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Systematics and phylogeography of a threatened tortoise, the speckled padloper

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Abstract

This study investigated the systematics and phylogeography of a threatened tortoise of South Africa, the speckled padloper *Homopus signatus*. Sixty three specimens were collected from 17 localities that covered the distributional range of the two subspecies in western South Africa and a north-eastern population that was recently discovered near Pofadder. The Pofadder sample could not be assigned to either subspecies based on morphology. The samples were sequenced for two partial mtDNA fragments, nicotinamide adenine dinucleotide dehydrogenase component four and cytochrome *b*, which yielded ~1.1 kb, while a subset of the samples were sequenced for a 390 bp nuclear DNA (nDNA) fragment of prolactin. Phylogenetic analyses of mtDNA using minimum evolution, maximum parsimony and Bayesian inferences supported the monophyly of *H. signatus* and revealed that the Pofadder specimen was basal in the topology and sister to the remainder. The phylogenetic analyses did not support the recognition of two subspecies; there was statistical support for a *Homopus signatus signatus* clade but *Homopus signatus cafer* was not monophyletic. The nDNA analysis showed no difference between the subspecies and placed the Pofadder sample distant but not distinct from *H. s. signatus*. The mtDNA and the nDNA data suggest that the subspecies are invalid taxonomic units. The structure of the mtDNA network corresponded to the geographical distribution of populations. The north-western populations formed one haplocluster, corresponding to *H. s. signatus*, whereas the south-western populations formed three haploclusters, corresponding to *H. s. cafer*. The Pofadder sample was unconnected to the network. The morphology of the northern and southern morphotypes probably reflects selection for crypsis on the different substrate types of the regions, granites and sedimentary rocks, respectively. These results highlight that subspecies designations should be authenticated by molecular techniques because morphological plasticity can obfuscate phylogenetic relationships. We consider the western *H. signatus* populations as one taxonomic unit and recommend wider sampling of the Pofadder locality to clarify the taxonomic status of this lineage.

Introduction

Globally, a large number of chelonian species are considered close to extinction, highly endangered or vulnerable in the wild due to overexploitation, habitat fragmentation and destruction, or as a consequence of illegal trade (Fritz & Havaš, 2007; Ives, Spinks & Shaffer, 2008). The conservation status of terrestrial tortoises (Testudinidae) is particularly dire, with 25 of the 43 extant species (Fritz & Havaš, 2007) listed as Threatened in the 2008 IUCN Red List (http://www.redlist.org/, accessed 24 April 2009). Despite the fact that the Testudinidae constitutes a small group, the taxonomic designation of a large number of tortoises remains uncertain and in flux, hampering effective conservation within the group.

The application of molecular techniques has improved biodiversity estimates of chelonians in recent years. Morphology-based taxonomies can underestimate diversity, as has been shown for cryptic taxa *Emys* (*Emys trinacris*, Fritz *et al.*, 2005*a*; *Emys marmonata*, Spinks & Shaffer, 2005) and *Geochelone nigra* (Russello *et al.*, 2005). Conversely, several molecular studies indicated that morphological evaluations can inflate the taxonomy. Starkey *et al.* (2003) found that *Chrysemys picta* consists of two evolutionary lineages instead of four subspecies, as defined by morphology.

Similarly, Fritz *et al.* (2007) found major discrepancies between morphological and molecular lineages of the *Testudo graeca* complex and concluded that morphological plasticity masks genetic differentiation within this group. Because biodiversity assessments impact species conservation and management, it has become important to use molecular data to clarify species and subspecies boundaries of chelonians (Starkey *et al.*, 2003; Russello *et al.*, 2007).

Southern Africa contains one of the most diverse tortoise faunas globally (Branch, 1998); however, the region's tortoises have not been subjected to molecular systematic studies. Consequently, three dubious subspecies groups are recognized based on morphological variation (i.e. *Homopus signatus signatus* and *Homopus signatus cafer*; *Stigmochelys pardalis pardalis* and *Stigmochelys pardalis babcocki*; *Psammobates tentorius tentorius, Psammobates tentorius verroxii* and *Psammobates tentorius trimeni*) (Branch, 1998). Recently, a systematic study of all southern African tortoise taxa was initiated in order to delineate operational taxonomic units, determine systematic diversity and develop sound conservation management plans for species. In the present study, we investigate the molecular systematics and conservation of the smallest tortoise, the speckled padloper *Homopus signatus*.

The current IUCN listing of *H. signatus* as Lower Risk/Near Threatened (http://www.redlist.org/, accessed 24 April 2009) is based on assessments performed in the 1980s. In a recent conservation assessment of South African reptiles, *H. signatus* was listed as vulnerable due to observed population reductions, a decline in habitat quality and increased levels of exploitation (E. H. W. Baard & M. D. Hofmeyr, pers. comm.). The species has a limited distribution in the arid, western region of South Africa, where it is associated with rocky outcrops and mountain ranges (Branch, 1998; Boycott & Bourquin, 2000). Two subspecies are distinguished: a northern subspecies, *H. s. signatus*, which occurs in succulent Karoo vegetation in Namaqualand and the Richtersveld, and a southern subspecies, *H. s. cafer*, which is found in fynbos vegetation from Piketberg to Lamberts Bay in the west and Clanwilliam in the east (Branch, 1998; Boycott & Bourquin, 2000). Populations on the escarpment in the Calvinia district are believed to represent intergrades between the two subspecies (Boycott, 1986; Boycott & Bourquin, 2000). The north-eastern range of *H. signatus* was recently extended when Branch *et al.* (2007) found an individual near Pofadder in the Northern Cape. The authors refrained from assigning the specimen to either subspecies because its morphology was intermediate between the two subspecies.

Boycott (1986) examined the species' morphology and found that subspecies can be differentiated on colour pattern, marginal scute serration and carapacial sulcation, with specimens from the intergrade zone displaying characteristics of both subspecies. However, the reliability of these distinguishing features is uncertain because male and female *H. s. signatus* differ in colour, and colour as well as scute serration change with body size, or the age of individuals (Loehr, Henen & Hofmeyr, 2006). Several studies on chelonians have indicated discrepancies between morphological taxonomy and molecular lineages (Ives *et al.*, 2008; Stuart & Fritz, 2008), leading us to question the current taxonomy of *H. signatus*.

