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Amblyomma parvum Aragão, 1908 (Acari: Ixodidae): phylogeography and systematic considerations

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Abstract

The geographical distribution of *Amblyomma parvum* Aragão 1908 in the New World is disjunct, with two main clusters separated from each other by the Amazon basin. The main objectives of this study were to further investigate the systematic relationships within *A. parvum*, to determine whether or not populations from different geographical areas might represent cryptic species, and to reconstruct the phylogeographical evolutionary history of the species. The genetic diversity of *A. parvum* collected throughout its distributional range was analyzed by using 6 molecular markers: 5 mitochondrial [(the small and the large ribosomal subunits 12rDNA and 16SrDNA, the cytochrome oxidase I (COI) and II (COII) and the control region or d-loop (DL)], and one nuclear (ITS2, Inter transcribed spacer 2). Phylogenetic trees were inferred by using maximum parsimony and Bayesian analyses. In addition, node dating was attempted for the main lineages identified phylogenetically. Although mitochondrial and nuclear topologies were not totally congruent, they all identified at least two main supported clusters, a Central American lineage, and a Brazilian-Argentinian lineage. Clade support and divergence values strongly suggest that the two lineages correspond to different taxonomic entities. Node dating placed the split between the Central American and the Brazilian-Argentinian lineages at approximately 5.8 – 4.9 Mya, just after the progressive replacement of the dry areas that occupied the northern part of South America by the Amazon Basin in the early-mid Miocene. This event might be the cause of fragmentation and putative speciation within the ancestral relatively xerophilic *A. parvum* population.

Key words: *Amblyomma parvum,* phylogeography, phylogeny, systematics, cryptic species, molecular markers.

Introduction

The hard tick *Amblyomma parvum* Aragão 1908 (Acari: Ixodidae) has a broad geographic distribution, ranging from Mexico to Argentina (Guglielmone and Hadani, 1980; 1982; Guglielmone et al. 1990; 2003; Hoffman, 1962; Nava et al., 2008a). Although the presence of a single specimen was reported from Florida in 2004 (Corn et al., 2012), the tick is certainly not established in the United States. Throughout its geographical distribution, this species has a predilection for drier areas of Central America, the Northern coast of South America, and the dry diagonal region (Morrone, 2006) that extends from the Chaco and Monte in Argentina, to the Cerrado and Caatinga in Brazil (Fairchild, 1966; Guglielmone and Hadani, 1980; Nava et al., 2008a). Basically, with the exception of an isolated population in Roraima (Amazonia), *A. parvum* has a disjunct distribution with two main clusters, one northern and one southern, separated by the Amazon basin.

With respect to host range, adult *A. parvum* commonly parasitizes a variety of medium to large-sized domestic and wild animals, including humans (Jones et al., 1972; Guglielmone et al., 1991; Nava et al., 2006; 2008b), while the immature stages are commonly collected from rodents, in particular members of the Caviidae and Echimyidae families (Labruna et al., 2005; Nava, 2006; 2008b; Saraiva et al., 2012).

Because *A. parvum* can bite humans, it can be a potential vector of pathogens of public health importance. Specimens of this tick have been found to be naturally infected with *Coxiella burnetii, Ehrlichia chaffeensis,* and *Candidatus* 'Rickettsia andeanae' (Labruna et al., 2011; Pachecho et al., 2007; 2013; Tomassone et al., 2008).

Aragão described *A. parvum* in 1908 (Aragão, 1908) and further illustrated it in 1911 (Aragão, 1911). Ivancovich (1973) designated a subspecies, *A. parvum carenatus*, which differed from the original description of *A. parvum* by the presence of ventral plates (carenae) on the festoons, not realizing that in his

1911 publication, Aragão had previously mentioned the occurrence of carenae in *A. parvum* (Guglielmone et al., 1990). Later, Guglielmone and Hadani (1980) observed that males with carenae were more commonly found on cattle, whereas males without carenae were almost exclusively found on a wild rodent, *Dolichotis salinicola* (Burmeister, 1876). The taxonomic status of this species group was addressed by Guglielmone et al. (1990) through an in depth morphological analysis of specimens collected mainly in northwestern Argentina. The authors redescribed the adults and described the immatures of *A. parvum* and synonymized *Amblyomma parvum carenatus* with *A. parvum* because it corresponded with the tick described by Aragão in 1911. In addition, they erected a new species, *Amblyomma pseudoparvum* Guglielmone, Mangold, and Keirans 1990, consistently found to lack carenae and to parasitize *D. salinicola*.

Although the systematic status of *A. parvum* appeared to have been clarified, recent molecular studies suggested that *A. parvum* could represent a species complex (Nava et al., 2008a). Increasing evidence shows that tick species with wide geographic distributions may in fact be clusters of more or less cryptic species (Szabo et al., 2005; Labruna et al., 2009; Mastropaolo et al., 2011; Beati et al., 2013; Nava et al., 2014).

Analysis of 16SrDNA sequences of *A. parvum* from Argentina and Brazil revealed significantly higher divergence values between (3.7%) than within populations (0 to 1.1%) suggesting the possible occurrence of two species (Nava et al., 2008a), one in each country. Nevertheless, divergence values are relative numbers, which cannot be used to define species, particularly when based on the analysis of a single gene. Multiple sources of morphological, molecular, and biological evidence should be combined in order to reliably delimit species. When Gerardi et al. (2013) compared feeding and reproductive parameters of *A. parvum* colonies from Argentina and Brazil infesting different host species, they could not reveal any significant difference between ticks from the two countries.

Nonetheless, these studies were based on specimens collected from southern South America only. If samples from Brazil and Argentina are genetically different enough to suggest incipient speciation, samples from Central

America are more likely to have diverged earlier and to constitute a different species. This would be particularly meaningful, if we consider that another tick taxon, *Amblyomma cajennense* Fabricius sensu lato, with a similar geographical distribution and also associated with relatively drier areas, was found to represent six different species, one of them with a distribution extending from southern Texas to Ecuador (*Amblyomma mixtum* Koch, 1844) and five confined to continental South America (Beati et al., 2013; Nava et al., 2014).

