

ANCESTRAL AREA FOR THE MAJOR CLADES IN TESTUDINES
(PAN-TESTUDINES:TESTUDINATA):
AN APPROACH BY TEMPORAL AND GEOGRAPHIC RANGES

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A Thesis for the Degree of MSc. in Biology

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RESUMEN

TÍTULO: Área ancestral para grandes clados en Testudines (Reptilia: Testudinata): Una aproximación desde rangos temporales y geográficos*

AUTOR: Leidy Viviana Romero Alarcon**

PALABRAS CLAVE: Evidencia Total, Testudines, Testudinata, Filogenia, Área ancestral.

DESCRIPCIÓN: Las tortugas son uno de los grupos más interesantes dentro de Amniota. Aunque es claro que las tortugas (Testudinata) forman un grupo monofilético, sus relaciones internas están en constante revisión. Como resultado de esta inestabilidad filogenética, las preguntas sobre el patrón biogeográfico y de diversificación llegan a ser difícil de resolver. Por tanto, proponer nuevas hipótesis sobre el patrón evolutivo de este tipo de grupos filogenéticamente complejos, requiere de la inclusión de toda la información viable y del empleo de diseños experimentales exhaustivos para poder soportar las nuevas propuestas. Mi objetivo en esta tesis de Maestría en Biología fue reconstruir las relaciones filogenéticas dentro del clado Testudinata bajo la aproximación de evidencia total y reconstruir su patrón de divergencia a partir de un análisis estadístico biogeográfico por capas estratificadas de tiempo. Para lograr mis objetivos, compilé toda la información viable de secuencias moleculares y matrices morfológicas para 335 especies vivas y 85 fósiles. Reconstruí la filogenia de Testudinata bajo pesos iguales e implícitos de parsimonia y Máxima verosimilitud. Con la filogenia de esta ultimo método, calibré los tiempos de divergencia de los nodos bajo el método de datación en terminales y el uso del modelo de Fossilización-Nacimiento-Muerte. Creé un conjunto de reglas para asignar las probabilidades de dispersión que mejor representara la historia geológica desde el presente hasta 240 Ma, y así reconstruir el área ancestral del grupo usando capas de tiempo en el modelo Dispersión, Extinción y Cladogénesis (DEC). Con esto soporté la monofilia de grandes grupos y sus nodos internos, excepto por Americhelydia, lo cuál cambia completamente el patrón biogeográfico de las tortugas modernas. Finalmente, generé una hipótesis en la que el origen de Testudines parece haber sido en el Triásico cuando su rango ancestral abarcaba un amplio rango distribucional antes de la completa fragmentación de Pangea.

* Trabajo de grado

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ABSTRACT

TITLE: Ancestral area for the major clades in Testudines (Reptilia: Testudinata): An approach by temporal and geographic ranges^{*}

AUTHOR: Leidy Viviana Romero Alarcon^{**}

KEY WORDS: Total evidence, Testudines, Testudinata, Phylogeny, Ancestral area.

DESCRIPTION: Turtles form the most striking group into amniotes. From the evolutionary biology perspective, they have intrigued and inspired researches for a long time. Although it is clear that turtles (Testudinata) are a monophyletic group, their internal relationships are under review. As a result of the phylogenetic instability, questions about biogeographical and diversification patterns had been obscured to solve. Thus, proposing an updated hypothesis about the evolutionary patterns in this kind of challenged group requires to include all the most workable data, and employing a comprehensive experimental design to ensure strength and coherent results. My main aim in this master's thesis was to reconstruct the phylogenetic relationships inside Testudinata clade under a total-evidence approach, as well as to reconstruct the biogeographic history of the Testudines clade, using tip-calibration approach, large-scale sampling and a stratified ancestral area analysis. To achieve aforementioned aims, I compiled all workable molecular and morphological information for 337 living species and 83 fossils. I reconstructed Testudinata's phylogeny under Equal and Implicit-Weight Parsimony and Maximum Likelihood. From the ML topology, I calibrated the divergence time of the group under the tip-dating technique, using the Fossilized Birth-Death model. I created a set of rules to assign dispersal probabilities that represented geological history from 240 Ma up today and, then reconstructed the ancestral area employing slice time, under the Dispersal-Extinction-Cladogenesis model (DEC). The results supported major clades and its internal ones as monophyletic groups. Also, I failed to retrieve Americhelydia as a monophyletic one, which brought consequences on the understanding of the Testudines' evolutionary pattern. Finally, I drew the hypothesis that Testudines was a Late Triassic group with a widespread ancestral geographical range, before the complete breakup of Pangea.

^{*} Degree work

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Chapter 1 A total evidence phylogeny of the crown and stem-groups of turtles (Reptilia: Testudinata)

Journal Format: Cladistics – Submitted

ABSTRACT

Testudinata is the clade that represents all extinct and living turtles. This group exhibits an extensive fossil record and molecular sampling in comparison with other amniotes, nevertheless, most studies have focused on reconstructing the phylogenetic hypotheses using genomic or morphology data, but not combining both. Therefore, it has caused phylogenetic instability because every type of data could depict different evolutionary histories. Despite the advantage that turtles have over other vertebrates with their extensive molecular and fossil sampling to implement the total evidence approach, there are scarce studies that have used this technique, and most of them using few taxa but none on a large scale. Our objective was to reconstruct the phylogenetic relationships inside Testudinata clade, under a total-evidence approach, comparing different partitions and reconstruction methods. We put together the most workable morphology and molecular datasets for most living species and some extinct ones. Using Implicit and Equal Weights of Parsimony and Maximum Likelihood methods, we reconstructed the molecular, morphology, and total evidence phylogenies. Finally, we compared all topologies using the Robinson-Foulds distance. We recovered the monophyly of all major clades, and most genera, being them stable through most of our analyses and comparable with previous hypotheses. A remarkable outcome was to recognize Chelydroidea but fail to retrieve the Americhelydia clade, a clade that has been thought as a reliable one. In the total evidence cases, we also noticed that Maximum Likelihood and Implicit Weights topologies were the most similar between methods. Although the resolution

improved, the support values declined when the analyses included fossils. Here we give a new insight into the turtles' phylogeny and point out some methodological considerations that could encourage future studies about total evidence reconstructions.

INTRODUCTION

Despite the instability of the position into amniotes, turtles have been considered a well-supported monophyletic taxon (See: Gaffney, 1975; Joyce, 2007). Nevertheless, its internal relationships among suborders, families, and genera have been in continuous review. To study turtles, authors usually divide them into two groups (e.g. Benson et al., 2011; Bever et al., 2015; Pérez-García, 2020). The first one is the crown-group (Testudines), a monophyletic clade with all modern species. And the second one, the Stem-group, which is an artificial non-monophyletic group with the most extinct lineages from the Mesozoic; both gathered in a solo clade called Testudinata (Joyce, 2007; 2020; Joyce et al., 2020). It is unclear the number of extinct species or clades into the Testudinata, but the Paleobiology Database (<https://paleobiodb.org>) reports around 1060 species, 492 genera, 52 families, and 44 unranked clades. Whereas into Testudines, Rhodin et al. (2017) reported 356 living species, belonging to 89 genera, 14 families, and two suborders plus four species described after 2017 (Vargas-Ramírez et al., 2017, 2020; López-Luna et al., 2018; Farkas et al., 2019).

Several studies have resolved the tree of turtles at different levels, with distinct types of data, and including or not fossils as tips. For the first time, Hay (1908) plotted on a tree the relationships among living and extant groups. Although, it was not until Gaffney (1975) when the phylogenetic analysis, as an objective method, began to play a significant role in the taxonomic classification of turtles, allowing the reassessment of groups under the premise of monophyly. Recent studies as Krenz et al. (2005) proposed the relationship among large clades into Testudines order, using large molecular sampling. Thomson and Shaffer (2010) and Guillón et al. (2012) gathered all workable molecular data up to that time, resolving not only the

relationship of the large groups but also at the species level. Barley et al. (2010) and Crawford et al. (2015) provided new mitochondrial and nuclear sequences to solve positions at the level of families. Posteriorly, Crawford et al. (2015) became the reference to constraint clades in current paleontological studies. Other authors have reconstructed a large-scale turtles' phylogeny; however, they have resolved other issues such as biogeographic patterns and diversification processes (Pereira et al., 2017; Colston et al., 2020).

Most phylogenetic analyses have focused their issues on living species, molecular data, and based-model methods, relegating fossil information to calibration points inside of an evolutionary tree. Unlike the phylogenomic era, morphology has concentrated its use on solving the positions of extinct lineages into the stem-group and sometimes into the crown-group (e.g. Shaffer et al., 1997), often under Parsimony analysis. Accumulated efforts from multiples researches have allowed building up the bases for the modern morphological matrices (Gaffney, 1975; Gaffney and Meylan, 1988; Meylan and Gaffney, 1989; Rougier et al., 1995; Shaffer et al., 1997; Hirayama et al., 2000). In the last years, the benchmark has been Joyce (2007), who presented a compilation and a complete review of all known turtle's characters. This work reconstructed the most comprehensive Testudinata's tree using living and extinct species as tips. Recently, Evers and Benson (2019a) presented a reviewed morphological matrix, improving the previous Testudinata hypothesis by providing new characters from cutting-edge technologies, and evaluating the evolutionary implication of marine lifestyle groups through the Testudinata clade.

Thus, both fields, phylogenomic and paleontology, have introduced significant advances in solving phylogenetic relationships of living and extinct turtles' lineages. However, some groups' positions and the monophyly of others remain uncertain because their resolution has depended on the type of data or the type of analysis performed (e.g. Paracryptodira: Zhou et al. (2014), Angolachelonia: Evers and Benson (2019a); Cryptodira: Sterli (2010), Americhelydia: Crawford et al. (2015), Chelydroidea: Joyce and Bourque (2016)). The total-evidence is an approach that

enhances the interpretation of the evolutionary history, helping to estimate the divergence times (Ronquist et al., 2012) and relocate rogue taxa (Sterli, 2010). It works combining molecular, morphological data, and including fossils as part of the analyses as tips and not as nodes (Eernisse and Kluge, 1993). Taxa such as seals, sea lions, and walruses (Paterson et al., 2020), Pilosa (de Melo-Casali et al., 2020), Arctoidea (Finarelli, 2008), Gymnotiformes (Tagliacollo et al., 2016), or even turtles (Shaffer et al., 1997; Sterli, 2010) has evidenced the advantages of using this technique. However, in the turtles instance, these studies were focused on fossil positions and the effect of their inclusion or the rooting effect, without emphasize on an extensive sampling of living fossils to test the monophyly of large groups and the relationships among them. Although the use of total evidence has depended on the availability of molecular sequences, fossil accessibility, and the computational power to perform extensive analyses, given its qualities, turtles as a group is an excellent model to reconstruct its evolutionary history under this approach. Therefore, we aimed to reconstruct the phylogenetic relationships into the Testudinata clade, using the total-evidence technique, comparing different partitions and reconstruction methods.

1.1 MATERIALS AND METHODS

1.1.1 Collecting Data and building matrices We obtained every sequence of Testudines order stored in GenBank® (Benson et al., 2015). We excluded sequences flagged as unverified, predicted, or hybrids. For every entry, we tracked and updated nomenclatural changes, using the Catalogue of Life (Roskov et al., 2019) and the annotated checklist of turtles of the world (Rhodin et al., 2017). We omitted sequences without specific epithet and did not take into account the infraspecific level. We separated the sequences by locus, performing preliminary alignments in Muscle 3.8.31 (Edgar, 2004a) and comparing suspicious arrangements in Basic Local Alignment Search Tool (BLAST 2.10.1) (Altschul et al., 1990). It allowed us to find loci and taxa misidentified or possibly misassembled. We

kept sequences that were unique for a species in a specific marker. In the cases where a species had two or more entries, we run an Uclust analysis in Usearch 11.0.667 (Edgar, 2010) to verify the specific epithet assigned and to eliminate sequences with identity lower than 98%. To minimize the noise content in the final database, we did not correct the flags from discarded sequences; thus, we would prevent human error introduced by ourselves. Finally, we kept the longest molecular sequences per species; and maintained markers with more than six sampled species. There were incompatibilities between diverse morphological matrices that did not allow us an easy integration among them (e.g. Evers and Benson, 2019b and Vlachos, 2018). Thereby, we put together only two matrices: Evers and Benson (2019b) and Sterli (2010), because these studies examined relevant fossil material in both the stem and the crown-group. They also presented morphological characters for some living species and compiled almost all previous studies in this field. We defined the equivalences between matrices' characters and re-coded unmatched states. Matching states kept the original codification (Supplementary material: Appendix 1).

1.1.2 Aligning and selecting substitution molecular model We performed definitive alignments using Muscle 3.8.31 (Edgar, 2004a), aligning each marker independently. We used 16 iterations, constructing two trees per iteration, and calculating the diagonal on the first iteration. We did not limit the time of the process. Following Edgar (2004b), we did not use 'anchor optimization' because our objective was to get the best accuracy on this analysis. We used Gblock 0.91b (Castresana, 2000) to decide which internal gapping blocks could be eliminated. Blocks that were not selected by the algorithm, but had the complete DNA sampling in all terminals, were kept. As the last step, we refined our alignments using the improvement tool proposed in the Muscle algorithm.

We chose the best nucleotide substitution model for every marker. In Jmodeltest 2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012), we used 203 schemes of substitution, gamma as the heterogeneity rate, and a tree optimized in Likelihood

with the best searching scheme. We used the Akaike Information Criterion (Akaike, 1973, 1974) for selecting the best model. After the complete process, we got three sets of data: molecular (hereafter labelled as ml), morphological (=mr), and total evidence matrix (=te), which we used for the phylogenetic analyses.

1.1.3 Phylogenetic analyses We run a Parsimony analysis in TNT 1.5 (Goloboff et al., 2008; Goloboff and Catalano, 2016), implementing two techniques: Implicit Weights (IW) and Equal Weights (EW). We employed a single search scheme. We run 50 Wagner's replicas for the morphological matrix, 100 for the molecular one, and 200 for the total evidence. In addition to Wagner's searching, we performed new technologies such as 50 ratchet's iterations, 30 iterations of tree-drifting, and 10 of tree-fusing. We implemented TBR as the branch swapping technique, the strict consensus technique, the collapsing rule one and bootstrapping as a node support measure (mr: 150, ml: 300 and te: 600 pseudo-replicas). Following Arias and Goloboff (2019), the K constant for Piwe in IW analysis was equal to N taxa/2 to soften the curve slope. The seed was equal 1 in every Parsimony run.

We implemented the Maximum Likelihood method (ML) using RaXML-NG 1.0 (mpi version: Kozlov et al., 2019). We run a partitioned analysis for the molecular and total evidence matrices. Each locus was considered as an independent partition with its best nucleotide substitution model, allowing the re-optimization parameter, as such as the software's author suggested. For the morphological partition/matrix, we selected the best model, running under Mkv and GTR models, and choosing between them, employing the Likelihood Ratio test (Edwards 1972). We carried out the analysis using 50 random trees + 50 Parsimony trees for morphology, 75 + 75 for molecular, or 100 + 100 for total evidence, without using a started tree. We did not constrain any node. We used the SPR branch swapping technique with the ratio selected by the software (auto-detect). Attending the recommendation by Duchêne et al. (2018), we assigned *scale* as a parameter for the branch length linkage between partitions. We employed the bootstrapping technique (auto-detect of the number of replicas) for support analysis. The number of replicas ranged between 1

and 1000, with 0.03 as a cut of convergence. Following, in part to Hillis and Bull (1993) about a good-support definition, we categorized bootstrapping values as: Strong (100-70), middle (69-40), and weak (39-0).

At the end, we compared the final topologies for each analysis (strict consensus for each Parsimony analysis with the best-fit tree from Maximum Likelihood), using the Normalized Robinson-Foulds distance (Robinson and Foulds, 1981), using the Phangorn 2.5.5 package (Schliep, 2011) written for the R programming language (R Core Team, 2020). Among couples of trees, we dropped unmatched tips to make the comparisons equivalent, using the Phytools 0.7-47 package (Revell, 2012), also written in R programming language.

1.2 RESULTS

We downloaded 7392 sequences for 100 different loci, and we kept 86 loci with 5070 sequences after the refining data. It was for 335 living species, and 15 sequences for the two extinct species in modern times (*Aldabrachelys grandis* (Vaillant 1885), and *Chelonoidis alburyorum* Franz & Franz, 2009). Supplementary material about Genbank® accesses, total-evidence matrix, evolutionary substitution models and phylogenies from every analysis are available in: bitbucket.org/alarconvv/testudinata_phylogeny/src/master.