Recent climate modelling suggests that the range of *H. signatus* will contract and shift by $>50\%$ over the next decades (Erasmus *et al.*, 2002) in response to predicted aridification of the western region of South Africa (Rutherford *et al.*, 1999). Furthermore, an increase in drought frequency may impact the fecundity of *H. signatus*, by prolonging the time that females require to reach sexual maturity and reducing their reproductive output (Loehr, Hofmeyr & Henen, 2007). In light of concerns about the current conservation status of the species and future threats to its existence, it became important to clarify the systematic status of the two *H. signatus* subspecies in order to aid the conservation management of the taxon.

Materials and methods

Sample collection

A total of 63 specimens were collected by hand between 2000 and 2008 from 17 localities that encompass most of the species distribution in the Northern and Western Cape provinces in South Africa (Fig. 1, Table 1). This included 37 *H. s. signatus*, 25 *H. s. cafer* and one indeterminate specimen from Pofadder. The geographic coordinates of each sample were recorded in the field, or in a few instances, estimated from maps. Taking gender, approximate age and geographic location into consideration, we used the colour pattern of the shell (primarily speckle size) and the degree of marginal scute serration to assign each specimen, apart from the specimen from Pofadder, to a particular subspecies. For a detailed list of diagnostic subspecies features, consult Boycott (1986) or Branch (1998). Blood of live tortoises was collected from either the jugular or the subcarapacial vein and stored in liquid nitrogen in the field and at $-20\,^{\circ}C$ in the laboratory, or alternatively, it was diluted with absolute ethanol in the field and stored at $-20\,^{\circ}C$ in the laboratory. All live animals were marked, photographed for a reference collection and released on site. Fresh and dried carcasses collected in the field were biopsied for bone or muscle tissue. These tissue samples were stored directly in absolute ethanol in the field and kept at $-20\,^{\circ}C$ upon return to the laboratory. In total, 10 samples were obtained from bone or dried connective tissue; two from fresh muscle tissue while the remaining 51 samples were blood.

DNA extraction, polymerase chain reaction (PCR) and sequencing

For a detailed outline of DNA extraction, PCR conditions and DNA sequencing protocols, see Daniels *et al.* (2007). Briefly, DNA extraction and PCR from blood and fresh muscle tissue samples were performed in the main laboratory, whereas DNA from dried tissue and bone, collected from carcasses, were extracted in an adjacent laboratory

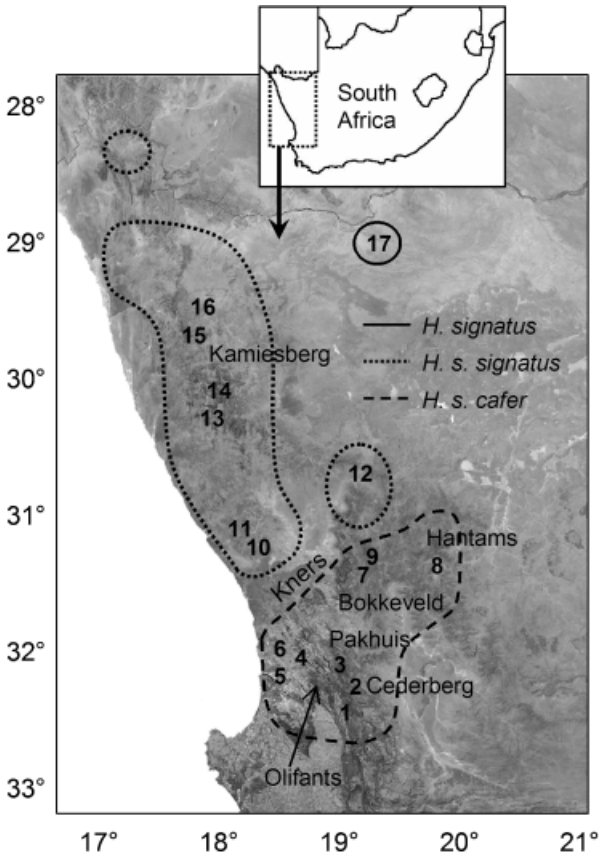

Figure 1 Map of localities sampled for *Homopus signatus* in relation to major mountain ranges and a broad plain, the Knersvlakte (Kners). Distribution ranges for *Homopus signatus signatus* and *Homopus signatus cafer* are according to the South African Reptile Conservation Assessment database. No samples were obtained from the northernmost populations of *H. s. signatus* in the Richtersveld. A recently discovered specimen at Pofadder (17; Branch *et al.*, 2007) could not be assigned to either subspecies according to the morphological criteria of Branch (1998). The numbers of the localities correspond to Table 1: (1) Piekenierskloof Pass, (2) Kriedouwkrans, (3) Clanwilliam, (4) Rietfontein, (5) Wadrif, (6) Bosduifklip, (7) Papkuilsfontein, (8) Calvinia, (9) De Lande, (10) Elandsfontein, (11) Komkans, (12) Loeriesfontein, (13) Kharkams, (14) Arkoep, (15) Kamieskroon, (16) Springbok and (17) Pofadder.

dedicated to ancient DNA research. Dried tissue was cut into small pieces before the tissue and bone were submerged in liquid nitrogen and macerated. The resulting powder was then subjected to standard DNA extraction. We sequenced two mtDNA loci, a 405 bp fragment of cytochrome *b* (cyt *b*) and a 791 bp fragment of nicotinamide adenine dinucleotide dehydrogenase component four (ND4), because these are the two rapidly evolving mtDNA markers that have been used in several systematic studies on tortoises (Austin, Arnold & Bour, 2003; Fritz *et al.*, 2005*b*; Daniels *et al.*, 2007; Ives *et al.*, 2008). The primer pairs tRNA-Glu A and J was used to amply the cyt *b* fragment, the incorrect primer pairs were listed in Daniels *et al.* (2007). We sequenced all samples for cyt *b*, because the amplification of the short fragment from dried samples could be undertaken with ease, whereas all fresh and selected bone samples were sequenced for ND4. Because mitochondria are inherited maternally and mtDNA comprises a single linked locus, we combined the sequence data from the two loci into a single data matrix and performed all analyses on the combined dataset and the haplotypes derived from these results. In addition, we investigated the utility of the nuclear DNA (nDNA) marker prolactin, because it is one of the fastest-evolving nDNA markers described to date. Primer pairs for this locus were obtained from Townsend *et al.* (2008). A subset of the samples representing all mtDNA haplotypes was sequenced for the nDNA marker.