Herein, we evaluate the genetic diversity of samples of *A. parvum* collected from the tick's main areas of distribution (Central-North America, Brazil, and Argentina), by analyzing and comparing DNA sequences of five mitochondrial and one nuclear gene. The phylogenetic reconstructions are also used to develop phylogeographical evolutionary scenarios involving tentative node dating.

Material and Methods

Sampling

Our samples included 87 adult specimens identified as *A. parvum* from the following countries: Argentina, Brazil, Costa Rica, El Salvador, Panama, Paraguay and Mexico (Figure 1). For Argentina and Brazil, specimens from several localities were included in order to consider variation between and within different eco-regions. Our tick sample included specimens from 24 localities, corresponding to 7 countries across the geographic distribution of *A. parvum,* thus covering a wide range of latitudes (Table 1 and Figure 1). The collection sites shown in Figure 1 and Table 2, and are designated as follows: Argentina (Chamical, San Antonio, Esquiu, Quilino, San Jose de las Salinas, Villa Ojo de Agua, Cejolao, Uratau, and Dragones); Brazil, (Nhecolandia, Araguapaz, Formoso, and Gilbues); Costa Rica, (Palo Verde: PV; Hacienda Orosi: HO); El Salvador, (Tejutepeque); Mexico, (Chochola); Panama, (Parque Summit, Los Santos, Santa Rosa, and Puerto Limon); Paraguay, (Filadelfia). In addition,

specimens of *A. pseudoparvum* included in the analyses were from Rivadavia Banda Sur, Salta, Argentina.

DNA extraction, PCR, and sequencing

Tick DNA was extracted and exoskeletons were preserved for further morphological analysis following previously published protocols (Beati and Keirans, 2001; Beati et al., 2012). A small portion of the postero-lateral idiosoma of each tick was cut off using a disposable scalpel and the entire specimen was incubated overnight in 180 µl Qiagen ATL lysis buffer (Qiagen, Valencia, CA) and 40 µl of a 14.3 mg/ml solution of proteinase K (Roche Applied Sciences, Indianapolis, IN). After complete lysis of the tick tissues and repeated vortexing, the whole exoskeleton was stored in 70% ethanol and kept as voucher specimen. The lysed tissues were further processed as previously described (Beati and Keirans, 2001; Beati et al., 2012). Five mitochondrial gene sequences, 12SrDNA (ribosomal small-subunit RNA gene sequence), 16SrDNA (ribosomal small-subunit RNA gene sequence), COI (Cytochrome oxidase subunit I), COII (Cytochrome oxidase subunit II), and d-loop (DL, control region) were amplified by using previously reported sets of primers (Barret and Hebert, 2005; Beati and Keirans, 2001; Beati et al., 2012; Mangold et al., 1998). In addition, a portion of the nuclear ribosomal internal transcribed spacer 2 (ITS2) was amplified by modifying previously published methods, with 35 instead of 27 cycles of annealing (Beati et al., 2012; McLain et al., 1995). PCRs were performed using a MasterTaq kit (5-Prime, Gaithersburg, MD). Each reaction contained 2.5 µl of tick DNA, 2.5 µl of 10 × Taq buffer, 5 µl of 5 × TaqMaster PCR Enhancer, 1.5 µl of MgAc (25 mM), 0.5 µl of dNTP mix (10 mM each), 0.1 µl of Taq polymerase (5U/ µl), 1.25 µl of each primer from a 10 pmoles/µl stock solution (Invitrogen, Life Technologies Corporation, Grand Island, NY), and 14.6 µl of molecular biology grade H_2O. The two DNA strands of each amplicon were purified and sequenced at the High-Throughput Genomics Unit (HTGU, University of Washington,

Seattle, WA) and were assembled with Sequencer 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic analyses

Sequences were aligned with McClade 4.07 OSX (Sinauer Associates, Sunderland, MA) (Maddison and Maddison 2000). Secondary structure was considered in aligning 12SrDNA (Beati and Keirans 2001) 16SrDNA and DL (Zhang and Hewitt 1997). Codon organization was taken into account when aligning the COI and COII data sets. Each data set was analyzed by maximum parsimony (MP) using PAUP (Swofford, 2000), and by Bayesian analysis (BA) with MrBayes 3.1.2 and 3.2.4 (Huelsenbeck and Ronquist 2001; Ronquist et al., 2011). Branch support was assessed by bootstrap analysis (1000 replicates) with PAUP for MP, and by posterior probability with MrBayes for BA. MP heuristic searches were performed by branch-swapping using the tree bisection-reconnection (TBR) algorithm, ACCTRAN character optimization, with all substitutions given equal weight. Gaps were treated either as a 5th (DL) or as a missing character (12srDNA, 16SrDNA, COI, COII, ITS2, and both concatenated datasets). The ML nucleotide substitution model best fitting the data was selected by JModeltest v2.1.7 (Darriba et al., 2012; Guindon and Gascuel, 2003). Maximum likelihood pairwise sequence distances were calculated based on the selected model by using PAUP. Two analyses, with four chains each, were run simultaneously for BA analyses (1,000,000 generations). Trees were sampled every 100 iteration. Trees saved before the average standard deviation of split fragments converged to a value < 0.01 were discarded from the final sample. The 50% majority-rule consensus tree of the remaining trees was inferred and posterior probabilities were recorded for each branch. A partition homogeneity test in PAUP (Swofford, 2000) (100 replicates, with maxtrees set to 1000) was run with the datasets to establish whether or not the character partitions were heterogeneous (significance threshold value $p = 0.05$) (Bull et al., 1993).

Mitochondrial data sets were combined for total evidence analyses in MacClade (Sinauer Associates, Sunderland, MA) (Maddison and Maddison 2000) and analyzed following the same procedure applied to the separate individual genes. Outgroup species used for all phylogenetic analyses were: *A. cajennense, A. sculptum* Berlese*, A. mixtum,* and *Amblyomma interandinum* Beati, Nava and Caceres*.* The species *A. pseudoparvum* was included in the analyses because it corresponds to the closest morphological relative of *A. parvum*.

