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Northern genetic richness and southern purity, but just one species in the *Chelonoidis chilensis* complex

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The *Chelonoidis chilensis* complex, the sister group of the famous Galápagos tortoises, is a widely distributed group of South American land tortoises, ranging from the dry Chaco of Bolivia, Paraguay and northern Argentina to northern Patagonia. Within this complex, up to three distinct species have been recognized. Using sequence data of the mitochondrial cytochrome *b* gene and length polymorphisms of 10 microsatellite loci, we investigate genetic differentiation among all three nominal species. We find only negligible differentiation, with decreasing genetic diversity from north to south. We conclude that only one species, *Chelonoidis chilensis* (Gray, 1870), is valid, with *C. donosobarrosi* (Freiberg, 1973) and *C. petersi* (Freiberg, 1973) as its junior synonyms. Morphological variation within *C. chilensis* sensu lato is in accord with the observation that size variation in chelonians follows Bergmann's rule, with body size increasing with latitude. The observed phylogeographic differentiation inverses the well-known pattern of southern genetic richness and northern purity from the northern hemisphere, resulting from dispersal from glacial refugia. This implies that in higher latitudes of both hemispheres genetic diversity may decrease with increasing distance from the refugium. For *C. chilensis* sensu lato, it seems likely that long-distance dispersal via rafting on the Desaguadero River led to the foundation of the southernmost populations in northern Patagonia during the Holocene.

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Introduction

Chelonoidis is a genus of land tortoises endemic in the Galápagos Islands, South America and southernmost Central America. Originally, its species were placed, like most land tortoises, in the genus *Testudo* sensu lato and later, since the revision by Loveridge & Williams (1957), in the genus *Geochelone* sensu lato. Within *Geochelone*, Auffenberg (1974) distinguished several subgenera, some of which were recognized as full genera by Bour (1980), among them *Chelonoidis*. Using mitochondrial and nuclear DNA sequences, Le *et al.* (2006) and Fritz & Bininda-Emonds (2007) confirmed the monophyly and generic distinctness of *Chelonoidis*. This genus embraces, besides the famous giant tortoises of the Galápagos Islands, some continental species reaching only small to medium body sizes. Two species, *Chelonoidis carbonaria* and *C. denticulata*, occur mainly in savannah and rainforest habitats in northern and central South America and Panama (Pritchard & Trebbau 1984; Ernst & Barbour 1989; Ernst *et al.* 2000; Vargas-Ramírez *et al.* 2010a). It is debated how many additional

continental species exist. For many decades, all remaining South American tortoises ranging from the dry Chaco of Bolivia, Paraguay and northern Argentina southwards to the Monte region of northern Patagonia (Argentina) were placed in the species *C. chilensis* (Gray, 1870). In 1973, Freiberg described two additional species, the small-sized *C. petersi*, from within the northernmost part of the former range of *C. chilensis*, and the large-sized *C. donosobarrosi*, from the southernmost part of the former range of *C. chilensis*. Besides size, the two species were diagnosed by minor colouration and shell differences, with *C. donosobarrosi* having smooth carapacial scutes with black centres and growth rings on the plastral scutes and *C. petersi* having yellow central carapacial scutes and smooth plastral scutes (Freiberg 1973). The same author reported for *C. donosobarrosi* a maximum straight-line carapace length of 43.3 cm and for *C. petersi*, 17.0 cm, while *C. chilensis* was thought to reach 36 cm (Freiberg 1973, 1981).

Cei (1986) treated *C. donosobarrosi* and *C. petersi* as full species along with *C. chilensis*. However, Buskirk (1993) failed to differentiate *C. chilensis* and *C. donosobarrosi* morphologically and synonymized the two species. Also, Fernández (1988), Cabrera (1998) and Richard (1999) recognized only two species. However, Fernández (1988) and Cabrera (1998) applied the name *C. petersi* to northern populations and *C. chilensis* to southern populations (with having *C. donosobarrosi* as a junior synonym of *C. chilensis*). By contrast, Richard (1999) used the name *C. chilensis* for northern populations (with having *C. petersi* as its junior synonym) and used *C. donosobarrosi* for southern populations. Ernst & Barbour (1989) and Ernst *et al.* (2000) recognized only one species, *C. chilensis*, and proposed that the putative diagnostic characters of the three species *C. chilensis*, *C. donosobarrosi* and *C. petersi* might reflect only a north-south cline or sexually dimorphic characters. Fritz & Havaš (2007) endorsed this point of view, acknowledging that pronounced phenotypic plasticity has been suggested as the source of similar morphological variation in a number of other tortoise species (*Aldabrachelys gigantea*: Austin *et al.* 2003; *Testudo graeca*: Fritz *et al.* 2007; *T. marginata*: Fritz *et al.* 2005; and since then: *Homopus signatus*: Daniels *et al.* 2010; *Stigmochelys pardalis*: Fritz *et al.* 2010; *T. horsfieldii*: Fritz *et al.* 2009; *T. kleinmanni*: Attum *et al.* 2007; Široký & Fritz 2007). Nevertheless, Rhodin *et al.* (2010) continued to treat *C. petersi* as a valid species along with *C. chilensis*.

Phylogenetically, two well-supported clades occur within *Chelonoidis* (Caccone *et al.* 1999). One of these clades comprises the species *C. carbonaria* and *C. denticulata*, and the other the *C. chilensis* complex plus the giant tortoises from the Galápagos Islands (*C. nigra* complex). However, all previously published sequences of the *C. chilensis* complex (Caccone *et al.* 1999; Le *et al.* 2006) originated from one or two zoo animals of unknown provenance, so that nothing is known about genetic differentiation within this group.

