TITLE: Phylogenetic and Divergence Times Analysis of the *Chelonoidis chilensis* Complex (Chelonia: Testudinidae)

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ABSTRACT

We present a phylogenetic and divergence times analysis of the *Chelonoidis chilensis* complex, the southernmost tortoises of South America. We compiled a dataset of 1118 bp cytochrome b (cytb) sequences derived from 111 individuals sampled across all the known geographic range, and performed a phylogenetic analysis employing Maximum Parsimony, Maximum Likelihood and Bayesian Inference methods. The resulting trees showed similar topologies and support values. The *Chelonoidis chilensis* complex was always recovered as a monophyletic group, and in turn composed by two major clades (i.e. haplogroups) that mirrors the biogeographic distribution: one clade is composed by individuals derived from

the Dry Chaco eco-region while the other assembles those from the Monte eco-region. In order to date the origin and diversification times of these two clades, we employed a previously published two-steps molecular clock method. In the first step we dated the time of origin of *Chelonoidis chilensis* as a clade within the Testudinidae family using available sequences, the fossil record and the Fossilized Birth-Death (FBD) model. In the second step we dated the divergence between the haplogroups of *C. chilensis* based in the time of origin estimated in the first step and a coalescent evolution model. Our results suggest that divergence between Dry Chaco and Monte tortoises may have occurred about 2.47 millons of years ago. We interpret these results in the light of the environmental and geological changes occurred during the late Pliocene to Middle Pleistocene of South America.

KEYWORDS: Tortoises; Testudines; Mitochondrial DNA; Haplotypes; Phylogeny; Molecular clock.

INTRODUCTION

Chelonoidis chilensis is a tortoise which inhabits the southern Neotropics (Turtle Taxonomy Working Group 2014). It is considered the most closely related species to *Chelonoidis nigra* species complex, the Galapagos Islands giant tortoise (Caccone *et al*. 1999, Le *et al*. 2006, Fritz & Bininda-Emonds 2007, Vargas-Ramírez *et al*. 2010). For many years, there has been much debate concerning the taxonomic status of these tortoises. In order to clarify this issue, a number of genetic tools have been recently applied to specimens of the genera *Chelonoidis* (Martínez *et al*. 2009, Fritz *et al*. 2012, Sánchez *et al*. 2015).

C. chilensis is widespread throughout the Argentina country, especially in the Dry Chaco and Monte of Steps and Plains Eco-regions, and extends northwards into Bolivia and Paraguay (Cabrera 1998, Burkart *et al*. 1999). This tortoise is currently included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and is considered *Vulnerable* by the IUCN Conservation Commission (Red List 2015) and by national assessments (Prado *et al*. 2012). Several factors put *C. chilensis* populations at risk: multipurpose extraction (mostly for sale as pets) (González-Acuña *et al*. 2005), habitat loss due to agricultural expansion, and subsistence hunting by local inhabitants (Barbarán 2003, Noss *et al*. 2013). In this context, delimiting the *C. chilensis* putative species within the *Chelonoidis chilensis* complex, together with identification of the main threats affecting this species, are one of the most important advances that impact its conservation in a positive manner (Joshi 2012). For many years, the *Chelonoidis chilensis* complex was considered consisting of one, two or three species, depending on the author (Freiberg 1973, Fernández 1988, Buskirk 1993, Cei 1993, Cabrera 1998, Richard 1999). All these studies were carried out considering morphological and ethological characters. This scenario changed in 2012, when Fritz and co-workers made a significant contribution to the systematic problem of the *C. chilensis* complex, based on analyses of mtDNA sequences of 45 specimens. These authors based their work specifically in samples from the typical localities of the three putative species of the *Chelonoidis chilensis* complex, and concluded that *Chelonoidis chilensis* is the only valid species. More recently, Sánchez *et al*. (2015) analyzed the chromosomal variation within *C. chilensis* and found evidence supporting the existence of two karyomorphs that could suggest a possible speciation event for *C. chilensis*. We consider that an exhaustive analysis

of the *C. chilensis* complex genetic variability over the entire distribution range is required to resolve this systematic problem.

