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Living with the genetic signature of Miocene induced change: Evidence from the phylogeographic structure of the endemic angulate tortoise *Chersina angulata*

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Abstract

The phylogeographic structure of the monotypic endemic southern African angulate tortoise *Chersina angulata* was investigated throughout its distribution with the use of partial sequences from three mtDNA loci (COI, cyt *b* and ND4). Phylogeographic and phylogenetic structuring obtained for the three mtDNA markers were highly congruent and suggested the presence of two genetically distinct, reciprocally monophyletic evolutionary lineages. Group one contained two subclades with haplotypes from the north-western Cape and south-western Cape, respectively, while haplotypes from the southern Cape comprised group two. The two major clades were separated by nine and eight mutational steps for COI and ND4, respectively. Of the three mtDNA gene regions examined, the ND4 partial sequence contained the most phylogenetic signal. Haplotype diversity was generally low and we recovered 34 haplotypes for the 125 animals sequenced for the ND4 subunit. Nested clade analyses performed on the variable ND4 partial sequences suggested the presence of two major refugial areas for this species. The demographic history of the taxon was characterised by range expansion and prolonged historical fragmentation. Divergence time estimates suggest that the temporal and spatial distribution of the taxon was sculpted by changes in temperature and rainfall patterns since the late Miocene. Corroborative evidence from other reptiles is also suggestive of a late Miocene divergence, indicating that this was a major epoch for cladogenesis in southern Africa. Apart from the genetic differences between the two major clades, we also note morphometric and behavioural differences, alluding to the presence of two putative taxa nested within *C. angulata*.

Keywords: mtDNA loci; Conservation; Nested clade analyses

1. Introduction

Global biodiversity is generally sculpted by the interplay of spatial and temporal isolation of taxa allowing for the slow or rapid accumulation of genetic distinctiveness, genetic isolation and speciation. Changes in paleoclimate influenced the distribution and evolution of species (Hewitt, 2000; DeMenocal, 2004) while geographical heterogeneity probably contributed to the high biodiversity of some regions (Hewitt, 2000; Goldblatt and Manning, 2002). South Africa has two biodiversity hotspots, the Cape Floristic Region and Succulent Karoo (Myers et al., 2000). Some floral diversity of these hotspots dates back to the Oligocene, but most modern lineages appear to have diversified in response to progressive aridification that occurred during the late Miocene and the Pliocene–Pleistocene (Midgley et al., 2001; Richardson et al., 2001; Klak et al., 2004; Linder, 2005). While late Neogene cli-

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mate change apparently provided the ideal setting for cladogenesis within floral assemblages, processes of divergence among the vertebrate fauna remain poorly understood. The reptiles of southern Africa contain marked levels of alpha diversity, with recent molecular systematic studies on the group suggesting a wealth of hidden diversity (e.g., Bauer and Lamb, 2003; Daniels et al., 2004, 2006a; Tilbury et al., 2006). Reptiles thus represent an ideal taxon with which to examine biogeographic patterning in the subcontinent.

Southern Africa harbours the highest diversity of terrestrial tortoises globally, with five genera (*Chersina*, *Stigmochelys*, *Homopus*, *Kinixys* and *Psammobates*) comprised of 14 described species, of which 12 species are endemic (Branch et al., 1995; Branch, 1998). Some taxonomic affinities remain unresolved, causing uncertainty of the chelonian diversity of the region. With the exception of the geometric tortoise, *Psammobates geometricus* (IUCN—Endangered, CITES Appendix 1), the remaining southern African tortoises (CITES Appendix 2) have received limited conservation attention. Consequently, little is known about the genealogical affinities and demographic history of most southern Africa tortoise species, severely impeding effective conservation and management.

The angulate tortoise *Chersina angulata* is a highly abundant, monotypic and medium-sized southern African endemic (Branch, 1998). This taxon has a broad, virtually contiguous contemporary coastal distribution extending from southern Namibia, along the west and south coasts of South Africa into the eastern Cape, with isolated populations in the interior (Branch, 1998). Few populations are found beyond the Great Escarpment, which separates the coastal margin (50–200 km wide) from the elevated inland plateau. Their range spans the Succulent Karoo and Cape Floristic Region, where the climate changes from arid, with winter rainfall in the west, to mesic, with all-year rainfall in the southeast. The distribution of *C. angulata* covers a geologically complex and heterogeneous landscape dissected along the western and southern coastal regions by the ancient Cape Fold Mountain Range and six perennial drainage systems.

Paleontological evidence shows that *Chersina* has been present at Langebaanweg in the south-western Cape in the early Pliocene (4.5 Myr; Meylan and Auffenberg, 1986; Lapparent de Broin, 2000) but the extent of its distribution and age of the taxon are unknown. *Chersina* appears to be older than the fossil record indicates because *Chersina* diverged from its sister group, *Chersobius* (*Homopus boulengeri* and *H. signatus*), approximately 18 Myr ago (Cunningham, 2002). The climate and landscape of southern Africa changed substantially during the Miocene–Pliocene epochs. Warm and humid conditions, which supported woodland and forest vegetation, characterised the early Miocene. Approximately 14 Myr ago, the expansion of the Antarctic ice sheet introduced a new cold and arid phase. Warm-mesic conditions returned in the early Pliocene, after which the high-latitude glaciation cycles caused repeated fluctuations between cold-arid and warm-mesic conditions (Partridge, 1997; Scott et al., 1997). These climatic cycles caused marine regressions and transgressions, with sea levels up to 110 m higher and 130 m lower during warm and cold periods, respectively (Partridge, 1997). Epeirogenic uplift, which occurred in the early Miocene and again in the early Pliocene, raised the interior plateau of southern Africa substantially, particularly in the south-east. The uplift reduced rainfall in the interior and the west, while the development of the cold Benguela Current along the west coast intensified the east to west aridity gradient and established rainfall seasonality (Partridge, 1997; McCarthy and Rubidge, 2005). In response to these climatic changes, fynbos vegetation became established in the south-west and south, and a succulent shrubland developed in the north-west (Scott et al., 1997). It is likely that the climate and landscape changes of the Miocene–Pliocene caused significant population contractions to refugia during adverse periods, followed by population expansions when favourable periods returned, the signature of which may be present in the genetic structure of *C. angulata*.