Phylogenetic analyses

Sequence Navigator (Applied Biosystems, Foster City, CA, USA) was used to compute a consensus sequence for both forward and reverse strands. The sequences were aligned in CLUSTAL X (Thompson *et al.*, 1997) using the default parameters of the program. No insertions or deletions were observed in either of the two protein-coding genes. All subsequent analyses were performed on the combined mtDNA. Maximum parsimony (MP), minimum evolution (ME) and Bayesian approaches were used to estimate the relationships among haplotypes. Phylogenetic analyses of the southern African tortoise fauna suggest that *Homopus solus* and *Homopus boulengeri* are sister to *H. signatus* (M. D. Hofmeyr & S. R. Daniels, unpubl. data); hence these two *Homopus* species were used as outgroups. For MP and ME, phylogenetic analyses were executed in PAUP*4 version beta 10 (Swofford, 2002). For the MP analyses, trees were generated using the heuristic search option with tree bisection and reconnection branch swapping using 1000 random taxon additions. Phylogenetic confidence in nodes was estimated by bootstrapping (Felsenstein, 1985), analysing 1000 pseudoreplicates of datasets for MP and ME. Bootstrap values $>75\%$ were considered statistically well supported. Bayesian inferences were used to investigate the optimal tree space using the program MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003) and the models selected from MODELTEST version 3.06 (Posada & Crandall, 1998). For each analysis, four Markov chains were run, with each chain starting from a random tree and run for five million generations, sampling each chain every 10 000th tree. This process was repeated four times for each gene fragment to ensure that all consensus trees converged on the same topology. A 50% majority rule consensus tree was generated from the trees retained (after the burn-in trees were discarded – using likelihood plots) with posterior probabilities ($_pP$) for each node estimated by the percentage of time the node was recovered. Posterior probability values >0.95 $_pP$ were considered statistically well supported. Uncorrected (p) sequence distance values calculated among sample sites were calculated in PAUP.

Table 1 List of sampled localities for *Homopus signatus*

Localities	Subspecies	GPS coordinates		*N*	Tissue type
1) Piekenierskloof Pass	*Homopus signatus cafer*	32.62918 S	18.94535 E	1	Blood
2) Kriedouwkrans	*H. s. cafer*	32.37100 S	18.99033 E	1	Blood
3) Clanwilliam	*H. s. cafer*	32.22071 S	18.92760 E	2	Blood and bone
4) Rietfontein	*H. s. cafer*	32.14411 S	18.52045 E	4	Blood and bone
5) Wadrif	*H. s. cafer*	32.21036 S	18.37919 E	3	Blood
6) Bosduifklip	*H. s. cafer*	32.08660 S	18.35700 E	8	Blood and bone
7) Papkuilsfontein	*H. s. cafer*	31.55983 S	19.19481 E	3	Bone
8) Calvinia	*H. s. cafer*	31.50000 S	19.75000 E	2	Blood
9) De Lande	*H. s. cafer*	31.48434 S	19.20339 E	1	Bone
10) Elandsfontein	*Homopus signatus signatus*	31.29050 S	18.26611 E	2	Blood and bone
11) Komkans	*H. s. signatus*	31.20210 S	18.08157 E	1	Blood
12) Loeriesfontein	*H. s. signatus*	30.91444 S	19.07444 E	2	Muscle tissue
13) Kharkams	*H. s. signatus*	30.35222 S	17.88500 E	5	Blood
14) Arkoep	*H. s. signatus*	30.14948 S	17.93128 E	1	Blood
15) Kamieskroon	*H. s. signatus*	29.83978 S	17.85251 E	2	Blood
16) Springbok	*H. s. signatus*	29.69166 S	17.88333 E	24	Blood
17) Pofadder	*Homopus signatus*	29.08083 S	19.41388 E	1	Muscle tissue

N represents the number of samples collected at each locality. The numbers next to the localities correspond to the locality numbers on the map (Fig. 1). Subspecies were recognized on the morphological criteria and distribution contained in Branch (1998).

Population genetic analyses

A haplotype network was constructed using TCS (Clement, Posada & Crandall, 2000) with a 95% parsimony probability for the combined mtDNA loci, and the nDNA. We performed an analysis of molecular variation (AMOVA) on the data using ARLEQUIN version 3.0 (Excoffier, Laval & Schneider, 2005) to calculate population differentiation over all localities (Φ_{ST}) and to calculate the degree of genetic variation between the two subspecies for the mtDNA. Permutation procedures used 10 000 randomizations to test for significance. Deviations in neutrality were investigated with Tajima's *D*-test (Tajima, 1989) using ARLEQUIN version 3.0 for each of the two subspecies.