1.2.1 Morphological reconstructions We based our morphological analysis on 355 characters for 83 fossils, 33 extant species, and two outgroups (*Owenetta kitchingorum* Reisz and Scott 2002, and *Anthodon serrarius* Owen 1876). We found 26 most parsimonious trees with 1507 steps (Supplementary Material: Appendix 3 A), using the Parsimony-Equal Weights (mrEW) technique. In the case of the Parsimony-Implicit Weights (mrlW), we found eight trees with a cost of 6.25 (Supplementary Material: Appendix 3 B). Both techniques allowed us to recover most clades at the superfamily level, but not the relationships among them. We also found genera and families nodes in both analyses; however, clades such as Pleurosternidae, the total group of Kinosternoidea (Pan-Kinosternoidea),

Testudinidae, or the total group of Chelydridae (Pan-Chelydridae) were only recovered from the IW analysis. We found some unexpected positions such as *Platysternon megacephalum* Gray 1831 as the sister of Pan-Chelydridae, and *Peltochelys durlstonensis* Dollo 1884 as the sister of Pan-Pleurodira. The Maximum Likelihood topology (mrML) did not get the same groups as the Parsimony techniques (mrEW or mrlW), but some nodes such as Testudines and the total group of Chelonioida were exclusive from this method. Inside Pan-Chelonioida, we found the Chelonioida superfamily relative to *Toxochelys latiremrys* Cope, 1873, and *Mesodermochelys undulatus* Hirayama and Chitoku, 1996. Both sisters of *Ordosemys leios* Brinkman and Peng, 1993. Other species with an unstable position were *P. durlstoensis* that was the sister of Trionychia, and *Pla. megacephalum* that fell into Chelydridae (Supplementary Material: Appendix 4 A). Although this method had found Testudines as a clade, it failed to recover Cryptodira as a monophyletic group, instead Pan-Pleurodira was monophyletic but it took part of Testudinoidea, a superfamily considered as a Cryptodire group.

1.2.2 Molecular reconstructions The molecular matrix had a length of 54179 pairs of bases the 335 living, two extinct species in the Quaternary period, and one outgroup (*Sphenodon punctatus* Gray 1842). Under molecular analysis Parsimony Equal Weights (mlEW), six trees were retained, with 655974 steps (Supplementary Material: Appendix 3 C). Parsimony-Implicit Weights (mliW) kept six trees with a cost of 7344.09 (Supplementary Material: Appendix 3 D). In both techniques, we did not obtain any monophyletic clade that made sense with previous studies. In contrast, the Maximum Likelihood tree (mlML) showed valid clades at different taxonomic levels (genera, subfamilies, or superfamilies). Even, it resolved the sisterhood of Cryptodira and Pleurodira as monophyletic suborders. However, clades such as *Apalone* Rafinesque 1832, *Mesoclemmys* Gray 1873, *Hydromedusa* Wagler 1830, or Americhelydia were the exception, being non-monophyletic “groups” (Supplementary Material: Appendix 4 B).

1.2.3 Total evidence reconstruction The total evidence matrix consisted of 54534 characters per 418 species of turtles, and three outgroups (*Sphenodon punctatus*, *Owenetta kitchingorum*, and *Anthodon serrarius*) (Supplementary Material: Appendix 1). Parsimony-Equal Weights (teEW) retained 59 trees with 138016 steps, a large number of trees compared to the six trees maintained by the Parsimony-Implicit Weights (telW) technique, these with a cost of 470.87 steps. In both techniques, we recovered all large nodes at superfamily and family level, which formed a large polytomy with other clades and tips from the stem-group, causing the no-monophyly of Testudines (Supplementary Material: Appendix 3 E, F).

In general, we observed relevant changes in the positions at the species level. For instance, *P. durlstoensis* was the sister of Pan-Pleurodira, and *T. latiremys* + *M. undulatus* fell in the stem-group outside of Chelonioidea. Besides, *Platysternon megacephalum* kept its position as the sister of Emydidae, and *Mongolemys elegans* Khosatzky and Mlynarski 1971 was a relative of the Testuguria clade. Some genera were often paraphyletic or polyphyletic "groups". For instance, *Xinjiangchelys* Yeh 1986 (see: *Xinjiangchelys latimarginalis* (Young & Chow, 1953) (Fig. 1.1 A, B; 1.2 D), *Juditthemys* Parham and Hutchison 2003 (Fig. 1.2 E), *Notoemys* Cattoi and Freiberg 1961, *Hydromedusa*, *Mesoclemmys* (Fig. 1.2 F) and *Apalone* (Fig. 2 H). They were also non-monophyletic "groups" on the Maximum Likelihood tree (teML). Here, we either recovered Americhelydia as a group, but the (teML) analysis recovered the rest of subclades at all levels, from the Mesochelydia clade, including Perichelydia and Testudines, to internal nodes at the genus level (Fig. 1.2).

1.2.4 Resolution, similarities among topologies, and bootstrapping behavior
Robinson-Foulds' distances (rfD) revealed that the most similar analyses were between topologies from the same method. Being telW and teEW the pair with the lowest differences (rfD: 0.04), followed by mrEW - mrlW (rfD: 0.09) and mIML - TEML (rfD: 0.11). The most similar methods were teML and telW (rfD: 0.16); in second place mIML-telW (rfD: 0.18), and teEW - telW (rfD: 0.19) in the third place. The most different analyses were the reconstructions based on the molecular matrix using

Parsimony (mLEW/IW) against other partitions, exceeding 0.9 of distance. Given rFD and the fact that clades in mLEW/IW did not make sense with previous literature, we did not draw inferences about lineages using those topologies (Fig. 1.3 A). We noticed that support values were sensible to the status of the tips (extinct or extant), but not to the type of matrix. Thus, weak support values were more frequent in nodes that involved fossils into the group, and they were strong if the nodes only contained living species. However, although Parsimony kept the same tendency about fossils, the support values trended downward (Fig. 1.3 B).

1.3 DISCUSSION

The total-evidence is a philosophical principle that entitles the use of all available evidence or data collected over time (Carnap, 1947; Good, 1967; Kluge, 1989). It is the concept that has encouraged the work between paleontological and molecular phylogenetic disciplines, allowing to tell a unified evolutionary history about groups. That was the reason why we are presenting an update hypothesis of phylogenetic relationship analysis of the Testudinata clade, at the species level, in which we involved fossils as tips and combined the most workable morphological and molecular data. The fossils included, helped us to reorganize and mainstay groups that historically have been problematic (Eernisse and Kluge, 1993; Sterli, 2010). Thus, a noteworthy part of our study was to have included extinct species without constraining clades a priori and, hence the importance of our comprehensive search strategies.

1.3.1 Testudinata's Stem-group Inner the Testudinata clade and until Testudines clade, we got the all members of the stem-group of turtles (see stem-group definition in Jefferies, 1979 and Budd and Mann, 2020). There, we found the first monophyletic group, Mesochelydia (Fig. 1.1). It grouped species such as *Indochelys spatulata* Datta et al., 2000, *Condorchelys antiqua* Sterli 2008, *Kayentachelys aprix* Gaffney et al., 1987, *Eileanchelys waldmanni* Anquetin et al., 2009, *Heckerochelys romani* Sukhanov 2006 and Perichelydia, a monophyletic group that gathered most stem-

turtles and the crown-group (Joyce, 2017; Joyce and Bandyopadhyay, 2020). For these species, we recovered similar positions in all analyses that involved fossils (teML, te/mrIW, te/mrEW). These topologies always showed *I. spatulata* located rootward and *K. aprix* and *E. waldmanni* ahead. Except in teML, we found a sisterhood between *K. aprix* and *E. waldmanni*, being equivalent to the Evers and Benson's (2019) proposal. Few studies have involved these five species and have placed them as part of a polytomy (Sterli, 2008, 2010; Rabi et al., 2013; Joyce, 2017) or have located *K. aprix* rootward (Cadena, 2015; Gentry et al., 2019; Joyce and Bandyopadhyay, 2020). But most of those studies only have one or two species (often only *K. aprix*) (Joyce, 2007; Sterli and Joyce, 2007; Anquetin, 2012; Cadena, 2015; Joyce et al., 2016; Evers and Benson, 2019a), what made difficult to draw inferences about the interrelationships.

A similar situation occurred with Meiolaniformes (Fig. 1.1). Though this clade only appeared in teML and mrML, which was comparable with Rabi et al. (2013) and Sterli and de la Fuente's (2013) hypotheses; most of the time, we got Meiolaniidae (Fig. 1.2 A) as the sister of *Mongolochelys efremovi* Khosatzky 1997. Other studies also have recorded the same group. However, they did not include specimens beyond Meiolaniidae and *M. efremovi* (Sterli, 2010; Anquetin, 2012; Zhou and Rabi, 2015). Clades such as Baenidae (Fig. 1.2 B) and Macrobaenidae (Fig. 1.2 E) were akin to previous hypotheses (Sterli, 2010). Even *Sinemys* Wiman 1930 genus (Fig. 1.1), Xinjianchelyidae (Fig. 1.2 D), Angolachelonia (Fig. 1.2 E) and Paracryptodira (Fig. 1.1) that have fallen into Testudines (Joyce, 2007; Anquetin et al., 2009; Cadena, 2015), here was placed in the stem-group like in Zhou et al. (2014) and Evers and Benson (2019a). All of groups above were monophyletic aside from Xinjianchelyidae, which left outside to *X. latimarginalis*. Studies such as Anquetin (2012), Rabi et al. (2013), Cadena and Parham (2015) and Joyce et al. (2016), have found it as a monophyletic group. However, none of these studies included *X. latimarginalis* in their analyses, and other researches only have used this species as a single entry for the family or as a genus representative (Joyce, 2007; Sterli, 2010; Sterli et al., 2013). It is, maybe, the first time that *X. latimarginalis* is analyzed

together with other members of the genus. But considering the re-assignation of this species to *Xinjiangchelys junggarensis* Yeh 1986, and the genera monophyly proposed by Zhou et al. (2014), we believe that the specimen of *X. latimarginalis* included here and employed in other analyses such as Sterli (2010) and Sterli et al. (2013), should be reviewed in future issues.

1.3.2 Testudines, the crow-group of turtles Testudines was an exclusive clade of the Maximum Likelihood analysis (ml/mr/teML). Unlike the Parsimony techniques (mr/teIW, mr/teEW) that only recovered Pleurodira (in our case infraorder Pan-Pleurodira because of Platychelyidae), ML runs recovered two suborders in, Cryptodira and Pleurodira (Fig. 1.1 A, B).

Both suborders have been well-supported in studies from Gaffney (1975), who proposed their unambiguous synapomorphies, to Evers and Benson (2019a), who made the most recent review of phylogenetic relationships of the Testudinata clade. In the instance of Cryptodira, it is not the first time that some analyses reject its monophyly. For example, as in our mrML analysis, Sterli (2010) found Pleurodira in a polytomy with Trionychidae and both nested with the rest of Cryptodira's members. Sterli (2010) considered it as the effect of the root selection, indicating that the non-monophyly of Cryptodira was because other researchers had assumed a priori the monophyly of this group, rather than Parsimony issues or the type of data implemented in her study, as other studies have argued (e.g. Crawford et al., 2015). Even though our Parsimony outcomes did not resolve Cryptodira's monophyly, like in Rabi et al. (2013) and Evers and Benson (2019), we treated Cryptodira was a monophyletic group (Fig. 1.1 B). Resolving as an extensive polytomy could be the product of a possible rapid radiation event (Danilov and Parham, 2008; Sterli, 2010; Crawford et al., 2015). These rapid changes could be detected when analyses involved molecular data and a good substitution-model exploration (Sullivan and Swofford, 1997; Baurain et al., 2007). Just like in our Maximum Likelihood runs (ml/teML).

In each of our topologies, we recognized two groups into Pan-Pleurodira: Platychelyidae and Pleurodira. Platychelyidae was a family conformed by *Platychelys oberndorferi* Wagner 1853 and the non-monophyletic genus, *Notoemys*. Some authors underpin the monophyly of *Notoemys* (Cadena and Gaffney, 2005; Cadena et al., 2013; Cadena and Joyce, 2015), considering four nominal species: *Notoemys laticentralis* Cattoi and Freiberg 1961, and *Notoemys oxfordiensis* (= *Caribemys oxfordiensis* de la Fuente and Iturralde-Vinent 2001), redefined by Cadena and Gaffney in 2005. And two more, *Notoemys tlaxiacoensis* Lopez-Conde et al. 2017 and *Notoemys zapatocaensis* Cadena and Gaffney 2005, not included in this study. In the case of *N. zapatocaensis*, it is one of the best-preserved specimens from the *Notoemys* genus, and its discovery obliged a re-examination of the group (Cadena and Gaffney, 2005). Most studies had solely involved *N. laticentralis* and *N. oxfordiensis* in their analyses, where *N. oxfordiensis* always appeared relative to *Pt. oberndorferi*, showing the paraphyly of the ‘genus’ (Zhou and Rabi, 2015; Joyce et al., 2016; Shao et al., 2018). However, we notice that it happens only when studies do not include *N. zapatocaensis* as part of their analysis. Thus, the absence or presence of this species could be key to recovered or not the *Notoemys*’ monophyly, or even the absence of key characters that was not included in this study, considering the bias sampling toward Cryptodire given the matrices used here.

Besides, the Pleurodira suborder gathered four families; Chelidae that was relative to a clade formed by Podocnemididae and Pelomedusidae, which was the sister of Araripeomydidae (*Araripemys barretoi* Price 1973). We also recovered other clades at the subfamily level as Chelinae and Cheloninae, as well as the monophyly of most genera from this suborder (teML, telW, etEW). *Pelomedusa* Wagler 1830 was one of those genera that were not a monophyletic group. It happened given the misposition of *Pelomedusa galeata* (Schoepff 1792) that also caused the paraphyly of *Pelusios* Wagler 1830. Authors like Fritz et al. (2011) have documented the evident divergence between *Pelusios* and *Pelomedusa*, like in our analysis of mIML. Nevertheless, some authors hesitated about the complete bifurcation between both genera. For example, Thomson and Shaffer (2010) presented *Pelomedusa subrufa*

(Bonnaterre 1789) nested into *Pelusios*, and Vargas-Ramírez et al. (2010) found *Pelomedusa* as a paraphyletic group, including *Pelusios*. This latter study argued the *Pelusios*' position was yield of a possible introgression or a hybridization between both lineages. In the specific case of *Pel. galeata*, it was a well-defined *Pel. subrufra* lineage, which was described as a new species by Petzold et al. (2014). As this taxonomic change was made recently, it is the first time that an extensive phylogenetic analysis included *Pel. galeata* as part of its sampling. Thus, multiple reasons could be causing its position into *Pelusios*. For example, incomplete sorting lineage (Petzold et al., 2014), phylogenetic noise (Vargas-Ramírez et al., 2010), or even a taxonomic misidentification (Spinks et al., 2013), what is a frequent mistake in collections or public electronic databases (Fritz et al., 2012). The same reasoning could support the no monophyly of genera like *Mesoclemmys* and *Hydromedusa*, which neither resolved in other studies such as Thomson and Shaffer (2010), Pereira et al. (2017), and Cadena et al. (2019). Regarding the *Hydromedusa* genus, there are not deep studies in population genetics or phylogeographic analyses for its living species. However, *Hydromedusa* is a monophyletic group when analyses incorporate its fossil member *Hydromedusa casamayorensis* de la Fuente and Bona 2002 or include fossils from the *Yaminuechelys* De la Fuente et al. 2001 genus (Ferreira et al., 2018; Cadena et al., 2019; Holley et al., 2020). Then, such in the *N. zapatocaensis*, including fossils in phylogenetic analyses could help to improve the resolution of the nodes, no matter if they are living or extinct. An assumption that has already been pointed out by other researchers (Harvey et al., 1994; Sterli, 2010; Wiens et al., 2010).

Inner Cryptodira (ml/etML), we got Trionychia, a superfamily with a historically uncertain position. Some authors have treated it as a relative of all other Cryptodira superfamilies (Gaffney, 1975, 1996; Meylan and Gaffney, 1989; Joyce, 2007; Bardet et al., 2013; Tong and Meylan, 2013). Also, it has been the Pleurodira's sister (Sterli, 2010), or even a suborder into Testudines (Savage, 1957). Here, Trionychia was rootward of Cryptodira, as the sister of the remaining large groups. This position endorsed the most stable and supported hypotheses up to this moment (Thomson

and Shaffer, 2010; Guillon et al., 2012; Cadena and Parham, 2015; Crawford et al., 2015; Pereira et al., 2017; Evers and Benson, 2019a; Gentry et al., 2019; Luo et al., 2019; Kundu et al., 2020). Despite the stability, taxa such as the Adocusia group (Fig. 1.1) and *P. durlstonensis* did not return like in previous hypotheses (Joyce, 2007; Joyce et al., 2016). For instance, in teML, mrlW, and mrEW, *P. durlstonensis* was the sister of Pan-Pleurodira, but it was the sister of Trionychia in mrML, telW, and teEW. Even though Sterli (2010) related Trionychia with Pleurodira, and Joyce (2007: pp 34) recognized “Shifting the position of the vertebral II-III scute sulcus from the sixth to the fifth neural” is the only shared character between Pleurodira and *P. durlstonensis* (but with independent origin), we did not identify evidence or a reason why we found this species next to Pan-Pleurodira. It is also true for the Adocusia group, which never took part of the Trionychia’s stem-group in our analyses or in others as Rabi et al. (2013).