In this study, we address the question of how many taxa should be recognized within the *C. chilensis* complex. For doing so, we generate sequences of the mitochondrial cytochrome *b* gene of 45 samples from the range of all three nominal taxa, *C. chilensis*, *C. donosobarrosi* and *C. petersi*. The cyt *b* gene is highly informative for unravelling alpha-taxonomic differences of chelonians (e.g. Austin *et al.* 2003; Fritz *et al.* 2005, 2007, 2009, 2010; Široký & Fritz 2007; Daniels *et al.* 2010). To gain additional insights into population structuring, we combine this data set with evidence from 10 microsatellite loci. Furthermore, we discuss our findings in a general phylogeographic context and draw taxonomic conclusions.

Materials and methods

Sampling and laboratory procedures

Forty-five tissue and saliva samples were collected throughout the ranges of the three nominal species *Chelonoidis chilensis*, *C. donosobarrosi* and *C. petersi*, and preserved in absolute EtOH (Fig. 1; Table S1). In north-south direction, the study area encompassed a distance of approximately 1550 km; and in east-west direction, approximately 600 km. Samples originated from wild tortoises and assigned to one of the three nominal species according to their geographical provenance, because morphological identification is not trivial (see Introduction and Discussion). To facilitate communication, each of the putative species is treated below provisionally as valid. Among the studied specimens were tortoises sampled at the respective type locality of each taxon or in close proximity.

Total genomic DNA was extracted using the DTAB method (Gustincich *et al.* 1991). The mitochondrial cytochrome *b* gene (cyt *b*) was amplified using the primer pairs CytbG + mt-E-Rev (Spinks *et al.* 2004; Fritz *et al.* 2006) and mt-c2 + mt-f-na (Fritz *et al.* 2006), yielding two DNA fragments overlapping by approximately 300 bp. PCR was carried out in a total volume of 20 μL containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany), 1× buffer (as recommended by the supplier), 0.5 μM of each primer and 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), with initial denaturation at 94 °C for 5 min, followed by 40 cycles with denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s and extension at 72 °C for 90 s. After the last cycle, samples were incubated in a final extending step at 72 °C for 10 min. PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH,

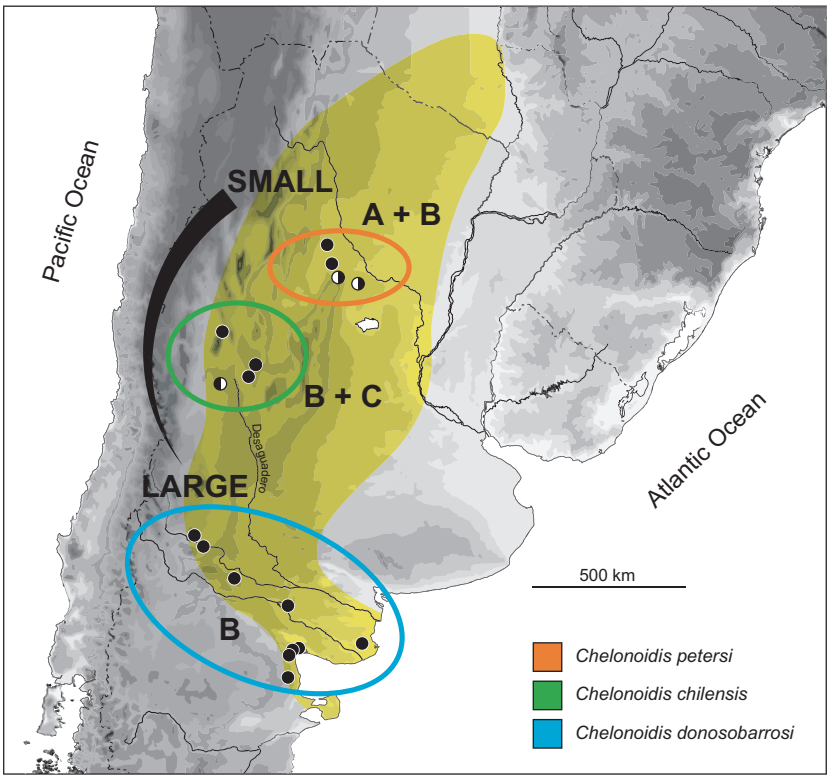

Fig. 1 Range (shaded in yellow) and sampling sites for the *Chelonoidis chilensis* complex. Colour code for species corresponds to Figs 2 and 4. Upper-case letters indicate mitochondrial haplotype clusters in the respective nominal species. Haplotypes of cluster B are shown as solid black circles; syntopic occurrences of haplotypes of clusters A + B or B + C, semi-open circles. Increase in body size from north to south is indicated. The Desaguadero River (see Discussion) runs from the range of *C. chilensis* to the south into the range of *C. donosobarrosi*.

Staufen, Germany; modified protocol: 30 min at 37 °C, 15 min at 80 °C) and sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers mt-E-Rev and mt-c2. The lengths of the resulting concatenated mtDNA sequences were 1040 bp; for GenBank accession numbers, see Table S1.

As the *C. chilensis* complex constitutes the sister taxon of Galápagos tortoises (*C. nigra* complex; Caccone *et al.* 1999), primers developed for 10 microsatellite loci of the latter group (Ciofi *et al.* 2002) were tested. Cross-amplifications were successful, and nine of the 10 loci turned out to be polymorphic (Table S2); the presence of microsatellites was confirmed by sequencing on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Microsatellites were individually PCR-amplified in a final volume of 10 μL using 0.25 units *Taq* polymerase (Bioron) with the buffer recommended by the supplier and a final concentration of 1.5 mM $MgCl_2$ (Bioron), 0.1 mM of each dNTP (Fermentas), 0.3 μM of each primer (forward primer fluorescent-labelled, Biomers, Ulm, Germany; Table S2), 0.2 μg of Bovine Serum Albumin (Fermentas) and approximately 10 ng of total DNA. The PCR cycling conditions were as follows: 35 cycles with denaturation at 94 °C for 40 s but 5 min for the first cycle; annealing for 45 s at the temperatures specified in Table S2 for each locus; and extension at 72 °C for 40 s but 5 min for the final cycle. Genotyping was performed on an ABI 3130xl Genetic Analyser using 0.5 μL of PCR product plus 0.25 μL GeneScanTM-600 LIZ® Size Standard, filled up with Hi-Di Formamide (Applied Biosystems) to a total volume of 10 μL. Up to three loci were analysed simultaneously. Allele scoring was performed with the software PEAK SCANNER 1.0 (Applied Biosystems).