Molecular clock and divergence dates

Mutation rates among lineages were compared by the relative mutation rates test, for all monophyletic clades, including the outgroup species, by using DAMBE5 (Xia, 2013). Saturation levels and the molecular clock hypothesis (likelihood ratio-test and the least-square method) were also tested by using DAMBE5 (Xia, 2013). Beast2 package (Bouckaert et al., 2014) was used to tentatively estimate the divergence time of the main nodes in the phylogenetic reconstruction. The dataset was partitioned by gene, and substitution models were estimated separately for each of them. The main clades were defined as being monophyletic according to the tree topology. The relaxed clock model was selected with uncorrelated lognormal distribution, and the tree prior was set to a calibrated Yule Process. For the MCMC analysis, chain lengths were set to 1,500,000 and data were sampled every 100 iterations with a random starting tree. Burnin was set to 10%, and the remaining trees were summarized as a maximum clade credibility tree with TreeAnnotator v2.3.0. FigTree v.1.4.2 was used to visualize tree topology, with mean divergence times and 95% confidence intervals. Node calibration was based on biogeographical information used in a recent publication on the biogeography of a closely related group of ticks, the *A. cajennense* complex (Beati et al., 2013). Species of that complex were purposely selected as outgroups in this study, because their previously evaluated divergence times can constitute additional calibration points for dating nodes within *A. parvum.* More than one calibration was performed in order to evaluate the robustness of our priors. The tested calibrations were: outgroup node set to

15 ± 4 million of years ago (Mya); *A. cajennense - A. mixtum* node set to 6 ± 2 Mya; *A. cajennense - A. mixtum -sculptum* node set to 9 ± 2 Mya. These calibrations corresponded to the estimated divergence dates within our outgroup taxa as previously reported (Beati et al., 2013).

Results

PCR amplification and sequence alignments

Due to variations in the amplification success, it was not possible to obtain sequences for all the genes for each sample (Table 1). However, we obtained sequences for *A. parvum* from all the geographic regions and localities. Only 1 12SrDNA, 2 16SrDNA, 1DL, and 1 COI sequence were obtained from *A. pseudoparvum.* Alignments were unambiguous, with the exception of a 66 bp hypervariable portion of DL which was eliminated from the matrix because it could not be aligned with sufficient confidence. The lengths of the final alignments were, therefore, as follows: 343 bp for 12SrDNA, 388 bp for DL, 444 bp for 16SrDNA, 604 bp for COI, 577 bp for COII, and 1161 bp for ITS2. GenBank accession numbers for the sequences generated in this study are as follows: *A. parvum;* KT820213-251 for 12SrDNA, KT820252-314 for 16SrDNA, KT820315-347 for DL, KT820348-355 for ITS2, KT820367-399, KT820415-16 for COI, KT820400-414 for COII; *A. pseudoparvum;* KT820356 for 12SrDNA, KT820357 for DL, KT820360 for 16SrDNA, KT820365 for COI; *A. interandinum;* KT820358 for 16SrDNA, KT820363 for COI; *A. mixtum;* KT820359 for 16SrDNA, KT820364 for COI; *A. sculptum;* KT820361 for 16SrDNA, KT820366 for COI; *A. cajennense;* KT820362 for COI. We also used GenBank sequences EU791593, EU791601/06/08, KM042849, KF787572, KF787607/18/27, KF527319/45/83, KF527405, JN866835/53/64, and JN866901 corresponding to outgroup taxa, and the sequence FJ627952 corresponding to *A. pseudoparvum*.

Sequence diversity

The 39 12SrDNA sequences were represented by 12 unique haplotypes; the 63 16SrDNA sequences by 21 haplotypes; the 35 COI sequences by 20 haplotypes;

the 15 COII sequences by 9 haplotypes; the 33 DL sequences by 27 haplotypes and the 8 sequences from the ITS2 by 7 genotypes (Supplementary file 1). There were no shared haplotypes between the countries or regions, according to the designations in Supplementary file 1, with the exception of one 12SrDNA haplotype (1K) which was found in both Central America and Mexico. The 16SrDNA haplotype 2R was also shared between Central America and Mexico. Because no other sequences were obtained from the Mexican samples, for practical purposes, we also assigned the CA designation to North America-Mexico in the following analyses.

Phylogenetic analyses

12SrDNA

MP analysis of the 12SrDNA sequences detected a total of 74 parsimony-informative characters, and the heuristic search found 8 equally parsimonious trees with relatively little homoplasy: length = 167; consistency index (CI) = 0.808; retention index (RI) = 0.858; homoplasy index (HI) = 0.192. The ML model that best fitted the data according to the Akaike Information Criterion was TIM2+ G with gamma shape (G) = 0.2340. Both the MP and Bayesian analyses (Supplementary file 2) revealed that the ingroup is not monophyletic due to the presence of *A. pseudoparvum* embedded within it. MP and BA analyses resulted in topologically identical trees. The ingroup and *A. pseudoparvum* were clustered in a well-supported (100%) polytomic clade. Within this group, the well-supported Brazilian (B) and Argentinian (A) lineages were clustered in a monophyletic group, and the Central American (CA) clade was resolved and separated from the other groups. The *A. pseudoparvum* branch stemmed from the polytomy and did not appear to be more or less related to any of the other groups. ML distance values within the three main clades (A, B, and CA) were always below 0.7%. Distances between either A or B and CA ranged from 6.3 to 8.2%, while between A or B and *A. pseudoparvum* they varied from 8.8 to 9.1%. *A. pseudoparvum* differed from CA by 8.8-9.1%. The distance separating the

ingroup from the outgroup ranged from 14.5 to 20.1%, and the distance within species of the outgroup varied from 8.0 to 13.2% (Supplementary file 3).

16rDNA

MP analysis of the 16SrDNA gene sequences detected a total of 81 parsimony-informative sites, and the search found 3 equally parsimonious trees. The length of the trees was 178 (CI = 0.764; RI = 0.883; HI = 0.236). The ML model that best fitted the data according to the Akaike Information Criterion was TVM+I+G with G = 0.2529 and the proportion of invariable sites (PI) = 0.1928. The MP and BA trees were totally congruent (Supplementary file 4). *A. pseudoparvum* constituted a basal lineage within a monophyletic group, and all *A. parvum* clustered in a well-resolved separate lineage. The first node within the ingroup divided the sequences into two resolved clades, CA and a cluster including all samples from Brazil, Argentina, and Paraguay. The second lineage was further subdivided into two supported groups. The first included the Brazilian samples and the second included the Argentinian and the Paraguayan samples (A + P). The CA clade was the first to diverge after the basal split between *A. pseudoparvum* and all other *A. parvum* lineages. Divergence values within the clades (A, B, and CA) were always below 1.4%. Distance between either A, B or Paraguayan, and CA samples ranged from 6.8 to 8.4%. *A. pseudoparvum* differed from the A, B or Paraguayan sequences by 10.0 to 12.0%. The divergence values between CA and *A. pseudoparvum* were 8.2 - 10.2%. The distance separating the ingroup from the outgroup ranged from 12.6 to 19.1%, and from 6.9 to 13.4% within species of the outgroup (Supplementary file 3).