Both groups could be considered as a case of ‘rogue taxa’ given they are not stable through our analyses. Although it might be advisable to remove those ‘rogue taxa’ from the dataset, in order to improve the resolution and the support values (Wilkinson, 1996; Thomson and Shaffer, 2010), we consider that if we accepted that assertion, we would prune almost all fossils from our study. For their nature of lacking information, fossils tend to be rogue taxa in a total-evidence analysis. However, they bring with itself worth evidence about the evolutionary patterns (Parham et al., 2012) that we could and should not ignore. We would think about them in terms of ‘friendly’ or ‘evil’ rogue taxa, such as in Westover’s et al. (2013) classification. Thus, the Adocusia group could be a ‘friendly’ taxon because it never was placed in a specific clade or interfered in any other node different to Cryptodira. Contrary to *P. durlstonensis*, that behaved like an ‘evil rogue’ taxon. Thus, future studies should reconsider their position or drop from the analyses until it could get better information to include them.

We found two monophyletic families into Trionychia, whose resolution and internal relationships were quite similar among our total-evidence analyses (ml/teML, telW, teEW). Within Trionychidae, we got two subfamilies, Cyclanorbinae and

Trionychinae. *Pelochelys cantorii* Gray 1864 was the only mispositioned species, leading to the paraphyly of *Apalone*. The relationship of *Plo. cantorii* as a member of the *Apalone + Rafetus* Gray 1864 clade only has been hypothesized in Viet Thanh et al. (2017), which looked for testing the taxonomy of the giant softshell turtles from the Hoankiem Lake. Different from this hypothesis, Zhang et al. (2018) presented a better statement to clarify the unfamiliar position of *Plo. cantorii*. Using mitogenomic analyses, they showed that GenBank® stored a genomic sequence from this species with an unknown origin. That sequence had a low similarity with other species of *Pelochelys* Gray 1864 but the highest affinity with *Apalone ferox* (Schneider 1783), concluding that the similarity of both species was a product of a misidentified sequence. Thus, the cleaning process of electronic databases is a vital step for large-scale studies, but feedback should also take part in this process (Chan et al., 1995). As GenBank® is almost the only referent to get molecular data for phylogenetic analysis, formal feedback could help to improve studies, minimizing the error associated with the reuse of data collected from electronic databases.

Separating from Trionychia, we got a clade that gathered the rest of Cryptodira's superfamilies (ml/teML) (Chelydroidea, Chelonioidea, and Testudinoidea). This clade had been identified as Durocryptodira by Crawford et al. (2015), Joyce et al. (2016), and Pereira et al. (2017). We found Chelonioidea was relative to Testudinoidea, and their join formed the sister node of Chelydroidea. Even though our outcome is unconventional, other authors such as Parham et al. (2006), Lourenço et al. (2012), and Luo et al. (2019) have supported these relationships. We failed to retrieve Americhelydia clade as a group that usually related Chelonioidea with Chelydroidea (Crawford et al., 2015). It is a clade that has been recorded in almost all extensive molecular phylogenetic analyses (Thomson and Shaffer, 2010; Crawford et al., 2015; Pereira et al., 2017), excluding Guillon et al. (2012), Kundu et al. (2020) and Colston et al. (2020). However, it is worth highlighting that this clade is hard to get when the analyses included fossils, or if they are focused on extinct groups (Joyce, 2007; Sterli, 2010; Anquetin, 2012; Cadena, 2015). This clade is present only when these studies use molecular hypotheses to constrain a priori

some groups (e.g. Cadena and Parham, 2015; Shao et al., 2018; Evers and Benson, 2019a). Not to have recovered this node change the topology that Crawford et al. (2015) propose as the relationship of the large clades of Testudines, implying that our understanding and our assumptions about how turtles evolve also would change. One example of this could be the conclusions that have been drawn about the biogeographic patterns (e.g. Pereira et al. 2017) or diversification processes (Colston et al. 2020). Thus, futures studies should be aware that constrain clade a priori could be forcing a probably misleading relationship.

In terms of Chelydroidea, we observed two lineages (ml/teML, telW, teEW). One corresponded to the total group of Chelydridae (Pan-Chelydridae), which was formed by Chelydridae and *Protochelydra zangerli* Erickson 1973. The second one coincided with the total group of kinosternoidea (Pan-kinosternoidea), a clade with *Hoplochelys crassa* Cope 1888 rootward plus Kinosternoidea clade. The monophyly of Chelydroidea has been widely questioned (Shaffer et al., 1997; Joyce, 2007; Thomson and Shaffer, 2010; Anquetin, 2012; Guillon et al., 2012; Rabi et al., 2013; Sterli et al., 2013). Even so, chelydrids as the sister of kinosternids is a node that has been observed by other authors (Parham et al., 2006; Barley et al., 2010; Knauss et al., 2011; Lourenço et al., 2012; Cadena, 2015; Crawford et al., 2015; Zhou and Rabi, 2015; Joyce et al., 2016; Joyce and Bourque, 2016; Lyson et al., 2017; Pereira et al., 2017; Gentry et al., 2019; Kundu et al., 2020). Joyce and Bourque (2016) remarked that the no recognition of Chelydroidea as a superfamily could be the product of a circumstantial situation, possibly because Baur died shortly after having proposed the clade (Baur 1893), without providing evidence enough to support the group. Currently, Kinosternoidea (Engstrom et al., 2007; van Dijk et al., 2011; Knauss, 2014; Spinks et al., 2014; Rhodin et al., 2017; Vlachos, 2020), and Chelydroidea (Hirayama and Chitoku, 1996; Sasaki et al., 2004; Karl, 2007) are considered as superfamilies. Considering that Kinosternoidea is a clade nested into Chelonioidea, keeping both clades at the same taxonomic level could be imprecise. Thus, we treated Kinosternoidea as an epifamily like in Shaffer et al. (1997) and Bourque et al. (2015). A taxonomic rank that, although it is not regulated by the ICZN

(International Commission on Zoological Nomenclature, <https://www.iczn.org/>), has been widely used in zoological studies (de la Fuente, 2003; Gaffney et al., 2006; Eggleton et al., 2007; Barej et al., 2014). In this way, we could maintain the original definition from Joyce et al. (2004), but retypifying the name with its correspondent suffix [-iodae] (Fig 1.2 H).

In all our analyses, we found the Kinosternoidae epifamily as a node that contained two families, Dermatemidae and Kinosternidae. Intergeneric relationships inside both families were stable and followed the Spink's et al. (2014) hypothesis, but it was not the same with the fossils' relationships. Regarding *H. crassa* and *Emarginachelys cretacea* (Whetstone, 1978), there had been diverse hypotheses about their position. For example, they have found both species as part of Pan-kinosternoidae (Knauss et al., 2011; Zhou and Rabi, 2015; Joyce et al., 2016), or resolving *Hoplochelys* Hay 1908 genus into Kinosternoidea but placing *E. cretacea* outside of it (Joyce, 2007; Sterli, 2010; Anquetin, 2012), or even including *Hoplochelys* species in the Pan-Dermatemys and *E. cretacea* like a Pan-kinosternoids (Joyce and Bourque, 2016; Lyson et al., 2017; Joyce and Claude, 2020). We recovered all these hypotheses, which makes us suspect a possible case of rogue taxa, making it difficult to define a precise position for any of these two species.

The last two superfamilies were Chelonioidea and Testudinoidea. This first was the superfamily with the most fossil sampling into Testudines and it was the direct relative of Testudinoidea. The broad fossil sampling was because Ever and Benson (2019) focused their study in groups with marine style life. We expected to get similar relationships into this superfamily, or at least in the Parsimony, because their matrix was the base of our analyses. Paradoxically, we only found the relationship proposed by them in teML. Despite we also found the three monophyletic families that conformed this group (Cheloniidae, Dermochelyidae, and Protostegidae), we did not recover the Dermocheloidae node (= Dermocheloidea, see discussion above) in Parsimony, like in Ever and Benson (2019) or Cadena and Parham (2015). In contrast, Dermochelyidae was the sister clade of Cheloniidae, forming the sister node

of Protostegidae (mr/teEW, etIW). This structure was largely consistent with recent studies in which new fossils were included to re-examine the phylogenetic internal relationships of the sea turtles (Evers et al., 2019; Chatterji et al., 2020), what re-evaluates the recognition of the group.

Likewise, in this superfamily, *M. undulatus* and *T. latimeris* are species that usually are recovered as part of Chelonioidea. For example, *M. undulatus* often fall into Dermatemidae (Joyce, 2007; Cadena, 2015; Cadena and Parham, 2015; Joyce et al., 2016), or as part of the total group of Chelonioidea, forming Pan-Chelonioidea (Sterli, 2010; Anquetin, 2012). Similar case of *T. latimeris*, which have represented the transition from mainland to marine life in this superfamily (Kear and Lee, 2006; Anquetin et al., 2009; Evers and Benson, 2019a). We recovered *M. undulatus* and *T. latimeris* as sisters of Chelonioidea in the mrML analysis, such as the previous hypothesis. In opposition to it, in the rest of the analyses, both species formed a node into the large polytomy of Perichelydia (mr/telW, mr/teEW), or even outside of Testudines in teML. Recovering this misleading position could be a result of a lack of information (Harvey et al., 1994) of *Toxochelys*' species. This genus does not have good-preserved fossil material (Evers and Benson et al. 2019), which coincided with our high amount of missing data for *T. latimeris*. Although including large amount of missing data could not have dangerous for the reconstructions (Wiens and Morrill 2011), it sometimes could generate rogue taxa (Westover et al. 2013), as possibly happened in this case.

Finally, Testudinoidea was a superfamily that we could find in all our analyses (ml/teML, telW, teEW). Despite the abundant convergent characters among Testudinoidea's families (McLaughlin and Stayton, 2016; Garbin et al., 2018), lineages within the superfamily were clear, and their internal groups were stable and equivalent to others large-scale phylogenetic analyses of turtles (Thomson and Shaffer, 2010; Guillon et al., 2012; Crawford et al., 2015; Pereira et al., 2017). Into this superfamily, we recognized two clades: Emysternia and Testuguria. Emysternia was a node formed by the sisterhood of Platystenidae and Emydidae. The first one was a monophyletic family with a single living species, *Pla. megacephalum*. However,

a current study pointed out that one of the three subspecies from *Pla. megacephalum*, specifically *Pla. m. peguense* Gray 1870, should be considered as an independent lineage, suggesting updating its status (Luo et al., 2019). The position of Platysternidae has been concerned for a long time. It has taken different places through Cryptodires, the most common as a relative of Chelydidae (see Barley et al., 2010), as such as in mrEW and mrlW. But now, it is clear that this monospecific family is the sister of Emydidae (since Parham et al., 2006), which was supported by our Parsimony and Maximum Likelihood's results (ml/mr/teML, telW, teEW).

Emydidae was the second family in the Emysternia clade. It was divided into Emydinae and Deirochelynae subfamilies (ml/teML, telW, teEW). Although Emydidae is one of the turtles' families with the most phylogenetic changes (Stephens and Wiens, 2003; Spinks et al., 2009; Wiens et al., 2010; Guillon et al., 2012; Parham et al., 2013), our internal relationships matched completely with the Pereira's et al. (2017) hypothesis. In this family, incomplete lineage sorting and methodological artifacts had historically made difficult species and genera delimitation (Spinks et al., 2016). For example, *Emys* Duméril 1805 is a monophyletic clade that Spinks and Shaffer (2009) proposed to separate into three genera, *Emys*, *Actinemys* Agassiz 1857, and *Emydoidea* Gray 1870. This scheme was followed by Fritz (2011) and used in the turtles' checklist (van Dijk et al., 2011). But it was Van Dijk et al. (2011) who suggested keeping *Emys* as a unique genus until new analyses were performed. In 2016, Spink et al. presented a well-supported hypothesis, where they stated that the three-genera scheme should be avoided. Considering Spink's et al. (2016) recommendations and our outcomes, we kept the solo-genus scheme for the four species of the monophyletic genus, *Emys*. Despite Van Dijk's et al. (2011) suggestion and the well-supported hypothesis by Spink's et al. (2016), the three-genera scheme is still being used (e.g. Rhodin et al., 2017).

On the other hand, we got two families into the Testuguria clade (ml/teML, telW, teEW). The first family was Testudinidae, a family without internal subfamilies. Although our intergeneric relationships differed from other studies, our analyses

agreed with the latter deep study of the family (Kehlmaier et al., 2019). One remarkable difference was the monophyly of *Aldabrachelys* Loveridge and Williams 1957, a genus composed of some extinct species and a living one, *Aldabrachelys gigantea* (Schweigger 1812), which is considered a species complex (Rhodin et al., 2017). This extant species was re-typified as *Dipsoschelys dussumieri* (Palkovacs et al., 2002; Palkovacs et al., 2003). But Austin and Arnold (2001) found that *A. gigantea* was a relative to *A. grandidieri*, an extinct Mascarenche tortoise. In this way, Pereira et al. (2017) included both taxa as *Aldabrachelys* genus, but they failed in recovering its monophyly, even they placed each species in separate clades. Although we found the non-monophyly of *Aldabrachelys* in teEW, we also recovered their sisterhood in three of the four conclusive analyses (ml/teML and telW). That endorsed Austin and Arnold's (2001) statements and could encourage reconsideration of the *D. dussumieri* name, taking into account the nomenclatural recommendation for Testudinids by Fritz and Bininda-Emonds (2007).

Through the time, *Homopus* Duméril and Bibron 1834 and *Testudo* L 1758 have been subject to taxonomic changes that we could confirm in our analysis (ml/teML, telW, teEW). *Homopus* has been a paraphyletic 'genus' that was resolved by Hofmeyr et al. (2017). They proposed to keep the *Homopus* name for the clade *Homopus areolatus* (Thunberg 1787) + *Homopus femoralis* Boulenger 1888, and treated *Homopus signatus* (Gmelin, 1789), *Homopus boulengeri* Duerden 1906, and *Homopus solus* Branch 2007 as *Chersobius* Fitzinger 1835 (*Chersobius signatus* (Gmelin 1789), *Chersobius boulengeri* (Duerden 1906) and *Chersobius solus* Branch 2007, respectively). However, although these changes were used in the last edition of the checklist of turtles of the world (Rhodin et al., 2017), it is still considered as a tentative. We agreed and followed Hofmeyr's et al. (2017) proposal, given that *Homopus* and *Chersobius* were found as independent monophyletic groups in our outcomes (ml/teML, telW, teEW).

Likewise, *Testudo* has been a genus with a complex taxonomy. Although some hypotheses had proposed to split the genus into several groups (e.g. de Lapparent de Broin et al., 2006), recently, Luján et al. (2016) maintained *Testudo* as a genus

but divided it into three subgenera (*Testudo* (*Chersine*) Merrem 1820, *T.* (*Agrionemys*) Khosatzky and Mlynarski 1966, and *T.* (*Testudo*) Linnaeus 1758). That proposal had been broadly accepted, even when the genus appeared as a polyphyletic ‘group’ (Rhodin et al., 2017; Kehlmaier et al., 2019). Here, we only recovered this genus as a monophyletic group in mIML, and as a paraphyletic one with teML. Contrary to the Parsimony analyses (mr/telW, mr/teEW), where it formed a polytomy including *Indotestudo* Lindholm 1929. Given the monophyly found in the molecular analysis and the no-rejection from Parsimony analyses, we kept the nomenclature proposed by Luján et al. (2016) and followed the recommendation by Fritz and Kraus (2008). Nevertheless, we suggested reviewing the monophyly of *Testudo* or the possibility of splitting *Testudo* into two groups with *Testudo horsfieldii* Gray 1844 and *Testudo hermanni* Gmelin 1789 as the first group, and *Testudo graeca* Linnaeus 1758, *Testudo kleinmanni* Lortet 1883, and *Testudo marginata* Schoepf 1789 as the second one.

Finally, following Rhodin’s et al. (2017) classification, we found the two subfamilies proposed for Geoemydidae, the Rhinoclemmyninae, and Geoemydinae clades. The monophyly of the genera and most of their relationships matched the latest large-scale turtles’ phylogeny (Pereira et al., 2017), with the most comprehensive family evaluation (Spinks et al., 2004), and partially with Diesmos et al. (2005), who checked the *Heosemys* Stejneger 1902 status. Here, *Siebenrockiella*’s Lindholm 1929 position and its monophyly were the most notable difference between the mentioned hypotheses above and our analyses (ml/teML, telW, teEW).

Historically, morphological similarities inside and between Emydidae and Geoemydidae have made difficult the delimitation of the clades (McDowell, 1964; Hirayama, 1985; Gaffney and Meylan, 1988; Yasukawa et al., 2001; Sasaki et al., 2006), but molecular advances and methodological developments have helped to elucidate the relationships among these enigmatic groups (Thomson and Shaffer, 2010; Crawford et al., 2015). Currently, the monophyly of the genera in this family and their positions are relatively stable (Pereira et al., 2017; Ascarrunz et al., 2019). Despite it, *Siebenrockiella* is a genus that has fallen in different places inside

Geooemydidae. Our outcomes recovered this genus relative to *Batagur* Gray 1856, *Pangshura* Gray 1856, *Morenia* Gray 1870, *Geoclemys* Gray 1856, *Malayemys* Lindholm 1931, and *Orlitia* Gray 1873, all as the sister clade of *Geoemyda* Gray 1834 (ml/teML). Contrary to our ML analyses, we got this genus as a non-monophyletic group (telW and teEW). The idea that this genus was not the sister of *Geoemyda*, but near it, is not new (Yasukawa et al., 2001; Diesmos et al., 2005; Le et al., 2007; Praschag et al., 2009). However, since the assessment of their monophyly (Diesmos et al. 2005), it has never been recovered as a non-monophyletic group. Although *Siebenrockiella*'s position and its monophyly were not precise, it is evident that both species *Siebenrockiella leyteensis* Taylor 1920, and *Siebenrockiella crassicollis* Gray 1830 were related to the genera named above. Its relationships with batagurids allow to form three clades that could correspond to three subfamilies into *Geoemydidae*, which partially endorse the hypothesis from Gaffney and Meylan (1988) and Le and McCord (2008). The first authors proposed two subfamilies and the seconds separate another one from Gaffney and Meylan's (1988) hypothesis. We could distinguish the three clades in all our analyses, which we treated as the tentative subfamilies 1, 2, and 3. However, this propose is not an official one, we believe that it should be tested and formally validated in a future study.