Results

Combined mtDNA topology

The combined mtDNA comprised 405 bp of cyt *b*, 791 bp of ND4, yielding a total of 1196 bp for 63 samples. For the partial cyt *b* fragment, the substitution model selected using the Aikake information criterion (AIC) (Akaike, 1973) criteria was TrN + I ($-\ln L = 834.16$, AIC = 1678.33). The base frequencies were A = 32.27%, C = 25.92%, G = 12.22% and T = 29.14%, the rate matrix was R(a) [A–C] = R(c) [A–T] = R(d) [C–G] = R(f) [G–T] = 1.00, R(b) [A–G] = 11.30 and R(e) [C–T] = 21.91, while the proportion of invariable sites (I) was 0.65. The substitution model selected using the AIC criteria for the partial ND4 fragment was TrN + I ($-\ln L = 1880.69$, AIC = 3771.38). The base frequencies were A = 27.99%, C = 12.28%, G = 24.01% and T = 35.71%, the rate matrix was R(a) [A–C] = R(c) [A–T] = R(d) [C–G] = R(f) [G–T] = 1.00, R(b) [A–G] = 10.45 and R(e) [C–T] = 12.50, while the proportion of invariable sites (I) was 0.56. Both these protein-coding gene regions were A and T rich, exhibiting a pattern typical of protein-coding mtDNA loci (Daniels *et al.*, 2007; Ives *et al.*, 2008; Stuart & Fritz, 2008). The two substitution models were used during the partition analyses of the combined mtDNA fragment in the Bayesian analysis. All phylogenetic analyses (MP, ME and Bayesian inference) produced congruent tree topologies; hence, we only show the Bayesian topology. For MP, we retrieved a total of 227 trees, with a CI = 0.89 and RI = 0.91 from 72 parsimony informative characteristics. The analyses provided strong statistical support (100%/1.00 $_pP$) for the monophyly of *H. signatus*. The tree topologies for ingroup samples were characterized by short internal branches and statistically poorly supported nodes (<75%/<0.95 $_pP$) (Fig. 2). The *H. signatus* sample from Pofadder was basal in the topology and sister to all other haplotypes. Samples of *H. s. signatus* were present in a single, statistically well-supported clade (>60%/1.00 $_pP$) and contained individuals from Elandsfontein, Springbok, Arkoep, Kamieskroon, Kharkams, Loeriesfontein and Komkans. *H. s. cafer* samples were not monophyletic, and occurred in a basal polytomy with poor statistical support (<75%/<0.95 $_pP$). The basal group comprised samples from Papkuilsfontein, Bosduifklip, Rietfontein, Clanwilliam and Wadrif, whereas the second group comprised samples from Papkuilsfontein, Calvinia and De Lande, sister to samples from Kriedouwkrans, Clanwilliam and Piekenierskloof Pass. The mean uncorrected sequence divergence between the Pofadder sample and the two *H. signatus* subspecies ranged from 1.77 to 2.40% for ND4, while for cyt *b* it ranged from 1.97 to 2.46%. Similarly, the mean uncorrected sequence divergence between the two subspecies ranged from 0.88 to 1.51% for ND4, and from 0.49 to 0.74% for cyt *b*.

TCS collapsed the 63 sequences into a network that contained 20 haplotypes (Fig. 3, Table 2). These haplotypes have been

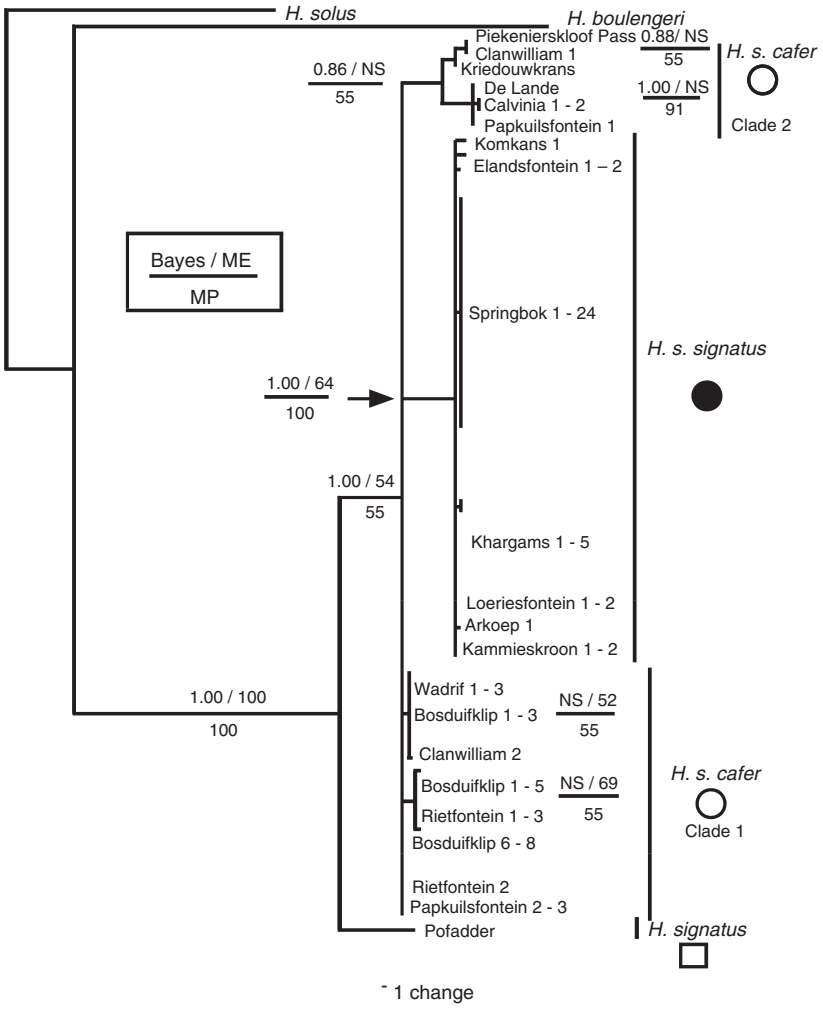

Figure 2 A Bayesian topology derived from the partitioned analyses of the combined mtDNA data for cytochrome *b* and nicotinamide adenine dinucleotide dehydrogenase component four of 63 *Homopus signatus* specimens. Posterior probability ($_pP$) values for Bayesian analyses and bootstrap (%) values for minimum evolution (ME) shown above the nodes. Bootstrap values for maximum parsimony (MP) are shown below the nodes. The black circles (●) represent *H. s. signatus* localities, open circles (○) represent *H. s. cafer* and an open square (□) represents Pofadder. NS indicates nodes that were not supported. Numbers after the site names represent specimen numbers.