Thus, the main objective of the present work is to gain further insight into the systematics of the tortoises of the *Chelonoidis chilensis* complex, using the mitochondrial gene cytochrome b (cytb) as a molecular marker. To carry out this goal, we sequenced mtDNA from 66 individuals and performed phylogenetic analyses under three different criteria (see Materials and methods section below). In addition, based on the molecular data obtained and the temporal information from the fosil record, we evaluated the possible date of origin and diversification of this taxon, and discuss our results in the context of the develop of a monitoring process across its range of geographical distribution in Argentina.

MATERIALS AND METHODS

Geographical area of study and samples collected

We collected samples from 92 specimens of *C. chilensis* in six field travels made during the summers of 2009, 2011 and 2012 in different locations included in the Monte of Step and Plains and Dry Chaco Eco-regions of Argentina. Sampling localities were distributed in 17 Argentina provinces (Río Negro, Neuquén, Mendoza, La Pampa, Buenos Aires, San Juan, La Rioja, San Luis, Córdoba, Catamarca, Tucumán, Jujuy, Salta, Santiago del Estero, Chaco, Formosa and Santa Fe) and included the Natural Protected Areas of Lihué Calel National Park (La Pampa), Copo National Park (Santiago del Estero), Talampaya National Park (La Rioja) and Formosa Nature Reserve (Formosa) (see Fig. 1). We collected samples

of saliva from live animals, and muscle and bone from dead specimens. No specimes were found in the provinces of Jujuy, Tucumán, and Santa fe.

DNA extraction, amplification and sequencing

DNA was extracted from blood and muscle samples following Gemmell & Akiyama (1996) and from bone samples following Rohland & Hofreiter (2007a, b). The complete cytb gene was PCR-amplified in a Biometra T3000 thermocycler with the following conditions: 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at T° annealing (51-56°C), 3 min at 72°C, and a final extension of 10 min at 72°C. We initially employed primers that have previously been reported for other Testudinidae species (Palumbi *et al*. 1991, Spinks *et al*. 2004) but then designed specific primers for the cytb gene of *C. chilensis* (see Table 1). The fragment amplified (1120 bp) was sequenced by Macrogen Inc. in a ABI3730XL sequencer.

Data matrices building

We obtained 66 good quality sequences (from the 92 samples collected), wich were combined with other 45 sequences available in GenBank (Fritz *et al*. 2012) to compile a *Chelonoidis chilensis* cytb matrix of 111 sequences. This matrix was then collapsed to 21 haplotypes (9 sequences belonging to the "Monte" haplogroup and 12 belonging to the "Dry Chaco" haplogroup, see Supp Material 2) using the DnaSP 5.1 (Librado & Rozas 2009). The alignments were performed using ClustalX (Larkin *et al*. 2007) letting default parameters and the molecular evolution models were selected with jModelTest 2.1.4 (Posada 2008) using the following parameters for the Likelihood scores estimation: 11 subtitution schemes, optimized ML base tree, estimation of base frequencies, invariant sites

and Gamma (with 4 categories); and the "Best" search of the base tree. Finally the model selection was based on the Bayesian Information Critera (BIC).

In order to follow the McCartney & Barreto (2010) protocol to date divergences in population level studies, we compiled previously published sequences from 12 taxa of the family Testudinidae, including four mitochondrial (rRNA 12S, rRNA 16S, cytb and ND4) and four nuclear (R35, Cmos, RAG1 and RAG2) genes. These sequences (Table 2) were added to our matrix of *C. chilensis* haplotypes (Supplementary Material 2 and 3) in order to perform a MP analysis (as indicated below) and select from the most parsimonious tree (MPT) three divergent haplotypes from each haplogroup. Once the six haplotypes were selected, its corresponding cytb sequences along with the outgroup sequences were used in the first step of the divergence time estimation analysis, in order to date the origin of the *C. chilensis* clade.

Phylogenetic analysis

The phylogenetic analysis was performed under three different criteria: Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). In order to balance the differences in the evolutionary rates between the highly divergent ingroup and outgroups, which is a common situation in population-level phylogenetic studies (Ho & Larson 2006, Endicott *et al*. 2009), we constructed a matrix (Supplementary Material 4) containing the haplotype sequences plus the remaining sequences of the genus *Chelonoidis* and as outgroup the taxon *Gopherus agassizii*.