The life histories of tortoises are particularly conducive to evaluate the importance of past geological and climate events on vertebrate phylogeography and evolution (Caccone et al., 2002; Austin et al., 2003; Lesia et al., 2003; Beheregary et al., 2004). Angulate tortoises seem to prefer coastal sandy soil and associated vegetation cover, and have small home ranges (ca 2 ha; Branch, 1998), indicating a limited dispersal potential. Female *C. angulata* require a long time to mature (9–12 years; Branch, 1984) and have a relatively low fecundity (one egg at a time, with a clutch frequency of one to six per year; Hofmeyr, 2004). Given the species' ecology and the region's complex landscape and climate history, we hypothesized that the genetic structure of *C. angulata* would reflect signals of the climate history and heterogeneous environment over its range.

A previous, small-scale genetic study on *C. angulata* revealed limited genetic structure among three west coast populations (Kleinsee, West Coast National Park and Dassen Island) for a short base-pair fragment of cyt *b* (Lesia et al., 2003). Evolutionary relationships among conspecific populations throughout the range of the species, however, remain unexplored and unquantified. In the present study, we examined the phylogeographic and demographic history of *C. angulata* in order to elucidate the genealogical affinities among populations.

2. Materials and methods

2.1. Sample collection

Chersina angulata samples were collected from throughout the range of the species. Tissue samples were blood, muscle, connective tissue or bone. Blood was collected from the jugular vein (Jacobson et al., 1992) in lithium heparin syringes, transferred to cryo-vials and

stored in liquid nitrogen in the field and at −20 °C in the laboratory or alternatively, blood was transferred directly to tubes filled with absolute ethanol or Queens Lysis buffer (Seutin et al., 1991) and stored at 4 °C. Following the collection of blood samples, wild caught animals were marked, photographed for reference collection and released immediately on site. Road kills or dried carcases of *C. angulata* were collected, their geographic positions recorded and tissues biopsied. These tissue samples were stored directly in absolute ethanol at 4 °C. Museum samples were sourced from areas for which no fresh material could be collected (generally the eastern Cape, South Africa). In this regard specimens deposited in the South African Museum of Natural History, Cape Town (IZIKO Museum of Cape Town, South Africa) were sampled for dry connective tissue or/and bone using dissecting tools and drill bits respectively. Between each sample, dissecting tools and drill bits were cleaned in 70% ethanol and stringently sterilised by burning the ethanol. A total of 125 *C. angulata* samples were collected from 27 localities that encompass most of the species' distribution in South Africa (Table 1 and Fig. 1).

2.2. DNA extraction, PCR and sequencing

Whole blood samples were thawed and 20 μl of blood was used to extract DNA. Prior to DNA extraction the blood was centrifuged and the supernatant removed. Nucleated erythrocytes were then used in the DNA extraction. For the road kill samples a small part of muscle tissue or connective tissue was generally used for DNA extraction. DNA was extracted using a Qiagen DNEasy kit, following the manufacturer's protocol for the blood and biopsy samples. Museum samples (bone or other tissue) were extracted, using a Qiagen DNEasy kit, in a separate room and area dedicated to museum sample research. No fresh tortoise material was ever handled in this room. Museum samples were incubated for four days and 20 μl proteinase K was added daily, because the maximum lysis of tissue samples appears to improve the overall yield of DNA. Extracted DNA was then stored in a fridge until required for PCR. Generally, a 1 μl DNA in 20 μl water dilution of DNA was performed prior to use for the fresh material. DNA from the museum samples was used without prior dilution. The following primer pair COIA (5′-

Table 1
The localities, sample sizes (*N*), geographic coordinates and mtDNA locus sampled for each *Chersina angulata* population

Locality	*N*	GPS coordinates	Locus		
			COI	cyt *b*	ND4
(1) Lekkersing	1	29°15′00″S 17°10′00″E	—	—	1
(2) Kleinsee	8	29°47′30″S 17°07′04″E	1	7	5
(3) Springbok	3	29°41′31″S 17°52′59″E	3	3	3
(4) Danskraal	21	29°42′29″S 17°48′06″E	15	20	21
(5) Nuwerus	1	31°06′33″S 18°20′51″E	1	1	1
(6) Lutzville	1	31°30′16″S 18°17′48″E	1	1	1
(7) Doorn Bay	1	31°46′57″S 18°13′58″E	1	1	1
(8) Lamberts Bay	2	32°00′43″S 18°20′28″E	2	1	2
(9) Graafwater	1	32°09′00″S 18°36′45″E	—	1	1
(10) Algeria	1	32°21′00″S 19°04′00″E	—	1	1
(11) Kriedouwkrans	2	32°22′14″S 18°59′17″E	3	2	2
(12) Citrusdal	2	32°23′45″S 18°57′02″E	1	1	1
(13) Kardoesie	1	32°37′43″S 18°56′54″E	1	1	1
(14) WCNP	25	33°14′03″S 18°08′01″E	8	15	25
(15) Dassen Island	15	33°25′21″S 18°05′17″E	13	15	13
(16) Vrolijkheid	6	33°55′21″S 19°53′28″E	—	6	6
(17) Kleinmond	3	34°19′00″S 19°02′00″E	—	3	3
(18) Moddervlei	4	34°36′00″S 19°49′00″E	—	4	4
(19) Vlooikraal	2	34°40′00″S 19°46′00″E	—	2	2
(20) Potberg	1	34°23′00″S 20°32′00″E	—	1	—
(21) EPNR	3	33°25′41″S 19°01′37″E	3	2	3
(22) Witteberge	2	33°18′40″S 20°35′18″E	1	1	2
(23) Still Bay	8	34°15′24″S 21°26′41″E	6	7	8
(24) Tierberg	1	33°10′13″S 22°15′55″E	1	1	1
(25) Port Elizabeth	18	34°01′08″S 25°29′51″E	18	17	17
(26) Verekraal	3	33°23′41″S 23°54′26″E	—	3	—
(27) Fort Brown	5	33°08′17″S 26°40′48″E	—	5	—
Total samples sequenced per locus			79	122	125

The locality numbers correspond to those on the map (Fig. 1).
The abbreviations EPNR stands for Elandsberg Private Nature Reserve while WCNP stands for West Coast National Park. Samples from Fort Brown ($N = 5$), Verekraal ($N = 3$) and Kleinsee ($N = 1$) were all museum samples based at South African Museum, Cape Town (IZIKO museums of Cape Town). Accession numbers are SAM 48103, 48090, 48103, 48092, 4893; SAM 48111–48113 and SAM 48161, respectively.