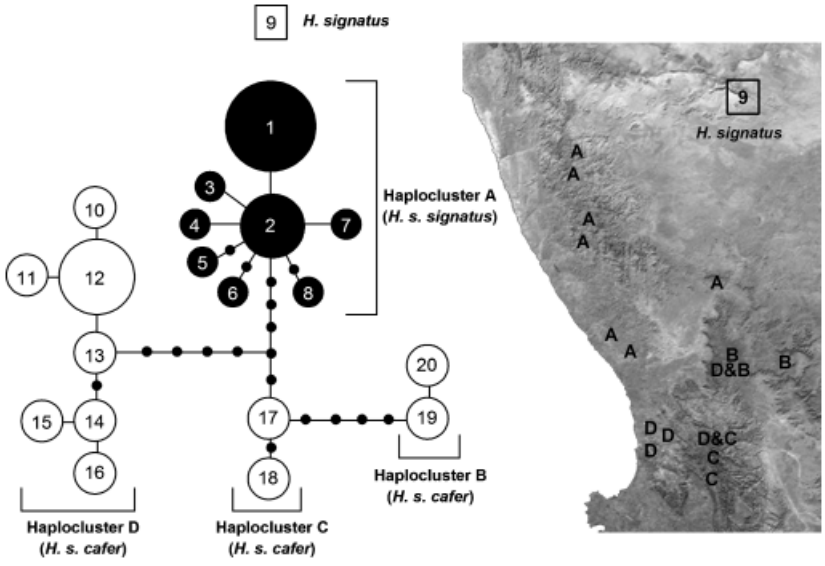

Figure 3 Haplotype network for the combined mtDNA data of *H. s. signatus* with the geographic localities of haploclusters A, B, C and D shown on the map. Black circles (●) represent *H. signatus* (haplotypes 1–8), open circles (○) represent *H. s. cafer* (haplotypes 10–20) and an open square (□) represents Pofadder (haplotype 9). Numbers inside each circle correspond to the haplotypes, while the size of the circle is an indication of haplotype frequency. Consult Table 2 for details of the haplotypes' geographic distribution.

Table 2 List of the haplotype frequencies for each sampled locality of *Homopus signatus*

Haplotype																					
Localities	Subspecies	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Springbok	*H. s. signatus*	17	5					2													
Kharkams	*H. s. signatus*		5																		
Kamieskroon	*H. s. signatus*		2																		
Elandsfontein	*H. s. signatus*			1			1														
Arkoep	*H. s. signatus*				1																
Komkans	*H. s. signatus*					1															
Loeriesfontein	*H. s. signatus*					1			1												
Pofadder	*Homopus signatus*									1											
Bosduifklip	*H. s. cafer*										1		4	1	1	1					
Clanwilliam	*H. s. cafer*											1							1		
Wadrif	*H. s. cafer*												3								
Papkuilsfontein	*H. s. cafer*												1	1						1	
Rietfontein	*H. s. cafer*													1	2		1				
Kriedouwkrans	*H. s. cafer*																	1			
Piekenierskloof Pass	*H. s. cafer*																		1		
De Lande	*H. s. cafer*																			1	
Calvinia	*H. s. cafer*																				2

Haplotypes 1–8 are present among the *H. s. signatus* localities, while the remaining haplotypes, except haplotype 9, are present *in H. s. cafer*.

Table 3 Diversity measures for *Homopus signatus*

Locality	Subspecies	*N*	N_h	N_p	*h*	π_n
Piekenierskloof Pass	*H. s. cafer*	1	1	N/A	N/A	N/A
Kriedouwkrans	*H. s. cafer*	1	1	N/A	N/A	N/A
Clanwilliam	*H. s. cafer*	2	2	2	1.0000 ± 0.50000	0.010033 ± 0.010443
Wadrif	*H. s. cafer*	3	1	N/A	N/A	N/A
Rietfontein	*H. s. cafer*	4	3	3	0.8333 ± 0.2224	0.001254 ± 0.001119
Bosduifklip	*H. s. cafer*	8	5	5	0.7857 ± 0.1508	0.001583 ± 0.001146
Papkuilsfontein	*H. s. cafer*	3	3	12	1.0000 ± 0.2722	0.006689 ± 0.005347
Calvinia	*H. s. cafer*	2	1	N/A	N/A	N/A
De Lande	*H. s. cafer*	1	1	N/A	N/A	N/A
Elandsfontein	*H. s. signatus*	2	2	3	1.0000 ± 0.50000	0.002508 ± 0.002896
Komkans	*H. s. signatus*	1	1	N/A	N/A	N/A
Loeriesfontein	*H. s. signatus*	2	2	4	1.0000 ± 0.50000	0.003344 ± 0.003739
Kharkams	*H. s. signatus*	5	1	N/A	N/A	N/A
Arkoep	*H. s. signatus*	1	1	N/A	N/A	N/A
Kamieskroon	*H. s. signatus*	2	1	N/A	N/A	N/A
Springbok	*H. s. signatus*	24	3	2	0.4674 ± 0.1025	0.000421 ± 0.000413
Pofadder	*Homopus signatus*	1	1	N/A	N/A	N/A

N, the sample size; N_h, the number of haplotypes; *Np*, the number of polymorphic sites; *h*, the haplotype diversity; π_n, the nucleotide diversity; N/A, not applicable.

deposited in GenBank. The accession numbers for cyt *b* is GU 139194-GU 139213, while for ND4 it is GU 139214-Gu 139233.

The haplotype network revealed that *H. s. signatus* was separated from *H. s. cafer* by a maximum of eight mutational steps (Fig. 3). The Pofadder sample could not be connected to the main network at either 95 or 90% confidence. *H. s. signatus* exhibited little genetic variation (Table 3); eight haplotypes (haplotypes 1–8 – Table 2) were separated by single mutation steps. In contrast, *H. s. cafer* possessed 11 haplotypes (haplotypes 10–20), greater genetic variation than within *H. s. signatus* (Table 3), and was comprised of three haploclusters. Haplocluster D (haplotypes 10–16) comprised samples from the south-western region, from Bosduifklip, Clanwilliam, Papkuilsfontein, Rietfontein and Wadrif, and was separated by six mutation steps from haplocluster C (Fig. 3). Haplocluster C (haplotypes 17 and 18) contained the southernmost samples (Clanwilliam, Kriedouwkrans and Piekenierskloof Pass), and was separated by four mutation steps from haplocluster B (haplotypes 19 and 20). The latter contained samples from localities further inland

on the escarpment, at Papkuilsfontein, De Lande and Calvinia. These localities have been described by Boycott (1986) as an intergradation zone of the two subspecies.