Alignment and analyses of mtDNA sequences

DNA sequences were aligned and examined using MEGA 5.05 (Tamura *et al.* 2011). No internal stop codons were detected, and because the two DNA fragments resulting from different PCR primers yielded consistent sequences closely resembling the cyt *b* gene of other testudinid species (Le *et al.* 2006; Fritz & Bininda-Emonds 2007; Fritz *et al.* 2010), we conclude to have amplified and

sequenced authentic mitochondrial DNA and not insertions of mtDNA in the nuclear genome (numts) as known to occur in testudinids (Fritz *et al.* 2010).

Cyt *b* sequences were further analysed with the parsimony network algorithm as implemented in TCS 1.21 (Clement *et al.* 2000). In addition, phylogenetic relationships were inferred with the maximum likelihood (ML) approach of RAxML 7.2.6 (Stamatakis 2006), using the GTR + G model and the graphical user interface RAXMLGUI 0.93 (Silvestro & Michalak 2011). Sequences of *Chelonoidis carbonaria* (GenBank accession number FN185755), *C. denticulata* (FN185758), *C. nigra* (DQ497300) and *Testudo graeca* (FR686466) were included as outgroups; for tree rooting, the distantly related *T. graeca* was used. To explore the robustness of the branching patterns, five independent ML searches were performed using the fast bootstrap algorithm. Subsequently, 1000 thorough bootstrap replicates were calculated and plotted against the tree with the highest likelihood value.

Genetic differentiation among clusters (as identified by microsatellite data, see below) was assessed with ARLEQUIN 3.11 (Excoffier *et al.* 2005) using an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) and pairwise F_{ST} values (Weir & Cockerham 1984). Haplotype diversity (h) and nucleotide diversity (π) were calculated in DNASP 5.0 (Librado & Rozas 2009) range-wide and per cluster. Furthermore, to check for population expansion, a mismatch distribution test was run in ARLEQUIN and the expansion parameters τ, θ_1 and θ_0 were calculated for the clusters. ARLEQUIN estimates parameters of a sudden demographic (or spatial) expansion using a generalized least-square approach (Schneider & Excoffier 1999). Tajima's D and Fu's F_S values were also calculated in ARLEQUIN. Pairwise mismatch distribution graphs were produced in DNASP. Uncorrected p distances were obtained with MEGA.

Cluster analyses using microsatellite data

Population structuring was inferred using STRUCTURE 2.3.2 (Pritchard *et al.* 2000; Hubisz *et al.* 2009), without information on sampling sites or any other *a priori* assignment. STRUCTURE searches in the data set for partitions corresponding to populations in Hardy–Weinberg and linkage equilibrium and clusters individuals accordingly. Population structure was simulated with three different upper bounds ($K = 5$, $K = 10$ and $K = 20$), assuming admixture with allele frequencies correlated. The admixture model allows for recent or current gene flow, so that individuals can have ancestors from more than one population. Correlated allele frequencies are expected to occur in populations with common origin. The burn-in was set to 100 000 and the number of further MCMC repetitions to 200 000; calculations were repeated 20 times for each K. Convergence of likelihood values was reached after the burn-in (values were not decreasing or increasing). For the estimation of the best K, posterior probabilities [highest lnP(D)] and the ΔK method (Evanno *et al.* 2005) were used. Obtained clusters and individual admixture were visualized using the software DISTRUCT 1.1 (Rosenberg 2004). Genetic differentiation among clusters was estimated in ARLEQUIN using an AMOVA (Excoffier *et al.* 1992) and pairwise F_{ST} values (Weir & Cockerham 1984).

To assess genetic diversity within clusters, Hardy–Weinberg equilibrium and pairwise linkage disequilibrium were calculated for all loci using the exact test implemented in ARLEQUIN. Observed heterozygosity (H_O) and Nei's unbiased expected heterozygosity (H_E) under Hardy–Weinberg equilibrium were also calculated in ARLEQUIN. Allelic richness (A_R), observed allelic diversity (A_{ob}) and the number of alleles per population and locus were computed in FSTAT 2.9.3.2 (Goudet 2001). GENALEX 6.1 (Peakall & Smouse 2005) was used to reveal the presence and the number of private alleles and MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004) to test for null alleles. All significance levels for tests involving multiple comparisons were Bonferroni-corrected (Rice 1989).

To examine whether a recent reduction in effective population size occurred in the southernmost cluster, a test for heterozygosity excess was conducted with the software BOTTLENECK 1.2.02 (Piry *et al.* 1999) using the two-phase mutation model. Test parameters were as follows: 95% single-step mutation and 5% multiple-step mutation, and the variance among multiple steps was set to 12, as suggested by Piry *et al.* (1999). Statistical significance was determined using a Wilcoxon test (Cornuet & Luikart 1996).

Results

Mitochondrial DNA

Among the 45 cyt *b* sequences, only weak divergence was detected, corresponding to 10 distinct haplotypes differing by a maximum of 24 mutation steps and a global sequence divergence (uncorrected p distances) of $0.63 \pm 0.14\%$ across all sequences.