COI

MP analysis of COI gene sequences detected 137 parsimony informative sites and the heuristic search produced 6 equally parsimonious trees. The length of the trees was 386 (CI = 0.681; RI = 0.811; and HI=0.319). The ML model that

best fitted the data according to the Akaike Information Criterion was GTR+I+G with PI = 0.5420 and G = 0.7690. In the MP results, three lineages arose from a polytomy: *A. pseudoparvum,* CA (100%), and the A – B lineage (87%). The A-B clade was further resolved into two monophyletic sister lineages, A and B. The BA results (Supplementary file 5) revealed that the ingroup was paraphyletic because of the position of *A. pseudoparvum,* located between the supported CA and A-B clades. The latter was further split into A and B (100% and 94% respectively). The CA lineage was basal to everything else, followed by the *A. pseudoparvum* branch. ML divergence values within B ranged from 0.2 to 0.5%, within A from 0.2 to 3.5%, and within CA from 0.2 to 1.3%. Clades A and B were separated from each other by 4.3 - 6.2%; and each of them differed from CA by 9.8 – 11.2%. Distance between CA and *A. pseudoparvum* was 11.6 – 12.3%. The ingroup was separated from the outgroup sequences by 14.7% to 18.5%. The variation within the outgroup ranged from 11.6 to 15.6% (Supplementary file 3).

COII

MP analysis of the COII dataset identified a total of 120 parsimony-informative characters, with 8 best trees (length = 304; CI= 0.799; RI= 0.840; HI= 0.201). The ML model best fitting the data, according to the Akaike Information Criterion, was TPM3uf+G with G = 0.1670. The MP and BA results (Supplementary file 6) were fully congruent. The ingroup was monophyletic and resolved into two clades: B - A (100%, 100%) and CA (100%, 96%). Of those, the first one was further subdivided into two supported lineages, B and A. Divergence values within the clades were below 0.8%. The distance separating B from A ranged from 3.3 to 4.2%. The divergence values between either A or B and the CA clade varied from 11.2 to 11.8%, while between the ingroup and the outgroup they differed by 16.6 - 21.9%. Within outgroup species divergence values were 10.7 to 19.3% (Supplementary file 3).

DL

MP analysis of DL sequence detected 79 parsimony-informative sites and the heuristic search produced 102 equally parsimonious trees. Tree lengths were 198; CI = 0.739; RI = 0.858 and HI = 0.263. The ML model best fitting the data, according to the Akaike Information Criterion, was TPM3uf+I+G with PI = 0.444 and G = 0.531. The two analyses led to topologically identical trees. The *A. pseudoparvum* branch was always embedded within the monophyletic *A. parvum* (Supplementary file 7). Two clades were well resolved, the CA - *A. pseudoparvum* (91%, 76%) and the A - B (97%, 100%). In the former, the CA samples were monophyletic. In the second, only B was supported (100%, 99%). ML pairwise distances within the clades were always below 1.1%. CA differed from A or B by 7.1 to 8.1%, and *A. pseudoparvum* differed from A or B by 8.4 to 9.2%. Divergence values between CA and *A. pseudoparvum* ranged from 6.8 to 7.1%. The distances separating the ingroup from the outgroup ranged from 11.8 to 16.2%, and within the ougroup from 6.8 to 11.8% (Supplementary file 3).

ITS2

MP analysis of the ITS2 gene sequences resulted in 121 parsimony-informative sites and the length of the best trees was 226 (CI=0.907; RI=0.931; and HI=0.093). The ML model best fitting the data by the Akaike Information Criterion was GTR+G with G=0.5300. The MP and BA reconstructions were fully congruent (Figure 2). The ingroup was monophyletic, and initially split in two clades: CA - GO Brazil (77%, 98%) and A – PA (MGS), MG Brazil (88%, 94%). With this molecular marker, the Brazilian sequences were paraphyletic. The supported CA lineage (98%, 100%), constituted the sister group of the Brazilian samples from Goiás. The ML divergence value within Brazilian samples from PA and MG was 0.2%, and differed from Brazilian samples from GO, by 3.8 to 4.1%. Thus, the overall variation within Brazilian samples was from 0.2 to 4.1%. The distance between CA samples and Brazil from either MG – PA (MGS) or GO was similar and ranged from 3.3 to 5.9%. Divergence values between Argentinian and Brazilian samples from MG and PA varied from 0.1 to 0.4%, whereas the distance between Argentinian samples and Brazilian samples from GO was

3.9%. Divergence values between A and CA sequences ranged from 3.6 to 5.5%. Between the ingroup and outgroup, the distances varied between 12.4 and 16.4%, and within the outgroup, between 1.8 and 6.7% (Supplementary file 3).

Concatenated datasets

Partition homogeneity tests revealed that the mitochondrial matrices provided congruent information, with the exception of the 12SrDNA and 16SrDNA partitions which were heterogeneous (p = 0.04). However, the significance level was not high, the overall topology of the trees obtained with separate analyses only differed by node support of some of the clades, and the test is considered to be very conservative (Cunningham, 1997). Therefore, mitochondrial datasets were concatenated with the exception of COII, a gene for which we did not have enough representative amplicons. The partition homogeneity tests comparing ITS2 and mitochondrial datasets were consistently significant (p = 0.01) indicating that the nuclear information is not fully congruent with mitochondrial data, as was amply illustrated by the differences in tree topologies. Taking this into account we did not concatenate the nuclear and mitochondrial matrices. The concatenated mitochondrial matrix included representative sequences of all countries included in the study, for a total of 76 sequences (1736 bp) corresponding to 14 unique haplotypes, 1 sequence of *A. pseudoparvum,* and 4 outgroup taxa. MP analysis of the concatenated mitochondrial dataset detected 352 parsimony-informative characters and the heuristic search found 16 equally parsimonious trees. The length of the trees was 896; CI = 0.733; RI = 0.807; and HI = 0.267. The ML model best fitting the data, according to the Akaike Information Criterion, was GTR+I+G with PI = 0.5355 and G = 0.8367. The MP and BA reconstructions were identical (Figure 3), and revealed a polytomic *A. parvum* with *A. pseudoparvum* arising from the same polytomy. The monophyly of *A. parvum* is not supported. Two clades were well-resolved, CA (100%, 100%) and B - A (100%, 100%). The latter was further resolved into two monophyletic lineages, B and A, both with 100% bootstrap and 100% posterior probability support. The Panama samples constituted a

