Org Divers Evol (2010) 10:161–172
DOI 10.1007/s13127-010-0016-0

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Red- and yellow-footed tortoises, *Chelonoidis carbonaria* and *C. denticulata* (Reptilia: Testudines: Testudinidae), in South American savannahs and forests: do their phylogeographies reflect distinct habitats?

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Received: 31 July 2008 / Accepted: 6 May 2009 / Published online: 30 March 2010

Abstract Using sequence data of the mitochondrial cytochrome *b* gene, we investigated phylogeographic differentiation of the Amazonian tortoise species *Chelonoidis carbonaria* and *C. denticulata*. While *C. carbonaria* is generally restricted to savannah habitats and adjacent forests, *C. denticulata* is associated with wet tropical and subtropical forests. Our study suggests a correlation between distinct habitat preferences and phylogeography of the two species. In Maximum Parsimony, Maximum Likelihood and Bayesian analyses, haplotypes of *C. carbonaria* cluster in several distinct clades reflecting the species' patchy distribution in savannah habitats. By contrast, haplotypes of *C. denticulata* are only weakly differentiated; a finding also confirmed by parsimony network analysis. This suggests that the contiguous Amazonian rainforest allows gene flow between populations of the forest-dwelling *C. denticulata* throughout the range, but significantly impedes gene flow in *C. carbonaria*. The phylogeographic structure and extant distribution pattern of *C. carbonaria* is supportive of former Amazonian rainforest fragmentation, enabling the dispersal of savannah species. Based on fossil calibration, we dated divergence times for the *C. carbonaria* clades using a relaxed molecular clock, resulting in average estimates ranging from 4.0–2.2 mya. This implies that the onset of rainforest fragmentation could predate the Pleistocene considerably. Furthermore, our findings call for further research on geographic and taxonomic variation in *C. carbonaria* and for a reassessment of the conservation status of the distinct genetic units.

Keywords Phylogeography · Cytochrome *b* gene · Testudinidae · Rainforest · Forest refugia hypothesis

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Introduction

Phylogeographic investigations can provide valuable insights about historical and geographic factors shaping genetic diversification across a species' range and improve our understanding of shifting environments over time (Avise 2000; Haig et al. 2004; Miller et al. 2006; Ripplinger and Wagner 2004). For Amazonian forest species, genetic evaluations and dating of their divergence have been used for inferring the biogeographic history of the Amazonian rainforest (Moritz et al. 2000), while non-forest species have been relatively neglected (Pennington et al. 2000, 2004). However, such taxa can potentially provide more information about historical rainforest fragmentation than rainforest species (Pennington et al. 2004).

Red-footed and yellow-footed tortoises, *Chelonoidis carbonaria* (Spix, 1824) and *C. denticulata* (L., 1766), with mainly Amazonian distributions, seem to be ideally suited models for gaining additional insights into the Amazonian history. *Chelonoidis carbonaria* occurs primarily in savannah habitats and adjacent forest areas, whereas *C. denticulata* is associated with wet tropical and subtropical forests (Pritchard and Trebbau 1984). Both *C. carbonaria* and *C.*

denticulata are large-sized tortoise species, reaching maximum shell lengths of approximately 50 cm and 80 cm, respectively (Ernst et al. 2000). However, most adult *C. denticulata* are below 45 cm (Pritchard and Trebbau 1984). A further, smaller species of the genus *Chelonoidis*, *C. chilensis* (Gray, 1870), is more or less confined to the dry Chaco of Argentina, Bolivia, and Paraguay (Ernst et al. 2000). Together with the giant tortoises of the *C. nigra* (Quoy & Gaimard, 1824) complex from the Galápagos Islands, *C. chilensis* represents the sister group of a clade comprising *C. carbonaria* and *C. denticulata* (Caccone et al. 1999; Fritz and Bininda-Emonds 2007; Le et al. 2006).

Phylogeographic studies of *C. carbonaria* and *C. denticulata* have not been undertaken until now. As the two species occupy complementary ecological niches, an investigation comparing their phylogeographies is promising for a better understanding of the history of the Amazonian rainforest. Therefore, here we use sequence variation in the mitochondrial cytochrome *b* (cyt *b*) gene, a phylogeographically highly informative marker in chelonians (e.g. Caccone et al. 1999; Fritz et al. 2005, 2006, 2007, 2008, 2009a, b; Lenk et al. 1999), to examine *C. carbonaria* and *C. denticulata*. Furthermore, based on relaxed molecular clock analyses, we estimate for the savannah species *C. carbonaria* the approximate dates of population divergence.