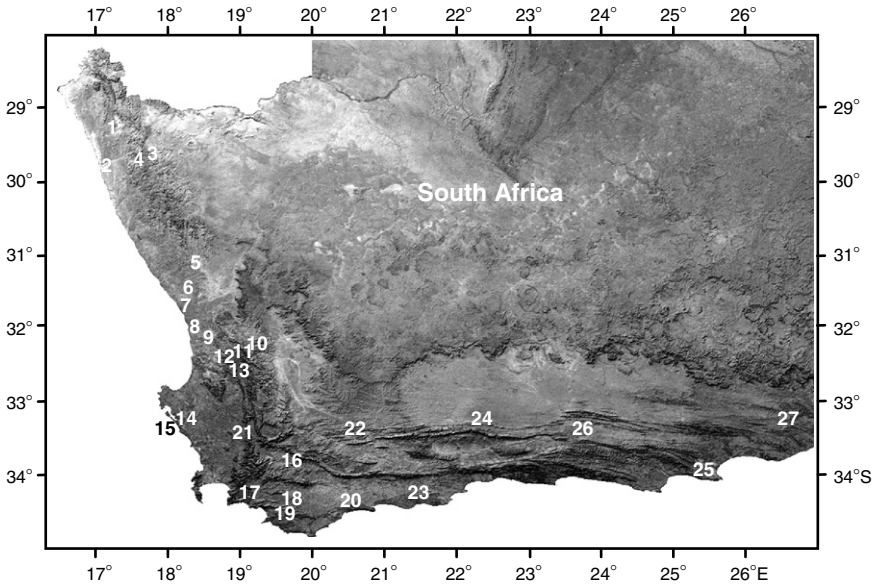

Fig. 1. Localities of the 27 *Chersina angulata* populations sampled throughout South Africa. Locality numbers represent the following populations, (1) Lekkersing, (2) Kleinsee, (3) Springbok, (4) Danskraal, (5) Nuwerus, (6) Lutzville, (7) Doorn Bay, (8) Lamberts Bay, (9) Graafwater, (10) Algeria, (11) Kriedouwkrans, (12) Citrusdal, (13) Kardoesie, (14) West Coast National Park, (15) Dassen Island, (16) Vrolijkheid, (17) Kleinmond, (18) Moddervlei, (19) Vlooikraal, (20) Potberg, (21) Elandsberg Private Nature Reserve, (22) Witteberge, (23) Still Bay, (24) Tierberg, (25) Port Elizabeth, (26) Verekraal and (27) Fort Brown.

AGT ATA AGC GTC TGG GTA GTC-3′) and COIF (5′-CCT GCA GGA GGA GGA GAT CC-3′) was used to amplify a partial fragment of the cytochrome oxidase subunit one (hereafter COI) from Palumbi et al. (1991); the primer pair tRNA-Glu-F (5′-AAG TCA TCC GTA TTG TAC GTC TCG-3′) and tRNA-Glu-J (5′-CCC TCA GAA TGA TAT TTG TCC TCA-3′) was used to amplify a partial fragment of cytochrome *b* (hereafter cyt *b*) from Austin and Arnold (2002); and the primer pair H-Leu (5′-ATT ACT TTT ACT TGG ATT TGC ACC A-3′) and L-ND4 (5′-GTA GAA GCC CCA ATC GCA G-3′) was used to amplify a partial fragment of nicotinamide adenine dinucleotide dehydrogenase component four (hereafter ND4) from Stuart and Parham (2004). For each PCR, a 25 μl reaction was performed that contained 14.9 μl of Millipore water, 3 μl of 25 mM $MgCl_2$, 2.5 μl of 10× Mg^{2+} free buffer, 0.5 μl of a 10 mM dNTP solution and 0.5 μl of each of the respective primer pairs at 10 mM, 0.1 U of *Taq* polymerase and 1–3 μl of template DNA. The PCR temperature regime for all three mtDNA loci was 95 °C for 3 min, 95 °C for 30 s, 50 °C for 40 s, 72 °C for 1 min, and then 34 cycles for the last three steps followed by a final extension of 10 min at 72 °C. Products were visualized under UV light. PCR products were purified using a PCR purification kit (QiaColumn kit) followed by gel purification using the QIA quick gel extraction kit. Purified PCR products were cycle sequenced using standard protocols. Unincorporated dideoxynucleotides were removed by gel filtration using sephadex G-25 (Sigma). Sequencing was performed on an ABI 3730 XL automated machine.

We conducted an exploratory analysis to examine the utility of partial sequences from three mitochondrial loci (mtDNA hereafter), comparing the general level of sequence divergence among the three loci. The COI gene evolved the slowest, followed by cyt *b* and the most rapid divergence was detected by the ND4. Consequently, we sequenced most samples for ND4 while sequencing a limited number of individuals for the cyt *b* and COI. Spinks and Shaffer (2005) also reported that ND4 was highly variable among populations of the western pond turtle (*Emys marmorata*). For the cyt *b,* we were able to successfully amplify museum samples because of the short fragment being employed. All three loci have been used extensively for population genetic studies among tortoises and have revealed significant phylogeographic structuring (e.g., Caccone et al., 1999, 2002; Beheregary et al., 2004; Austin et al., 2003).

2.3. Phylogenetic analyses

Sequence navigator (Applied Biosystems) 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 for any of the three protein coding genes. A 95 % probability haplotype network was constructed using the method of Templeton et al. (1992) implemented in the software TCS version 1.13 (Clement et al., 2001). All subsequent analyses were performed on the number of haplotypes for COI, cyt *b* and ND4. Maximum parsimony (MP), Minimum evolu-

tion (ME), Maximum likelihood (ML) and Bayesian approaches were used to estimate the relationships among haplotypes. MP, ME and ML 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 TBR branch swapping using 1000 random taxon additions. For the ML analysis, the best-fit substitution model for each fragment was calculated using MODELTEST version 3.06 (Posada and Crandall 1998). The best-fit maximum likelihood score was chosen using the Akaike information criteria (AIC) (Akaike, 1973), since this approach reduces the number of parameters that contribute little to describing the data by penalizing more complex models (Posada and Buckley, 2004; Nylander et al., 2004). For the ML analyses heuristic searches with TBR branch swapping and 100 random additional taxa were also performed. Phylogenetic confidence in nodes were estimated by parametric bootstrapping (Felsenstein, 1985), analysing 1000 pseudoreplicates of data sets, while due to time constraints only 100 pseudoreplicates were performed for ML. For ME the ML model was used, followed by parametric bootstrap with 1000 pseudoreplicates. Bayesian inferences were used to investigate optimal tree space using the program MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) and the models selected from MODELTEST. For each analysis, four Markov chains were run, with each chain starting from a random tree and run for 10 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 (*p*P) for each node estimated by the percentage of time the node was recovered. Uncorrected ('p') sequence distance values were calculated between haplotypes to allow comparison with published data.