supported lineage within CA. The CA clade was basal to both, A and B. ML pairwise distances within the clades were always below 1.8%. CA differed from A or B by 8.2 to 8.8%, and *A. pseudoparvum* differed from A or B by 10.2 to 10.8%. The divergence value between CA and *A. pseudoparvum* was between 9.5 and 9.7%. The distances separating the ingroup from the outgroup ranged from 13.9 to 16.8%, whereas the distance between *A. pseudoparvum* and the outgroup varied from 15.4 to 18.2%. The distances between species belonging to the outgroup differed by 9.7 to 13.1% (Supplementary file 3).

Molecular clock and divergence dates.

Tentative node dating was performed on the mitochondrial combined dataset only, because the partition homogeneity test precluded us from combining nuclear and mitochondrial information, and because the relative mutation rate tests rejected the molecular clock hypothesis for ITS2.

Relative mutation rate tests between mitochondrial monophyletic lineages were not significant. They were also not significantly different between ingroup and outgroup sequences, suggesting that the overall rates of variation were similar. Therefore, rate variation across lineages would not be a reason for low accuracy when estimating divergence dates. The molecular clock hypothesis, tested with DAMBE 5, could not be rejected by using either the likelihood rate or the least square test. The tree generated by Beast was congruent with the concatenated dataset, but placed *A. pseudoparvum* as sister group to *A. parvum.* Divergence dates obtained with the three different calibrations were similar to each other. The range of the mean estimated ages obtained with the three calibrations is shown in Figure 4. The dating placed the root of the tree between 16.2 – 12.2 Mya, the basal radiation of the outgroup taxa between 13.8 – 8.8 Mya, and the divergence between *A. parvum* and *A. pseudoparvum* between 7.2 – 6.0 Mya. Within *A. parvum* the split between CA and A - B was placed at about 5.8 – 4.9 Mya, and the most recent divergence event, between A and B, at approx. 2.3 – 1.8 Mya.

Discussion

The overall structure of the trees obtained with the 6 genes was different. When *A. pseudoparvum* was included in the analyses, the *A. parvum* lineages did not cluster in a monophyletic clade (16S rDNA excepted), with the CA and the A-B clades being consistently deeply separated. When the position of *A. pseudoparvum* was resolved, it did not cluster consistently with either the CA or the BA clade. Between CA and A-B, CA and *A. pseudoparvum*, and A - B and *A. pseudoparvum*, the ML pairwise distance values are similar to values recorded between outgroup species and between the outgroup and the ingroup. This strongly suggests that we are dealing with three species, *A. parvum* (from Brazil, Paraguay, and Argentina), a distinct species from Central America- Mexico which needs to be described and characterized, and *A. pseudoparvum.* Distance values between A and B are compatible with these geographically distant lineages being conspecific, particularly when compared to distance values within the outgroup cluster of recognized different species. Nevertheless, distance values must be considered with caution when delimiting species. Phylogenetic reconstructions, particularly those based on ITS2 and DL gene sequences, do not always identified Brazilian and Argentinian samples as discrete monophyletic lineages, with some Brazilian and Argentinian sequences clustering in the same clade and the Brazilian Goiás lineage sharing its closest common ancestor with the CA clade. Although these results, combined with the observed distance values, might indicate that the Goiás lineage represents a different species, as is the case with CA, biological comparisons (Geradi et al., 2013) and recent cross-breeding experiments (Nava et al., unpublished data) have shown that ticks from Argentina and Goiás are similar in feeding behavior and are reproductively compatible, thus supporting the mitochondrial, rather than the nuclear data. Despite of being geographically distant, and possibly currently prevented from interbreeding, their recent geographical isolation (2.3 – 1.8 Mya, Fig. 4) has not yet led to speciation. Additional cross-breeding studies involving the Goias ticks,

B, and CA ticks should be conducted in order to corroborate the hypothesis that Goias ticks can also interbreed with B, but not with CA ticks.

In contrast, the systematic status of the CA clade deserves additional scrutiny. The data presented herein, strongly suggest that it should be considered to be a separate species that is distinct from the more southern ticks, which correspond to *A. parvum,* because the type specimen was described from the State of Bahia, Brazil (Aragão, 1908). In order to fully ascertain the taxonomic status of the Central American lineage, it would be informative to attempt to cross-breed colonies ticks from CA, B, and A in order to verify whether or not they are reproductively compatible. In addition, it would be important to include three other *Amblyomma* taxa in future analyses, *Amblyomma auricularium* (Conil, 1878), *Amblyomma pseudoconcolor* (Aragão, 1908), and *Amblyomma inornatum* (Banks, 1909), species closely related to our ingroup (Nava et al., 2008a), and additional *A. parvum* samples from other South-American regions (Bolivia, Venezuela, and the Roraima area of Brazil).