Materials and methods

Laboratory techniques

The complete cyt *b* gene was sequenced in 34 known-locality samples of *Chelonoidis carbonaria* and six known-locality samples of *C. denticulata* (Fig. 1; Appendix 1). Blood or tissue samples were preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol and kept at −20°C until processing. Frozen blood samples from Colombian tortoises are permanently housed in the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá; DNA of the Colombian specimens and DNA, tissue, or blood samples from the tortoises from French Guiana and Paraguay are stored at −80°C in the tissue sample collection of the Museum of Zoology, Dresden. Total genomic DNA was extracted by overnight incubation at 37°C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, 1% SDS) including 1 mg of proteinase K (Merck), followed by the standard phenol/chloroform protein extraction. DNA was precipitated from the supernatant with 0.8 volumes of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Using polymerase chain reaction (PCR), two mitochondrial DNA fragments were amplified, corresponding to the cyt *b* gene (primers: mt-c-For2, mt-f-na, mt-E-Rev2, mt-a-neu3; Fritz et al. 2006; Praschag et al. 2007). PCR was performed in a 50 μL volume (Bioron PCR buffer or 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of Taq DNA polymerase (Bioron), 10 pmol dNTPs (Fermentas) and 5 or 10 pmol of the respective primer. The thermo-cycling conditions were: Initial denaturing for 5 min at 94°C, 35–40 cycles with denaturing for 45 s at 94°C, annealing for 52 s at 50–60°C, and primer extension for 80 s at 72°C, followed by a final elongation of 10 min at 72°C. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30 μL), 1 volume 4 M NH_4Ac (30 μL) and 12 volumes EtOH (100%; 360 μL). DNA was pelleted by centrifugation (15 min at 13,000 rpm). The pellet was washed with 70% ethanol, then dissolved in 20 μL H_2O. PCR products were sequenced directly on both strands on an ABI 3130 (Applied Biosystems) sequencer using the primers mt-c-For2 and mt-E-Rev2. For GenBank accession numbers, see Appendix 1.

Data analyses

Cyt *b* sequences for 41 Brazilian specimens each of *C. carbonaria* and *C. denticulata* were downloaded from GenBank and aligned with our sequences in BIOEDIT 7.0.5.2 (Hall 1999). Also all other cyt *b* sequences of *C. carbonaria* and *C. denticulata* available from GenBank were included, although their geographic provenance is unknown (Appendix 1). This was done to test how variation in these sequences, which could originate from other regions, corresponds to our known-locality samples. Using TCS 1.21 (Clement et al. 2000), all sequences were collapsed into haplotypes, resulting in 30 haplotypes for *C. carbonaria* and 15 for *C. denticulata* (Appendix 1). Most sequences downloaded from GenBank were much shorter than the ones obtained in this study (392–430 bp vs. 1179 bp).

Phylogenetic relationships of haplotypes were inferred with Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian analyses (BA) using an 1179-bp-long alignment. Previously published sequences for the other two species of the genus *Chelonoidis*, *C. chilensis* and *C. nigra*, were included in the analyses. *Manouria impressa* (Günther, 1882), *Pyxis arachnoides* Bell, 1827 (Testudines: Testudinidae) and *Rhinoclemmys melanosterna* (Gray, 1861) (Testudines: Geoemydidae) served as outgroups (Appendix 1). MP and ML analyses were performed in PAUP* 4.0b10 (Swofford 2002) with heuristic searches with 100 random addition sequences using the tree bisection-reconnection branch swapping option; bootstrap support values were calculated for MP with 1000 and for ML with 100 replicates. Under MP, 824 of 1179 characters were constant; 128 variable characters were parsimony-uninformative and 227 variable characters were

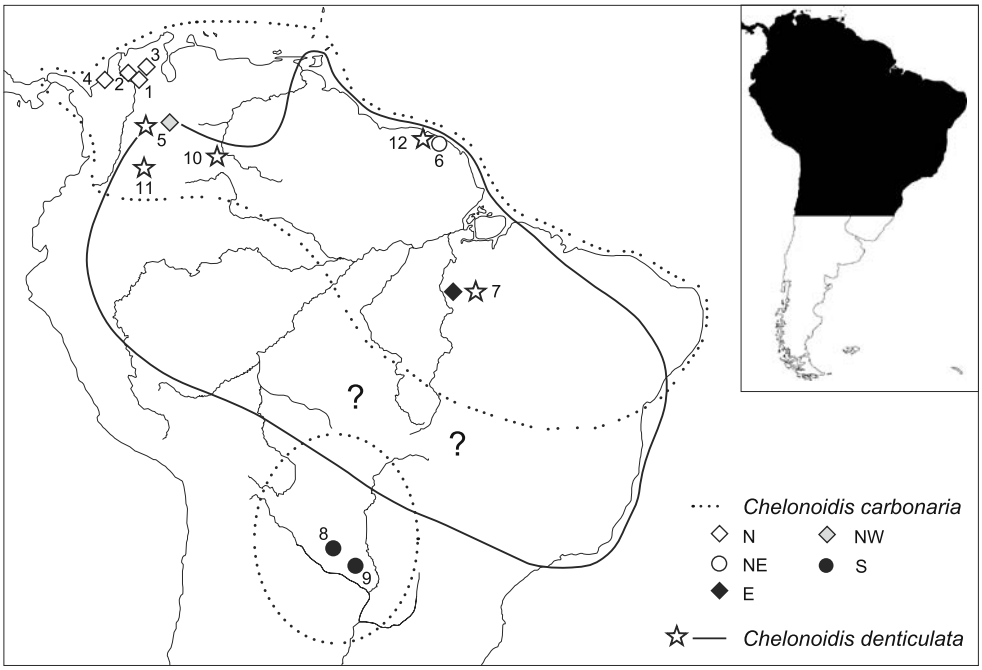

Fig. 1 Approximate ranges of *Chelonoidis carbonaria* and *C. denticulata* (modified from Iverson 1992) and geographic distribution of haplotypes. Locality numbers refer to Appendix 1. Question marks indicate that the southern part of the range of *C. carbonaria* might be connected with the northern part. Symbols for *C. carbonaria* correspond to haplotype clades (Fig. 2); upper-case letters indicate geographic origin within the range (N, north; NE, northeast; etc.)