Maximum Parsimony

The MP analysis was performed using TNT (Goloboff *et al*. 2008a, b) with equal weights in a heuristic search of 100 replicates of Random Addition Sequences (RAS) and Tree Bisection-Reconnection (TBR), keeping 100 trees per replicate. Branch supports, referred to the MPT, were calculated using the bootstrap method with absolute frequencies over 1000 replicates.

Maximum Likelihood

The ML analysis was performed using RAxML v7.4.2 (Stamatakis 2006) with the ML+rapid bootstrap option, to estimate the maximum likelihood tree and the bootstrap branch supports simultaneously. The number of bootstrap replicates was 1000 and the model of molecular evolution used was the GTR+G, since RAxML only works under GTR-based models and internally implements a likelihood-based algorithm to optimize the model. The parameters for the analysis were defined in the platform raxmlGUI v1.3 (Silvestro & Michalak 2012).

Bayesian Inference

The BI analysis was carried out using the software BEAST v2.3.2 (Drummond & Rambaut 2007, Bouckaert *et al*. 2014) under the molecular evolution model TrN+G (AC = 1, AG = 10.5501, AT = 1, CT = 19.6717, Gamma Shape = 0.238). As in the molecular clock analyses, the values of the molecular model parameters estimated with jModelTest were used as priors in the Bayesian inference. The analysis was run assuming the Yule speciation

model (Gernhard 2008) during 20E 6 generations, sampling every 1,000 generations and discarding the first 2E 6 generations as burn-in.

Divergence time estimation

In order to estimate the divergence time between the haplogroups "Monte" and "Dry Chaco" the two steps protocol described in McCartney & Barreto (2010) was followed. In the first step six divergent haplotypes were added on a matrix containing several outgroup sequences and this matrix (Supplementary Material 5) was used to date the origin of *C. chilensis*. In the second step the age previously estimated was used to constraint the root of the haplotypes tree, obtained from the haplotypes matrix (Supplementary Material 2).

Molecular clock analyses

The Bayesian molecular clock analyses were performed using BEAST v2.3.2 (Drummond & Rambaut 2007, Bouckaert *et al*. 2014) with an uncorrelated Lognormal relaxed molecular clock model. All priors for the molecular clock analysis were defined in the platform BEAUti (Drummond *et al*. 2012) and the posterior values of the model parameters were visualized in Tracer v1.5.0 (Rambaut & Drummond 2009).

First step: The six individual matrices (rRNA 12S, rRNA 16S, cytb, ND4, R35, Cmos, RAG1 and RAG2) containing sequences previously published and the sequences from the six divergent selected haplotypes of cytb, were concatenated with SequenceMatrix 1.7.8 (Vaidya *et al*. 2011) (Supplementary Material 5) and the best partition scheme was found using PartitionFinder 1.1.0 (Lanfear *et al*. 2012). Then each partition was inspected for the best molecular model with jModelTest as previously described (Supplementary Table 1).

Both the clock and the tree model were fixed through the partitions. The clock model was the uncorrelated Lognormal and the tree speciation model was the Fossilized Birth-Death (FBD) process (Heath *et al*. 2014). Divergence time estimations were carried out under the method of Sampled Ancestor (Gavryushkina *et al*. 2014), which employs the ages of fossil taxa to calibrate the phylogeny and these are considered as tips even when lack of morphological information. The fossils selected to calibrate the analysis were located in congruent positions within well known clades of testudinids, based on previous phylogenetic studies and available information (e.g., Le *et al*. 2006, Guillon *et al*. 2012, Lourenço *et al*. 2012, Zacarías *et al*. 2013). The age of each fossil was defined incorporating the uncertainty corresponding with the age range of its respective stratum (Table 3). The MCMC chains were run during 100E 6 generations, sampling every 1,000 generations and discarding the first 10E 6 generations as burn-in. This procedure ensured values of ESS above 200.