Le et al. (2006) suggested recently that all southern African tortoise taxa represent a distinct regional radiation (exclusive of *Kinixys*) with *Chersina angulata* being a highly derived taxon. Hence, we used two southern African taxa, *Stigmochelys pardalis* (previously *Geochelone*) and *Psammobates geometricus,* as well as the Malagasy *Astrochelys radiata* (previously *Geochelone*) as outgroup taxa to determine relationships among *C. angulata* haplotypes.

2.4. Nested clade analyses (NCA)

The haplotype network (constructed with TCS) was converted into a nested statistical design as outlined in Templeton et al. (1992); and Crandall and Templeton (1996) for each of the three partial gene fragments (COI, cyt *b* and ND4). Haplotypes were connected into a single network with a 95% parsimony probability. Geographic and genetic associations were tested using the software GeoDIS version 2.0 (Posada et al., 2000) under a null hypothesis of no geographic association among haplotypes using 10,000 permutations. We performed the NCA analyses exclusively on the ND4 partial sequence fragment since this locus exhibited the largest degree of genetic differentiation compared to the other two loci (COI and cyt *b*) and reflect the impact of most recent patterns that could have sculpted the population structure within *C. angulata*. We estimated the clade distance (D_C), which measures the geographic spread of a clade, and the nested distance (D_N), which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category (Posada et al., 2006). Templeton's (2004) inference key was then used to interpret patterns of population structure. The conclusions of NCA have recently been debated (Knowles and Maddison, 2002; Templeton, 2004) and are not infallible, and we are cognisant of this fact. Indeed, we have implemented a variety of other approaches to test for corroborating support for the NCA inferences. In addition to the NCA, we performed an analysis of molecular variation on the data (AMOVA) using ARLEQUIN version 3.0 (Excoffier et al., 2005) to calculate gene and nucleotide diversity and to assess population differentiation based on the observed clades within the haplotype network for the ND4 data set. Permutation procedures used 10,000 randomisations to test for significance. Deviations in allele frequencies were investigated with Fu's F statistic (Fu, 1997) using ARLEQUIN version 3.0. We also assessed the demographic history of groups by performing a mismatch distribution. This method explores the signature of population fluctuation in DNA sequence data. In this regard, DnaSP (Rozas et al., 2006) was used to obtain an estimate of the population expansions.

2.5. Molecular clock

A number of recent authors have raised concerns about the utility of a molecular clock (Graur and Martin, 2004; Hugall and Lee, 2004). To guard against the assumption of a strict molecular clock we used a relaxed Bayesian clock (Thorn et al., 1998) as implemented in the package PAML version 3.14 (Yang, 1997). The ML tree topology was used as in the input for the 'baseml' model using PAML with the model F81+Γ as the only available option. We then estimated the branch lengths using ''stebranches'' module. The topology with branch lengths was input into Multidivtime module and used to produce estimates of node divergence. We used two calibration points based on fossil records: a fossil *Chersina* species that dates to the Pliocene (4.0–4.5 Myr) (Meylan and Auffenberg, 1986; Lapparent de Broin, 2000) and an early Pleistocene fossil of *Psammobates* (with a maximum age of 2 Myr) (Lapparent de Broin, 2000). As the upper limit for the divergence of *Chersina*, we included Cunningham's (2002) estimate of divergence date of *Chersobius* and *Chersina* (18 Myr), the dates were not fixed following Near et al. (2005). This analysis was repeated thrice to make sure that the estimates of the divergence values converged.

Table 2
Summary of the phylogenetic information contained within each of the three mtDNA loci sequenced among *Chersina angulata* populations

Locus	Fragment size (bp)	*N*	Number of haplotypes	GenBank Accession numbers	ML MODEL selected	Rate matrix	Base pair frequency	MP analyses
COI	599	79	31	EF120479–EF120509	TrN + I + G ($-\ln L = 1748.04$) (AIC = 3510.11)	*R*(a)[A − C] = 1.00 *R*(c)[A − T] = 1.00 *R*(d)[C − G] = 1.00 *R*(f)[G − T] = 1.00 *R*(b)[A − G] = 15.74 *R*(e)[C − T] = 6.59 Invariable sites (I) = 0.37 Gamma shape (G) = 0.80	A = 31.37% C = 17.37% G = 13.61% T = 29.53%	204 Trees recovered CI = 0.78 RI = 0.82 Tree length 177 steps 60 parsimony informative characters
cyt *b*	320	122	35	EF120510–EF120544	TrN + G ($-\ln L = 1031.49$) (AIC = 2074.98)	*R*(a)[A − C] = 1.00 *R*(c)[A − T] = 1.00 *R*(d)[C − G] = 1.00 *R*(f)[G − T] = 1.00 *R*(b)[A − G] = 5.61 *R*(e)[C − T] = 6.59 Gamma shape (G) = 0.40	A = 30.86% C = 25.36% G = 15.09% T = 28.70%	1770 Trees recovered CI = 0.74 RI = 0.75 Tree length 109 steps 32 parsimony informative characters
ND4	791	125	34	EF120545–EF120578	TrN + I + G ($-\ln L = 2420.84$) (AIC = 4851.69)	*R*(a)[A − C] = 1.00 *R*(c)[A − T] = 1.00 *R*(d)[C − G] = 1.00 *R*(f)[G − T] = 1.00 *R*(b)[A − G] = 14.60 *R*(e)[C − T] = 11.12 Invariable sites (I) = 0.37 Gamma shape (G) = 0.80	A = 26.57% C = 11.55% G = 26.26% T = 35.62%	504 trees recovered CI = 0.79 RI = 0.80 Tree length 272 steps 97 parsimony informative characters

N is the number of individuals sampled.