parsimony-informative. The optimal model of sequence evolution (K81uF+G) was established in MODELTEST 3.06 (Posada and Crandall 1998). BA was run using MrBAYES 3.1 (Ronquist and Huelsenbeck 2003) with four Markov chains of 10^7 generations and every 1000th generation being saved. The burn-in was set to sample only the plateau of most likely trees. The remaining trees were used for generating a 50% majority rule consensus tree. The posterior probability of any individual clade in this consensus tree corresponds to the percentage of all trees containing that clade, thus is a measure of clade frequency and credibility.

Within the same species or between closely related species, ancestral haplotypes may persist and relationships of haplotypes are likely to be reticulate, which is why gene evolution may be only imperfectly reflected by dichotomous trees (Posada and Crandall 2001). To explore this ambiguity, parsimony networks were constructed with TCS 1.21. The networks were built using alignments of 420 bp, corresponding to the lengths of the shorter GenBank sequences. In addition, uncorrected *p* distances of haplotypes were calculated in MEGA 4.0 (Tamura et al. 2007).

To evaluate the impact of geographic distances on genetic variation in *C. carbonaria*, a Mantel test was performed with the software IBD (Isolation-by-Distance; Bohonak 2002) using uncorrected *p* distances of known-locality sequences and geographic distances (km). To test also for the influence of long-term historical processes, a categorical matrix was produced that reflected phylogenetic association of haplotype clades. The haplotype clusters NE, NW and E, which the phylogenetic analyses placed in a superordinated clade, were coded as associated (1); the remaining haplotype clades were coded as not associated (0). This matrix was used in a second Mantel test to determine whether the phylogenetic association covaried with the genetic distances. Furthermore, partial Mantel tests (Legendre and Legendre 1998) were run, simultaneously evaluating the effects of long-term divergence and isolation-by-distance. Genetic variation shaped by isolation-by-distance is thought to occur on a much smaller time-scale than long-term historical isolation or deep-time coalescence (Telles and Diniz-Filho 2005).

Molecular dating

Divergence times of the known-locality clades of *C. carbonaria* were estimated by a Bayesian relaxed molecular clock approach (MULTIDISTRIBUTE package; Thorne et al. 1998; Thorne and Kishino 2002). As backbone served a Bayesian tree also including one representative of each remaining *Chelonoidis* species, rooted with *Rhinoclemmys melanosterna*. Fossil evidence was used for inferring minimum ages of two nodes within the *Chelonoidis* phylogeny. The age of the type stratum of *C. hesterna* (Auffenberg, 1971), the ancestral species of *C. carbonaria* and *C. denticulata* (Auffenberg 1971), served as prior constraint for the split between the latter two species. *Chelonoidis hesterna* originates from the Middle Miocene deposit of La Venta, Colombia (mean age 12.55 mya; Flynn and Wyss 1998). In addition, the estimate for the divergence between *C. chilensis* and *C. nigra* (6–12 mya) from Caccone et al. (1999) was used as constraint for this node.

In order to determine the appropriate nucleotide substitution model parameters, the data set was analyzed using the program PAML 3.13 (Yang 1997). Subsequently, the branch lengths and their variance-covariance matrix were estimated with the program ESTBRANCHES. Markov chains in the application MULTIDIVTIME were run with numsamps=1000000, sampfreq=1000 and a burn-in of 1×10^7 (three independent runs to test the stability of the result). The prior for the mean of the ingroup root age (rttm) was set to 12.55 mya with a standard deviation (rttmsd) of 1.10 mya, corresponding to the mean age of the type stratum of *C. hesterna*. Mean and standard deviation of the rate of molecular evolution at the ingroup root node (rtrate and rtratesd) were 0.001127 substitutions per site in 1 mya with 1 time unit=1 mya (calculated with the mean of the branch lengths from ESTBRANCHES); mean and standard deviation of the Brownian motion constant (brownmean and brownsd) were set to 0.08. Bigtime was set to 220 mya to match the age of the oldest known chelonian, *Odontochelys semitestacea*, described by Li et al. (2008).

Results

Phylogeny, intraspecific variation and geographic distribution of haplotypes

All phylogenetic analyses yielded similar results. Under MP, 7032 equally parsimonious solutions (616 steps; CI=0.6786, RI=0.8545) were found that differed mainly with respect to the allocation of short terminal branches within *Chelonoidis denticulata* and within the clades of *C. carbonaria*. While the branching pattern within *C. denticulata* received virtually no statistical support, *C. carbonaria* shows a clear structure, with several moderately to well-supported clades corresponding to well-delimited geographic regions. GenBank haplotypes of unknown provenance (U1–U4) cluster either within these clades or appear as their sister. The clade formed by haplotypes from Paraguay, the southernmost part of the range of *C. carbonaria*, constitutes the sister of all other haplotypes of this species (Fig. 2). In some trees, this clade is associated with haplotype U4 of unknown origin. The difference between the two species is obvious, in particular when it is considered that support values for the clades of *C. carbonaria* would increase if all sequences were of the same length as ours. The distinct pattern of the two species is also confirmed by parsimony network analyses of data sets of sequences trimmed to the lengths of the short GenBank sequences. Due to the short sequence lengths, several haplotypes were lumped together (Fig. 3). Nevertheless, each of the clades of *C. carbonaria* returned by phylogenetic analyses corresponds to a well-defined haplotype cluster, whereas within *C. denticulata* no clear structure occurs. Accordingly, the number of mutational steps is distinctly higher in *C. carbonaria*. This pattern is also reflected by uncorrected *p* distances (Table 1), as the within-group divergence in *C. carbonaria* is about twice that observed in *C. denticulata* (1.953% vs. 0.926%).