Because the main lineages identified in this study, *A. pseudoparvum*, CA, and BA correspond roughly, in terms of geographical distribution, to clades identified within the *A. cajennense* species complex (Beati et al., 2013), we hypothesized that the evolution of the *A. parvum* group might have followed a similar phylogeographical temporal pattern. The decision of using only mitochondrial gene sequences was not only justified by the results of the partition homogeneity test, but also by the visible topological contrast observed between the ITS2 and the mitochondrial reconstructions, and by the fact that mitochondrial DNA, with its lower population size, putative neutrality, and elevated mutation rate, is considered to be a robust marker for developing hypotheses on population history and phylogeographical evolutionary patterns (Zink and Barrowclough, 2008). The deepest split between lineages in this group of taxa separates *A. pseudoparvum*, a tick strictly associated with the Chaco area (northern Argentina and Paraguay), a Brazilian-Argentinian clade mostly found in the Chaco/Cerrado/Caatinga regions, and a clade found in the seasonally dry tropical forest of Central America. The results of our tentative node dating

revealed that these three main lineages diverged almost simultaneously, approx. 7.2 - 4.9 Mya. The inconsistent placement of *A. pseudoparvum* in our phylogenetic reconstructions, and the often polytomic basal split of the three main lineages, also concur in suggesting that these clades radiated very rapidly. Therefore, we can surmise that, in the early-middle Miocene, their common ancestor occupied a relatively dry wide area spanning from present time northern South America to Argentina. This area was fragmented when the Amazon Basin progressively developed at its center, a biogeographical event that was completed approximately 6 Mya (Hoorn et al., 2010).

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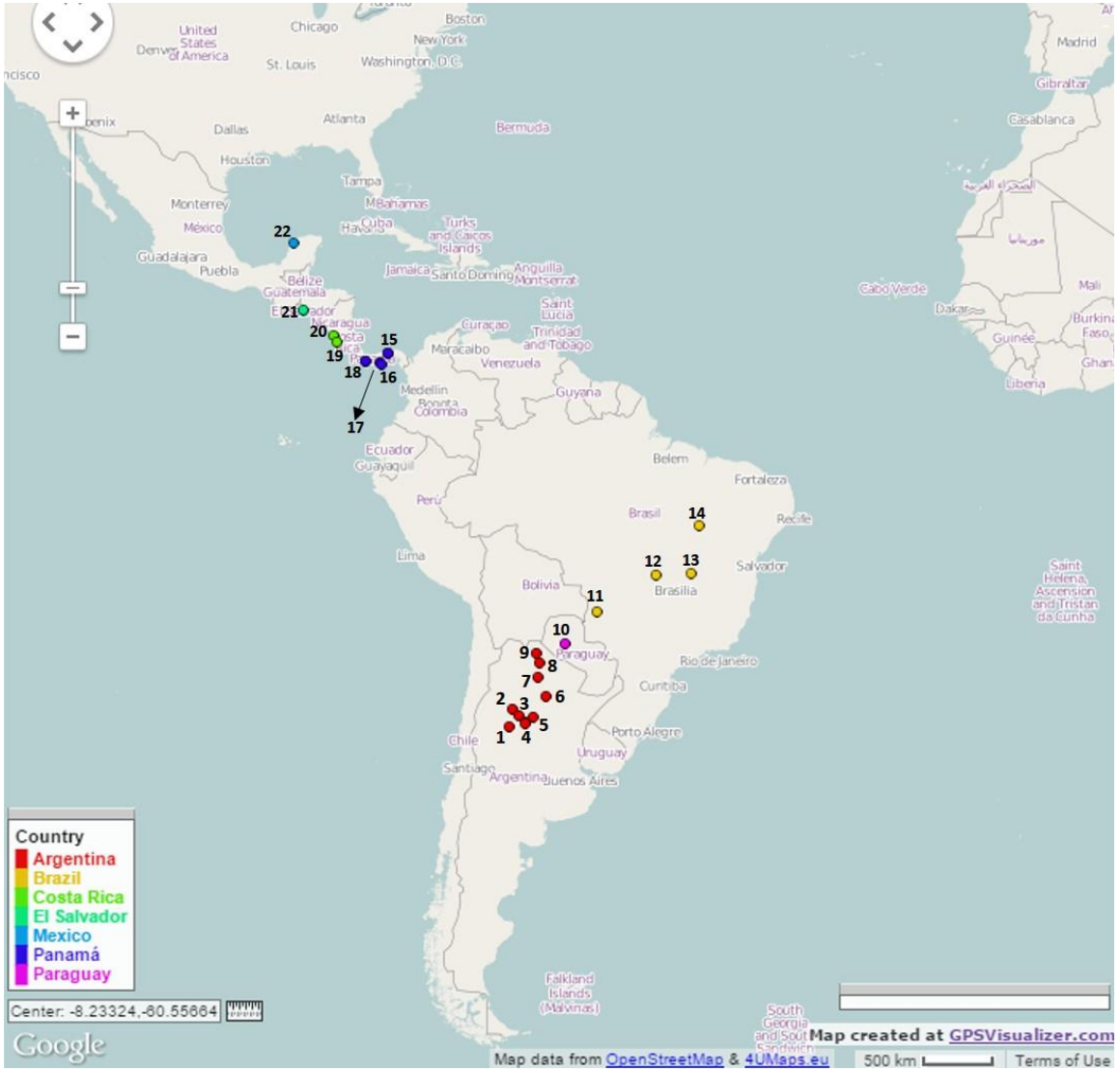

Figure 1. Political map of the southern parts of North America, Central and South America showing the collection sites. Each circle corresponds to a locality, and the different colors correspond to the countries, as indicated in the figure. Localities are indicated as follows; Argentina: 1, Chamical; 2, San Antonio; 3, Esquiu; 4, Quilino and San Jose de las Salinas; 5, Villa Ojo de Agua; 6, Cejolao; 7, Uratau; 8, Rivadavia banda Sur; 9, Dragones. Paraguay: 10, Filadelfia. Brazil: 11, Nhecolândia; 12, Araguapaz; 13, Formoso; 14, Gilbues. Panama: 15, Parque Summit and Santa Rosa; 16, Los Santos; 17, Puerto Limon; 18. San Lorenzo. Costa Rica: 19, Palo Verde; 20, Hacienda Orosi. El Salvador: 21, Tejutepeque. Mexico: 22, Chochola. The map was built using gpsvisualizer.