AMOVA results over all sampled localities revealed that 80.44% ($\Phi_{ST} = 0.8219$, d.f. = 15, Va = 2.87%, $P < 0.001$) of the variation occurred among localities, and 19.56% of the variation occurred within localities (d.f. = 46, Vb = 0.69, $P < 0.001$). When we compared the degree of genetic differentiation between the two *H. signatus* subspecies (excluding the sample from Pofadder), we detected 57.10% of the variation between the two groups (d.f. = 1, Va = 2.65, $P < 0.001$), 27.88% of the variation among populations within groups (d.f. = 30, Vb = 0.398, $P < 0.001$) and 15.01% of the variation within populations (d.f. = 46, Vc = 0.69, $P < 0.001$). For *H. s. signatus*, the mean Tajima's *D* was −0.018, while for *H. s. cafer*, the mean Tajima's *D* was −0.093. The negative Tajima's *D* values suggest either population size expansion or positive selection.

nDNA (figure not shown)

A 520 bp fragment of the prolactin locus was amplified for 14 of the haplotypes identified in the mtDNA network. Haplotypes have been submitted to GenBank (accession numbers are GU 139234-GU 139237). Ambiguities in the first 60 bp and the last 70 bp were deleted and a 390 bp fragment was used in the analyses. A TCS network of the 14 sequences revealed four haplotypes. Haplotype 1 (f = 9) was present in samples from Springbok, Loeriesfontein, Wadrif, Bosduifklip, Kriedouwkrans and Piekenierskloof Pass, haplotype 2 (f = 2) comprised samples from Elandsfontein, Komkans and Calvinia, haplotype 3 comprised of a single sample from Springbok and haplotype 4 contained the Pofadder sample and one sample from Springbok (Fig. 1). The latter haplotype was one unsampled mutation apart from the central haplotype. These results suggest that no nDNA genetic differences exist between the two subspecies.

Discussion

Our molecular analysis indicated the monophyly of *H. signatus*, with the Pofadder specimen being basal in the topology and sister to the remaining *H. signatus* populations. The morphotype of the Pofadder tortoise could not be assigned to either of the two subspecies (Branch *et al.*, 2007), further distinguishing this lineage from the remainder. All other populations could be assigned to either *H. s. signatus* or *H. s. cafer* subspecies, although there was substantial morphological variation within regions, as has been shown by Loehr *et al.* (2006) for *H. s. signatus* near Springbok. The molecular data, however, did not support the division of *H. signatus* into two subspecies; *H. s. cafer* was not monophyletic and consisted of three haploclusters, with *H. s. signatus* nested within this group but monophyletic.

The division of *H. signatus* into two subspecies is based on morphology, but the rates of molecular and morphological change do not necessarily correspond (Bromham *et al.*, 2002), and genetic bottlenecks or environmental pressure can alter the morphology of tortoises within a relatively short period (Fritz *et al.*, 2005*b*). Carapace morphology in tortoises often exhibits large intraspecific variation that may be influenced by diet, physical environmental conditions (such as the slope and the elevation of the habitat), pathology and age (Austin *et al.*, 2003; Fritz *et al.*, 2005*b*; Ives *et al.*, 2008). Colour variation of the dorsal body surface has often been interpreted as an adaptation for crypsis (Rosenblum, 2005), and experimental studies have shown that substrate matching strongly influences avian predation rates (Kaufman, 1974; Hoekstra, 2006). Colour polymorphism in reptiles may be caused by genetic variability, for example, variation in the *Mc1r* gene (Rosenblum, Hoekstra & Nachman, 2004), or may be a consequence of an ontogenetic change in response to environmental stimuli (Dunn, 1982; Rosenblum, 2005). Fritz *et al.* (2007) found major inconsistencies between morphological taxa and mtDNA clades of the *T. graeca* complex and reported a strong correlation between substrate and tortoise colour in some regions.

Homopus signatus is rupicolous and the shell colour patterns may relate to camouflage on different rock substrates over the species' range. Intrusive igneous rock underlies the north-western region of South Africa (McCarthy & Rubidge, 2005), where the coarse speckled pattern of *H. s. signatus* blends well with the pattern of granite–gneiss rock slabs and gravel (Fig. 4a and b). Granite–gneisses also underlie the substrate at Pofadder (McCarthy & Rubidge, 2005), but too little is known about the ecology of this population to make inferences about its association with a particular substrate. South and east of the Knersvlakte and Olifants River, the substrate changes to sedimentary rocks (McCarthy & Rubidge, 2005). The fine speckled pattern of *H. s. cafer* populations provides an excellent camouflage against sandstone or shale substrates (Fig. 4c and d). It seems likely that improved camouflage, particularly against avian predators, provided a strong selective force for the divergent colour patterns of *H. signatus* morphotypes. South African tortoises are known to face predation from a wide range of avian predators (Branch, 2008).

The mtDNA haplotype network revealed a close correspondence between haplotypic distribution and geography (see Fig. 3). The Pofadder sample is geographically isolated from the main distribution of *H. signatus* by the vast plains of Bushmanland, with no known populations connecting them. This geographic separation is clearly reflected in the mtDNA structure and to a limited extent in the nDNA structure. The four haploclusters of the network correspond to different geographical regions. Haplocluster A (*H. s. signatus*) is associated with rocky outcrops and mountain ranges of the north-west and extends along the Kamiesberg Mountain to the furthest point east, at Loeriesfontein. Haplocluster D is present on rocky outcrops along the south-western coast and extends eastwards along the Olifants River Mountains towards Clanwilliam and northwards along the Pakhuis Mountains onto the escarpment and Bokkeveld Mountains at Nieuwoudtville (see Figs 1 and 3). Haplocluster C occurs in the south-east, in the southern

Figure 4 Colour variants of *Homopus signatus* with two *Homopus signatus signatus* morphs, respectively, from Springbok (a) and Kamieskroon (b) in the north-west and two *H. s. cafer* morphs (c, d) from Bosduifklip in the south-west. The morphotypes were distinguished mainly by speckle size on the shell, which camouflage them on granite and sandstone/shale substrates, respectively, north and south of the Knersvlakte.