4. Results

4.1. COI (see Appendix 1 for the frequency and distribution of haplotypes)

Table 2 contains a summary of the phylogenetic information for the COI locus. All methods retrieved the same general topology, however, clades were poorly supported. Phylogenetic analyses derived from the haplotype data set revealed two highly divergent clades, with Clade 2 being recovered with good statistical support (bootstrap >84 for ML and ME) (Fig. 2a). Within both subclades 1A and 1B the maximum uncorrected sequence divergence was 0.9%, while in clade 2 the maximum uncorrected sequence divergence was 1.3%. The maximum uncorrected sequence divergence between the two major phylogroups (Clades 1 and 2) was 3.1%. The network obtained from TCS revealed the presence of three distinct phylogroups comprising the north-western Cape (with individuals from Danskraal, Kleinzee, Springbok, Nuwerus, Doorn Bay, Lutzville and Lamberts Bay) the south-western Cape (with individuals from Citrusdal, Lamberts Bay, Witteberge, Dassen Island, West Coast National Park—(hereafter WCNP), Kardoesie, Elandsberg Private Nature Reserve (hereafter EPNR) and Kriedouwkrans) and southern Cape (with individuals from Port Elizabeth, Still Bay and Tierberg) clades (Fig. 2b). The two main phylogroups were separated by nine mutational steps.

4.2. cyt b (results not shown—see Appendix 2 for the frequency and distribution of haplotypes)

Table 2 contains a summary of the phylogenetic information for the cyt *b* locus. Phylogenetic analyses based on the haplotypes recovered a poorly supported topology, however the overall pattern of genealogical differentiation observed using COI was also present. The lack of phylogenetic signal in this gene region is probably attributable to the short, relatively conserved section that was amplified. Within subclade 1A and 1B, as well as within Clade 2, the maximum uncorrected sequence divergence was 1.8%. The maximum uncorrected sequence divergence between the two major clades was 2.8%. The network obtained from TCS recovered three major phylogeographic groups, including the north-western Cape clade (subclade 1A—with individuals from WCNP, Kleinzee, Springbok, Danskraal, Dassen Island, Kriedouwkrans, Witteberge, Citrusdal, Lambertsbay, Nuwerus, Doorn Bay and Lutzville), south-western Cape clade (subclade 1B—with individuals from Graafwater, Algeria, Vlooikraal, Vrolijkheid,

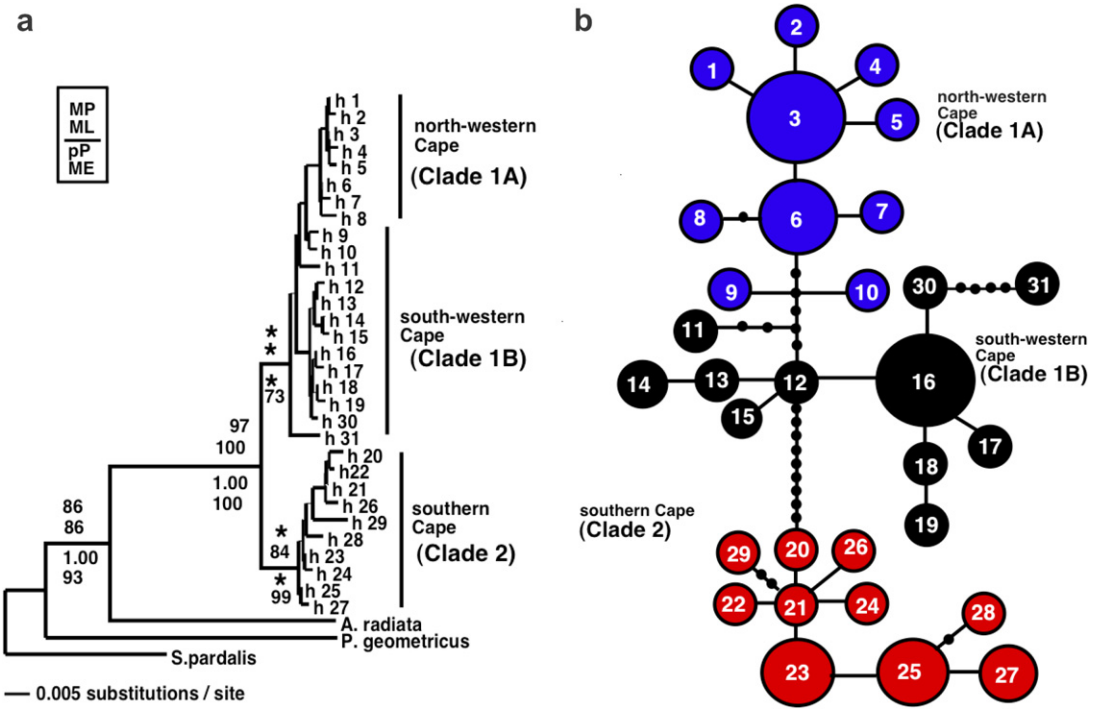

Fig. 2. (a) ME phylogram for the COI sequence data of the 31 haplotypes (h1–h31) detected among *Chersina angulata* populations. Bootstrap values for MP, ML and ME are given above the branch, while posterior probability values from Bayesian analysis are given below the branch with asterisks represent bootstrap values <70%. Clade 1 is comprised of two subclades (subclade 1A = north-western Cape, subclade 1B = south-western Cape); Clade 2 is comprised of southern Cape populations. (b) A statistical parsimony network showing the distribution of the 31 haplotypes, also demonstrating the two major phylogeographic units separated by nine mutational steps. The number inside each circle corresponds to the haplotypes in Appendix 1.

Dassen Island, WCNP, Kleinmond and EPNR) and southern Cape clade (Clade 2—with individuals from Moddervlei, Vlooikraal, Port Elizabeth, Tierberg, Fort Brown, Verekraal, Kleinmond, Potberg and Still Bay).

4.3. ND4 (see Appendix 3 for the frequency and distribution of haplotypes)

Table 2 contains a summary of the phylogenetic information for the ND4 locus. In contrast to results from the previous two loci (COI and cyt *b*), the phylogenetic results derived from ND4 recovered two highly distinct reciprocally monophyletic and statistically well-supported clades (Fig. 3a). Clear geographic structure was evident in the distribution of the haplotypes. Clade 1 (retrieved with 76% bootstrap and 0.95 *p*P) contained all the haplotypes from the north-western Cape (subclade 1A—with 86% bootstrap support and 1.00 *p*P—with individuals from Kleinzee, Springbok, Danskraal, Lekkersing, Lamberts Bay, Lutzville, Nuwerus, Doorn Bay, Citrusdal, Witteberge) and the south-western Cape groups (subclade 1B—with 83% bootstrap support and 1.00 *p*P—contained individuals from EPNR, WCNP, Dassen Island, Kardoesie, Kleinmond, Vlooikraal, Vrolikheid, Algeria, Graafwater and Kriedouwkrans). Clade 2 was comprised of all the samples collected from the southern Cape (retrieved with 71% bootstrap support and 0.95 *p*P—with individuals from Still Bay, Moddervlei, Kleinmond, Tierberg and Port Elizabeth). The topology is characterised by short internal branches, corroborative of recent population divergence within each clade. At Kleinmond and Vlooikraal, we observed haplotypes of the highly divergent south-western Cape and the southern Cape clades. Within subclade 1A and 1B the maximum uncorrected sequence divergence was 1.5%, while within Clade 2 the maximum sequence divergence was 1.6%. The maximum uncorrected sequence divergence between the two major clades was 3.0%.

