human population than Namibia, and not only does *P. gariepinum* occur on farmland but there is extensive mining that occurs along the coast, more so than in Namibia. It is likely therefore that anthropogenic influences here will be greater. North of the Olifants River human habitation is known from approximately 320 years ago, so the overall effect of man, habitat transformation, destructive farming practices, and collection of beetles on *P. gariepinum* is not known and points to a need for monitoring these effects through the collection of species census data over a number of years.

Lineage Age Estimates

The estimation of divergence times has been a topic of considerable debate over the last two decades (for reviews, see Avise 2000; Arbogast et al. 2002; Bromham and Penny 2003, and references therein; Graur and Martin 2004; Pulquério and Nichols 2006). Factors affecting divergence times include the variability of substitution rate across lineages and among loci, multiple substitutions at one site, ancestral polymorphism, change in effective population size over time, evolutionary models selected, and the unpredictable nature of genetic change (Avise 2000; Zheng et al. 2003). By using multiple loci, a decrease in variance of coalescent time and an increase in the accuracy of divergence time could be expected. With this in mind, lineage age estimates are interpreted with caution and are intended only to provide an indication of a time line. The divergence of the South African lineage within *P. gariepinum* probably occurred around 4.7 mya with the individual lineages (Holgat River and Langhoogte/Kommagas)

dating back to 2.28 and 2.22 mya, respectively, and the Namibian lineage dating back to around 5.77 mya. These dates coincide with the late Miocene into the Plio-Pleistocene era. It is well documented that the age of the Namib desert, ~10 mya, is related to the present climatic and oceanic patterns which in turn developed after intensive cooling in the Antarctic (Van Zinderen Bakker 1975). Oceanic patterns have resulted in the formation of cold surface water upwellings (Benguela current), which condense when they come into contact with the warm surface air off the land leading to the formation of fog (Van Zinderen Bakker 1975, 1980; Pickford and Senut 1999). In an area of low and unpredictable rainfall, this fog which can be wind blown up to 100-km inland represents a constant and reliable source of water to the organisms living along the arid west coast, allowing them to inhabit previously inhospitable areas (Sole et al. 2005).

Species Boundaries and Implications for Conservation

It has been suggested that the taxonomy of a group should be consistent with its evolutionary history (Wiley 1981; Frost and Hillis 1990). Every species in nature consists of numerous local populations, which raises the problem of how to treat them taxonomically. Adding dimensions of geography and time poses numerous additional problems (Mayr and Ashlock 1991). The mitochondrial DNA of *P. gariepinum* reveals 3 distinct genetically isolated assemblages reflecting different demographic histories. All 3 assemblages are unique in that they do not share haplotypes and have probably been isolated for more than a million years. They may, therefore, warrant distinct taxonomic status under various species concepts. In principle, the 3 assemblages could be defined as separate species based on the phylogenetic species concept (Nixon and Wheeler 1990) and Templeton's cohesion species concept (Templeton 2001). Because it appears that there has been no recent gene flow and that the time to coalescence of the Namibian and South African populations predates that of the recognized sister-species within this genus (Sole et al. 2005)

Moritz (1994a, 1994b) identified units or targets for conservation by applying the principle of conserving ecological and evolutionary processes in an attempt to conserve biodiversity (Moritz 1999). It may be optimistic to attempt to conserve all localities and lineages of a species, so one would ideally like to target areas that will ensure that a species remains viable and able to survive in the short term and diversify in the long term. Moritz (1994a, 1999) describes these units or targets as evolutionary significant units (ESUs) and management units (MUs). ESUs he defines as having to be reciprocally monophyletic for mtDNA alleles and to have shown significant divergence of allele frequencies at nuclear loci. MUs are recognized as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of phylogenetic distinctiveness of the alleles and are the units used for monitoring and demographic-based studies (Moritz 1994a). According to these definitions, *P. gariepinum* could be described as containing 3 distinct MU. The areas are connected by low levels of historical gene flow but appear functionally independent and would therefore need to be managed as individual entities, forming part of an inclusive species.

Concluding Comments

This study shows that mtDNA can be useful for phylogeographic studies of flightless beetles such as *P. gariepinum* over a limited spatial scale. Added to this by using *P. gariepinum* as a model, broader scale studies on different taxa occurring along the arid gradient of southwest Africa may also be addressed through COI analyses. There are statistically significant associations of haplotypes with geography for the entire cladogram. From these associations and other analyses, range expansion, past fragmentation, and reduced vagility could be inferred as processes possibly responsible for the haplotype distributions. Inferences are congruent with recently documented ecological, geographical, and geological events. However, because this is a single-locus approach evolutionary stochasticity and locus-specific evolutionary forces, such as natural selection, may erode the power of the analyses or be misleading (Templeton 1998), and future studies should include a multilocus approach. Genetic structuring within a limited spatial scale aids in the detection and understanding of pattern and process that in turn provides tools for decision making in a conservation framework. Common patterns and the genetic variation in different species and localities occurring along the southwest African coastline need to be obtained which may help conservation authorities implement conservation plans.

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