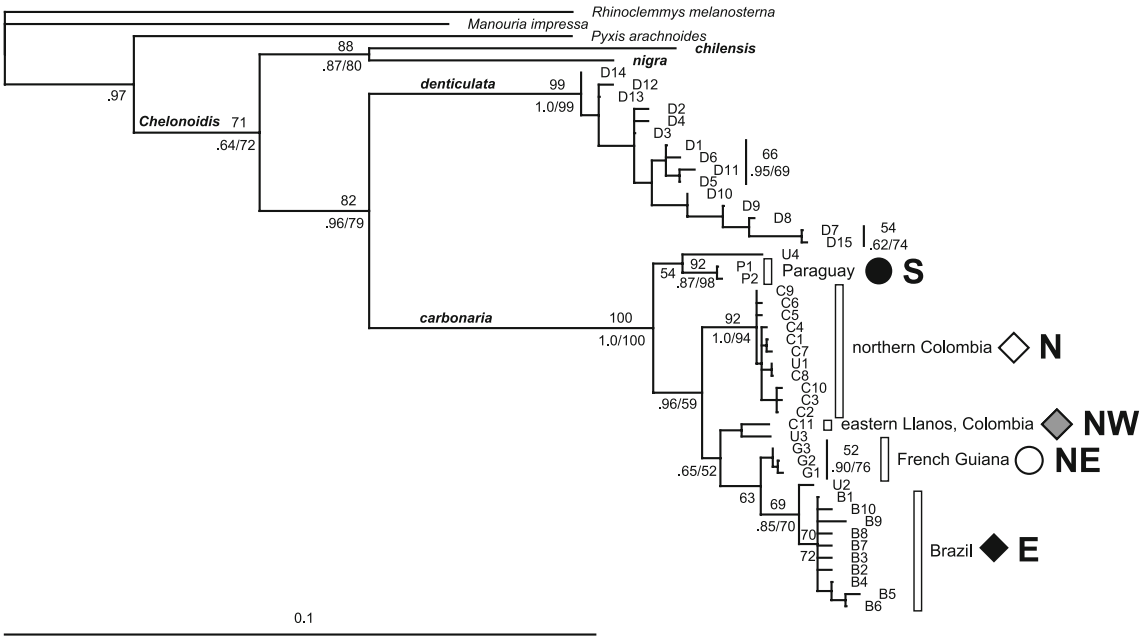

Fig. 2 Maximum Likelihood tree for haplotypes of *Chelonoidis carbonaria* and *C. denticulata*, including sequences of *C. chilensis* and *C. nigra*. Support values above branches are ML bootstrap values; below branches, Bayesian posterior probabilities and MP bootstrap values. The few nodes with higher support values within *C. denticulata* refer to very similar haplotypes (cf. Fig. 3). For haplotypes of *C. carbonaria*, geographic origin is indicated on the right; symbols correspond to Fig. 1