The network obtained from TCS recovered the same three clades observed using COI and cyt *b* (Fig. 3b). The north-western Cape (subclade 1A) and south-western Cape (subclade 1B) clades were separated by five mutational steps while Clade 1 was separated by eight mutational steps from the southern Cape group (Clade 2) (Fig. 4). For the NCA, the 34 haplotypes were partitioned into 30 one-step clades, eight two-step clades, four three-step clades and two four-step clades. Contingency analyses showed no significant association between nested clades and geography for the one step clades, with the exception of clade 1–2, for which geographic sampling was inadequate to discriminate between fragmentation and isolation. For the two-step clades, three clades were significant; these were clades 2–1, 2–2 and 2–7. The first two-step clade (2–1) showed a pattern of long distance colonization coupled with fragmentation or past fragmentation following range expansion, while in the case of both two-step clades (2–2 and 2–7), geographic sampling was inadequate to discriminate

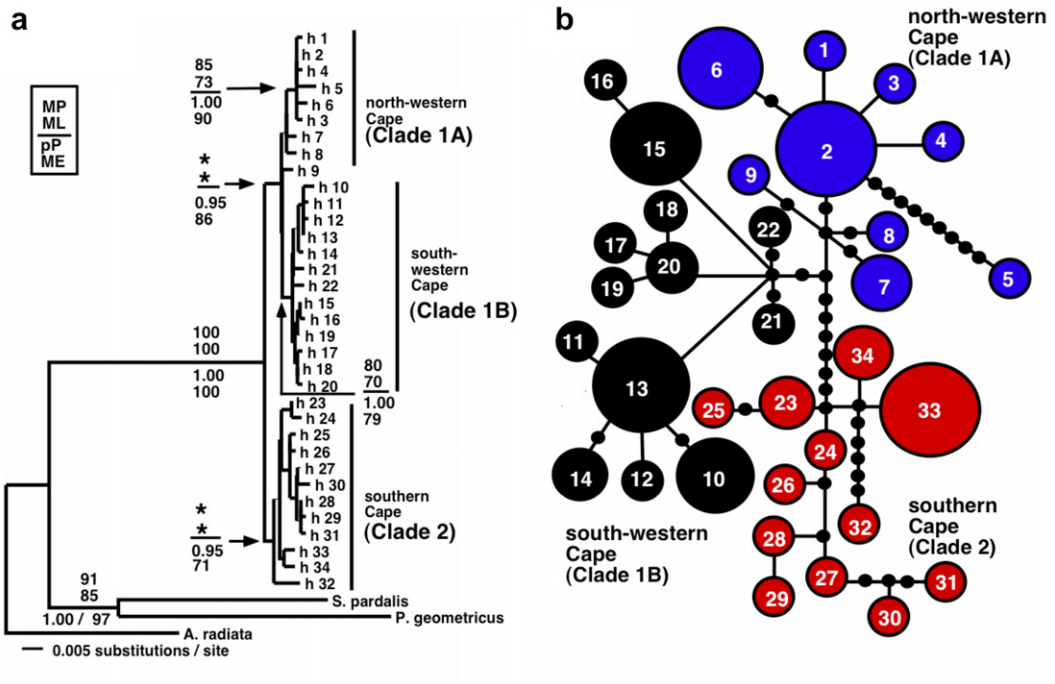

Fig. 3. (a) ME phylogram for the ND4 sequence data of the 34 haplotypes (h1–h34) detected among *Chersina angulata* populations. Bootstrap values for MP, ML and ME are given above the branch, while posterior probability values from Bayesian analysis are given below the branch with asterisks represent bootstrap values <70%. Two well-supported reciprocally monophyletic clades are evident. Clade 1 is comprised of two subclades (subclade 1A = north-western Cape, subclade 1B = south-western Cape); Clade 2 is comprised of southern Cape populations. (b) A statistical parsimony network showing the distribution of the 34 haplotypes, also demonstrating the two major phylogeographic units with eight mutational steps separating the two major groups. The number inside each circle corresponds to the haplotypes in Appendix 3.

between fragmentation and isolation by distance. Between the two three-step clades (3–1 and 3–4) past fragmentation and/or isolation by distance was observed. For the total cladogram, the results suggest long distance colonization with subsequent fragmentation or past fragmentation followed by range expansion (Table 3).

When comparing the three phylogeographic groups, the AMOVA revealed highly significant values ($F_{ST} = 0.52$; $P < 0.001$) with 52% of variation among groups and 48% of variation within groups suggesting significant structure in the data. The gene diversity for the north-western Cape, south-western Cape and southern Cape was 0.74, 0.86 and 0.82, respectively, while the nucleotide diversity within the three groups (in similar order) was 0.003, 0.004 and 0.010, respectively. In addition, Fu's FS values indicated that all three groups appear to be in Hardy-Weinberg equilibrium; north-western Cape −0.28 ($P = 0.48$), south-western Cape −2.98 ($P = 0.13$) and the southern Cape groups 1.32 ($P = 0.73$). The mismatch distribution showed clearly that the data were highly structured with a bi-modal distribution and suggested population expansion is currently occurring (result not shown). The divergence time estimations (±SD) indicate that the clades diverged in the late Miocene: Clade 1 is between 9.9 and 9.71 Myr (±3.6 Myr), with subclade 1A between 8.5 and 8.4 Myr (±3.3 Myr) and subclade 1B between 8.7 and 8.6 Myr (±3.3 Myr), and Clade 2 is between 10.4 and 10.2 Myr (±3.5 Myr).

5. Discussion

5.1. Phylogeographic structure

The phylogeographic pattern detected among *C. angulata* populations was highly congruent for the three mtDNA loci sequenced (COI, cyt *b* and ND4). Two major clades were detected, with Clade 1 comprised of two subclades, 1A and 1B, comprising samples from the north-western Cape and south-western Cape, respectively, while Clade 2 was exclusively comprised of southern haplotypes (Fig. 5). These results show a clear demographic genetic structure when superimposed onto the geographic landscape, as supported by the NCA for the ND4 locus, rejecting the null hypothesis of no geographic genetic structure within *C. angulata*.