In parsimony network analysis, the haplotypes clustered in the three groups A, B and C, being different from one another by a minimum of 8–20 mutation steps (Fig. 2). Within group A, a maximum of five mutations occurred; within group B, a maximum of three mutations; and within group C, only one mutation. While only haplotypes of group B occurred in tortoises from the southernmost localities (*Chelonoidis donosobarrosi*), tortoises from the centre and north of the distribution range of the *C. chilensis* complex yielded haplotypes of two distinct clusters. Tortoises from the centre (*C. chilensis* sensu stricto) had

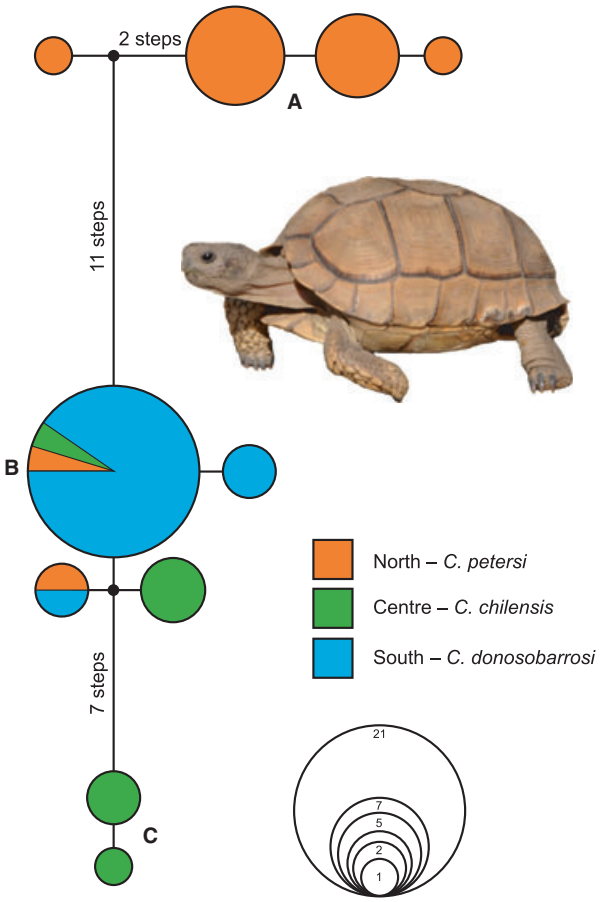

Fig. 2 Parsimony network based on 1040 bp of the mitochondrial cyt *b* gene of 45 tortoises of the *Chelonoidis chilensis* complex (95% connection limit = 14 steps). Each circle corresponds to a distinct haplotype and lines connecting haplotypes to one mutational step, if not otherwise specified. Symbol size indicates haplotype frequency; inset shows individual haplotype frequencies. Small black circles are missing node haplotypes. Upper-case letters denote haplotype clusters. The greatest outgroup probability (0.4107) has the most frequent haplotype of cluster B.

haplotypes of clusters B and C; tortoises from the north (*C. petersi*) had haplotypes of clusters A and B. Private haplotypes were found in all of the three nominal species (Fig. 2; Table 1). However, although the largest sample was available for *C. donosobarrosi*, only one private haplotype (differing by one mutation step from the most frequent haplotype shared with the two other species) was detected, whereas in *C. chilensis*, corresponding to the smallest sample, three of the four haplotypes were unique, and in *C. petersi*, four of six.

In phylogenetic analyses, *C. nigra* constituted the sister taxon of the *C. chilensis* complex, and the successive sister was a clade embracing *C. carbonaria* and *C. denticulata* (Fig. S1). Monophyly of the *C. chilensis* complex was maximally supported; within the complex, the three haplotype clusters of the network analysis appeared as weakly to well-supported clades. None of the three nominal species was reciprocally monophyletic because sequences of every species occurred in a weakly supported clade corresponding to haplotype cluster B. This clade was with high support sister to another clade corresponding to haplotype cluster C, and a clade containing only sequences of *C. petersi* (= haplotype cluster A) was the successive sister.

Uncorrected *p* distances (Table 2) among the nominal species ranged from 0.40% between *C. chilensis* and *C. donosobarrosi* over 1.05% between *C. donosobarrosi* and *C. petersi* to 1.21% between *C. chilensis* and *C. petersi*. Sequence divergences within each species ranged from 0.03% (*C. donosobarrosi*) over 0.37% (*C. petersi*) to 0.45% (*C. chilensis*), reflecting that in *C. donosobarrosi* only haplotypes of a single cluster were detected, whereas *C. petersi* and *C. chilensis* had haplotypes of two distinct clusters. When sequences of *C. petersi* and *C. chilensis* were lumped, as suggested by the ΔK method for microsatellites (see below), the difference to *C. donosobarrosi* (0.85%) resembled the divergence within *C. petersi* + *C. chilensis* (0.75%). Sequence divergences among the three haplotype clusters (being incongruent with any species) ranged from 0.74% to 1.37%, and within the clusters from 0.06% to 0.10% (Table 2). Haplotype (*h*) and nucleotide diversities (π) are summarized in Table 1.

For three clusters corresponding to the nominal species, the AMOVA revealed an F_{ST} value across clusters of 0.762 ($P < 0.0001$), indicating that the majority of variation (76.2%) occurs among clusters and 23.8% of variation within clusters. For two clusters (*C. petersi* and *C. chilensis* lumped), an F_{ST} value across clusters of 0.535 ($P < 0.0001$) suggests that 53.5% of variation occurs among clusters and 46.5% within clusters, reflecting that the two clusters possess a higher number of shared haplotypes. The same pattern is echoed by pairwise F_{ST} values (Table 3).

To explore demographic events, Tajima's *D* and Fu's F_S values were computed and mismatch distribution analyses were performed using two and three clusters. For *C. donosobarrosi*, the unimodal distribution of pairwise nucleotide differences was consistent with the expectation for population expansion (Fig. 3), whereas the multimodal distributions for the other clusters suggested stationary, non-expanding populations. Also, the significantly negative *D* value and the negative, albeit non-significant, F_S value suggested population expansion (Table 4). Among the other clusters, only for *C. petersi* moderate support for population expansion was obtained by the non-significant goodness-of-fit test and the negative Tajima's *D* value. No evidence for population expansion was found for *C. chilensis* and the cluster embracing *C. chilensis* and *C. petersi*.