Olifants River Mountains and mountains of the Cederberg, while haplocluster B is found only on the escarpment, east of the Bokkeveld Mountains towards the Hantams Mountain range. We observed sympatric haplotypes (11 and 18) that belonged to two distinct haploclusters (D and C) in the Pakhuis Mountains near Clanwilliam, suggesting a potential refugial area or recent range expansions of either or both haploclusters (Figs 1 and 3). The Cederberg Mountain range has been suggested to be a refugial area for other reptile taxa, including angulate tortoises, *Chersina angulata* (Daniels *et al*., 2007), and the fossorial skink *Acontias meleagris meleagris* (Daniels, Heideman & Hendricks, 2009). There were also sympatric haplotypes (12, 13 and 19) of haploclusters D and B in the Bokkeveld Mountains south of Nieuwoudtville. It appears that the range of haplocluster D expanded considerably towards the east. The relatively high genetic divergence in *H. s. cafer* is probably attributable to the topographically heterogeneous nature of the southern distribution range, where populations are isolated on mountain ranges and separated by low-lying valleys that appear to restrict gene flow among allopatric populations.

The question now arises as to which biogeographic factors could have sculpted the genetic divergence within *H. signatus*. The Knersvlakte and Olifants River drainage currently separate the subspecies, but these phylogeographic breaks date back to the Miocene and the lower Cretaceous/Tertiary period, respectively (Dingle & Hendey, 1984; Moon & Dardis, 1988). The relatively small genetic distances among haploclusters (0.5–1.3%), and between them and the Pofadder sample (1.8–2.7%), indicate that *H. signatus* diverged more recent than in the Miocene, if their mtDNA mutation rate is similar to that of other chelonians (*c*. 0.5% per million years; Caccone *et al*., 2002). Co-distributed rock-dwelling vertebrate taxa such as the agamid lizard *Agama atra* (Matthee & Flemming, 2002), two gecko genera (*Goggia* and *Pachydactylus* – Bauer, 1999; Lamb & Bauer, 2000) and the elephant shrew species *Elephantulus edwardii* (Smit, Robinson & van Vuuren, 2007) all exhibit a phylogeographic break in the same region. It is likely that recurrent wet–warm and dry–cold cycles since the late Pliocene (Partridge, 1997) influenced the genetic structure of rupicolous species from this region.

Subspecies designations within *H. signatus* do not correspond with the mtDNA or nDNA patterns. There is no subspecies designation for the distinct Pofadder population and *H. s. cafer* is not a valid taxon. If *H. s. signatus* is recognized as a valid subspecies, the three other haploclusters should receive equal recognition, which would result in

unwarranted taxonomic inflation. There are strong indications that selection for crypsis on two different substrate types explains the existence of two morphotypes in the western region. These morphotypes do not reflect the genetic differentiation within the species and we conclude that the western populations of *H. signatus* form one taxonomic unit. We thus recommend that the current subspecies designations be abandoned, but that more samples should be collected from the north-eastern population (Pofadder) to clarify the taxonomic status of this population.

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References

Akaike, H. (1973). Information theory as an extension of maximum likelihood principle. In *Second international symposium on information theory*: 267–281. Petrov, B.N. & Csake, F. (Eds). Budapest: Akademiai Kaido.

Austin, J.J., Arnold, E.N. & Bour, R. (2003). Was there a second adaptive radiation of giant tortoises in the Indian Ocean? Using mitochondrial DNA to investigate speciation and biogeography of *Aldabrachelys* (Reptilia, Testudinidae). *Mol. Ecol.* **12**, 1415–1424.

Bauer, A.M. (1999). Evolutionary scenarios in the *Pachydactylus* group geckos of southern Africa: new hypotheses. *Afr. J. Herpetol.* **48**, 53–62.

Boycott, R. (1986). A review of *Homopus signatus* (Schoepff) with notes on related species (Cryptodira: Testudinidae). *J. Herpetol. Assoc. Afr.* **32**, 10–14.

Boycott, R.C. & Bourquin, O. (2000). *The southern African tortoise book. A guide to southern African tortoises, terrapins and turtles*. Revised expanded edition. Pietermaritzburg: Interpak, 228pp.

Branch, W.R. (1998). *Field guide to the snakes and other reptiles of southern Africa*. Cape Town: Struik Publishers.

Branch, W.R. (2008). *Tortoises, terrapins & turtles of Africa*. Cape Town: Struik Publishers.

Branch, W.R., Bauer, A.M., Jackman, T. & Marias, J. (2007). Geographic distribution, Testudinidae, *Homopus signatus*, speckled padloper. *Afri. Herp. News.* **43**, 26–27.

Bromham, L., Woolfit, M., Lee, M.S.Y. & Rambaut, A. (2002). Testing the relationship between morphological and molecular rates of change along phylogenies. *Evolution* **56**, 1921–1930.

Caccone, A., Gentile, G., Gibbs, J.P., Fritts, T.H., Snell, H.L. & Powell, J.R. (2002). Phylogeography and history of Galápagos tortoises. *Evolution* **56**, 2052–2066.

Clement, M.D., Posada, D. & Crandall, K.A. (2000). TCS. A computer program to estimate gene genealogies. *Mol. Ecol.* **9**, 1657–1660.

Daniels, S.R., Heideman, N.J.L. & Hendricks, M.G.J. (2009). Examination of evolutionary relationships in the Cape fossorial skink species complex (Acontinae: *Acontias meleagris meleagris*) reveals the presence of five cryptic lineages. *Zool. Scr.* **38**, 449–463.

Daniels, S.R., Hofmeyr, M.D., Henen, B.T. & Crandall, K.A. (2007). Living with the genetic signature of Miocene induced change: evidence from the phylogeographic structure of the endemic angulate tortoise *Chersina angulata*. *Mol. Phylogenet. Evol.* **45**, 915–926.

Dingle, R.V. & Hendey, Q.B. (1984). Late Mesozoic and Tertiary sediment supply to the eastern Cape basin (SA Atlantic) and palaeo-drainage systems in southwestern Africa. *Mar. Geol.* **56**, 13–16.

Dunn, R.W. (1982). Effects of environment upon tortoise pigmentation. *Aust. Zool.* **21**, 105–107.