The western and southern clades of *C. angulata* diverged between 10.4 and 8.4 Myra in the late Miocene. This divergence estimate predates the early Pliocene fossils of *Chersina* at Langebaanweg in the south-western Cape (4.5 Myr; Meylan and Auffenberg, 1986; Lapparent de Broin, 2000). Cunningham (2002) estimated that *Chersina* dates back to the early Miocene (approximately 18 Myra), which theoretically leaves sufficient time for *Chersina* to have spread throughout the southern, south-western and north-western regions of southern Africa before the clades diverged in the late Miocene. About 14 Myra, after the Drake Passage opened, the Antarctic ice sheet expanded

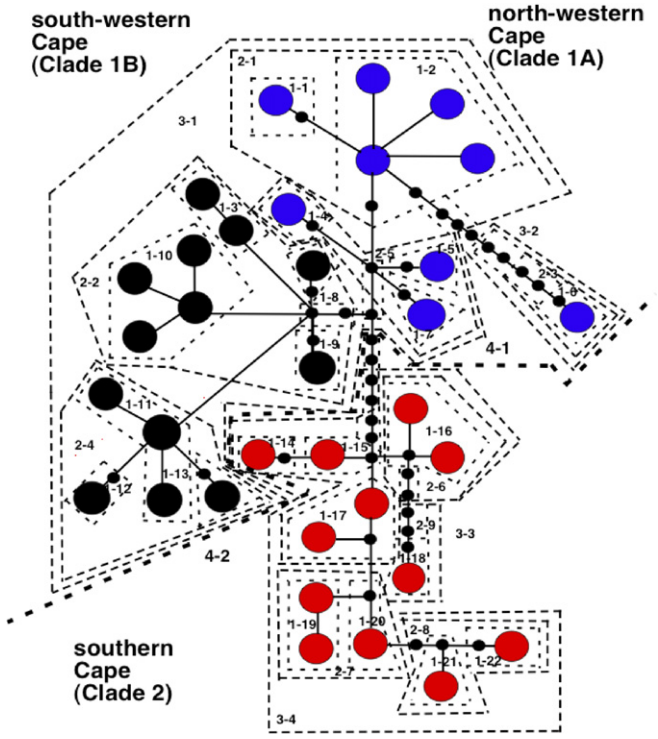

Fig. 4. Nested haplotype network for the 34 haplotypes detected using ND4 for *Chersina angulata* populations sampled. The level of nesting is 1–*x* for one-step clades, 2–*x* for two-step clades, 3–*x* for three-step clades and 4–*x* for the four-step clades. The dark closed circles represent the missing or unsampled haplotypes.

and the Benguela Current developed along the west coast. Subsequently, the climate became colder and drier, an east-west climate gradient became established, and summer droughts became increasingly severe in the west (Partridge, 1997). The divergence date for *C. angulata* clades thus corresponds to a time of differential climate change across the range of *C. angulata*; the populations in the western regions became exposed to increasing summer aridity and low rainfall, while rainfall in the south remained higher and aseasonal. Tortoises are ectotherms and low temperatures during the cold-dry periods, such as in the late Miocene, may have caused populations to contract from coastal regions to inter-montane valleys where temperatures would have been more favourable. Recurrent contractions and expansions throughout the Pliocene–Pleistocene glaciation cycles probably enhanced divergence among and within the clades. Corroborative evidence exists that vicariant events associated with climate change in the late Miocene have also influenced cladogenesis in other taxa with a co-distributional pattern, not only for reptiles, e.g., the cordylid lizards *Cordylus cordylus-niger-oelofseni* complex (Daniels et al., 2004), the limbless fossorial Cape skink complex, *Acontias meleagris meleagris* (Daniels unpubl. data), and the chameleon genus *Bradypodion* (Tolley et al., 2006), but also for plants, e.g., *Phylica* (Richardson et al., 2001).

The north-western and south-western subclades of *C. angulata* formed distinct and cohesive units, but the genetic distance between the two subclades was not substantial. Although haplotypes of subclades 1A and 1B were not found at the same localities, the distribution of the two subclades overlapped in the Citrusdal area (see Fig. 1). The phylogeographic pattern for these subclades is suggestive of secondary contact between two previously isolated groups. However, the sample sizes for *C. angulata* were small in the overlap zone and wider sampling in this region would indicate if recurrent gene flow occurs between the two subclades.

The Cape Fold Mountains form a formidable physical barrier between the south-western and the southern clades, with many populations known from the inter-montane valleys. Three populations on the south-eastern side of the mountains contain haplotypes of the south-western Cape clade (Vrolijkheid, Kleinmond and Vlooikraal). The haplotypes from Vrolijkheid are distinct to the south-western clade and the Riviersonderend Mountains separate the Vrolijkheid population from the coastal populations. Kleinmond and Vlooikraal were areas of sympatry for haplotypes of the south-western and southern clades. These areas of sympatry are at the westernmost limit of the

Table 3
Significant results of the nested clade analyses (NCA) for the 34 ND4 haplotypes of *Chersina angulata*

Clade	χ^2	Probability	Inference	Outcome
1–2	24.28	0.02	1–2–3–4–9–10–No	Geographic sampling inadequate to discriminate between fragmentation and ILD
2–1	7.79	0.03	1–2–3–5–6–13–Yes	LDC possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
2–2	66.00	0.00	1–2–3–5–15–16–18–Yes	Geographic sampling inadequate to discriminate between fragmentation and ILD
2–7	25.00	0.00	1–2–3–5–15–16–18–No	Geographic sampling scheme inadequate to discriminate between fragmentation and range expansion and ILD
3–1	222.00	0.00	1–2–3–5–15–No	Past fragmentation and/or ILD
3–4	36.00	0.00	1–19–20–No	Inadequate geographic sampling
Total Cladogram	118.87	0.00	1–2–3–5–6–13–Yes	LDC with subsequent fragmentation or past fragmentation followed by range expansion

Nested contingency results based on 10,000 permutations in GEODIS (Posada et al., 2000). Inferences were made using Templeton's (2004) key. ILD, isolation by distance, LDC, long distance colonization.