1.3.3 Methodological considerations For many years, the assessment of properties such as robustness, accuracy, sensibility to the type of data sets, or sensibility to lack of characters or taxa, among others, has been the key to the advance and development of the phylogenetic reconstruction and support techniques. However, currently, most of the studies are empirical analyses, and their main concern is only the topology, avoiding the comprehension of the methods or parameters that might influence their conclusions.

Although our study is an empirical analysis, some patterns among partitions and methods are relevant to broaden and rekindle methodological discussions from the total-evidence perspective. Our outcomes showed an empirical and cursory

comparison among Maximum Likelihood and two techniques in Parsimony, Implicit, and Equal Weights. As well as individual reconstructions, using every partition (molecular and morphology) against the total-evidence approach. In them, we looked that the internal resolution of large clades (i.e. superfamilies) did not depend on the method in total-evidence, but on the relationships among them, getting the sisterhood only in ML. In contrast, Parsimony analyses showed extensive polytomies that were not exclusive to our study. Authors such as Anquetin (2012), Cadena (2015), Evers and Benson (2019a), and partially Guillon et al. (2012) showed the problems to resolve relationships among superfamilies in Testudines, or even within clades in Testudinata. Despite it, total-evidence topologies had the highest grade of similarities in their resolution (See: rFD in result section), following the pattern found by DeBry and Lawrence (1995).

We also noticed that among methods, IW and ML topologies were the most similar, coinciding with the Goloboff and Arias' (2019) premise. We are aware that our best outcomes, IW and ML, had been considered as inaccurate methods. But it had been under particular scenarios such as reconstruction with morphological matrices with a low number of characters (Puttick et al., 2017). The method's performance is also associated with the type of data, and some studies had suggested that molecular data operated better in based-model analyses (Felsenstein, 1981; Gadagkar and Kumar, 2005; Yang and Rannala, 2012). Something similar, we recognized here. Molecular data in ML (mlML) and morphological data in Parsimony (mrIW, mrEW) were closed to all total evidence reconstructions (teML, teIW, teEW); all of them recovering comparable resolution at the superfamily/family/genus level. In contrast, mrML recovered unorthodox relationships among large clades. However, this performed better than Parsimony with the molecular matrix (mrIW, mrEW), which did not get any clade at any taxonomic level. One could consider that just including fossils would increase congruence between molecular and morphological partition (Legg et al., 2013). But we found that exists congruence if molecular data is analyzed by based-model analysis and the morphology with Parsimony. But also, we point out

that it is indifferent when the total evidence approach is employed as we have exposed above.

On the other side, the effects on support measures, when analyses include fossils, or they reconstruct under total evidence, has scarcely been studied. In an empirical study, van der Wal et al. (2019) showed a significant increase in bootstrapping support values when the phylogeny is inferred by combining different datasets. Although our experimental design was similar to van der Wal et al. (2019), we never got this tendency in our support values. However, the difference between both studies could be a product of the type of taxa used. In contrast to van der Wal et al. (2019), we included fossils as terminals, which could determine the difference in behavior among support values.

Although it is undeniable the advantages of reconstructing phylogenies combining different datasets for living and extinct taxa (Harvey et al., 1994; Rabosky, 2010; Ronquist et al., 2012; Legg et al., 2013; Losos et al., 2013; Dos Reis et al., 2016), including fossils in the analyses will increase the uncertainty in the nodes (Huelsenbeck, 1991). It could explain nodes that nested fossils often got a weak support level. Shaffer et al. (1997), for the first time, recorded this effect of decreasing support values when fossils are added. Interestingly, we confirm Shaffer et al. (1997) statements, which suggested that there was a cost that we should balance between the great benefice of including fossils in total-evidence and the risk of striking the statistical support. As well as supporting claims from Lemoine et al. (2018), who declared that deep nodes tended to get low bootstrapping values when extensive datasets were implemented. As a result, in a total evidence matrix, fossils usually would have partial morphological information and just missing data in the molecular part, which usually is the largest partition. Thus, matrices reconstructed in the bootstrap process could contain more molecular characters than morphological ones. Even some replicas could not have a representation of morphology, causing that fossils been represented only by missing data. Fossils would not have information enough to define their position in some or most of the replicas, behaving as rogue taxa, and then, resulting in the loss of structure and the increase of the

uncertainty. This behavior could be explained by the effect of the combination among the support technique nature, the few data that we could get from a fossil, and the size of the total evidence matrix (Soltis and Soltis, 2003).

1.4 CONCLUSION

In this study, we reconstructed the phylogenetic hypothesis of the Testudinata clade. It is the first attempt to gather most living and extinct taxa with morphological and molecular workable data in a single matrix. Our results suggested several stable groups at different taxonomic levels, for instance, the monophyly of Paracryptodira, Baenidae, Angolachelonia, Testudines, Pan-Pleurodira, Cryptodira, Testudinoidea, Chelonioidea, Trionychia, and their respective families, subfamilies, and genera. All of them well-supported by literature. Moreover, we endorse some hypotheses that have been controversial for a long time, as the recovering of the superfamily Chelydroidea. However, we failed to retrieve the Americhelydia clade, which many authors have considered as a stable clade. Our phylogenetic hypothesis linked Chelonioidea and Testudinidae as the sister clade of Chelydroidea. We remarked about the “unresolved clade Americhelydia” because this is one of the clades that paleontological studies had wrongfully constrained a priori, and its non-recognition could change some evolutionary inferences about the history of turtles. On the other hand, although our objective was an empirical study of turtles, we cannot ignore some particular technical patterns. We found that combining multiple datasets under a total evidence approach the resolution could increase, independently of the method. Finally, our results suggested a possible decrease in bootstrapping values given the inclusion of extinct taxa (as terminals with missing data). However, finding a reason to explain these methodological behaviors challenged us, and there are scarce technical studies about the effects of the total evidence approach over the analysis’ support values. We hope our findings could help to resolve some taxonomical issues but also to reopen other ones, as well as to encourage methodological discussions from the total-evidence perspective.

FIGURES

Figure 1.1 Phylogenetic reconstructions under the total evidence approach. A: The strict consensus of Parsimony Implicit- Weights. B: the best-fit hypothesis from the Maximum Likelihood method. Bootstrapping values represented by the color in nodes. Black: Strong (100-70), Gray: Middle (69-40), White: Weak (39-0). Black arrows show monophyletic groups recovered in both methods and gray ones in one of both methods. Bold names indicate tips found on different positions in every phylogeny.

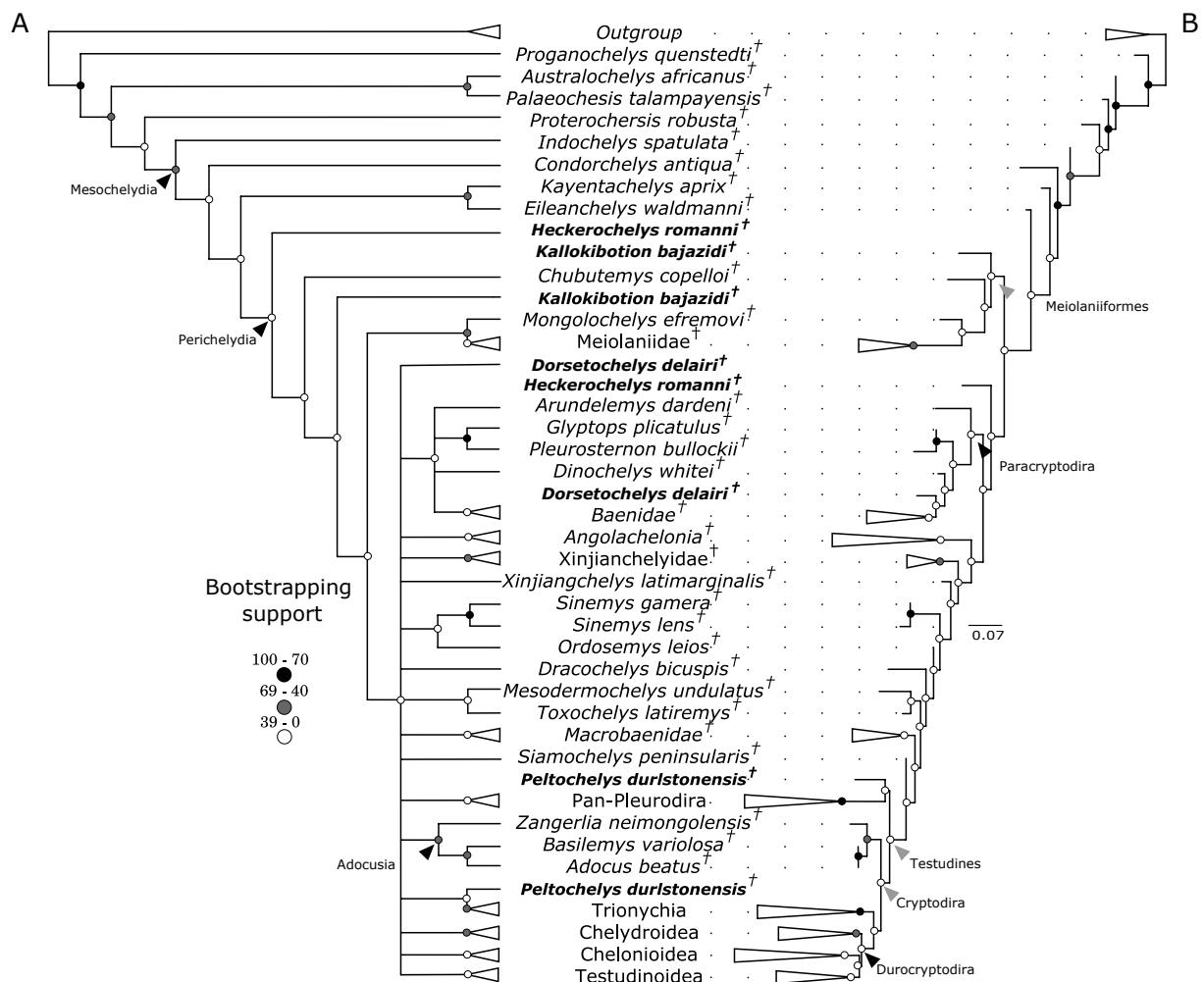
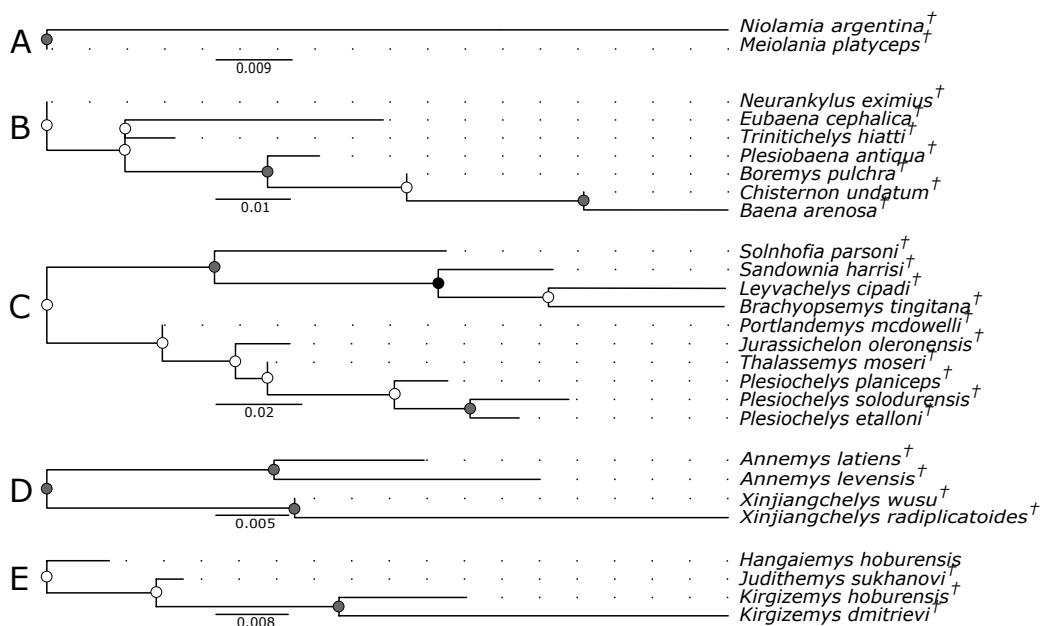
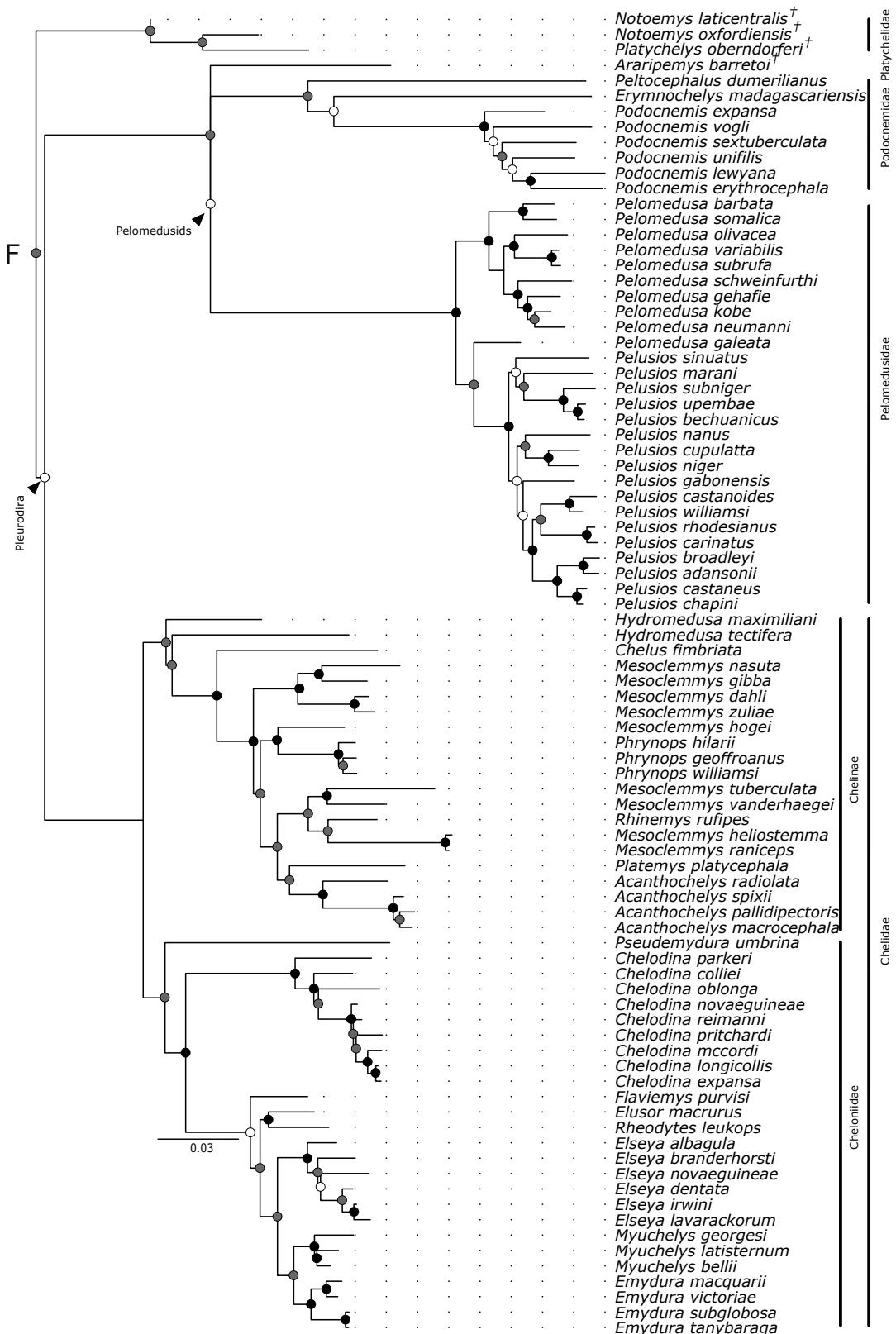
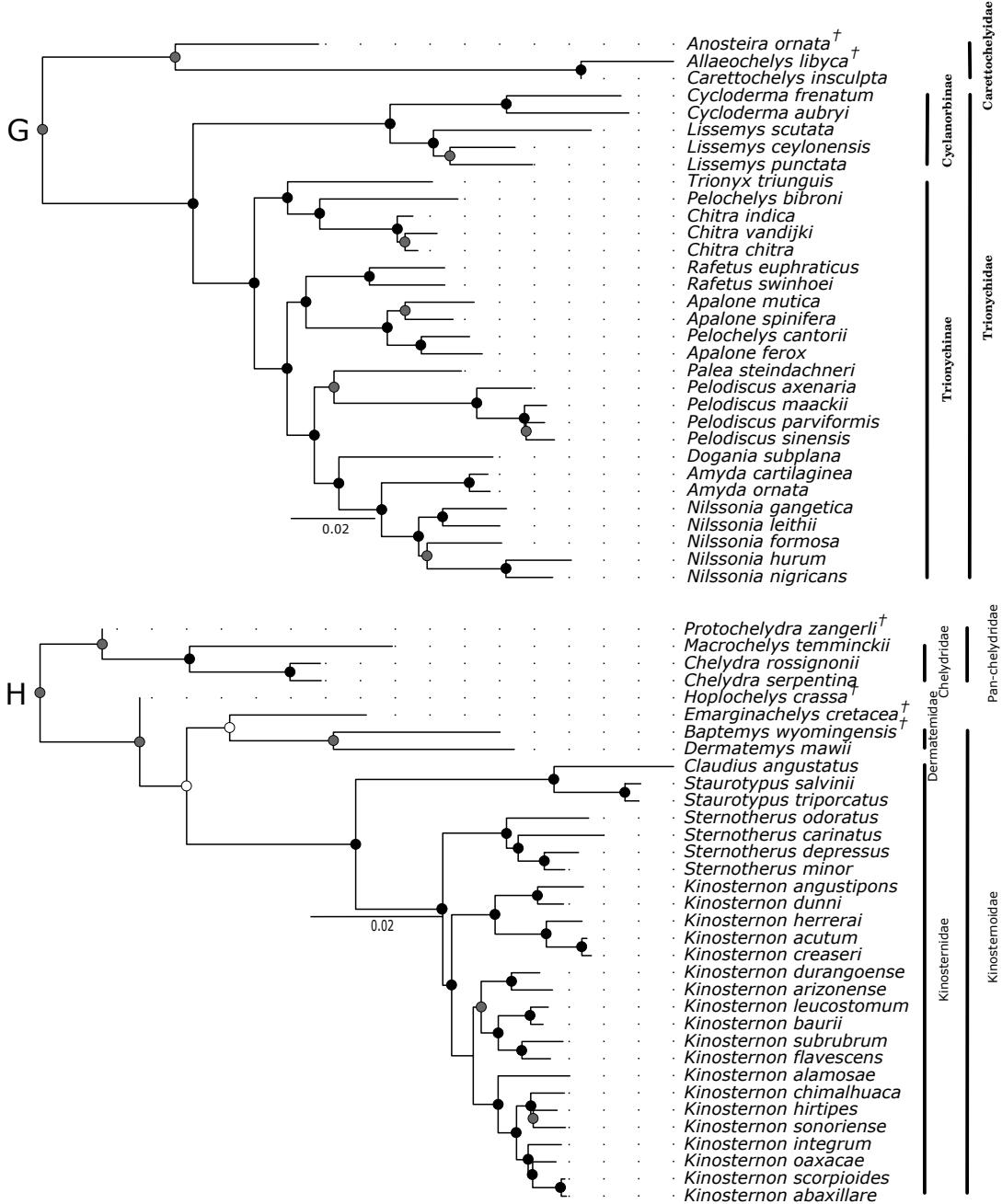
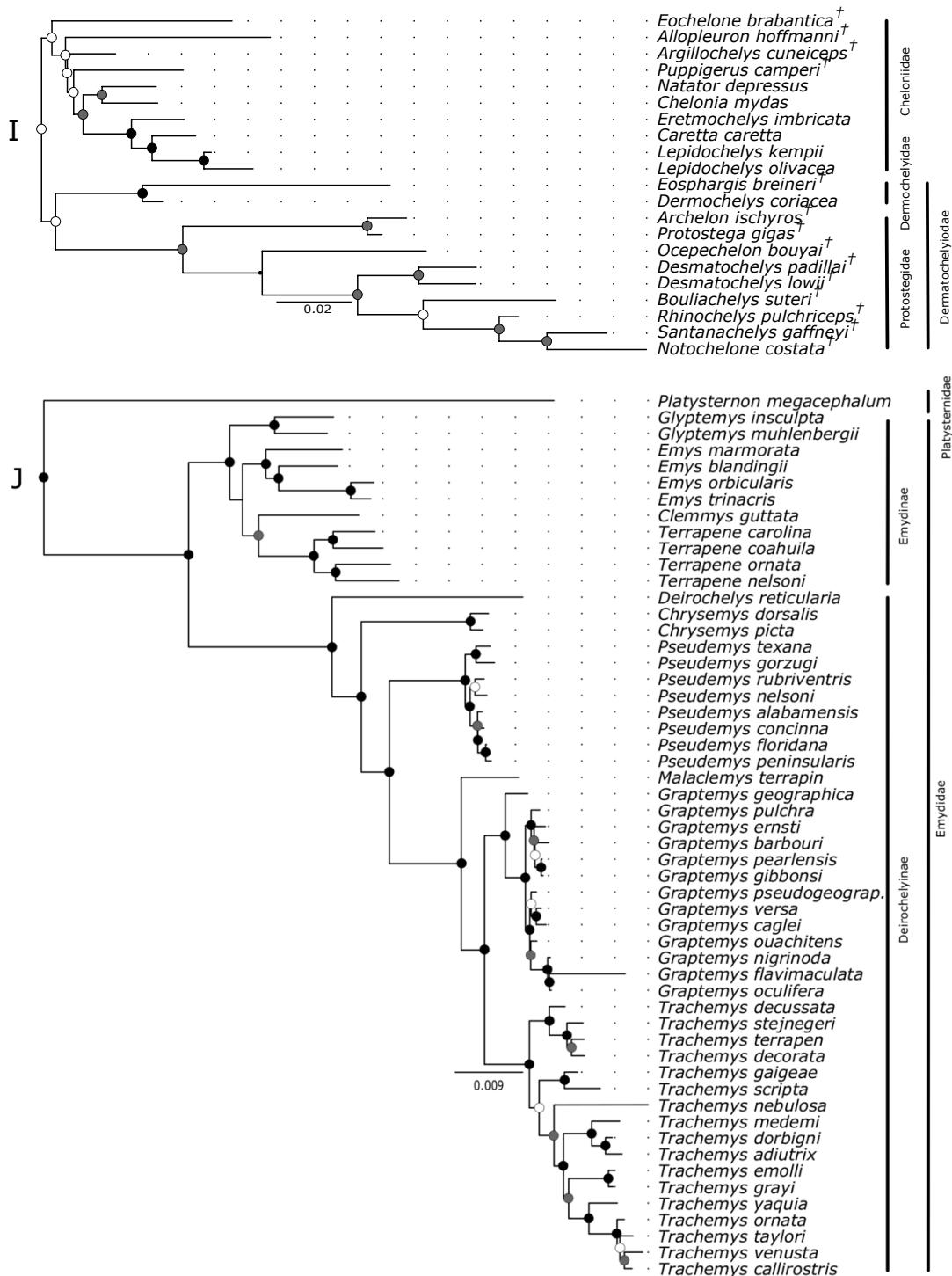