Second step: The best model for the haplotypes matrix as indicated by jModelTest was the HKY (kappa = 5.3307). The age estimated for the root of the haplotypes tree (calibration point estimated in the previous step) was assumed to follow a Lognormal distribution with the following parameters: mean = 2.9, Log(s.d.) = 0.25, offset = 0.0. The analysis was run under a coalescent model with constant population size (Kingman 1982) during 10E 6.

RESULTS

Phylogenetic analysis and genetic divergence

The phylogenetic analyses performed under the three different criteria here considered produced very similar topologies (Fig. 2). The MP analysis resulted in ten MPTs of 390

steps (CI = 0.79, RI = 0.72) and the strict consensus of these trees increased the length in four steps. Nevertheless, the main aspects of the results under the three methods are: i) as previous studies suggested, *C. chilensis* was consistently recovered as the sister group of *C. nigra*, with high support scores (MP bootstrap = 87, ML bootstrap = 92, BI posterior probability = 0.99), ii) the *C. chilensis* complex was always recovered as a monophyletic group with the highest support scores (MP bootstrap = 100, ML bootstrap = 100, BI posterior probability = 1) and iii) the *C. chilensis* cytb sequences are distributed in two major, allopatric clades consistent with the Eco-regions Monte and Dry Chaco, which were recovered with low to high support scores depending on the clade and the method of phylogenetic inference (MP $bootstrap_{Monte}$ = 93, ML $bootstrap_{Monte}$ = 84, BI posterior $probability_{Monte}$ = 1; MP $bootstrap_{Dry\ Chaco}$ = 45, ML $bootstrap_{Dry\ Chaco}$ = 46, BI posterior $probability_{Dry\ Chaco}$ = 0.97). Even when the resolution about the relationships between haplotypes inside each clade is poor, the three phylogenetic methods recovered with high support (MP bootstrap = 96, ML bootstrap = 100, BI posterior probability = 1) a clade containing the haplotypes Monte_8 and Monte_9 as sister group of the remaining haplotypes included in the Monte haplogroup. Aditionally in the Dry Chaco haplogroup there were three clades recovered by the three methods with moderated to high support. The first clade was integrated by the haplotypes Dry_Chaco_2-5 (MP bootstrap = 46, ML bootstrap = 76, BI posterior probability = 1), the second was integrated by the haplotypes Dry_Chaco_6 and Dry_Chaco_7 (MP bootstrap = 62, ML bootstrap = 70, BI posterior probability = 1) and the third by the remaining haplotypes, which are represented by a polytomy in the MP analysis and have some better resolution in the ML and BI analyses.

The genetic divergence between species of Testudinidae (*C. chilensis, C. nigra, C. carbonaria* and *C. denticulata*) ranged between 9.1 and 13.4%, considering all the haplotypes of *C. chilensis* from the Monte and the Dry Chaco Ecoregions as a whole. At the intraspecific level, the genetic divergence between the Monte and the Dry Chaco subclades was 1.3% (Table 4).

Divergence times estimation

The first step of the molecular clock analysis dated the origin of the genus *Chelonoidis* in 31.73 million years ago (mya) with its 95% highest posterior density (95% HPD) ranging from 25.17 to 41.53 millon years (mys), the divergence between *C. carbonaria* + the fossil taxon *C. hesterna* and *C. denticulata* in 23.08 mya (95% HPD = 17.24-32.79 mys) and the divergence between *C. nigra* and *C. chilensis* (represented by six divergent haplotypes) in 21 mya (95% HPD = 12.64-27.3 mys). Furthermore this analysis dated the divergence between the haplogroups Monte and Dry Chaco between 1.62 mya and 4.92 mya (median = 2.99 mya), which represent the 95% limits of the probability distribution used as prior for the age of the root of the haplotypes tree in the second step of the molecular clock analysis (Table 5, Fig. 3A).

The second step of the molecular clock dated the divergence between the haplogroups Monte and Dry Chaco in 2.47 mya (95% HPD = 1.44-3.87 mys). The ages estimated for the origin of each individual clade were slightly different: 1.25 mya (95% HPD = 0.46-2.37 mys) for the Monte haplogroup and 0.78 mya (95% HPD = 0.27-1.52 mys) for the Dry Chaco haplogroup (Table 5, Fig. 3B).