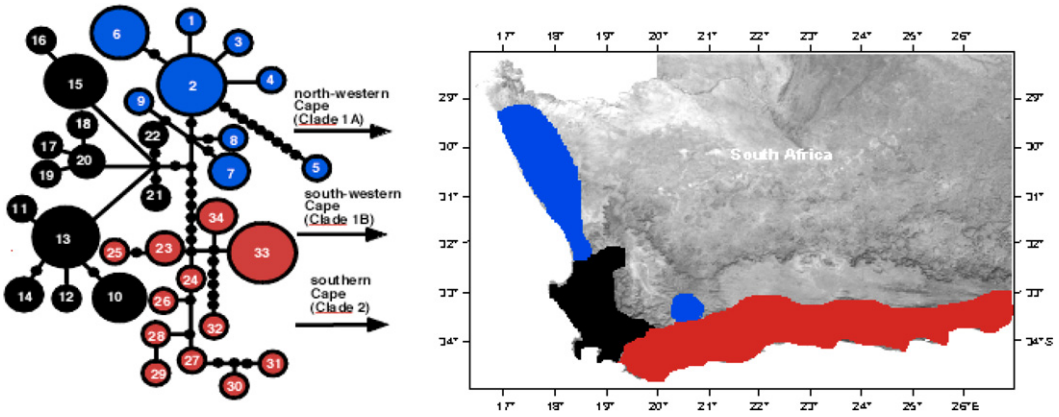

Fig. 5. Haplotype network derived from the ND4 data (as in Fig. 3b) superimposed on the geographic distribution of the *Chersina angulata* populations sampled. The blue shaded areas on the map represent the north-western Cape clade, the black shaded areas on the map represents the south-western Cape clade while the red shaded areas on the map represents the southern Cape clade. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

southern clade, with the Cape Fold Mountains forming a natural barrier to gene flow with the south-western clade. Despite the fact that these tortoises were sympatric, they were genetically highly discrete at the mtDNA level, suggesting genetic isolation of these lineages. Unpublished analyses from selected individuals for the control region yielded identical results. Those results will be incorporated into a study together with nDNA markers. The sympatry of south-western and southern haplotypes at Kleinmond and Vlooikraal most likely represent recent expansions of animals either from inter-montane valleys or along the coastal plain when sea levels were low. This region has also been shown to form a zone of secondary contact between genetically isolated units of the freshwater crab *Potamonautes perlatus* (Daniels et al., 2006b). The possibility that the sympatry reflects translocation of tortoises by humans, however, should be considered. We sampled mainly in remote areas to lower the risk of sampling translocated tortoises, but it is impossible to exclude this factor from a sampling regime. We nevertheless believe that the impact of human translocation on the population genetic structure of *C. angulata* is insignificant since we would have anticipated a more random distribution of haplotypes that are unrelated or uncoupled from geography.

5.2. Taxonomic considerations

Without proposing a direct correlation between genetic distance and taxonomic rank, we nevertheless compared the divergence between the two *C. angulata* clades (approximately 3%) with divergence values for other chelonians. For partial sequences of ND4, the sea turtle *Lepidochelys kempii* and its congener *L. olivacea* differ by only 1.5% (Bowen et al., 1998), while genetic distances range between 3.92% and 5.73% among *Terrapene* species (Feldman and Parham, 2002), and between 3.88% and 5.75% among *Cuora* species (Stuart and Parham, 2004). For partial sequences of the cyt *b* gene, the maximum divergence for the subspecies of *Geochelonia* (Galapagos tortoises) is 2.4% (Caccone et al., 2002), while divergence values range from 1.4% to 5.6% for *Testudo* species (Austin and Arnold, 2002), and from 3.6% to 5.9% for *Indotestudo* species (Iverson et al., 2001). The genetic difference between the two *C. angulata* clades clearly falls within the interspecific ranges reported for other chelonian taxa. However, since mutational rates, life history characteristics, age of the lineage, and the length, portion and polymorphism of the loci sequenced can influence interspecific divergence, caution is necessary when applying divergence values to assess species boundaries.

The question now arises if the two major clades of *C. angulata* represent distinct evolutionary lineages. Here, we adopt the phylogenetic species concept and recognise the two clades as diagnostic units (de Queiroz, 1998; Wheeler and Meier, 2000), which require that the clades should be reciprocally monophyletic and diagnosable on a number of addition characters. The phylogenetic results for ND4 recovered two highly distinct, reciprocally monophyletic and statistically well-supported clades, suggestive of two putative species for *C. angulata*. Additional support for differences between the clades includes regional differences in morphometric characters (van den Berg and Baard, 1994; Hofmeyr, unpubl. data), different activity patterns linked to different climates in the south-west and south-east (Ramsay et al., 2002), and indications that the reproductive behaviour of the two clades may differ. Observations of captive angulate tortoises over several years showed that south-western Cape males apparently did not recognise the sex of an eastern Cape female; during the mating season, the males showed agonistic behaviour towards this female while they persistently courted females of their own clade (Hofmeyr, pers. obs.). Collectively, these congruent character differences between the two main clades allude to potential diagnostic characteristics useful for identifying unique genealogically units. We regard this conclusion, however, as a working hypothesis that requires further testing.

In order to reach a final decision on the taxonomic status of *C. angulata* clades, we propose to sample wider in the contact zones of the clades and to include nuclear genes in the analyses. However, a number of researchers have noted that nDNA sequence markers evolve remarkably slow in chelonians, impeding the detection of recent genetic divergence and limiting the utility of these markers for phylogeographic inferences. For example, Caccone et al. (2004) found virtually no difference between taxa for a 4 kb nDNA fragment, and suggest that mtDNA evolves at least 30 times faster than nDNA. Similarly, Spinks and Shaffer (2005) reported limited sequence variation in a 1 kb fragment of nDNA for the western pond turtle (*Emys marmorata*). Consequently, Spinks and Shaffer (2005) argue that the sole use of mtDNA will remain the most powerful means of assessing phylogeographic structure in chelonians. These studies suggest that nDNA may simply be too slowly evolving to detect intraspecific differences between the two putative taxa nested within *Chersina*. Alternatively, a microsatellite library should be developed or an amplified fragment length polymorphism (AFLP) study should be undertaken and both are indeed planned, to gain a nuclear genetic perspective and fine scale genetic perspective into genealogical affinities between the two major divergent *C. angulata* clades. In an attempt to retain the genetic diversity and integrity of these divergent clades and aid conservation, we suggest that no translocations be undertaken between any areas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.08.010.

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