Erasmus, B.F.N., Van Jaarsveld, A.S., Chown, S.L., Kshatriya, M. & Wessels, K.J. (2002). Vulnerability of South African animal taxa to climate change. *Glob. Change Biol.* **8**, 679–693.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin ver 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinformatics* **1**, 47–50.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fritz, U., Fattizzo, T., Guicking, D., Tripepi, S., Pennisi, M.G., Lenk, P., Joger, U. & Wink, M. (2005). A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys* (Reptilia, Testudines, Emydidae). *Zool. Scr.* **34**, 351–371.

Fritz, U. & Havaš, P. (2007). Checklist of chelonians of the world. *Vertebr. Zool.* **57**, 148–368.

Fritz, U., Hundsdörfer, A.K., Široký, P., Auer, M., Kami, H., Lehmann, J., Mazanaeva, L.F., Türkozan, O. & Wink, M. (2007). Phenotypic plasticity leads to incongruence between morphology-based taxonomy and genetic differentiation in western Palaearctic tortoises (*Testudo graeca* complex; Testudines, Testudinidae). *Amphibia-Reptilia* **28**, 97–121.

Fritz, U., Siroky, P., Kami, H. & Wink, M. (2005*b*). Environmentally caused dwarfism or a valid species – is *Testudo weissingeri* Bour, 1996 a distinct evolutionary lineage? New evidence from mitochondrial and nuclear genomic markers. *Mol. Phylogenet. Evol.* **37**, 389–401.

Hoekstra, H.E. (2006). Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* **97**, 222–234.

Ives, I., Spinks, P.Q. & Shaffer, H.B. (2008). Morphological and genetic variation in the endangered Sulawesi tortoise *Indotestudo forstenii*: evidence of distinct lineages? *Conserv. Genet*. **9**, 709–713.

Kaufman, D.W. (1974). Adaptive coloration in *Peromyscus polionotus*: experimental selection by owls. *J. Mammal*. **55**, 271–283.

Lamb, T. & Bauer, A.M. (2000). Relationships of the *Pachydactylus rugosus* group of geckos (Reptilia: Squamata: Gekkonidae). *Afr. Zool*. **35**, 55–67.

Loehr, V.J., Hofmeyr, M.D. & Henen, B. (2007). Growth and shrinking in the smallest tortoise, *Homopus signatus signatus*. *Oecologia* **153**, 479–488.

Loehr, V.J.T., Henen, B.T. & Hofmeyr, M.D. (2006). Shell characteristics and sexual dimorphism in the Namaqualand speckled padloper, *Homopus signatus signatus*. *Afr. J. Herpetol*. **55**, 1–11.

Matthee, C.A. & Flemming, A.F. (2002). Population fragmentation in the southern rock agama, *Agama atra*: more evidence for vicariance in southern Africa. *Mol. Ecol*. **11**, 465–471.

McCarthy, T. & Rubidge, B. (2005). *The story of earth and life: a South African perspective*. Cape Town: Struik Publishers.

Moon, B.P. & Dardis, G.F. (1988). *The geomorphology of southern Africa*. Cape Town: CTP Book Printers.

Partridge, T.C. (1997). Evolution of landscapes. In: *Vegetation in southern Africa:* 5–20. Cowling, R.M. Richardson, D.M. & Pierce, S.M. (Eds). Cambridge: Cambridge University Press.

Posada, D. & Crandall, K.A. (1998). *MODELTEST*: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Ronquist, F. & Huelsenbeck, J.P. (2003). *MrBayes 3*: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Rosenblum, E.B. (2005). The role of phenotypic plasticity in colour variation of Tularosa basin lizards. *Copeia* **2005**, 586–596.

Rosenblum, E.B., Hoekstra, H.E. & Nachman, M.W. (2004). Adaptive reptile colour variation and the evolution of the *MC1R* gene. *Evolution* **58**, 1794–1808.

Russello, M.A., Glaberman, S., Gibbs, J.P., Marquez, C., Powell, J.F. & Caccone, A. (2005). A cryptic taxon of Galápagos tortoise in conservation peril. *Biol. Lett*. **1**, 287–290.

Russello, M.A., Hyseni, C., Gibbs, J.P., Cruz, S., Marquez, C., Tapia, W., Velensky, P., Powell, J.R. & Caccone, A. (2007). Lineage identification of Galápagos tortoises in captivity worldwide. *Anim. Conserv*. **10**, 304–311.

Rutherford, M.C., Midgley, G.F., Bond, W.J., Powrie, L.W., Roberts, R. & Allsopp, J. (1999). *Plant biodiversity: vulnerability and adaptation assessment*. *South African Country study on climate change*. Claremont: National Botanical Institute.

Smit, H.A., Robinson, T.J. & Van Vuuren, B.J. (2007). Coalescence methods reveal the impact of vicariance on the spatial genetic structure of *Elephantulus edwardii* (Afrotheria, Macroscelidae). *Mol. Ecol*. **16**, 2680–2692.

Spinks, P.Q. & Shaffer, B. (2005). Range-wide molecular analysis of the western pond turtle (*Emys marmorata*): cryptic variation, isolation by distance, and their conservation implications. *Mol. Ecol*. **14**, 2047–2064.

Starkey, D.E., Shaffer, H.B., Burke, R.L., Forstner, M.R.J., Iverson, J.B., Janzen, F.J., Rhodin, A.G.J. & Ultsch, G.R. (2003). Molecular systematics, phylogeography, and the effects of Pleistocene glaciation in the painted turtle (*Chrysemys picta*) complex. *Evolution* **57**, 119–128.

Stuart, B.L. & Fritz, U. (2008). Historical DNA from museum type specimens clarifies diversity of Asian leaf turtles (*Cyclemys*). *Biol. J. Linn. Soc*. **94**, 131–141.

Swofford, D.L. (2002). *PAUP* phylogenetic analysis using parsimony (and other methods), version 4.10*. Champaign: Illinois Natural History Survey.

Tajima, D. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. **24**, 4876–4882.

Townsend, T.M., Alegre, R.E., Kelly, S.T., Wiens, J.J. & Reeder, T.W. (2008). Rapid development of multiple nuclear loci for phylogenetic analysis using genomic resources: an example from squamate reptiles. *Mol. Phylogenet. Evol*. **47**, 129–142.