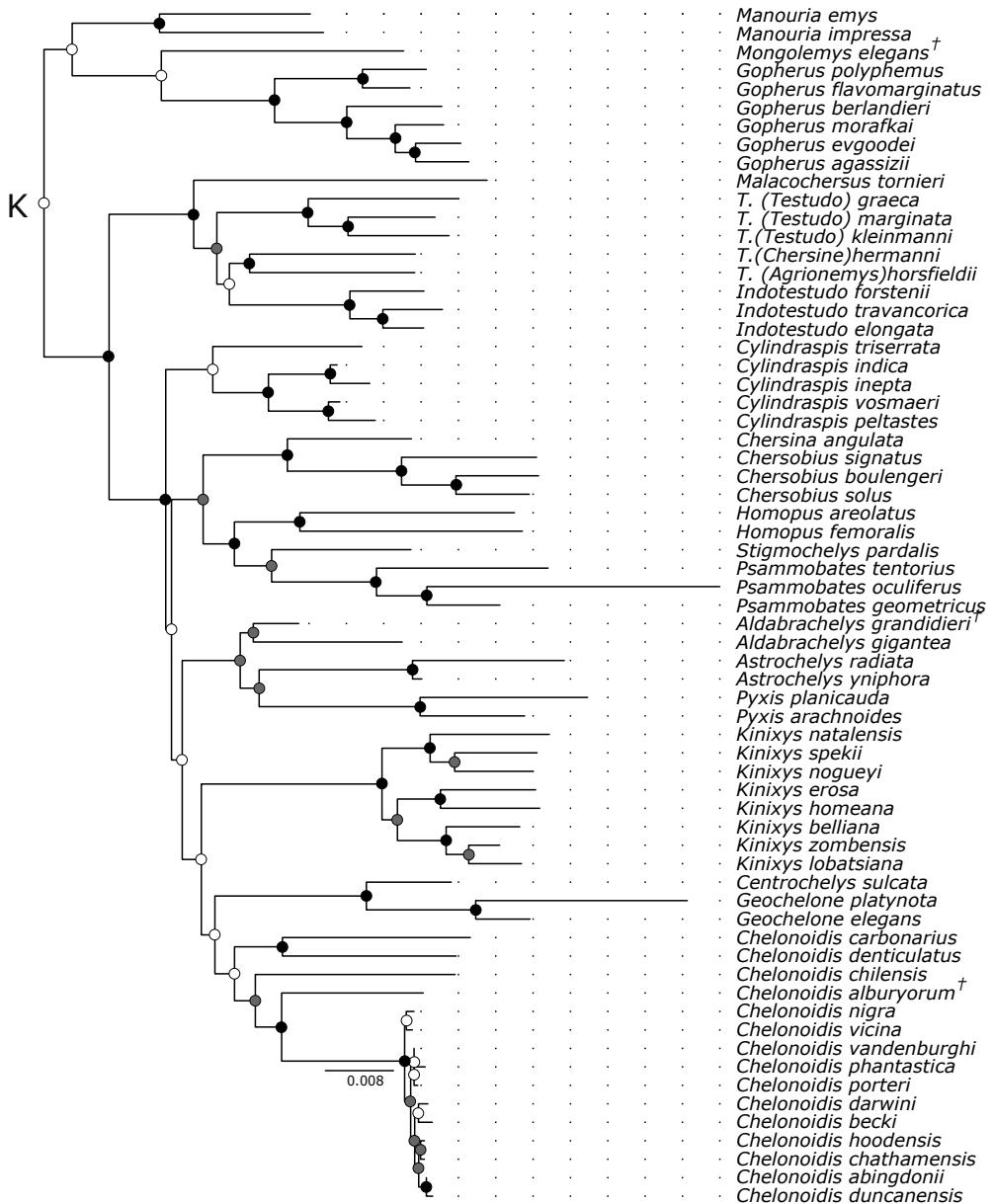
Figure 1.2 Internal resolution at the genus level from the total evidence of the Maximum Likelihood analysis. Color in nodes shows the range of bootstrapping support. A: Meiolaniidae, B: Baenidae, C: Angolachelonia, D: Xinjianchelyidae, E: Macrobaenidae, F: Pan-Pleurodira, G: Trionychia, H: Chelydroidea, I: Chelonioidea, J: Testudinoidea – Emysternia, K: Testudinoidea – Testudinidae, L: Testudinoidea – Geoemydidae. The cross in the species indicate that it is a fossil. Bootstrapping values represented by the color in nodes. Black: Strong (100-70), Gray: Middle (69-40), White: Weak (39-0).











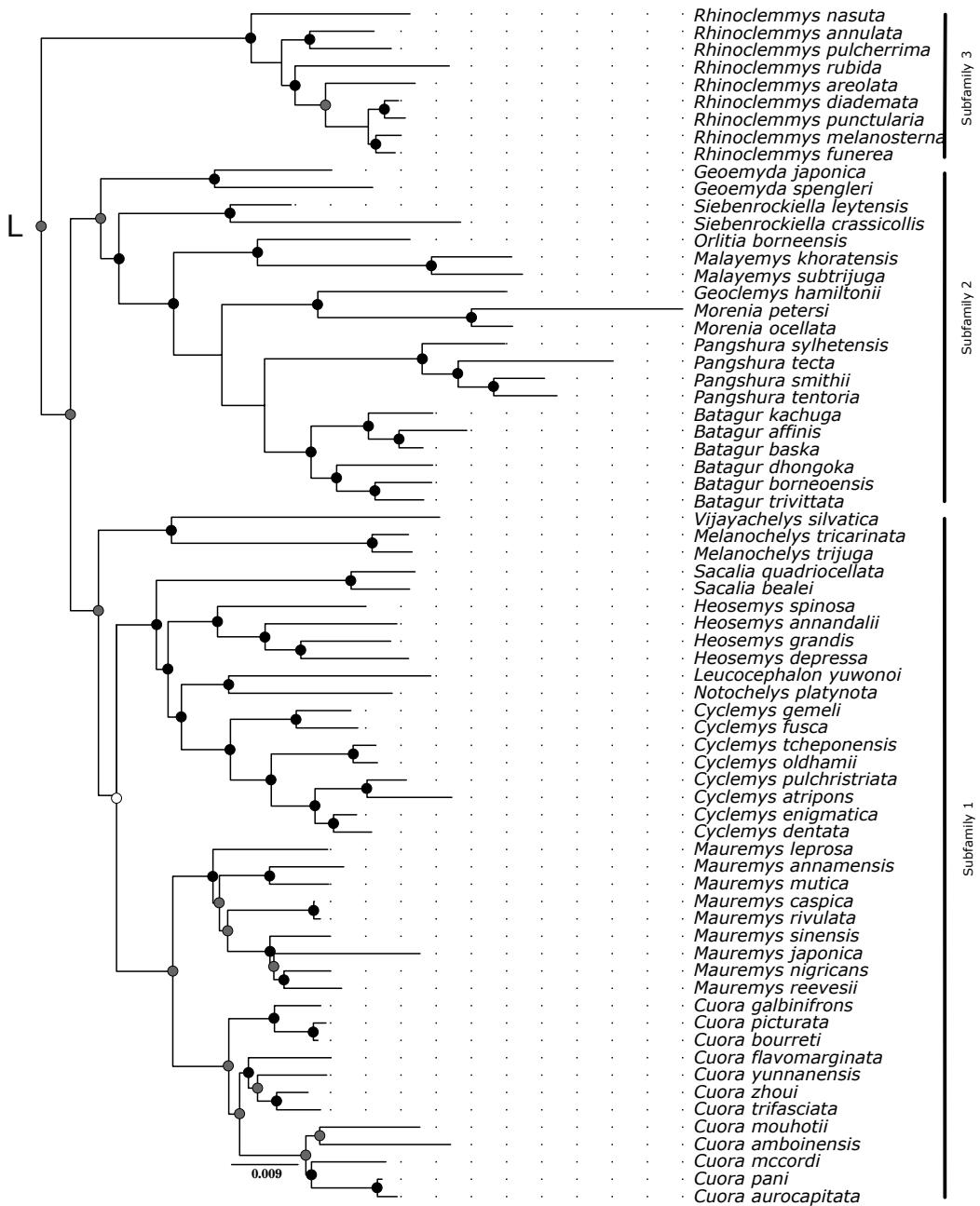
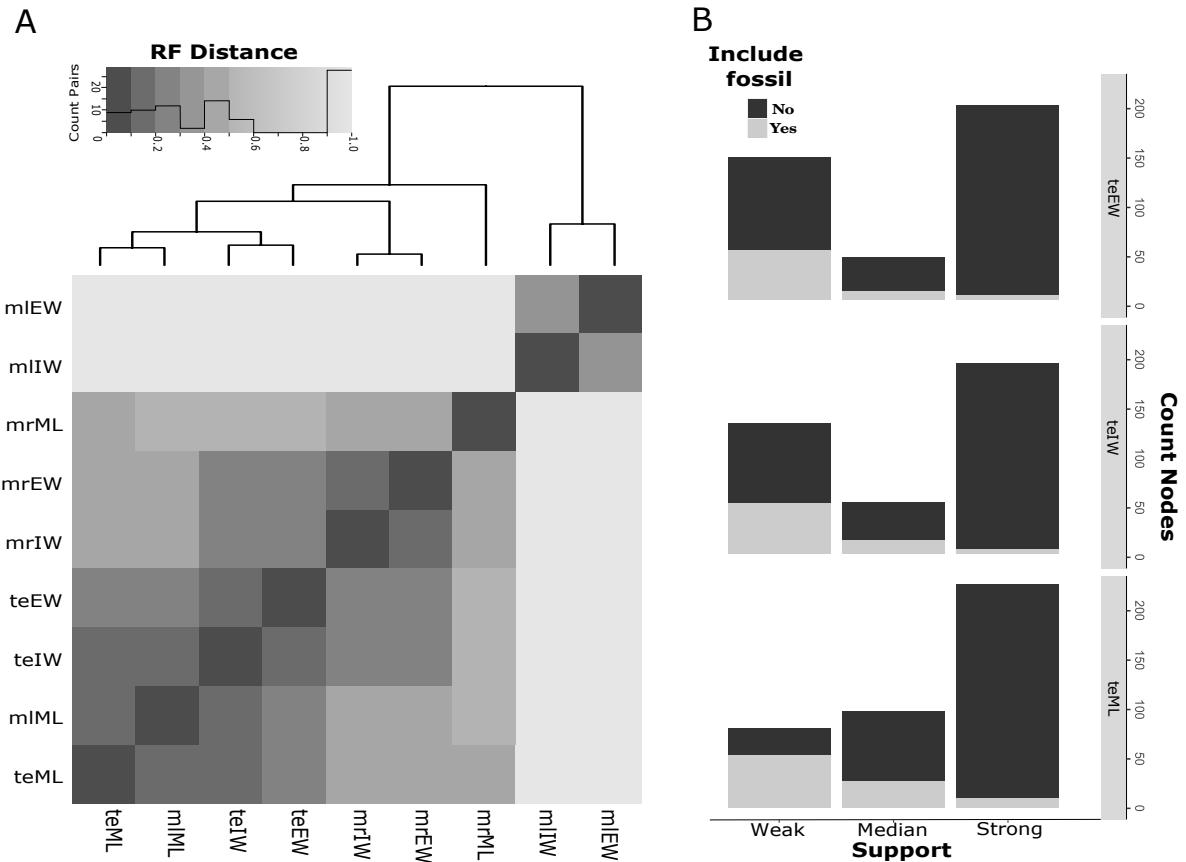


Figure 1.3 Robinson-Foulds distances and Bootstrapping values behavior. A: similarity among pairs of topologies. B: decrease in the support values when fossils are included in nodes, under the total evidence analyses.