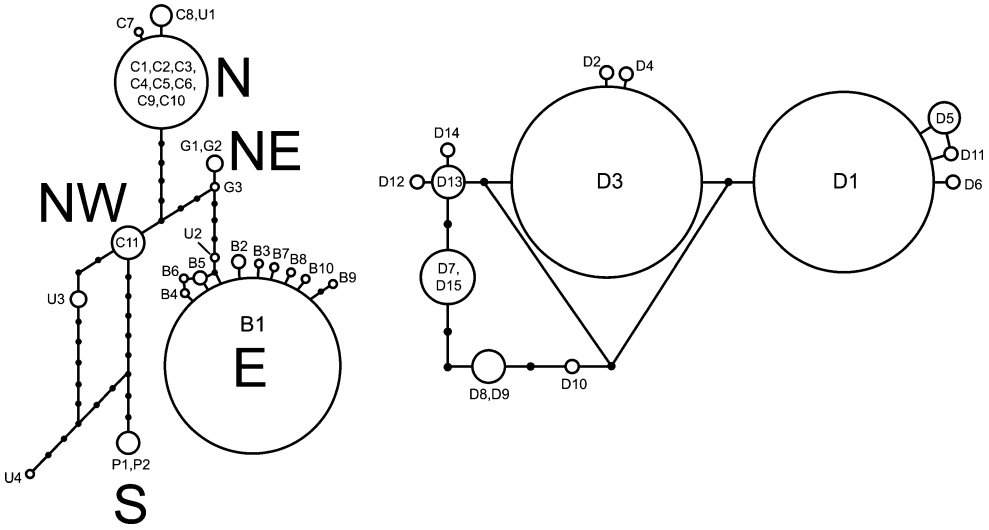

Fig. 3 Parsimony networks for mtDNA haplotypes of *Chelonoidis carbonaria* (left) and *C. denticulata* (right), based on alignments of 420 bp. Circle size approximately reflects haplotype frequency. Missing haplotypes are shown as small solid circles. Each line connecting haplotypes corresponds to one mutational step. Large upper-case letters indicate geographic origin of haplotypes within the range of *C. carbonaria* (N, north; NE, northeast; etc.). Haplotype frequencies for *C. carbonaria* are B1: n=30; (C1, C2, C3, C4, C5, C6, C9, C10): n=16; C11: n=6; (P1, P2): n=5; (C8, U1): n=4; (G1, G2): n=3; U3: n=3; B2: n=2; B5: n=2; other haplotypes: n=1. Haplotype frequencies for *C. denticulata* are D3: n=18; D1: n=17; (D7, D15): n=5; D5: n=3; (D8, D9): n=2; D13: n=2; other haplotypes: n=1. For further explanation, see text. In *C. carbonaria*, the haplotypes (P1, P2) and U4 are not connected with the others when a 95% threshold is used for the probability of parsimony, while in *C. denticulata* all haplotypes are connected. Connection of (P1, P2) and U4 can be enforced by lowering the threshold to 94%

Between the haplotype clusters of *C. carbonaria*, average values from 0.721% to 4.462% occur; the average difference between *C. carbonaria* and *C. denticulata* is 8.659%. For *C. carbonaria*, the Mantel tests revealed no correlation between genetic distances and geographic distances (Z=691.2, r=0.32, p=0.218), while a significant correlation between genetic distance and phylogenetic association was found (Z=0.22, r=0.60, p=0.029). This was also supported by the partial Mantel tests that found a significant correlation between genetic distance and phylogenetic association (r=0.558, p=0.022), but none between genetic and geographic distances (r=0.124, p=0.360). These results suggest that the divergence of the *C. carbonaria* clades was shaped by long-term historical processes rather than a microevolutionary process caused by isolation-by-distance.

Dating

Our relaxed molecular clock analyses suggest a divergence time between *C. carbonaria* and *C. denticulata* of 13.32 mya, closely resembling the mean age of the type-stratum of the fossil *C. hesterna* (12.55 mya) used as prior constraint for the minimum age of the split between the two extant species. *Chelonoidis chilensis* and *C. nigra* separated distinctly later (8.98 mya; Fig. 4). The branching event between (*C. carbonaria*+*C. denticulata*) and (*C. chilensis*+*C. nigra*) was estimated to 14.08 mya. Within *C. carbonaria*, the southern clade branched off 3.99 mya, followed by the northern clade (2.82 mya), and the split between the north-western, north-eastern, and eastern clades (2.19 mya). All average values for the splits between the *C. carbonaria* clades predate the onset of the Pleistocene, some of them considerably. Even when the 95% credibility intervals are considered, the youngest estimates for the southern clade terminate in the uppermost Pliocene (1.90 mya; Table 2), prior to the onset of the Pleistocene with its extensive glaciations.

Discussion

Despite incomplete locality sampling, our results provide evidence for a subdivision of *Chelonoidis carbonaria* in genetically distinct, geographically vicariant populations, while *C. denticulata* seems to represent a more or less homogenous species. This suggests a marked correlation between habitat preference and phylogeographic differentiation. We hypothesize that the contiguous Amazonian rainforest allows gene flow between populations of the forest-dwelling *C. denticulata* throughout the species' range. In contrast to *C. denticulata*, *C. carbonaria* prefers savannahs and open habitats, resulting in the observed genetic structure corresponding to its patchy distribution associated with such habitats.

To date, phylogeography has been examined in detail for only one open-habitat reptile that occurs north, south and within the Amazonian forests: the rattlesnake *Crotalus*

Table 1 Uncorrected *p* distances (percentages) within and between the *Chelonoidis* species and the haplotype clusters of *C. carbonaria*, based on a 420-bp-long alignment of cyt *b*

	n	*carbonaria*								*denticulata*	*chilensis*	*nigra*
		all	N	NW	NE	E	S	U3	U4			
carbonaria — all	20	**1.953 (0.371)**										
carbonaria N (northern Colombia)	3	–	**0.159 (0.157)**									
carbonaria NW (eastern Colombia)	1	–	1.508 (0.538)	–								
carbonaria NE (French Guiana)	2	–	2.103 (0.645)	1.071 (0.457)	**0.238 (0.227)**							
carbonaria E (Brazil)	11	–	3.155 (0.794)	2.361 (0.689)	1.527 (0.563)	**0.538 (0.157)**						
carbonaria S (Paraguay)	1	–	2.698 (0.784)	2.143 (0.699)	3.214 (0.808)	4.462 (0.993)	–					
carbonaria U3	1	–	2.003 (0.635)	0.721 (0.406)	1.803 (0.604)	1.399 (0.514)	2.885 (0.846)	–				
carbonaria U4	1	–	3.205 (0.823)	2.885 (0.800)	3.966 (0.892)	3.518 (0.802)	2.163 (0.723)	2.163 (0.661)	–			
denticulata	13	8.659 (1.145)	7.917 (1.197)	8.820 (1.280)	9.182 (1.269)	8.841 (1.214)	8.171 (1.288)	8.270 (1.230)	8.548 (1.309)	**0.926 (0.254)**		
chilensis	1	12.685 (1.479)	12.302 (1.565)	12.381 (1.604)	12.024 (1.603)	12.781 (1.588)	13.095 (1.618)	12.500 (1.613)	14.183 (1.711)	11.250 (1.403)	–	
nigra	1	10.324 (1.290)	9.444 (1.393)	10.000 (1.451)	10.119 (1.427)	10.571 (1.403)	10.238 (1.444)	10.096 (1.463)	11.298 (1.558)	7.896 (1.190)	7.143 (1.223)	–

n, number of haplotypes; see Fig. 3

On table diagonal, average within-group differences in bold type; below diagonal, average between-group differences