Table 1 Genetic parameters within the *Chelonoidis chilensis* complex for clusters identified by STRUCTURE using mitochondrial and microsatellite data

	Cytochrome *b* (mtDNA)				Microsatellites (nDNA)						
Cluster	*n*	*h*	π	Haplotypes	*n*	A_{ob}	A_R	H_O	H_E	PA	PAf
K = 3											
C. petersi	16	0.742	0.00367	6 (4)	16	10.0	6.625	0.731	0.739	54	0.031–0.281
C. chilensis	7	0.810	0.00451	4 (3)	7	4.5	4.500	0.571	0.588	8	0.071–0.429
C. donosobarrosi	22	0.255	0.00031	3 (1)	21	4.6	3.546	0.483	0.505	10	0.024–0.286
Total	45	–	–	10	44	–	–	–	–	72	–
Mean/overall	–	0.602	0.00283	–	–	6.4	4.890	0.595	0.611	–	–
K = 2											
C. petersi + *C. chilensis*	23	0.858	0.00748	9 (7)	23	11.0	10.470	0.682	0.726	74	0.022–0.217
C. donosobarrosi	22	0.255	0.00031	3 (1)	21	4.6	4.571	0.483	0.505	10	0.024–0.286
Total	45	–	–	10	44	–	–	–	–	84	–
Mean/overall	–	0.556	0.00389	–	–	7.8	7.520	0.582	0.616	–	–

n, sample size; *h*, haplotype diversity; π, nucleotide diversity; number of private haplotypes in brackets after total number of haplotypes; A_{ob}, mean observed number of alleles per locus; A_R, allelic richness; H_O, observed heterozygosity; H_E, unbiased expected heterozygosity; PA, number of private alleles per cluster; PAf, private allele frequency range.

Table 2 Mean uncorrected *p* distances (percentages) between and within mitochondrial DNA sequences (1040 bp, cyt *b*) of the three nominal *Chelonoidis* species (top), the two clusters corresponding to *C. petersi* + *C. chilensis* and *C. donosobarrosi* (centre) and the three incongruent haplotype clusters (bottom). Below the diagonal, divergences between groups; on the diagonal, within group divergences in boldface. In brackets, standard error estimates

	n	1 *C. petersi*	2 *C. chilensis*	3 *C. donosobarrosi*
1 *C. petersi*	16	**0.37** (**0.09**)		
2 *C. chilensis*	7	1.21 (0.27)	**0.45** (**0.15**)	
3 *C. donosobarrosi*	22	1.05 (0.27)	0.40 (0.13)	**0.03** (**0.02**)
		1 + 2	3 *C. donosobarrosi*	
1 + 2	23	**0.75** (**0.16**)		
3 *C. donosobarrosi*	22	0.85 (0.20)	**0.03** (**0.02**)	
		A	B	C
A	14	**0.10** (**0.05**)		
B	28	1.20 (0.03)	**0.07** (**0.03**)	
C	3	1.37 (0.33)	0.74 (0.25)	**0.06** (**0.06**)

Table 3 Pairwise F_{ST} values within the *Chelonoidis chilensis* complex using cyt *b* sequences (mtDNA; below diagonal) and 10 microsatellite loci (above diagonal). All values are significantly different from zero

K = 3	1 *C. petersi*	2 *C. chilensis*	3 *C. donosobarrosi*
1 *C. petersi*	–	0.097	0.209
2 *C. chilensis*	0.674	–	0.156
3 *C. donosobarrosi*	0.832	0.588	–
K = 2	1 + 2	3 *C. donosobarrosi*	
1 + 2	–	0.164	
3 *C. donosobarrosi*	0.535	–	

Microsatellites

In all three STRUCTURE runs with upper bounds of 5, 10 and 20, the highest mean posterior probabilities suggested three as optimal cluster number (*K* = 3), whereas the ΔK method favoured only *K* = 2. Accordingly, barplots showing population structuring and individual admixture are presented for both *K*s (Fig. 4). With *K* = 3, tortoises from the north (*Chelonoidis petersi*), centre (*C. chilensis*) and south (*C. donosobarrosi*) corresponded each to a distinct cluster, with only weak indication of individual admixture in the cluster from the central range. With *K* = 2, the clusters from the northern and central range were lumped together, with results being unchanged otherwise. However, low pairwise F_{ST} values suggested only weak differences among all clusters (Table 3).

None of the studied loci differed significantly from Hardy–Weinberg expectations. In addition, no significant linkage disequilibrium between any pair of loci was detected; neither for any cluster nor across clusters after Bonferroni correction. From a total of 120 alleles, 72 private ones were found for three clusters and 84 for two clusters, each at low frequency (Table 1). Null alleles were not detected. The highest number of private alleles was found in the northernmost cluster corresponding to *C. petersi* (*K* = 3) or in the cluster embracing *C. petersi* + *C. chilensis* (*K* = 2). The southern cluster, corresponding to *C. donosobarrosi* and represented by the largest sample size, had only few private alleles. Allelic richness (A_R) for three clusters ranged from 3.546 to 6.625 (mean: 4.890) and for two clusters from 4.571 to 10.470 (mean: 7.520). Observed allelic diversity