DISCUSSION

Implications of phylogenetic results

The analysis of phylogenetic relationships, under the three methods employed here (Maximum Parsimony, Maximum Likelihood and Bayesian Inference), supports the existence of two haplogroups for *C. chilensis* within the set of haplotypes found so far. In turn, these haplogroups are constant in terms of grouping sequences. Finally, these haplogroups have an almost perfect allopatric distribution: One of them contains sequences derived from tortoises that are distributed mostly in the Monte of Steps and Plains Ecoregion (with some tortoises reaching the southern part of the Dry Chaco Ecoregion), whereas the other includes the tortoises living in the Dry Chaco Ecoregion. This same separation was found by Fernández (1988), who analyzed morphometric and osteological characters of the three alleged species using phenetic and cladistic techniques found two distantly interlinked groups, one that includes the tortoises of the Chaco Seco Ecoregion (samples from Santiago del Estero, Córdoba and Chaco) and the other one with the tortoises from the Monte Plains and Plateaus Ecoregion (samples from La Pampa, Mendoza and Río Negro). The pattern based on morphological characters found by Fernández (1988) agrees well with the genetic data presented in this work. However, our analysis do not support the assignation of a specific category to each clade (as Fernández did), since the genetic divergence found between the two groups analyzed (1.3%) is much lower than the one found among the other species of the Testudinidae family (9.1-13.4%). The same appreciation was made by Fritz *et al*. (2012), who analyzed sequences of the cytochrome b gen and microsatellites of the *Chelonoidis chilensis* complex and found genetic distances values between groups that do not justify the separation into different species. Though Fritz

et al. (2012) does not recognize the existence of the two clades we identified in the present work, it is possible that two genetic lineages have diverged within the *C. chilensis* complex because of the existence of gene flow barriers which, once disappeared, gave rise to the generation of a secondary contact zone, allowing the admixture of vicariant groups (González-Porter *et al*. 2011). While the data presented here indicate a clear geographic matching of each clade, their isolation is incomplete, as shown by the fact that both lineages are present in the southern region of Santiago del Estero and Catamarca. In a previous work based on cytogenetic data, Sánchez *et al*. (2015) identified the same two groups of tortoises belonging to the Ecoregions mentioned above, each one showing a specific karyomorph. In addition, in the area of secondary contact of these two groups they identified a tortoise with an intermediate karyomorph between the two typical forms (i.e., from the Monte and the Chaco Seco Ecoregions) (Sánchez *et al*. 2015).

To explain this genetic pattern where two well-defined clades are identified, with a clear geographical matching but separated by low genetic divergence and a contact area in the central area of Argentina, we can consider two hypotheses: (1) the pattern observed is the result of a secondary contact that allows interbreeding of individuals, therefore the separation is incomplet, or (2) there exist two partially sympatric, genetically isolated lineages.

The morphological characters employed by Freiberg (1973) to classify the *C. chilensis* tortoises into three species were later considered as highly variable and without taxonomic value (Auffenberg 1971, Wermuth & Mertens 1977, Fernandez 1988). It is well known that external characteristics like shape, texture and color of the shell and size in tortoises are highly influenced by the environment, as it was shown for several cases in recent years

(Fritz *et al*. 2005, 2006, 2007, 2008, 2009, Attum *et al*. 2007, Široký & Fritz 2007). Concerning this point, it is worth mentioning the existence of intermediate morphs (*donosobarrosi-chilensis* or *petersi-chilensis*) (Sánchez *et al*. 2015) in areas where the Monte and the Chaco Seco Ecoregions contact. Another proof of the influence of the environment on the external characteristics of *C. chilensis* is that the young individuals of, for example, the *donosobarrosi* morph are very similar in shape and color of the shell to the young individuals of the *chilensis* morph, suggesting that the typical features of the *donosobarrosi* morph are acquired with age, and so with the greater exposure to certain environments.

Therefore, we consider the taxonomic classifications based on external characteristics that, as suggested, define each morphotype (Freiberg 1979, Cei 1986, 1993) have no systematic validity.