In brackets, standard error estimates (500 bootstrap replicates)

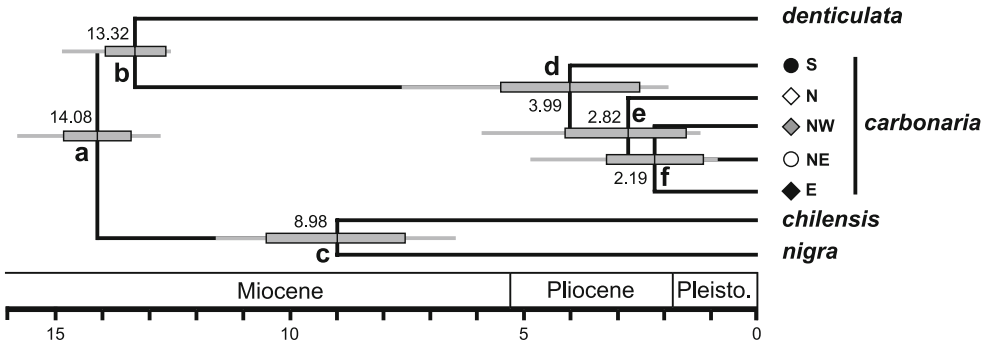

Fig. 4 Estimated divergence times for extant species of *Chelonoidis* and for clades of *C. carbonaria* (Bayesian tree). Grey lines correspond to credibility intervals, grey bars to one standard deviation above and below the mean divergence time. Upper-case letters in *C. carbonaria* indicate clades; symbols correspond to Figs. 1 and 2. Lower-case letters refer to nodes (see Table 2)

durissus (Quijada-Mascareñas et al. 2007; Wüster et al. 2005). These investigations suggest that the Amazonian rainforest was fragmented in the mid-Pleistocene, opening a central colonization corridor of dry forest or savannah habitat, and support that Pleistocene climatic and vegetation fluctuations played an important role in shaping the biodiversity of tropical South America. Basically, these studies fuel the ongoing debate over whether or not the so-called 'forest refugia hypothesis' is justified. According to this hypothesis, increasing aridity and cooling during the Pleistocene glaciations resulted in isolated rainforest patches being separated by savannahs and deserts (e.g. Haffer 1969, 1997; Hooghiemstra and van der Hammen 1998; Potts and Behrensmeyer 1992; Prance 1973). This hypothesis was widely accepted until a few years ago, but has been strongly criticized recently (e.g. Pennington et al. 2004), as follows: (1) Pollen cores show no evidence of reduced rainforest cover in the Amazon Basin (e.g. Colinvaux et al. 2001; Mayle et al. 2004); (2) dynamic vegetation model simulations reject the hypothesis of widespread savannahs in Amazonia during the last glacial maximum (e.g. Cowling et al. 2001); and (3) genetic data provide evidence that diversification in tropical rainforest animals generally predates the Pleistocene (Glor et al. 2001; Moritz et al. 2000).

Our data suggest that the extant diversity of *C. carbonaria* was shaped by dispersal enabled by retreating rainforest and subsequent vicariance caused by forest re-expansion, leading to population fragmentation in 'savannah islands'. This parallels the situation in *Crotalus durissus* (Quijada-Mascareñas et al. 2007; Wüster et al. 2005) and is consistent with significant past fluctuations in the distribution of Amazonian rainforest. However, according to our relaxed molecular clock calculations the onset of these events could considerably predate the Pleistocene – the separation of the southern clade of *C. carbonaria*, occurring south of the Amazon Basin, is estimated to date back to the early Pliocene (3.99 mya), followed by successive isolation of the other populations during the upper Pliocene (2.82–2.19 mya). This general pattern is

Table 2 Divergence time estimates for nodes in Fig. 4

Node	Average ± SD	95% credibility interval
a	14.08±0.78	12.84–15.81
b	13.32±0.64	12.58–14.92
c	8.98±1.41	6.39–11.65
d	3.99±1.51	1.90–7.69
e	2.82±1.23	1.20–5.95
f	2.19±1.04	0.85–4.89

SD, standard deviation

Fig. 5 *Chelonoidis carbonaria*; left: Brazil (Museum of Zoology Dresden MTD D 3620); right: Filadelfia, Chaco, Paraguay (Museum of Zoology Dresden MTD D 43485). Scale bars: 10 cm. Note distinct shell shapes and colorations

also confirmed when the 95% credibility intervals are considered. While the upper boundaries of the late Pliocene estimates reach well into the Pleistocene, the same is not the case for the southern clade. Here, the uppermost value still lies in the latest Pliocene (Table 2). Hence, it may be concluded that the cladogenetic vicariant events started in the Pliocene and continued in the Pleistocene.