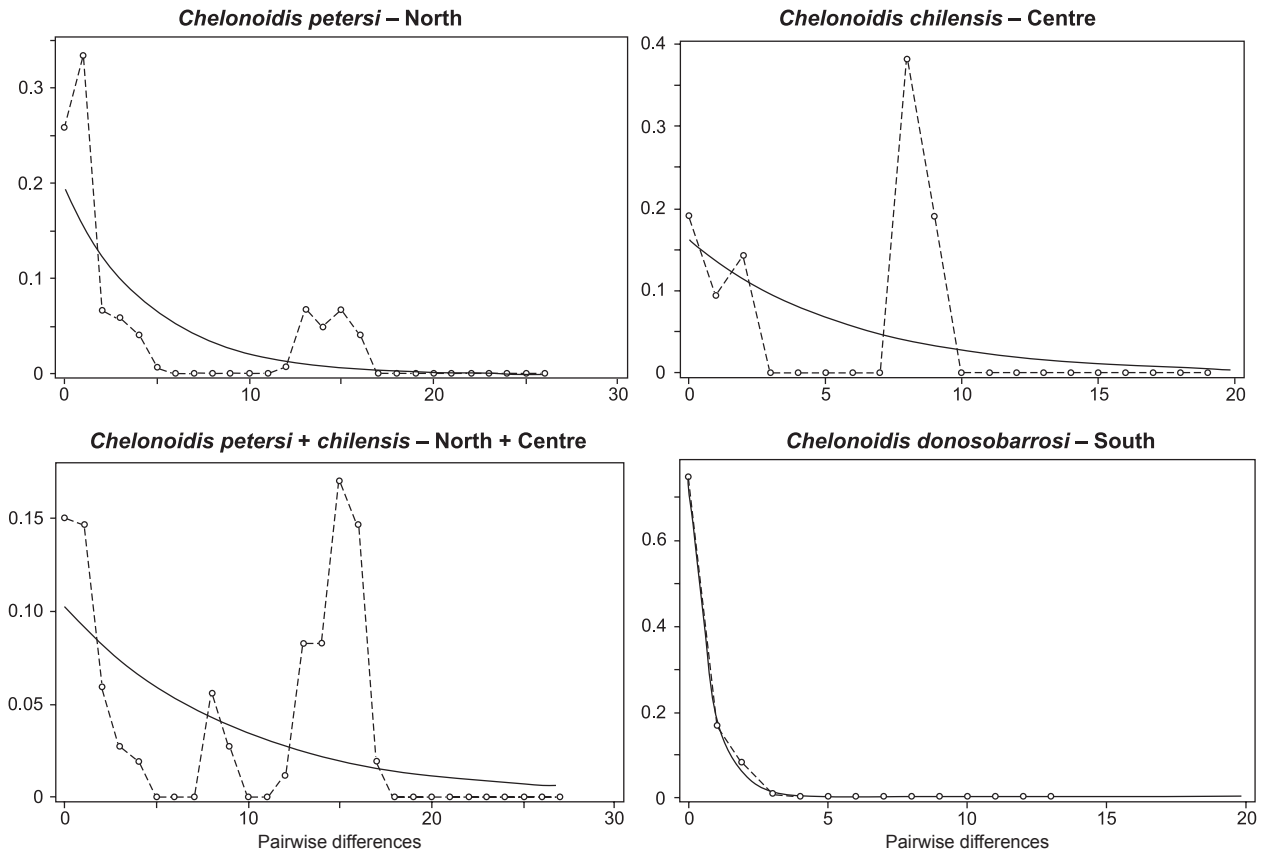

Fig. 3 Pairwise mismatch distributions for the *Chelonoidis chilensis* complex. Solid lines represent the expected frequencies of pairwise differences among haplotypes for demographic expansion; dotted lines, observed values. Only the mismatch distribution for *C. donosobarrosi* suggests population expansion.

Table 4 Using cyt *b* sequence data, mismatch distribution models, Tajima's *D* and Fu's F_S detect population expansion only in *Chelonoidis donosobarrosi*. The model of population expansion is significantly supported by a goodness-of-fit test (Rogers & Harpending 1992; Schneider & Excoffier 1999), Tajima's *D* (Tajima 1989) and Fu's F_S values (Fu 1997)

	C. petersi	*C. chilensis*	*C. chilensis* + *C. petersi*	*C. donosobarrosi*
Model parameters				
n	16	7	23	22
S	17	10	23	3
τ	0.434 (0–3.559)	9.633 (0.072–98.633)	16.498 (0.867–31.127)	3 (0.355–3.500)
θ_0	1.067 (0–2.210)	0.002 (0–16.310)	0 (0–13.846)	0 (0–0.007)
θ_1	99 999 (4.234–99 999)	16.910 (5.934–99 999)	17.615 (9.539–99 999)	0.338 (0.000–99 999)
Goodness-of-fit test				
SSD	0.03339	0.14459	0.06290	0.00180
P	0.25	0.03*	0.02*	0.44
Tajima's *D*	−0.72755	1.39511	1.37433	−1.47087
P	0.28	0.93	0.93	0.05*
Fu's F_S	1.38214	1.79170	2.41353	−0.77942
P	0.76700	0.80500	0.84800	0.18900

n, sample size; *S*, number of segregating sites. Parameters of the model of population expansion: τ, age of expansion; θ_0, population size before expansion and θ_1, population size after expansion. When sum of square deviation (SSD) values in the goodness-of-fit test for the mismatch distribution are non-significant ($P > 0.05$), the data do not deviate from the expectation of population expansion. Negative significant *D* and F_S values are expected when population expansion occurs. Significant values asterisked. The number of bootstrap replicates in the mismatch test was 1000.

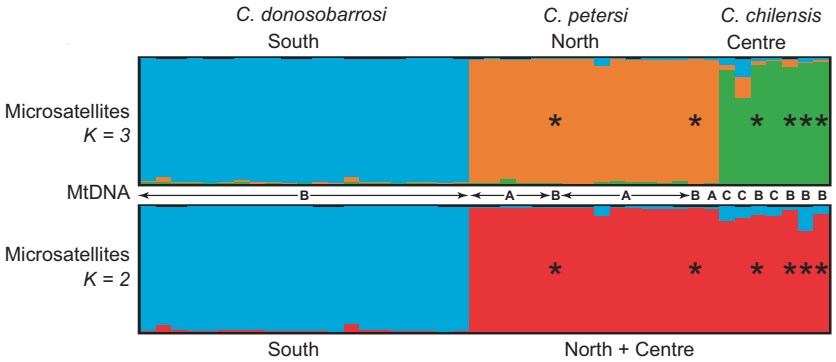

Fig. 4 Estimated population structure of the *Chelonoidis chilensis* complex for $K = 3$ (top) and $K = 2$ (bottom) from the STRUCTURE runs with the highest probability values. Distinct clusters are colour-coded. Within each cluster, an individual tortoise corresponds to a vertical segment that reflects its inferred ancestry. Asterisks indicate tortoises from the northern and central part of the distribution range with mitochondrial haplotypes of cluster B. Tortoises from the south (*C. donosobarrosi*) harbour only haplotypes of cluster B (see also the line indicating the mitochondrial haplotypes of each tortoise).