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Chapter 2 Ancestral area of Testudines (Reptilia: Testudinata), a biogeographical story told from a tip-calibration approach.

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ABSTRACT

Aim: Turtles are a group that has been an excellent biological model to explore theoretical and technical issues in the evolutionary field. Empirically, it also has been an intrigued clade given its autapomorphic body shape, early divergence and its biogeography pattern, which reflects the large geological events occurred through the Mesozoic and Cenozoic eras. Despite it, few studies have explored its divergence patterns under biogeographic statistical models. Here, our objective was to reconstruct the biogeographic history of the Testudines clade, using a tip-calibration approach, large-scale sampling, and a stratified ancestral area analysis.

Taxon: Testudines: Testudinata. **Methods:** From a total-evidence ML topology of the Testudinata clade, we calibrated the divergence time of the group under the tip-dating technique, using the Fossilized Birth-Death model. We created a set of rules to assign dispersal probabilities that represented geological history from 240 Ma up today and, then we reconstructed the ancestral area employing slice time, under the Dispersal-Extinction-Cladogenesis model (DEC) in Biogeobears R package.

Results and Main Conclusions: This performance allowed us reassigning the Testudinata lineage origin to the Permian period and the Testudines diversification in the Carnian age in the Late Triassic. We also refitted the Testudines' divergence pattern to the geological history of the earth in the last 230 Ma, finding events that could explain better the patterns given the dates proposed in our statement.

Keywords: Testudines, Testudinata, Tip-calibration, Ancestral Area, slice time.

INTRODUCTION

Mesozoic was the Golden era for the diversification of amniotes lineages. One lineage from this group was Testudinata. This clade has been characterized by an unambiguous fully developed shell, which has allowed to define it as the ‘true turtles’ clade (Gaffney and Meylan, 1988; Joyce and Gauthier, 2004). Given the fossil record, authors have assumed that Testudinata appeared in the Late Triassic (e.g. Joyce et al. 2004; Joyce, 2017; Sterli et al. 2018). This hypothesis has broad been supported by specimens as *Proganochelys quenstedti* Baur, 1887, and *Proterochersis robusta* Fraas 1913, which already showed the Testudinata condition for the time of the Norian age (Joyce, 2007; Anquetin, 2012; Joyce and Sterli, 2012; Sterli et al. 2013). As well as the presence of its undeniable sister group, *Odontochelys semitestacea* Li et al. 2008 (232-221 Ma) and the followed stem-taxa *Eorhynchochelys sinensis* Li et al. 2018 (237-227 Ma) and *Pappochelys rosinae* Schoch and Sues 2015 (242-235 Ma). Moreover, biogeographical hypotheses of the early turtles have been based on descriptions from their fossil record’s distribution (except by Sterli and de la Fuente in 2013, who used based-model methods to evaluate the Meiolaniformes’ biogeographic pattern). They have shown that rootward species of Testudinata were widespread in the Triassic, being more diverse in the North of Pangea than in the South, or at least given the fossil record up today (See: Joyce et al. 2016; de la Fuente et al. 2020). It demonstrates that turtles’ lineage begins to diversify in the Middle and the Late Triassic, being Jurassic and Cretaceous the periods of its flowering. However, it also indicates that the divergence of Testudinata could have occurred long before the authors’ expectations (de la Fuente et al. 2020).

In the same way, studies have often proposed that the origin of the crow-group of turtles (Testudines) took place between the Upper Triassic and the Early Jurassic. For instance, the more recent estimation showed the Early Jurassic (199.5 Ma) as the origin time of Testudines (Pereira et al. 2017). However, Dornburg et al. (2011) presented the divergence time of Testudines could be far older than estimations, such as in the Early Triassic (~249 Ma). In any case, it is a question in continuous review because its divergence’s dates have depended on the calibration method,

the type of data, and the taxa sampling (Rougier et al., 1995; Joyce, 2007; Naro-Maciel et al. 2008; Chiari et al. 2012; Heath, 2012; Lourenço et al. 2012; Joyce et al. 2013). Currently, Testudines is a taxonomic order with approximately 360 living species, which occur in warm areas around the world, including some oceanic islands (Rhodin et al. 2017). Most of them are endemic species, and some are widespread because of their sea living style, specifically the Chelonioidea superfamily (Rasmussen et al. 2011). Thus, its early divergence time and current distribution reflect a broad evolutionary history where large geological events have affected its distribution, have molded its diversification, and have defined the biogeographical pattern that today we observe (Joyce et al. 2016).

Interestingly, few studies have evaluated the biogeographic pattern of modern turtles. A vast part of them have proposed biogeographic hypothesis for specific subclades into Testudines (Le and McCord, 2008; Sterli and De La Fuente, 2013; Ferreira et al. 2018; Holley et al. 2020), but few studies have focused on the origin area/range of the large groups or the older clades. Hirayama et al. (2000) is one of the first studies that put in context the biogeography of Testudines' large clades under the light of the fossil record, showing that distribution pattern in turtles obeyed to the vicariant event product of the breakup of the Pangea, as well as to dispersal events given the constant connectivity and separation of the areas through the time (e.g. marine transgressions). Rodrigues et al. (2016) also presented a biogeographic analysis but for the complete extension of the Testudines clade. For this, they reconstructed the phylogeny of the group, finding Chelydridae and Dermatemyidae as part of Testudinoidea and Testudines appearing around ~158 Ma. Despite the unorthodox relationships, they reconstructed the ancestral area and obtained Asia and Australasia as the ancestral geographical range. In the same year, Joyce et al. presented a global biogeographic model that described the evolutionary pattern for Testudines and some stem-clades. They pointed out the breakup of Pangea as the promoter for the divergence of the main clades of the modern turtles, as set out Hirayama et al. (2000). However, they did not explore any biogeographic statistical methods to support their inferences. In contrast, Pereira et al. (2017) evaluated the

phylogenetic and biogeographic history of the extant turtles, performing a biogeographic statistical model to reconstruct the ancestral area and concluding that Asia and South America were the ancestral range of the living turtles. They achieved to endorse the phylogenetic hypothesis proposed by Crawford et al. (2015) and the inferences about temporal and spatial diversification by Joyce et al. (2016). Pereira et al. (2017) also identified aspects that could have improved their approach, suggesting the inclusion of fossil information to cover unrepresented areas and the clades. However, not only including fossils as tips in the phylogenetic reconstructions could help to get better the biogeographic patterns (Meseguer et al. 2015; Wood et al. 2013), but also using time slice that reflects the breakup, connectivity, and loss of areas over the time, and therefore to employ good-calibration practices (Turk et al. 2020). Thus, our objective was to reconstruct the biogeographic history of the Testudines clade, using a tip-calibration approach, large-scale sampling, and a stratified ancestral area analysis.

2.1 MATERIALS AND METHODS

2.1.1 Calibrating *Testudinata* tree From the Maximum Likelihood topology presented in the chapter one above, we run a tip-calibration analysis in Mrbayes 3.2.7a (Ronquist et al. 2012). We fixed the topology prior and the evolutionary substitution models, using the bases frequencies, the nucleotide substitution rates, and the gamma shape from the optimized models in Raxml-ng 1.0.1 (Kozlov et al. 2019). For the length branch estimation, we employed the Fossilized Birth-Death model (FBD), following Puttick and Thomas (2015) and Heath (2012). For this model, we used a diversified sampling strategy (see Zhang et al. 2016) with the probability of the extant taxa sampling equal to 0.001. We set speciation's prior using an exponential distribution with a lambda 10, and the extinction and fossilization priors as a beta distribution with mean and shape equal to 1. We assigned a normal distribution with a mean 0.0001 and a standard deviation of 0.1 for the clock rate prior.

We assigned the 83 fossils in the topology like calibration points, employing the range age presented in Evers and Benson (2019) and the Paleobiology Database (<https://paleobiodb.org/>). Besides, we assumed the rootage prior to a uniform distribution between 190 to 330 Ma and assigned two internal calibration points, taking into account dates estimated by Crawford et al. (2015) and Shaffer et al. (2017), Testudinata (min=200, max=232) and Testudines (min=151.3, max=200). Given the Warnock's et al. (2015) recommendations, we evaluated three different prior schemes to calibrate the tree, and we selected the best set of priors estimating the harmonic means differences (Supplementary Material: Appendix 1). Each prior set was run for 10 million generations, setting two chains, four runs, and the sampling frequency equal to 1000. The final run, with the best priors, was performed with 100 million generations, and the run kept the same parameters described above. Using Tracer 1.7.1 (Rambaut et al. 2018), we evaluated the mixing efficiency and effective sample size [ESS over 1000 or at least >200 (Kass et al 1998)].

Finally, to evaluate the tendency of the highest posterior density (HDP 95%), we extracted the mean, the minimum, and the maximum age per node and estimated the range size of the HDP 95%. We correlated the time in Million years (Ma) with every range size of HDP 95%, employing a Spearman correlation test using the Stats package. We also estimated the number of nodes between a specific node and the root as a distance into the phylogeny in Adephylo 1.1-11 package (Jombart et al. 2017). We also constructed in Phytools 0.7-70 (Revell, 2012) a Lineages Through the Time plot to compare the correlation with possible decline events of lineages number and plotted the dating tree in MCMCTreeR 0.1 (Puttick and Title, 2019) package; all packages written in the R programming language (R Core Team, 2020).

2.1.2 Reconstructing the ancestral area for the Testudines clade We extracted the Testudines clade and dropped the Chelonioidea superfamily because this is a clade with marine habits that could distort vicariance and dispersion patterns (Joyce et al. 2016). We sectioned the Testudines' tree in eight subclades to make the

ancestral area reconstruction computationally workable: Pelomedusidae, Cheloniidae, Trionychia, Chelydroidea, Emysternia, Testudinoidea, and Geoemydidae. At the end, we gathered the results from each clade, and used them to reconstruct the Testudines node. The ancestral geographic reconstruction was calculated on the Biogeobears 1.1.2 package (Matzke, 2014), employing the Dispersal-extinction-cladogenesis (DEC) model (Ree et al. 2005; Ree and Smith, 2008).

As our intention was to include earth history as slices of time, we reconstructed a global regionalization, taking into account geological events that occurred from 0 to 240 Ma (Fig. 2.1). For example, we kept land extensions that were dry during the marine transgression among ~50 and 100 Ma. We tracked these events and some other such as collisions, breakup, movements, and loss of landmasses, from the global paleo-maps project (Scotese, 2016) in Gplates (Müller et al. 2018). We created a set of rules that represented the connection among areas, assigning to them three different schemes of dispersal probability values (Fig. 2.2). We generated dispersal probabilities matrices each ~5 Ma from 0 to 240 Ma per probability scheme and analyzed clade. In each analysis, we selected the best-fit probability set under the Likelihood Ratio Test (Edwards, 1972), allowing all pairs of dispersal ranges and using the maximum number of areas involved in a run as the maximum areas to optimize per node (Supplementary Material: Appendix 2).

2.2 RESULTS

Exploring different prior or model schemes allowed us to ensure analyses with the most informative parameters (Rieux and Balloux, 2016). The best set of priors was the third configuration (Supplementary Material: Appendix 2). It consisted of an offsetgamma distribution for internal calibration points and a uniform distribution for the fossil tips, which followed the statements from Nowak et al. (2013) for using gamma distribution on the internal node (Soft bounds) and Harrington and Reeder (2017) for a uniform one in the tips. After 100 million generations, we got an effective sample size (ESS) from 1134 to 42634, getting a good posterior estimation our

parameters of interest. Moreover, we got the second dispersal probability scheme as the best to reconstruct the ancestral area of Testudines. Although, Emydidae, Chelidae, and Pelomedusides clades selected the third scheme as the best, the difference between the Likelihoods were lower than two units, showing us that both are quite the same (Burnham and Anderson, 2002). Supplementary material with Likelihood values from the dispersal probability schemes and prior sets, as well as the calibration points, dispersal probability matrices, and the dating tree of Testudinata are available in bitbucket.org/alarconvv/ancestral-area-testudines-uis/src/master.

2.2.1 Divergence time from the stem-group We found that the Cisuralian, at the beginning of the Permian, gave rise to the ‘true turtles’ clade (Testudinata) around ~298 Ma. Divergence of the individual lineages was kept through the Permian until the Early Triassic, arising the main stem-clades that posteriorly diversified between the Late Triassic and the Early Jurassic. For instance, in the Carnian age, appeared the branch that gave rise to the Testudines order (~231 Ma), followed by the Angolachelonia clade, which began to diversify at the end of the Carnian age (~227 Ma), and the Paracryptodira clade that appeared ~213 Ma in the Norian. The rest of the stem-node arose in the Early Jurassic. Some of them as Xinjanchelyidae (~196 Ma), Macrobaenidae (~164 Ma), and Meiolaniformes clade (~184 Ma), which lived in sympatry with modern turtles, through the Mesozoic and the Tertiary. Apparently, until the Pliocene when *Meiolania platyceps* Owen 1886 went extinct.

2.2.2 Ancestral area and divergence time into Testudines Testudines diversified in the Late Triassic (~229 Ma), beginning with the bifurcation that would generate the largest modern clades, the Pleurodira and Cryptodira suborders. At this time, Testudines was widespread, taking up in the Eastern border at the North and the South of Pangea. Important events of divergence through the time, and their correspondent ancestral area, could be tracked in the figures 2.3 and 2.4 below. We found that between both suborders, the first clade to diversify was the total-group of

Pleurodira (Pan-Pleurodira, ~214 Ma), followed by the Pleurodira origin (~212 Ma). Both events took place in the Western of the South of Pangea. Our outcomes also supported the origin of Cryptodira in the North of Pangea. This clade began to diversify (~196 Ma), giving rise to the branch of the Adocusia clade (~108 Ma), and the branch that gathered all modern Cryptodires (~185 Ma). Some Pleurodira's clades appeared in our record, finishing the Early Jurassic. It was the case of Platychelyidae, which diversified in the Toarcian (~181 Ma), over the North-West of Pangea, and Araripeomydidae that spitted from Pelomedusides (~174 Ma) on the North-Center of Gondwana. At the same epoch over the North strips of Gondwana, the Pelomedusides' node gave rise to Pelomedusidae and Podocnemididae lineages (~168.87 Ma). Simultaneously in the Cryptodira clade, the first superfamily appeared (~167 Ma) over a widespread ancestral area. This latter taking place at the Western Gondwana and the North strip of Australasia.

Unlike the Late Jurassic, the slice time where we did not find new lineages in Testudines, the Early Cretaceous gave rise to a vast number of large clades in Testudines. In this epoch, we got that Durocryptodira arose in the West-Center of Laurasia (~141 Ma), dividing it into two branches, one that would become Chelydroidea, and another one that became Chelonioidae+Testudinoidea. Even though we did not reconstruct this node under the ancestral area analysis because of the lifestyle of the Chelonioidae superfamily, possibly the divergence between Chelonioidae and Testudinoidea (~137 Ma) had occurred in the same area. Subclades in Pleurodira also diversified in the Early Cretaceous. For example, the Chelidae family (~136 MA) appeared in the Center of Gondwana and the Eastern of Australasia, and *Podocnemys* genus split from *Erymnochelys madagascariensis* (Grandidier, 1867) (~121 Ma) in the North of Gondwana. Onward in the Barrerimian, we obtain that Chelydroidea diversified over the Western of Laurasia, being the first superfamily that appeared into Durocryptodira (~128 Ma). Aptian age gave rise to the diversification of the Chelonioidae superfamily (~121 Ma), and also the Trionychidae diversification. In the case of the Trionychidae family, we found that the ancestral area was the same ancestral range of the superfamily Trionychia (~120

Ma). Different from the Chelyidae's subfamilies, which appeared around ~122 Ma with a restricted ancestral range in the Centre of Gondwana (Chelinae) and in the Eastern of Australasia (Chelodiinea).