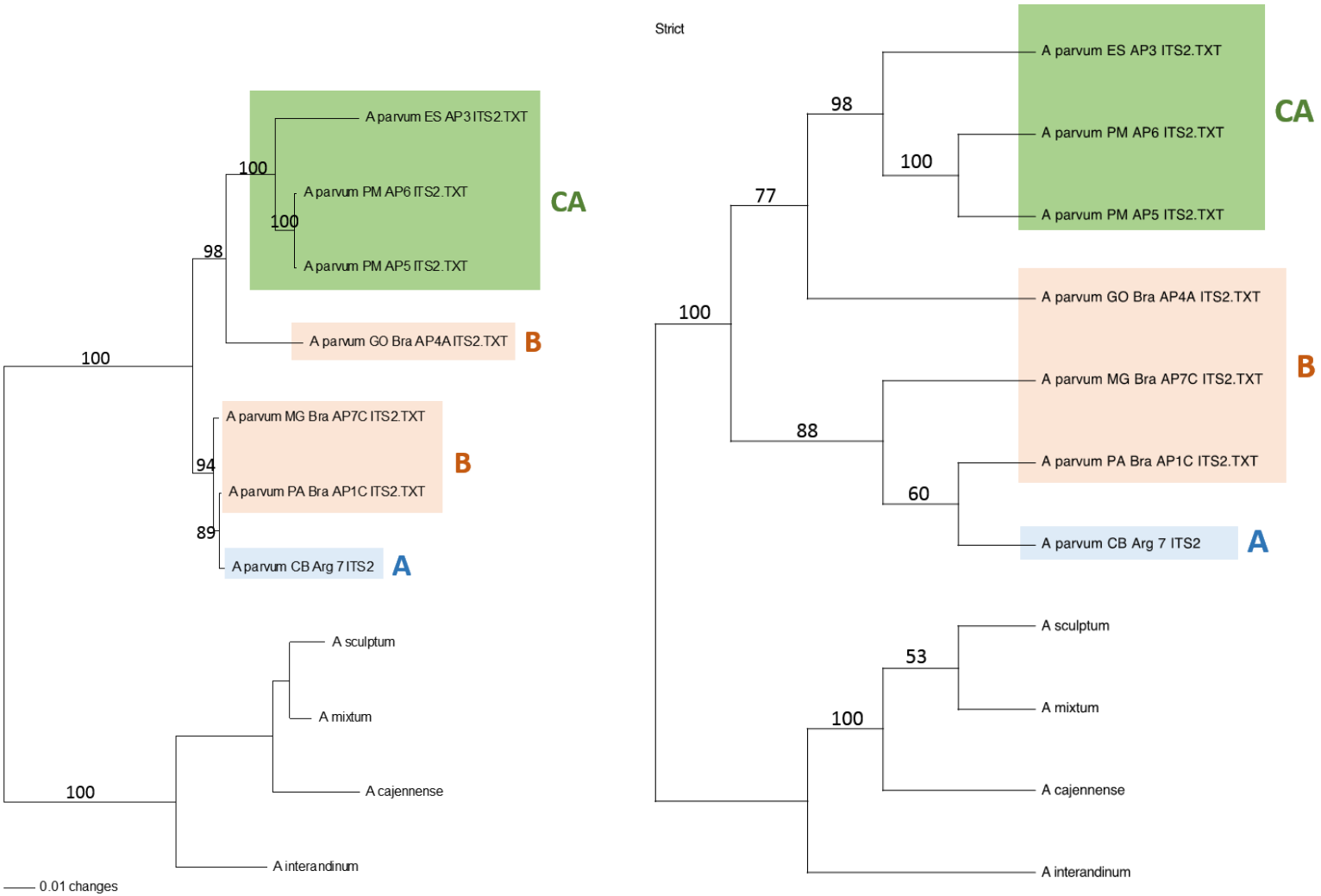

Fig. 2. Phylogenetic reconstructions for ITS2. On the left, Bayesian analysis and on the right Maximum parsimony analysis. The green box corresponds to the Central American clade (CA), the pink box to the Brazilian clade (B) and the blue box to the Argentinian clade (A). These color designations are used in the remaining images. Numbers in the branches indicate posterior probabilities for the Bayesian analysis and bootstrap support for Maximum parsimony.