(A_{ob}) ranged from 4.5 to 10.0 (mean: 6.4, $K = 3$) and from 4.6 to 11.0 (mean: 7.8, $K = 2$; Table 1). Expected heterozygosity (H_E) was similar for three and two clusters with means of 0.611 and 0.616, respectively. All of these parameters reflected the same pattern as the private alleles, with the lowest values consistently occurring in the southern cluster (*C. donosobarrosi*). However, according to the test for heterozygosity excess, there is no evidence for a bottleneck ($P = 0.9981$), suggesting that the observed low diversity in the south has another reason.

For three clusters, the AMOVA revealed an F_{ST} value across populations of 0.175 ($P < 0.0001$), indicating that 17.5% of variation occurs among populations and 82.5% within populations. This figure was also confirmed for two clusters, with a similar F_{ST} value of 0.164 ($P < 0.0001$).

Discussion

Until today, the number of species in the *Chelonoidis chilensis* complex is debated, with some authors (Freiberg 1973, 1981; Cei 1986) recognizing three species, and other authors two (Fernández 1988; Buskirk 1993; Cabrera 1998; Richard 1999; Rhodin *et al.* 2010) or only a single species (Ernst & Barbour 1989; Ernst *et al.* 2000; Fritz & Havaš 2007).

Uncorrected *p* distances of the mitochondrial cyt *b* gene have repeatedly been used as a tool for species delineation among turtle and tortoises. Between other congeneric chelonian species, uncorrected *p* distances range from 1.5% to 18.3% (see the review in Vargas-Ramírez *et al.* 2010b and Praschag *et al.* 2011; Stuckas & Fritz 2011; Fritz *et al.* 2012). For other tortoise species in particular (family Testudinidae), the following average values were observed: between the sister species *C. carbonaria* and *C. denticulata*, 8.7% (Vargas-Ramírez *et al.* 2010a); among the five species of the Western Palaearctic genus *Testudo*, 6.9% to 12.7% (Fritz *et al.* 2007); between *Aldabrachelys gigantea* and the extinct *A. grandidieri*, 5.8% (Austin *et al.* 2003); and among the three *Indotestudo* species, 3.7% to 5.9% (Iverson *et al.* 2001). By contrast, within the African tortoise species *Chersina angulata* and *Homopus signatus*, sequence divergences of up to 2.8% and 2.5% were reported, respectively (Daniels *et al.* 2007, 2010), and within *A. gigantea*, 0.9% (Austin *et al.* 2003). Within *Chelonoidis carbonaria* and *C. denticulata*, two species congeneric with the studied *C. chilensis* complex, average values of 2.0% and 0.9% occurred (Vargas-Ramírez *et al.* 2010a); within *Testudo graeca*, 3.4% (Fritz *et al.* 2007); and within *T. horsfieldii*, 1.1% (Fritz *et al.* 2009).

When these figures are compared to the values within the *C. chilensis* complex (Table 2), it is clear that the sequence divergences among the three putative species or the three incongruent haplotype clusters (maximum average values of 1.21% and 1.37%, respectively) fall into the range as observed within, but not among, other testudinid species. Nevertheless, species delineations should not be based on rigid thresholds alone, and obtaining additional evidence is advisable (compare Vieites *et al.* 2009; Padial *et al.* 2010; Vargas-Ramírez *et al.* 2010b; Praschag *et al.* 2011; Stuckas & Fritz 2011; Fritz *et al.* 2012). In fact, the sympatric occurrence of mitochondrial haplotypes of clusters A and B or B and C (Fig. 1; Table S1), respectively, suggests that distinct, recently split species could be involved, even though Freiberg (1973) claimed that only *C. petersi* may occur sympatrically with *C. chilensis*, while *C. donosobarrosi* was described as being allopatric. However, Richard (1999) mentioned some syntopic records for *C. chilensis* and *C. donosobarrosi*. These contradictory statements underline that morphological differentiation of the concerned taxa is difficult (see also Fernández 1988; Ernst & Barbour 1989; Buskirk 1993; Cabrera 1998; Richard 1999; Ernst *et al.* 2000).

The geographical distributions of clusters A and C match more or less the range of *C. petersi* and *C. chilensis*, respectively; haplotypes of cluster B occur there together with haplotypes of clusters A and C. In the putative range of *C. donosobarrosi*, only haplotypes of cluster B are present (Fig. 1). If the tortoises harbouring distinct mitochondrial haplotypes would represent distinct species, it were expected that each species is represented by a highly distinct microsatellite cluster, also in the case of sympatric tortoises. However, our STRUCTURE analyses indicate that the tortoises of each of the three sampling regions correspond to only one population each ($K = 3$) or that the tortoises from the north and centre of the range correspond to another cluster than the tortoises from the south ($K = 2$; Fig. 4), irrespective of their mitochondrial haplotype. Moreover, the pairwise F_{ST} values of the microsatellite clusters are low ($K = 3$: 0.097–0.209; $K = 2$: 0.164; Table 3) and fall into the range as observed, for instance, among eight populations of another tortoise species, *Gopherus polyphemus*, from southern Georgia and Florida (pairwise F_{ST} values based on 9 microsatellite loci: 0.060–0.511; Schwartz & Karl 2005). Consequently, the hypothesis of sympatrically (or allopatrically) occurring distinct species can be rejected, and the observed mitochondrial sequence variation must have another reason.