At the end of the Early Cretaceous, the widespread Testudinoidea superfamily (~114 Ma) split into two groups. The first group was Emysternia that diversified on the East and West of Laurasia (~101 Ma) in the Late Cretaceous, and Testuguria, which did it in South America, Madagascar, Central, and South Africa and Asia (~90 Ma). Another clade that diversified in the same epoch was the total-group of Carettochelyidae from Asia (~90 Ma) that arose in the same ancestral area of Emysternia. We got that the Campanian gave rise to the most diverse modern families, Testudinidae and Geoemydidae. The first one occupied a large geographical land extension such as South America, Central and South of Africa, and Asia around ~74 Ma. And Geoemydidae splitted (~78 Ma) between its American clade (~32 Ma), which diversified until the Oligocene, and its Asian one (~71 Ma) that arose in the last age of the Cretaceous. Pelomedusidae also appeared (~66 Ma) in the Maastrichtian, it took place in the Central and South of Africa. Finally, the diversification of the Emydidae family began earlier in the Thanetian, the last age in the Paleogene (~59 Ma).

2.3 Highest density posterior exploration (HDP 95%)

The Spearman correlation test between the size ranges of the HDP's and the mean age of the node showed us there is a strong non-linear relationship between both variables, size ranges of the HDP's and mean ages ($\rho = 0.7181285$; $p\text{-value} < 2.2\text{e-}16$). The pattern showed nodes in the middle of the tree (node distance: 100-200), getting the largest range size HDP's (20-80 Ma). They coincided with the diversification of some stem-turtles and some fossils clades into Testudines. This high uncertainty in the nodes matched loosely with the decline in the number of new lineages between the Middle and Late Jurassic (Fig. 2.5). Finally, we also noticed that the calibration of the deep-nodes close to the root (node distance: 0-32) got smaller sizes, which also concurred with our large concentration of fossil sampling.

2.4 DISCUSSION

2.4.1 Early Divergence of Turtles Paleontological studies have suggested that the Testudinata clade appeared in the Late Triassic, which ties in with the record of the oldest Testudinata, *P. quenstedti*, and the sister of the group, *O. semitestacea* (Li et al. 2008; Joyce et al. 2009). However, a recent study about the paleo-distribution of a new specimen from *Palaeochersis talampayensis* Rougier et al. 1995 stated that Testudinata could have had its origin at least 25 Ma before other authors have proposed (de la Fuente et al. 2020). Here, the diversification of the Testudinata clade began around ~298 Ma, just at the end of the Carboniferous and the beginning of the Permian periods. It supported de la Fuente's et al. (2020) hypothesis about an earlier origin, but with the double-time of their statement. It is the first time that Testudinata is dated far longer than expected, but it is not a new idea about an ancient turtles' origin. Authors have already assumed that the total-group of turtles appeared in the Permian ~265 Ma, in which *Eunotosaurus* Seeley 1892 is the older member of this group (Lyson and Bever, 2020). Following our outcomes, the turtles' origin coincided with the global temperature increases during the lower Permian, when the Paleo-Thetis sea got closed, and Pangea became the largest continent (Rose and Rose, 1985; Blakey, 2003). In this time, the new connectivity among large landmasses could have allowed the dispersion (Dunne et al. 2018), favoring the diversification of the Permian-biota, especially for Tetrapoda lineages that survived of the Carboniferous rain forest collapse (Sahney et al. 2010; Pardo et al. 2019). Here, we stated two hypotheses about the obtaining date in our analyses. The first one is the Testudinata clade diverged in the Permian, and the fossil record could not catch the event, or the second one is the lack of fossil sampling rootward in our analysis that could not discern between the origin of the Testudinata as a clade and the birth of the total stem-group of Turtles. In any case, our results endorsed the statements about the origin of turtles, in which some authors have argued that turtles were one of the first branches in emerging within the rapid Eureptilia radiation (Wang et al. 2013; Joyce, 2015; Lyson and Bever, 2020). Thus, although the first stem-turtle

fossil appeared toward the Middle and Late Permian (Bever et al. 2015), the divergence of the lineage took place back at the beginning of the Permian. Otherwise, it is not the first time that the tip-calibration approach and the Fossilized Birth-Death model extend the taxa origin dates. Studies such as Peijnenburg et al. (2020), Gustafson et al. (2017), and Gavryushkina et al. (2016) have shown earlier dates than suggested up that moment. Although we are aware the date extension could be a computational artifact, this issue has been recorded when dating has been performed only with calibrated points on tips (Puttick et al. 2016). Given our previous prior exploration and the combination of internal calibration points with soft-bounds priors, plus the calibration points on tips (see suggestions from Yang and Rannala, 2006; Nowak et al. 2013; O'Reilly and Donoghue, 2016; Barba-Montoya et al. 2020), we could support the performance of the dating with the stretch range size of the HDP in most our nodes (Fig. 2.5). Then, thinking about the good-HDP recovered, and the idea that fossils support better minimum ages rather the maximum one (Benton and Donoghue, 2007; Rieux and Balloux, 2016), we assumed that earlier origin of turtle was a possible result of its evolutionary history printed in its characters, and no from the method's sensibility.

2.4.2 Biogeographic History of the Major Clades of Testudines In contrast to the turtles stem-group, the molecular phylogenetics field has extensively explored the dating of the Testudines clade (See: Shaffer et al. 2017, Pereira et al. 2017), suggesting mean ages between Late Triassic and Early Jurassic (Naro-Maciel et al. 2008; Chiari et al. 2012; Heath 2012; Shaffer et al. 2017). Our study showed that the Testudines diversification, including the split that arose its suborders, began at the end of the Carnian around ~229 Ma over a wide range among the Eastern Eurasia and the North-East strip of Gondwana. This age matched with the arid climate that was getting warm at the end of the Carnian, which were gradually turning moister given the monsoonal seasons at the end of the Late Triassic (Preto et al. 2010). Conditions that were appropriate for the evolution of ectothermal reptiles as early dinosaurs (Martinez et al. 2011).

The biogeographic pattern showed us that this broad area was broken into two clades, the Pan-Pleurodires, and Cryptodires' lineages. Our results suggested the Pan-Pleurodira lineage appearing in the Late Triassic and passing through the Triassic-Jurassic boundary without affection like in other terrestrial-biota (Lucas, 2018).

Besides, Cryptodira diversified in the Early Jurassic. Although our outcomes Cryptodira's date coincided with Joyce et al. (2013) proposal, it is the first time that Pleurodira appeared before the Cryptodira group. However, we should point out that Cryptodira's HDP is larger than Pleurodira, even overcoming the maximum bound of Pleurodira's age estimation. The high uncertainty in the Cryptodira age could be a product of the lack of sampling of stem-Cryptodires. Different from Pleurodira, in which we included the Platychelydae family. Even so, both groups appeared before the Joyce's et al. (2016) and Pereira's et al. (2017) proposal, we recovered a clear diversification from Cryptodira in the North-East and Pleurodires in the South of Pangea. This pattern could correspond to the beginning of Eurasia and Gondwana breakup because of the Neo-Tethys sea's opening in the Late Triassic (Gómez and Goy, 2005; Schettino and Turco, 2011).

Pleurodira. Some studies have shown that Pleurodires had a broad distribution through the South hemisphere (Joyce et al. 2016), being South America the most likely ancestral area (Pereira et al. 2017). As a previous hypothesis, our results found the split of Platychelydae from Pleurodira, as well as Chelidae from Pelomedusoides (Araripeomydidae+Pelomedusides), taking place on the Western of West Gondwana, the area that today we know as South America. In contrast to the ancestral range of the group, our ages were discrepant from other authors (Joyce et al. 2016, Pereira et al. 2017; Ferreira et al. 2018; Holley et al. 2020), changing the internal biogeographic pattern of Pleurodira. Despite the incongruences, our dates fitted better to some biogeographical questions that remain obscure up today. For example, we recorded the split into Pelomedusides around ~168 Ma earlier than the latter proposes (Pereira et al. 2017, Holley et al. 2020) but congruent with the molecular dating from Luo et al. (2019). Pereira et al. (2017) recorded the

Pelomedusides divergence as a result of the early rifting between South America and Africa around 137 Ma. They stated that Podocnemididae remained in South America and Pelomedusidae in Africa, advocating that posterior records of Podocnemids in Africa and Europe were a result of dispersion events. In our case, the Podocnemididae family kept the ancestral areas over the North strip of Gondwana, and Pelomedusidae was restricted to the Central and South Africa.

A broad ancestral range in the north of Gondwana, before the rifting of America and Africa, could explain why after completing the land masses separation (Toon et al. 2010; Granot and Dyment, 2015) freshwater taxa appear in the Oligocene in Africa (e.g. *Dacquemys paleomorpha* Williams 1954 in Gaffney et al. 2011). Although the dispersal hypothesis became more supportive when fossil taxa are included (e.g. Ferreira et al. 2018), it is easier to think that turtles used short routes to spread instead large transoceanic ones. Thus, an earlier divergence could explain better the biogeography pattern of the Pelomedusides.

Likewise, Joyce et al. (2016) have stated the split into Chelidae as product of a vicariant event in the Early Cretaceous. This hypothesis was followed by Ferreira et al. (2018), who exposed an Australian origin followed by dispersion events after the breakup of West and East Gondwana. We also reinforced Joyce et al. (2016) ideas about a vicariant event in the Early Cretaceous. However, we differed from them, because we estimated the South strip of Gondwana as the ancestral area. Thus, a solo vicariant event as the Weddell sea opening (König and Jokat, 2006) would define the divergence between the South American and Australian lineages (~136). Holley et al. (2020), the widespread Pan-Chelids (Smith, 2010), and even some restricted South American Fossils (Lapparent de Broin and de la Fuente, 2001) support our outcomes. However, it is clear that conclusions about the biogeographic pattern of Chelidae could depend on the phylogenetic resolution. Thus, if American and Australian taxa mix among them, only dispersion events would be favored to explain the pattern (See Chelidae relationships: Pereira et al. 2017, Ferreira et al. 2018).

Cryptodira. As in other models about *Cryptodira*'s diversification, this group appeared in Asia and, from there, began to spread toward other areas (Li et al. 2017, Joyce et al. 2016, Pereira et al. 2017). From current lineages, the first superfamily that diversified was *Trionychia*. Authors have inferred its origin from the Early Cretaceous, with an Asian ancestral range (Hirayama et al. 2000; Joyce et al. 2013, 2016). We found that the *Trionychia* superfamily began to diversify at the end of the Middle Jurassic, taking place on a broad ancestral range with recent dispersion events toward Australia and surrounding areas. Our outcomes partially endorsed the hypothesis by Li et al. (2017). Even though we agreed about young insular dispersion events, Asia was not the only ancestral range. In contrast, our results showed that *Trionychidae* inherit the same ancestral area of the superfamily, *Trionychia*. Although our broad range was a product from the best-fit ancestral area outcome, we did not discard that differences with other authors could be because they may have restricted the max area optimization parameter to few areas (Pereira et al. 2017); whereas, we used all current distribution to estimate the ancestral range. Despite the differences, our results suggested an internal radiation that was driven by dispersion events as in previous hypotheses (Joyce 2014; Joyce et al. 2016; Li et al. 2017; Pereira et al. 2017). For instance, the dispersion of the *Apalone* Rafinesque 1832 lineage, which diverged from *Rafetus* Gray 1864 (~63 Ma) and diversified around 30 Ma in North America. *Apalone* could be a product of the opportunity to travel via Bering land bridge in the Late Cretaceous (Wen et al. 2016), or via the Eurekan route because of the approximation between Eurasia and Greenland in the Early Eocene (Torsvik and Cocks, 2016). Likewise, the *Lissemys* Smith 1931 and *Cycloderma* Peters 1854 divergence was leaded by a dispersion event that was possible via the Kohistan-Dras volcanic Arc, which favored the connection between Africa and India in the Late cretaceous (Chatterjee and Scotese, 2010).

The Durocryptodira lineage appeared over the East of North America. A place in which occurred the divergence of Chelydroidea from Chelonioidea + Testudinoidea;

and where probably the divergence of these latter clades also took place. In the Chelydroidea instance, its radiation occurred in North America and subsequent dispersion events took place toward the South, covering Centro America and even reaching South America (Cadena et al. 2012) as part of the Great American Biotic Interchange in the Late Miocene (Montes et al. 2015). Although Chelydroidea appeared in North America, members from the total group of this family were not exclusive from this area. Fossil record has confirmed that some Pan-Chelydrids went into Europe and Asia. However, the time of these facts remains in discussion (Joyce, 2016). We did not estimate the ancestral area of Chelonioidea and the clade that gathered this superfamily with Testudinoidea. However, we hypothesize that given the fossil record and the ancestral range from its sister, both nodes apparently could have taken place in North America. We suggested their divergence time ensued at the Early Cretaceous. In our analysis, Chelonioidea was the Cryptodira family with most fossil members and, therefore, with most calibration points. Although authors have proposed the origin of this group for the Early Cretaceous (Joyce et al. 2013, Pereira et al. 2017), other proposed have advised an earlier radiation, given the fossil record (Cadena and Parham, 2015; Evers and Benson, 2019). Indeed, Evers and Benson (2019) argued that taking into account fossil material to reconstruct the Chelonioidea relationships could have implications over the calibration. In this case, not only on the Chelonioidea clade but also in their back ancestor. Thus, our dates included and fit well to older members as *Desmatochelys padillai* Cadena and Parham 2015, and entailed the Evers and Benson (2019) assumption about the beginning of the Chelonioidea diversification in the Early Cretaceous (~121 Ma), as well as, the crown Cheloniidae's origin earlier than expected.

Cretaceous was a period with an intensive climate change characterized by several peaks of the warm global temperature (Steuber et al. 2005). It favored dispersal routes among large landmasses that eventually were interrupted by the high sea level, creating a perfect scenario of expansion, contraction, and speciation (Sanmarti et al. 2001; Föllmi 2012). This condition could favor the dispersal events that allowed the origin of Testudinoidea over a wide ancestral range.

Inner Testudinoidea, the first lineage in diversifying was Emysternia, which split around 101 Ma, into two branches, the North American Emydidae family and the Asiatic Platysternidae one. This divergence was the result of a vicariant event given the Western Interior Seaway that separated the East from the West North America + East Asia (Cumbaa et al. 2010), which were connected via the Bering land bridge by that time (Scotese, 2016; Graham, 2018). Likewise, Testuguria split into two lineages at the end of the Turonian, arising the Testudinidae and Geoemydidae families. It occurred in a broad range that posteriorly was inherited by Testudinidae family, around 74 Ma. A broad ancestral area and the subsequent pattern of radiation may be explained by a scenario of sympatric-subset speciation. In this scenario a broad area divides into unequal small ranges (Matzke, 2014), which fit to the pattern Testudinidae family. It could resolve how tortoises arrive from Africa to South America. Considering the terrestrial habit of the family, a diversification pattern via sympatric subsets could be a suitable hypothesis instead of the transoceanic dispersal events or a migration through Africa-Europe-Asia-North and South America. Although these hypotheses have been debated (de la Fuente et al. 2014; Kehlmaier et al. 2017), it has been the most available explanation up now. However, crossing the sea continues being the best explanation to support tortoises inhabiting oceanic islands (e.g. Gerlach et al. 2006).

Finally, authors have considered Geoemydidae as an Asian family (Le and McCord, 2008; Pereira et al. 2017). Our outcomes validated this hypothesis but including America in the range. We found this family diversified about 78 Ma, splitting in the American and the Asian clade. Although our proposal shows the North of South America has part of the range, it is clear that it is an artifact. We suggest the West of North America could be the actual area of Geoemydidae ancestral distribution. Current American Geoemydids (*Rhynoclemmys* Fitzinger 1835) take place in the Central and the South of the continent. However, genera such as *Echmatemys* Hay 1906 (~55 Ma) and *Bridgeremys* Hutchison 2006 (~49 Ma) confirm that the West of North America was part of the Geoemydidae's distribution in the Paleogene, and

also that *Rhynoclemmys* was a posterior diversification in the Southern, diversifying around 32 Ma and being part of the Pre-GABI biotic interchange (Agnolin et al. 2019). The Geoemydidae ancestral area between the East of Asia and the West of North America coincided with the free migration routes via the Bering land bridge and the greenhouse conditions in the Early Late Cretaceous (Gardner, 2000). But the divergence between both clades, the Asiatic and American ones, may have occurred because of the cooling of North Alaska in the Santonian-Campanian boundary, which is evident given the floristic differences between the margins of Asia and North America in this age (Zakharov et al. 2011; Herman, 2013).