From an eco-ethological and morphological approach, Richard (1999) considered the existence of two valid species within the *chilensis* complex: *C. chilensis* and *C. donosobarrosi*. The eco-ethological characters that supported his proposal were the type of shelter used by those morphs (construction with or without a cave), presence/absence of ectoparasites (ticks of the genus *Amblyomma*) and trophic spectrum. We propose that the eco-ethological characters employed by Richard (1999) to determine the existence of two species within the *C. chilensis* complex are questionable. In fact, it has been shown for other species of tortoises that they may show some phenotypic plasticity, that allows them to live in environments with different characteristics (Fritz *et al*. 2005, 2007, Fusco & Minelli 2010). Furthermore, the tick *Amblyoma argentinae* (Acari: Ixodidae) found in the tortoises from the Dry Chaco Ecoregion but absent in the tortoises from the Monte

310 Ecoregion, has also been found in other reptiles (*Crotalus terrificus*, *Boa constrictor*
311 *occidentalis*, *Eunectes notaus*, *Bothrops spp.*, *Phynops spp.*, etc.) and even in amphibians of
312 the genus *Bufo* (Guglielmone *et al*. 2001). For this reason, we reject the taxonomic validity
313 of the characters proposed by Richard (1999).
314
315 *Molecular clock analysis*
316 Our molecular clock analysis represents the first temporal approach to the evolutionary
317 history of *Chelonoidis chilensis* and, as far as we know, also for the family Testudinidae
318 although the conclusions at family level are beyond the scope of this paper. However, from
319 this analysis we could obtain some results on the divergence times within the genus
320 *Chelonoidis* and, specifically, the two-steps approach followed gave us a confidence
321 interval to estimate the divergence time between the two clades within the *C. chilensis*
322 complex.
323 The origin of the genus *Chelonoidis* was estimated in 31.73 mya, the divergence between
324 *C. carbonaria* + *C. hesterna* and *C. denticulata* in 23.08 mya and the divergence between
325 *C. nigra* and *C. chilensis* in 21 mya. These divergence times imply that diversification of
326 lineages within *Chelonoidis* occurred during the Oligocene and the early Miocene. On the
327 other hand, the time estimates for the divergence between the two clades of the *C. chilensis*
328 complex ranged from 1.44 to 3.87 mya if the 95% HPD of the second step of the molecular
329 clock analysis is considered (the putative more precise estimation), and from 2.99 to 4.92
330 mya if the 95% HPD of the first step of the molecular clock analysis is considered. Clearly,
331 if both age distributions are considered, the age interval would increase its size ranging
332 from 1.44 to 4.92 mya. If this is the case the diversification within *C. chilensis* would have

occurred since the middle Pliocene to the middle Pleistocene. However, as the results of the second step of the molecular clock are thought as more precise at intraspecific level, our conclusions on the diversification of the *C. chilensis* complex will be based on these. So, taking this into account, the divergence between the two haplogroups would have happened mainly during the late Pliocene-midle Pleistocene. This time was characterized by a trasition where the geological behavior switched from large stationary periods followed by short periods with rapid changes, which produced extinctions and cladogenesis (Pliocene), to recurrent environmental changes, which were produced more frequently and by longer time, generating expansions and contractions of populations ranges rather than cladogenesis, immigrations and extinctions (Pleistocene). These environmental changes, among which are glaciations and their coeval changes in the sea level, were accompanied by the arrival to South America of North American faunas troughout the Isthmus of Panamá, which began competing against native faunas for resources. In this moment, the cyclical increase and decrease of arid areas finally produced a savanna corridor in the eastern flank of the Andes Cordillera that connected the steppes and grasslands of Patagonia with the grasslands of Colombia (see Ortiz-Jaureguizar & Cladera 2006), allowing the interchange between biotas and as result offering good conditions for the expansion of the populations. It is worth noting that in Argentina this corridor had some overlap with the geographic area presently occupied by the Monte Eco-region.