Our findings also have implications for taxonomy and conservation. The uncorrected *p* distances between the haplotype clusters of *C. carbonaria*, ranging from 0.721% to 4.462%, resemble the differences observed between distinct subspecies of the Western Palaearctic tortoise species *Testudo graeca* (Fritz et al. 2007, 2009b), suggesting that *C. carbonaria* is also polytypic. It is well-known that different morphotypes exist in *C. carbonaria* (e.g. Pritchard and Trebbau 1984), and it is a future challenge to find out how these morphotypes correspond to the observed genetic variation. According to our own observations, the tortoises of the southern clade are morphologically highly divergent by lacking the 'waist' of the shell that is characteristic elsewhere; furthermore, their coloration resembles that in *C. denticulata* rather than other populations of *C. carbonaria* (Fig. 5). Considering their genetic distinctness, this could warrant taxonomic differentiation. Consequently, a rangewide reappraisal of morphology and correlation with genetic data is in urgent need. This is also underlined by the remote position of the GenBank haplotype U4 of unknown provenance in parsimony network analysis (Fig. 3), suggesting that even more distinct populations of *C. carbonaria* may occur. We wish to point out that only mitochondrial data are available at this time. Therefore, we cannot even rule out that the different clades of *C. carbonaria* represent distinct, reproductively isolated species.

In any case, the clades of *C. carbonaria* should be considered as representing evolutionarily significant units (ESUs; Moritz 1994) and their exact geographic ranges should be determined. Currently, *C. carbonaria* is not included in the IUCN Red List of Threatened Species (IUCN 2008). However, this assessment is based on the assumption that there is no genetic subdivision anywhere in the species' range. Moreover, there are regional differences with respect to conservation status. In Colombia, for instance, *C. carbonaria* is listed as 'critically endangered' (Castaño-Mora 2002). It is obvious that the genetic structuring of *C. carbonaria* revealed in the present study not only calls for further research on geographic and taxonomic variation, but also for a reassessment of the conservation status of the distinct genetic units.

Acknowledgements The work of Mario Vargas-Ramírez was made possible by a fellowship of the DAAD (Deutscher Akademischer Austausch Dienst). María Cristina Ardíla-Robayo and Willington Ortiz (Instituto de Biología Tropical Roberto Franco, Universidad Nacional de Colombia), Olga Castaño-Mora and Orlando Rangel-Ch. (Instituto de Ciencias Naturales, Universidad Nacional de Colombia), Wilson Pérez (CORPOCESAR, Colombia) and Juan Nicolas Vargas-Gonzales were of invaluable help in Colombia. Marcelo Sánchez-Villagra (Paleontological Institute, University of Zürich) provided information about the fossil record of *Chelonoidis*. Anke Müller, Anna Hundsdörfer and Christian Kehlmaier (Museum of Zoology Dresden) much assisted Mario Vargas-Ramírez during lab work.

Appendix

Appendix 1 Locality and sequence data of *Chelonoidis carbonaria*, *C. denticulata* and other taxa used in the present study

Taxon	*n*	Locality	Site[a]	Haplotype	MTD[b]	Source	Accession number[c]
Chelonoidis carbonaria	1	Colombia: César: Chimichagua; 09° 11.4′ 41.1″ N / 73° 43′ 55.6″ W	1	C1	T 4590	this study	FM165591
Chelonoidis carbonaria	1	Colombia: César: Chimichagua; 09° 11.4′ 41.1″ N / 73° 43′ 55.6″ W	1	C2	T 4591	this study	FM165592
Chelonoidis carbonaria	3	Colombia: César: Terraplen; 07° 52′ 58.2″ N / 73° 44′ 38.5″ W	2	C1	T 4593-4595	this study	FM165591
Chelonoidis carbonaria	2	Colombia: César: Valledupar; 10° 29′ N / 73° 15″ W	3	C3	T 4602-4603	this study	FM165593
Chelonoidis carbonaria	1	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C4	T 4604	this study	FM165594
Chelonoidis carbonaria	2	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C5	T 4605-4606	this study	FM165595
Chelonoidis carbonaria	2	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C6	T 4607, 4609	this study	FM165596
Chelonoidis carbonaria	1	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C1	T 4608	this study	FM165591

Appendix 1 (continued)