2.4.3 Highest density posterior ranges (HDP 95%) In phylogenetic calibration, the Highest Density Probability determines the node uncertainty, interpreted as the age range in which a taxon appeared (Lee et al. 2009). Thus, narrow HDP ranges translates into low uncertainty, and therefore, more accurate and precise dates (Warnock et al. 2011). Although uncertainty in nodes could be improved depending on, for example, the priors (Ronquist et al. 2012; Warnock et al. 2015), some authors have identified that the deeper the node, the higher the uncertainty; which could increase in the nodes when fossils are included in the analyses (Huelsenbeck, 1991; O'Reilly and Donoghue, 2016). Nevertheless, uncertainty could improve whether analyzes use the tip-dating approach with several calibrated tips close to the root (Püschel et al. 2020). Given this recurrent affirmation, we hoped rootward nodes with high HDP range size, similar to Dornburg et al. (2011), Heath (2012), Shaffer et al. (2016), or Joyce's et al. (2013) results. In contrast, our estimation disclosed that the HDP range sizes did not depend on the node depth. Our outcomes indicated that, even though HDP range size and the age mean were correlated, it was not in a linear way (Arcila et al. 2014; O'Reilly and Donoghue, 2016). Our pattern showed that nodes with medial age (150-200 Ma) got the highest intervals. However, they did not reflect ages or periods, with a low level of fossil record diversity (~150 Ma) or massive extinction events (Cleary et al. 2020). Instead, these wide HDP ranges were linked to clades with only fossils tips, especially nodes in the stem-group, as a

possible response to taxon sampling. Despite we got wide HDP ranges, they did not go up 80 Ma, and most of them did not outpoint 20 Ma. Thus, narrow HDP ranges made us infer that dating was precise given the parameters used here and, therefore, the gosh lineages suggested by our early ages could be a result of a taphonomic artifact rather than a computational one (Arcila et al. 2015, Evers and Benson 2019). Thus, although we could not confirm our assumptions about our narrow HDP ranges, given the empirical nature of our study, we wanted to show their behavior to enhance future studios evaluating uncertainty on tip-dating approach.

2.5 CONCLUSIONS

In this study, we achieved to state the possible turtles' origin and to describe the biogeographic history of the Testudines clade under a tip-calibration approach, large-scale sampling, and stratified ancestral area analysis. We hypothesized that turtles appeared in the Early Permian earlier than expected and far sooner than the fossil record could recognize as morphological forms relative to this group. Besides, we obtained the crown-group diversified in the Late Triassic, reinforcing the Testudines' origin proposed by previous assessments. Also, our study showed a broad range as the ancestral area of Testudines, covering the Eastern border at the North and the South of Pangea. The wide range and the early divergence of Testudines allowed fitting its biogeographic pattern to the major geological events that the Earth suffered between 230 – 0 Ma, such as the vicariant barriers arose because of the breakup of Pangea, together with the warm global periods and the formation of land bridges that favored intercontinental dispersal routes. Finally, although dispersion is considered a predominant event into the crown-turtles' radiation, vicariance could have developed an essential role in the divergence of the main clades of Testudinata. However, future studies should also focus on other events such as sympatric speciation and extinctions, which could be explaining some particular patterns found in clades at the family level; as well as future assessments about the effect of the fossil position, the number of fossils and both type of calibration, tip and node-dating, on empirical data.

FIGURES

Figure 2.1 Map of the regionalization used in this study. Letters indicate the units or areas, they were kept and fitted through the time from 0 – 240 Ma.

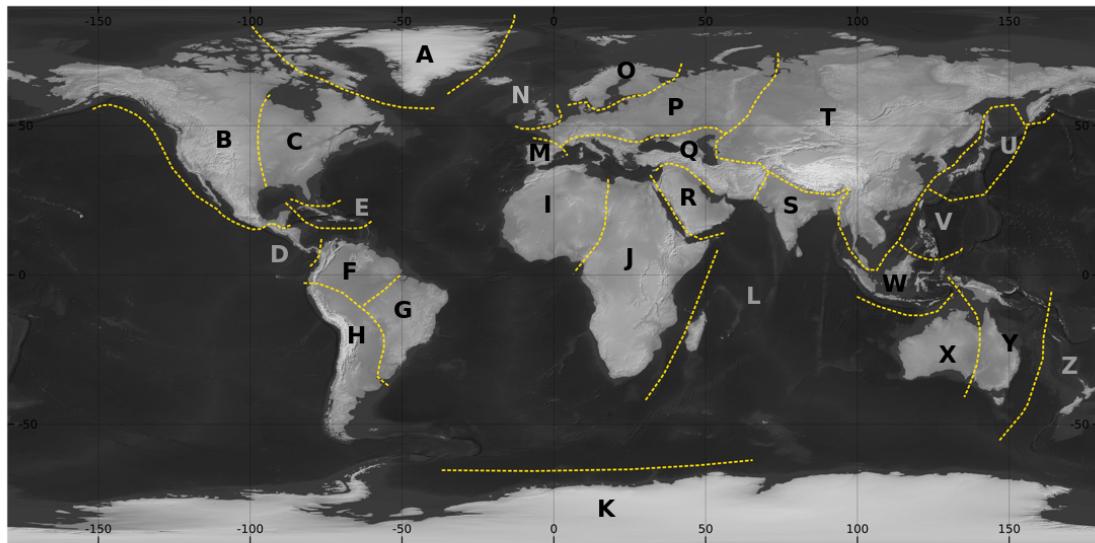


Figure 2.2 Rules of adjacency among areas. On the right, the upper triangular matrix indicates the rule used among the areas (yellow) connection. Lower triangular matrix indicates allowance used in this study. On the left, rules' description and the dispersal probabilities evaluated in this study.

F	E	Sea	A	B	Rules						Dispersal Probability Schemes		
					I	II	III	I	II	III	I	II	III
A	-	1 2 3 4 5			1 Adjacent area			1.0	1.0	1.0			
B	1 -	1 2 5 6			2 Areas separated by one area			1.0	0.5	0.7			
C	1 1 -	1 6 6			3 Adjacent area but these separated by the sea			1.0	0.3	0.5			
D	1 1 1 -	6 6			4 Areas separated by two or three areas			1.0	0.2	0.3			
E	1 1 1 1 -	1			5 Areas separated by the sea and an area			1.0	0.1	0.2			
F	1 1 1 1 1 -				6 Without any type of adjacency above or separated by frozen areas			1.0	0.01	0.001			
		Allowance											

Figure 2.3 Phylogeny of Testudines order. Letters below nodes show the ancestral area estimated under the second scheme of dispersal probability matrices. HDP 95% are showed as navy bars. NA indicates nodes with non-assigned ancestral area.

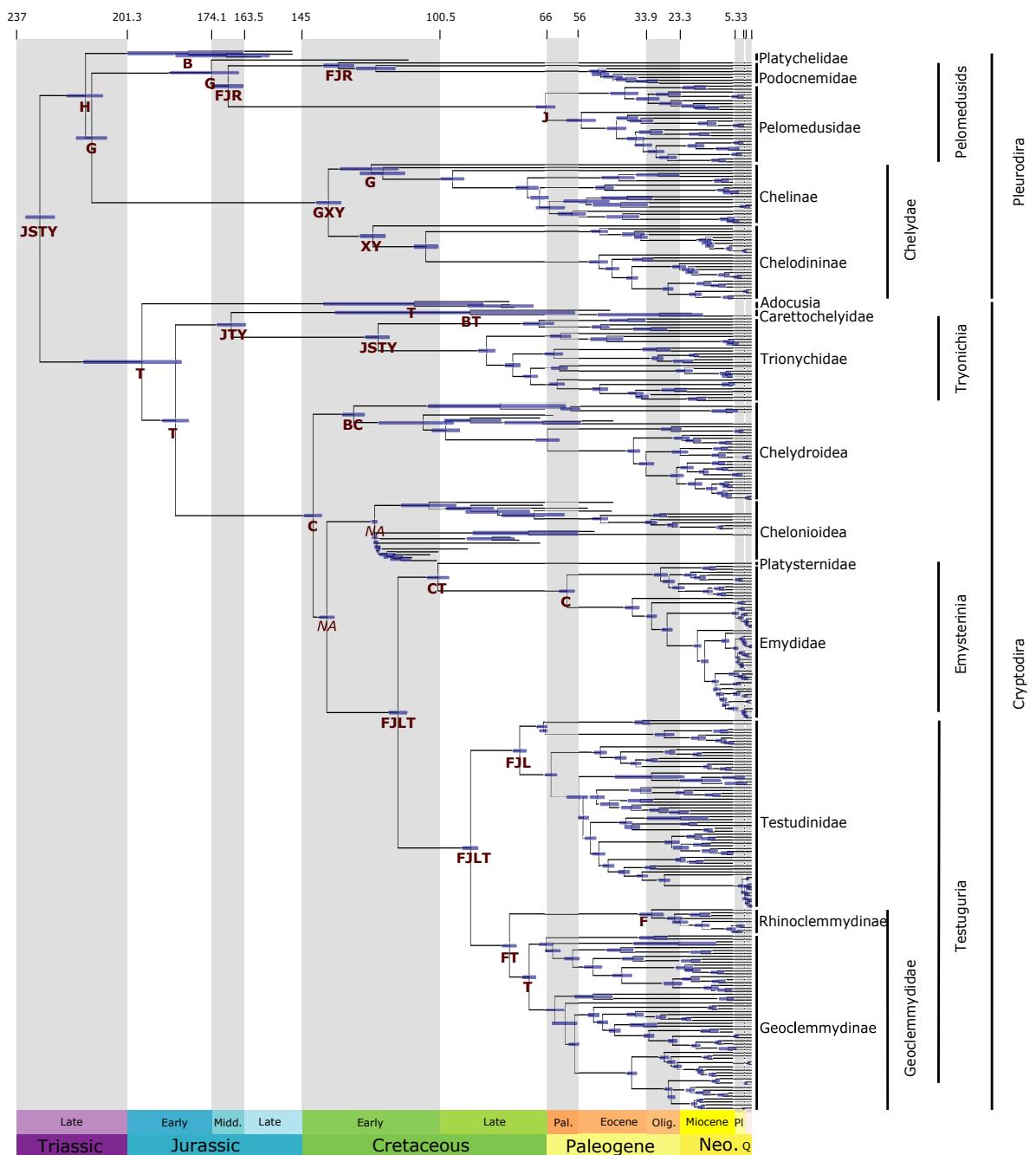


Figure 2.4 Landmasses movements through the geological epochs. Time coincide with the principal events of diversification and divergence of the large clade of Testudines. Letters indicates the equivalence with the area presented in the figure 2.1. Maps was taken from the paleo-maps project (Scotese 2016) and visualized in Gplates (Müller et al. 2018) using 3D orthographic projection.

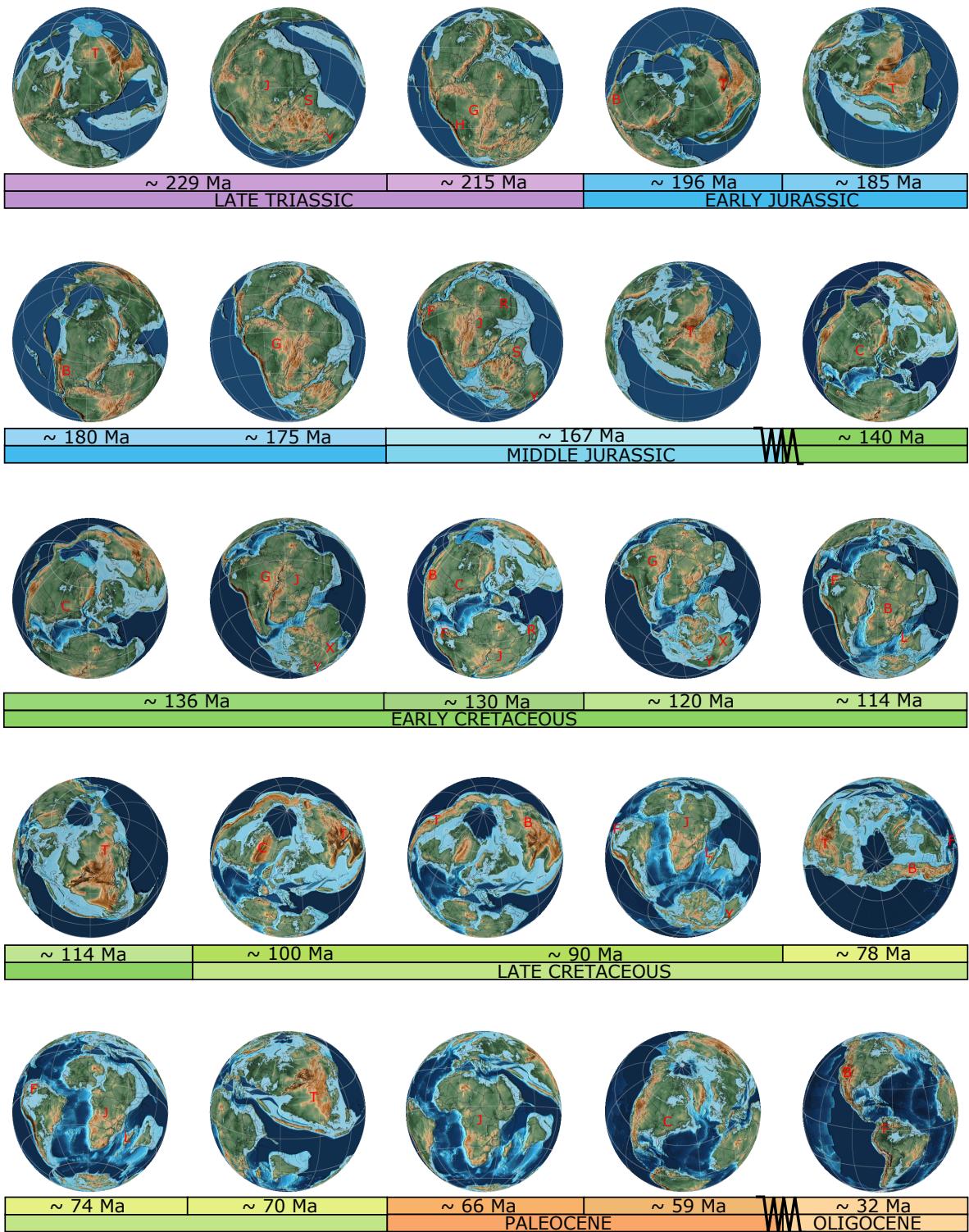
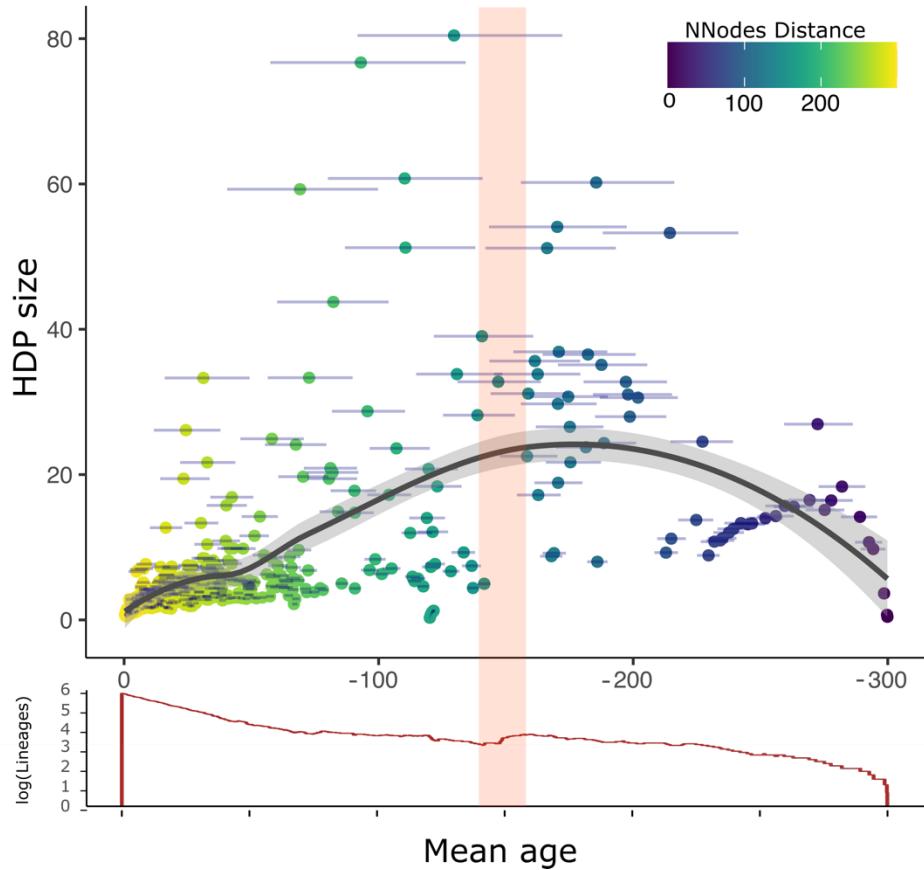


Figure 2.5 Range size of the HDP 95% per node. Ranges and time are in units of Ma, color in the points indicates the distance to the root in terms of the number of nodes. Red line shows the Lineages Through the Time plot, with number of lineages axe is in Log scale.



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