From the evidence and the reconstruction of the paleobiogeographic scenario during the late Pliocene-midle Pleistocene of Argentina (and South America) sketched above, the diversification of *C. chilensis*, with the split of the two clades at the estimated divergent time, distributed along the two Eco-regions named here, seems very likely. Nevertheless,

the fossil record assigned to the genus *Chelonoidis* is rich in both Eco-regions, with representatives from the upper Oligocene-lower Miocene to the Pleistocene (see Zacarías *et al*. 2013). This evidence implies that the diversification of *Chelonoidis* and its lineages (including *C. chilensis*) was accompanied by multiple geological, climatic and enviromental changes, such as sea introgressions (Paranean Sea), rising of the Andes Cordillera, the close of the Isthmus of Panamá, establishment of the Antartic Circumpolar Current, and others.

Overall, the results obtained in the present work support the monophyly of *C. chilensis,* its sister-group relationship with *C. nigra* and the intraspecific diversification in two mtDNA haplogroups during the Pleistocene. Furthermore, these results also support the hypothesis that *C. chilensis* is the only valid species of tortoise for arid regions of Argentina, and complement previous results presented by Fritz *et al*. (2012) and by ourselves (Sánchez *et al*. 2015) evidencing the existence of new genetic diversity. The description of two clades with almost complete disjunct distribution except for small area were overlap and probably hybridization has occurred (southern areas of Catamarca and Santiago del Estero) is relevant for conservation considerations concerning this tortoises and their environment. Further studies applying phylogeographic tools will be needed for a better understanding of the relationship between the geographic distribution and the genetic variability of the *C. chilensis* complex.

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Table 1. Primers of cytb used for the PCR amplification and sequencing in this study.

Name	Position	Sequence	Reference
GLUDGE	14358	5´-TGATCTTGAARAACCAYCGTTG-3´	Palumbi et al. 1991.
CytbJSi	15011	5´-GGATCAAACAACCCAACAGG-3´	Spinks et al. 2004.
CytbJSr	15030	5´-CCTGTTGGGTTGTTTGATCC-3´	Spinks et al. 2004.
THR-8	15585	5´-GGTTTACAAGACCAATGCTT-3´	Spinks et al. 2004.
Cit b-A	14411	5´-TTACGAAAAACCCACCCAAT-3´	This study
Cit b-B	14810	5´-GGCCTCATGGTAGGACGTAA-3´	This study
Cit b-C	14737	5´-CCTGAAACACAGGAATTACCC-3´	This study
Cit b-D	15135	5´-CCTAGGAGGTTTGGGGAGAA-3´	This study
Cit b-E	632	5´-CACCGACAAAATTCCTTTCC-3´	This study
Cit b-F	935	5´-GGTTGAGCGTTGTTTTGATG-3´	This study
Cit b-G	832	5´-TACGATCCATCCCAAACAAA-3´	This study
Cit b-H	1170	5´-GGTTTACAAGACCAATGCTT-3´	This study

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394

395 **Table 2.** Sequences from outgroup taxa used in the first step of the molecular clock

396 analysis.

	GB code Mitochondrial genes				GB code Nuclear genes			
Species	**rRNA 12S**	**rRNA 16S**	**ND4**	**cytb**	**R35**	**Cmos**	**RAG1**	**RAG2**
Astrochelys radiata	AF020883.1	AF020890.1	AY673595.1	DQ497304.1	NA	DQ497337.1	JQ073222.1	DQ497373.1
Centrochelys sulcata	AF175334.1	AY081788.1	AY673478.1	DQ497305.1	NA	DQ497338.1	NA	DQ497374.1
Chelonoidis carbonaria	AB090019.1	AF192926.1	AF351692.1	DQ497296.1	NA	DQ497329.1	EU930790.1	DQ497365.1
Chelonoidis denticulata	AF175336.1	AF192927.1	AF351693.1	DQ497298.1	AJ293980.1	DQ497331.1	EU930792.1	DQ497367.1
Chelonoidis nigra	NA	JN637273.1	AF351770.1	DQ497300	NA	NA	NA	NA
Geochelone elegans	AY081785.1	AY081786.1	AY673465.1	DQ497299.1	NA	DQ497332.1	NA	DQ497368.1
Gopherus agassizii	AY434630.1	NA	AY673591.1	AY434562.1	AY434646.1	NA	NA	NA
Homopus areolatus	NA	NA	AY673587.1	AY678323.1	NA	NA	NA	NA
Indotestudo travancorica	DQ497257.1	DQ497277.1	AY673472.1	DQ497311.1	NA	DQ497344.1	NA	DQ497380.1
Kinixys belliana	DQ497258.1	DQ497278.1	AY673583.1	DQ497312.1	HE662486.1	DQ497345.1	NA	DQ497381.1
Psammobates geometricus	NA	NA	AY673580.1	AY678376	NA	NA	NA	NA
Testudo hermanni	AF067503.1	NA	NC_007696.1	AJ888362.1	DQ386652.1	AM491036.1	NA	AM491038.1