In the northern hemisphere, a well-known and common phylogeographic pattern is the so-called southern richness and northern purity (Hewitt 2000), with southern populations harbouring much greater genetic diversity than their northern conspecifics. This pattern is caused by repeated range contractions and northern extinctions during the Ice Age. In thermophilic species, only southern populations survived the cold periods in glacial refugia, preserving there ancient genetic diversity. By contrast, formerly glaciated or otherwise inhospitable regions in the north were colonized only in the Holocene, often via long-range dispersal out of few refugia. Fostered by founder effects, this resulted in decreasing genetic variability with increasing distance from the respective refugia (Hewitt 1996). The phylogeographic structure of the *C. chilensis* complex reverses this geographical pattern, in agreement with the climatic conditions of the southern hemisphere. In the populations from the north-central part of its distribution range, the whole array of mitochondrial haplotypes and much higher haplotype and nucleotide diversities are present, while in the southernmost part of the range only three haplotypes were found that differ by a maximum of only three mutation steps (Figs 1 and 2; Table 1). Also microsatellite variation confirms decreasing variation from north to south (Table 1). Besides the Patagonian frog *Eupsophus calcaratus* (Nuñez *et al.* 2011), the *C. chilensis* complex is only the second South American case corresponding to this phylogeographic pattern of 'northern richness and southern purity' (compare the reviews in Hewitt 2004a,b, 2011; Sérsic *et al.* 2011). However, we predict it will be discovered in further wide-ranging species.

Generally, the climate in Patagonia was tundra-like during Quaternary glacial periods (Ruzzante & Rabassa 2011). During the Last Glacial Maximum, temperatures in northern Patagonia decreased by 5–6 °C (Clapperton 1993; Rabassa *et al.* 2011), rendering the range of *C. donosobarrosi* uninhabitable for tortoises, whereas less temperature-dependent reptile species, like the cold-tolerant viviparous lizards of the genus *Liolaemus*, survived Pleistocene cold periods there (Sérsic *et al.* 2011). Accordingly, it can be hypothesized that the extant southernmost tortoise populations result from a recent, Holocene, range expansion from the north and are not derived from a local remnant population. This is also supported by the signature of population expansion and lacking evidence for a bottleneck. However, when the great distance of about 1000 km between the centre and the south of the range, the low reproduction rate (Freiberg 1981) and the well-known low dispersal capacity of tortoises are considered, a peculiar dispersal mode must have enabled such rapid range expansion to the south. The few mitochondrial haplotypes present in the southernmost part of the range suggest no gradual or stepping stone dispersal (compare Hewitt 1996), but long-distance dispersal, a dispersal mode at first glance unlikely for a tortoise (even though there are several instances of evident trans-oceanic dispersal of tortoises: Caccone *et al.* 1999; Gerlach *et al.* 2006; Le *et al.* 2006). However, for the northern hemisphere, a parallel case exists. There, the rapid Holocene range expansion of the European pond turtle (*Emys orbicularis*) was facilitated by rafting on rivers debouching in the North and Baltic Seas (Sommer *et al.* 2007), and a similar scenario could explain the situation in the South American tortoises as well. During the transition from glacial to interglacial periods, the Patagonian rivers were very large and powerful, being fed by melting Andean glaciers, with water discharges up to ten times higher than at present (Martínez & Kutschker 2011). Taking this into account, it seems plausible that the southernmost populations of *Chelonoidis* could have been founded by tortoises rafting on the north-south flowing Desaguadero River, which was in the earlier Holocene much more water-rich than today.

Returning to diversity within the *C. chilensis* complex, Ernst & Barbour (1989) proposed that the morphological characters used by Freiberg (1973) to diagnose his new species *C. donosobarrosi* and *C. petersi* represent merely a morphological cline, with small-bodied northern tortoises (*C. petersi*) and large-bodied southern tortoises (*C. donosobarrosi*). This view is also supported by the observation that

morphological variation in turtles and tortoises generally follows Bergmann's rule, that is, size increases within species with latitude (Ashton & Feldman 2003). Moreover, a high degree of phenotypic plasticity has been described for some other tortoise species (*Aldabrachelys gigantea*: Austin *et al.* 2003; *Homopus signatus*: Daniels *et al.* 2010; *Stigmochelys pardalis*: Fritz *et al.* 2010; *Testudo graeca*: Fritz *et al.* 2007; *T. marginata*: Fritz *et al.* 2005; *T. horsfieldii*: Fritz *et al.* 2009; *T. kleinmanni*: Attum *et al.* 2007; Široký & Fritz 2007), among them two species in which body size variation resembles the extremes as observed in the *C. chilensis* complex (*S. pardalis*, *T. marginata*). This suggests that the morphological variation within the *C. chilensis* complex follows a general pattern.

Conclusions

Genetic variation in the South American *Chelonoidis chilensis* complex supports the existence of only one species, with *C. donosobarrosi* (Freiberg, 1973) and *C. petersi* (Freiberg, 1973) being junior synonyms of *C. chilensis* (Gray, 1870). The observed phylogeographic differentiation inverses the well-known pattern of southern genetic richness and northern purity from the northern hemisphere, resulting from dispersal from glacial refugia. This suggests that at higher latitudes of both hemispheres genetic diversity may decrease with increasing distance from the refugial area. For *C. chilensis* sensu lato, it seems likely that long-distance dispersal via rafting on the Desaguadero River led to the foundation of the southernmost populations in northern Patagonia during the Holocene. The size difference observed between *C. chilensis* from the northernmost and southernmost localities confirms that clinal size variation in chelonians follows Bergmann's rule and that phenotypic plasticity plays a major role for shaping external morphology of tortoises.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Maximum Likelihood tree for cyt *b* sequences of the *Chelonoidis chilensis* complex and other *Chelonoidis* species, rooted with *Testudo graeca*.

Table S1 Samples of the *Chelonoidis chilensis* complex and GenBank accession numbers of their mitochondrial haplotypes.

Table S2 Cross-amplified microsatellite loci in the *Chelonoidis chilensis* complex.

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