Taxon	*n*	Locality	Site[a]	Haplotype	MTD[b]	Source	Accession number[c]
Chelonoidis carbonaria	1	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C7	T 4610	this study	FM165597
Chelonoidis carbonaria	1	Colombia: César: Valledupar; 10° 29′ N / 73° 15′ W	3	C2	T 4611	this study	FM165592
Chelonoidis carbonaria	2	Colombia: Bolivar: Tolú; 9° 24′ N / 75° 37′ W	4	C8	T 4791-4792	this study	FN185746-47
Chelonoidis carbonaria	1	Colombia: northern Colombia	?	C9	T 4793	this study	FN185748
Chelonoidis carbonaria	1	Colombia: northern Colombia	?	C10	T 4794	this study	FN185749
Chelonoidis carbonaria	6	Colombia: Meta: eastern Llanos; 3° 50′ N / 72° 17′ W	5	C11	T 4795-4800	this study	FN185750-55
Chelonoidis carbonaria	2	French Guiana: Iracoubo; 5° 28′ 46″ N / 53° 12′ 14″ W	6	G1	T 5135, 5138	this study	FM165599
Chelonoidis carbonaria	1	French Guiana	?	G2	D 46958	this study	FM165598
Chelonoidis carbonaria	1	French Guiana	?	G3	D 46417	this study	FM165600
Chelonoidis carbonaria	30	Brazil; southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B1	–	Farias et al. (2007)	EF490386
Chelonoidis carbonaria	2	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B2	–	Farias et al. (2007)	EF490387
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B3	–	Farias et al. (2007)	EF490388
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B4	–	Farias et al. (2007)	EF490389
Chelonoidis carbonaria	2	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B5	–	Farias et al. (2007)	EF490390
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B6	–	Farias et al. (2007)	EF490391
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B7	–	Farias et al. (2007)	EF490392
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B8	–	Farias et al. (2007)	EF490393
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B9	–	Farias et al. (2007)	EF490394
Chelonoidis carbonaria	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	B10	–	Farias et al. (2007)	EF490395
Chelonoidis carbonaria	4	Paraguay: Chaco boreal; 20° 29′ 41″ S / 60° 18′ 18.2″ W	8	P1	T 4, 6–8	this study	FM165601
Chelonoidis carbonaria	1	Paraguay: Chaco boreal; 22° 13′ 88.4″ S / 59° 4′ 66.2″ W	9	P2	T 11	this study	FM165602
Chelonoidis carbonaria	1	unknown	?	U1	–	Le et al. (2006)	DQ497296
Chelonoidis carbonaria	1	unknown	?	U1	–	Caccone et al. (1999)	AF192928
Chelonoidis carbonaria	1	unknown	?	U2	–	Cunningham (2002)	AY678433
Chelonoidis carbonaria	1	unknown	?	U3	–	Cunningham (2002)	AY678332
Chelonoidis carbonaria	1	unknown	?	U3	–	Cunningham (2002)	AY678333
Chelonoidis carbonaria	1	unknown	?	U3	–	Cunningham (2002)	AY678351
Chelonoidis carbonaria	1	unknown	?	U4	–	Cunningham (2002)	AY678334
Chelonoidis denticulata	17	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D1	–	Farias et al. (2007)	EF490396
Chelonoidis denticulata	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D2	–	Farias et al. (2007)	EF490397

Appendix 1 (continued)

Taxon	*n*	Locality	Site[a]	Haplotype	MTD[b]	Source	Accession number[c]
Chelonoidis denticulata	18	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D3	–	Farias et al. (2007)	EF490398
Chelonoidis denticulata	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D4	–	Farias et al. (2007)	EF490399
Chelonoidis denticulata	3	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D5	–	Farias et al. (2007)	EF490400
Chelonoidis denticulata	1	Brazil: southern Para State; 7° 20′–7° 45′ S / 52° 00′–51° 40′ W	7	D6	–	Farias et al. (2007)	EF490401
Chelonoidis denticulata	1	Colombia: Puerto Inírida; 5° 51′ 55″ N / 67° 55′ 26″ W	10	D7	T 4519	this study	FM165604
Chelonoidis denticulata	3	Colombia: Meta: eastern Llanos; 3° 50′ N / 72° 17′ W	5	D7	T 4993-4995	this study	FN185756-58
Chelonoidis denticulata	1	Colombia: Caquetá: foothills of Cordillera Oriental; 1° 37′ N / 75° 37′ W	11	D15	T 4996	this study	FN185759
Chelonoidis denticulata	1	French Guiana: Apatou; 5° 9′ 12″ N / 54° 20′ 10″ W	12	D8	T 5108	this study	FM165603
Chelonoidis denticulata	1	unknown	?	D9	–	Le et al. (2006)	DQ497298
Chelonoidis denticulata	1	unknown	?	D10	–	Cunningham (2002)	AY678402
Chelonoidis denticulata	1	unknown	?	D11	–	Caccone et al. (1999)	AF192941
Chelonoidis denticulata	1	unknown	?	D12	–	Cunningham (2002)	AY678316
Chelonoidis denticulata	1	unknown	?	D13	–	Cunningham (2002)	AY678354
Chelonoidis denticulata	1	unknown	?	D13	–	Cunningham (2002)	AY678317
Chelonoidis denticulata	1	unknown	?	D14	–	Cunningham (2002)	AY678331
Chelonoidis chilensis	1	unknown	–	–	–	Le et al. (2006)	DQ497297
Chelonoidis nigra	1	unknown	–	–	–	Le et al. (2006)	DQ497300
Manouria impressa	1	unknown	–	–	–	Le et al. (2006)	DQ497317
Pyxis arachnoides	1	unknown	–	–	–	Le et al. (2006)	DQ497319
Rhinoclemmys melanosterna	1	unknown	–	–	–	Le et al. (2006)	AY434590

[a] Site numbers refer to Fig. 1

[b] MTD T numbers refer to samples in the tissue collection, MTD D numbers to voucher specimens in the herpetological collection of the Museum of Zoology Dresden

[c] Identical accession numbers refer to the same haplotypes

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