397 NA: Not Available

398

399 **Table 3.** Fossil taxa used to calibrate the phylogeny of the Testudinidae family during the

400 first step of the molecular clock analysis.

Fossil taxa	Age (mya)	Reference
Hadrianus majusculus	Early Eocene (56.0-47.8 Ma)	Hay (1908)
Oligopherus laticuneus	Early Oligocene (38.0-33.9 Ma)	Hay (1908)
Gigantochersina ammon	Late Eocene (36.0-35.0 Ma)	Andrews (1903)
Chelonoidis gringorum	Late Oligocene-Early Miocene (28-23 Ma)	Simpson (1942)
Testudo promarginata	Early Miocene (23.0-20.0 Ma)	Reinach (1900)
Kinixys sp.	Lower Miocene (23.0-20.4 Ma)	Meylan and Auffenberg (1986)
Chelonoidis hesterna	Late Miocene-Early Pliocene (7.25-5.33 Ma)	Auffenberg (1971)

401

Table 4. Genetic divergence between the South American species of the family Testudinidae and between the two haplogroups of *C. chilensis* studied in the present work (tortoises from Monte of Step and Plains and Dry Chaco Eco-regions of Argentina) expressed in percentage (SD is indicated between parentheses).

	***C. chilensis* Total Pop.**	***C. denticulata***	***C. Carbonaria***	***C. chilensis* Monte**
C. denticulata	13.1 (1.2)			
C. carbonaria	13.4 (1.5)	11.7 (1.2)		
C. nigra	9.1 (1.1)	11.8 (1.2)	12.8 (1.4)	
***C. chilensis* Dry Chaco**				1.3 (0.3)

Table 5. Divergence times estimated within the genus *Chelonoidis* in the two steps of the molecular clock analysis (see Fig. 3).

Molecular clock	**Node**	**Clade**	**Median of the age (mya)**	**95% HPD (mys)**
1st step	1	*Chelonoidis*	31.73	25.17-41.53
	2	(*C. denticulata*(*C.* carbonaria, *C. hesterna*))	23.98	17.24-32.79
	3	(*C. carbonaria*, *C. hesterna*)	6.35	5.33-8.74
	4	(*C. gringorum*,(*C. nigra*, *C. chilensis*))	25.93	23-29.83
	5	(*C. nigra*, *C. chilensis*)	21	12.64-27.3
	6	*C. chilensis* complex	2.99	1.62-4.92
2nd step	1	*C. chilensis* complex	2.47	1.44-3.87
	2	Monte haplogroup	1.25	0.46-2.37
	3	Dry Chaco haplogroup	0.78	0.27-1.52

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Legends of illustrations.

Figure 1. Map of Argentina showing the distribution of localities from which were taken the samples of tissues along the two Eco-regions. The Monte Eco-region is showed in brown and the Dry Chaco Eco-region is showed in green.

Figure 2. Phylogenetic trees of the genus *Chelonoidis*, including the *C. chilensis* complex, obtained by three different criteria. A: Maximum Parsimony, B: Maximum Likelihood, C: Bayesian Inference. The numbers above the nodes indicate bootstrap support scores (A and B) and posterior probability scores (C). Clade colors refer to the corresponding Eco-regions (see Fig. 1).

Figure 3. Ultrametric trees obtained by the two-step method of McCartney & Barreto (2010), showing the divergence times within the family Testudinidae (A) and the divergence times within the *C. chilensis* complex (B). Numbers above the nodes indicate median of node ages, red numbers below the nodes indicate the clades belonging to the genus *Chelonoidis* (see table 5).