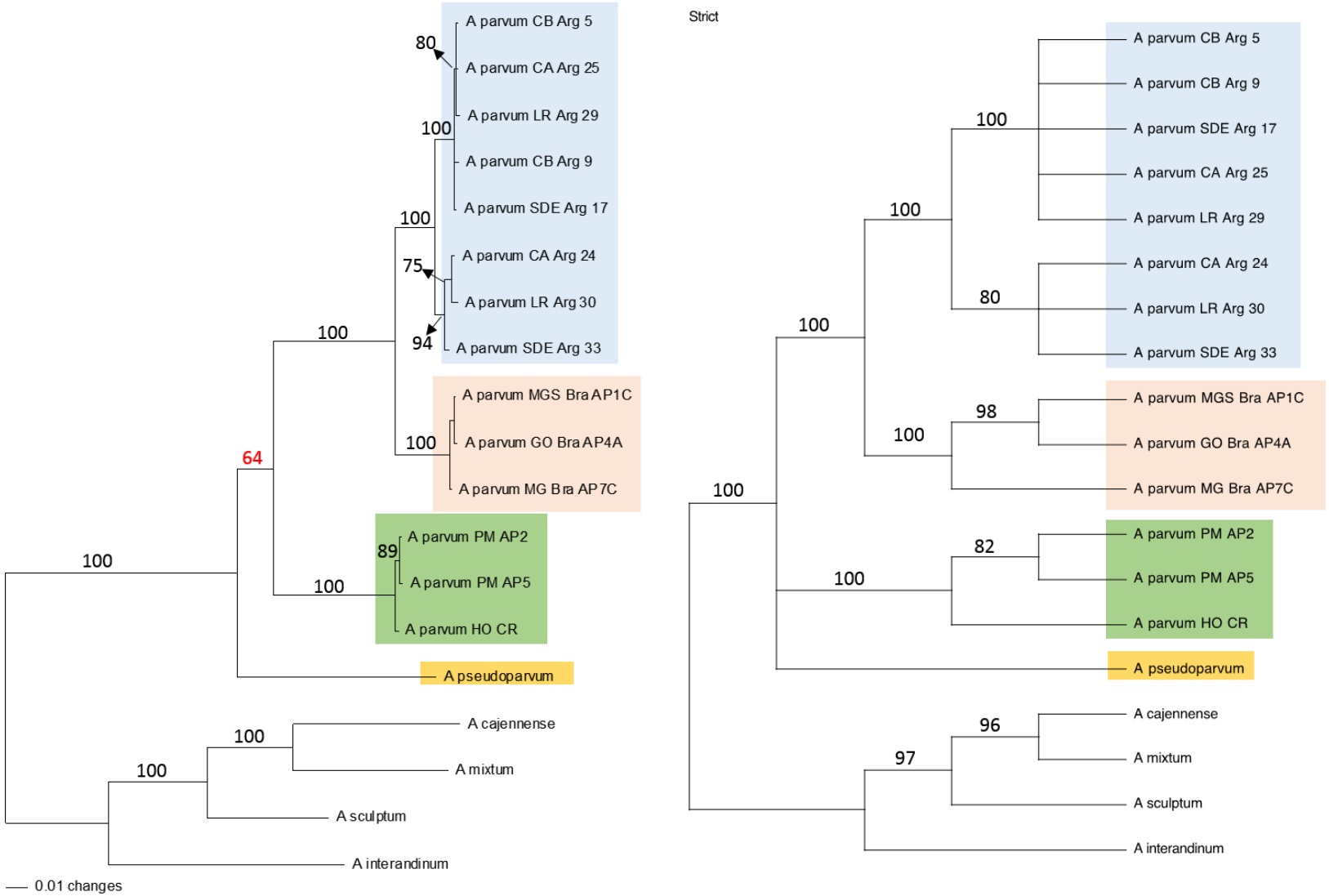

Fig. 3. Phylogenetic reconstructions for the mitochondrial concatenated dataset. On the left, Bayesian analysis and on the right Maximum parsimony analysis. The green box corresponds to the Central American clade (CA), the pink box to the Brazilian clade (B), the blue box to the Argentinian clade (A) and the orange box to *A. pseudoparvum*. These color designations are used in the remaining images. Numbers in the branches indicate posterior probabilities for the Bayesian analysis and bootstrap support for Maximum parsimony.

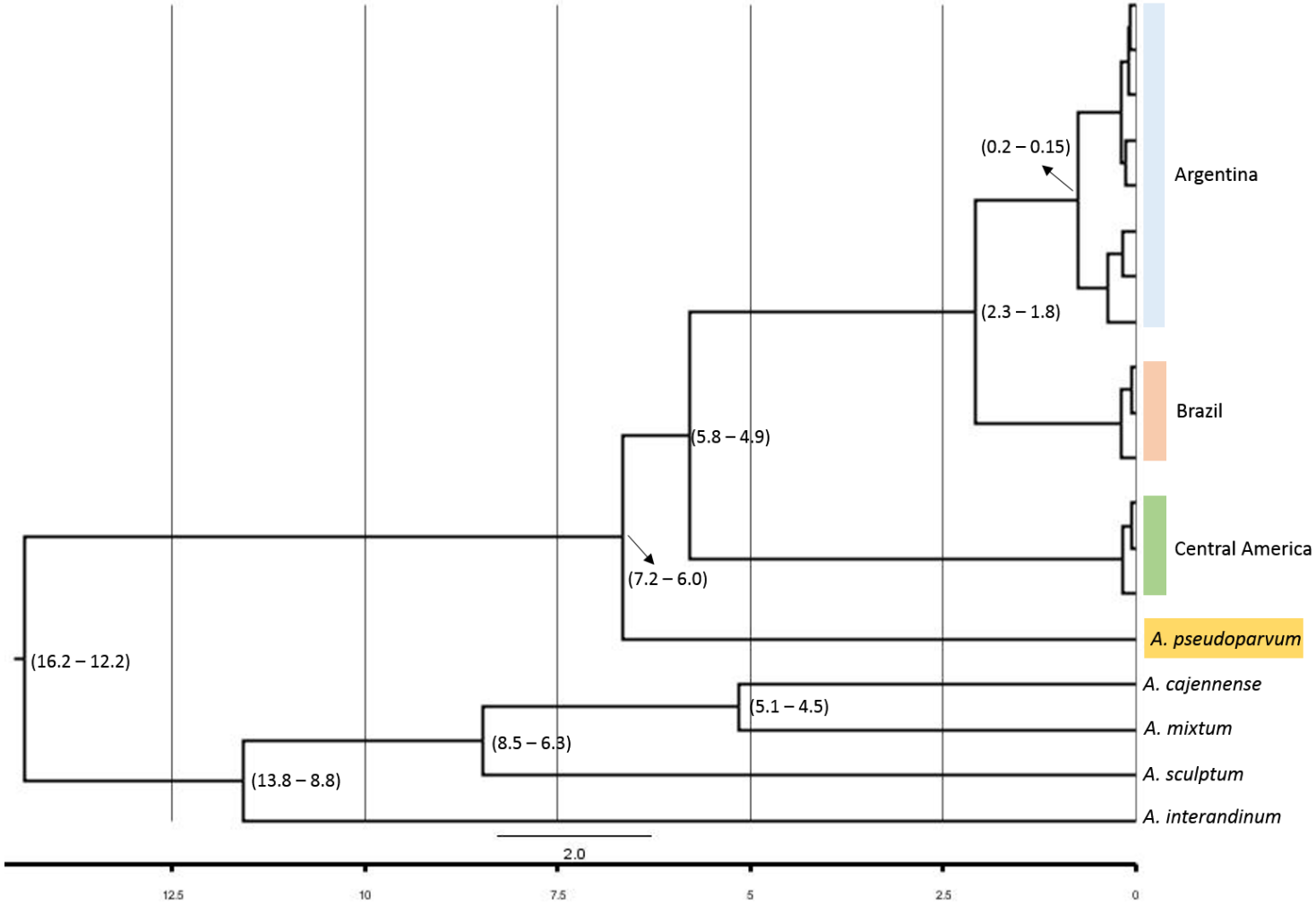

Fig. 4. Node dating. Tentative dating of the main nodes in the mitochondrial concatenated gene tree. The range presented in each main node corresponds to the values obtained through the three calibrations performed. The time scale is in Mya, shown as numbers for each node.

Country	*State/Province*	*Locality*	*# Samples per Locality*	*# Samples per Country*
Argentina	Catamarca	Esquiu	3	
	Catamarca	San Antonio	4	
	Cordoba	Quilino	5	
	Cordoba	San Jose de las Salinas	2	
	La Rioja	Chamical	4	
	Salta	Dragones	3	
	Santiago del Estero	Cejolao	4	
	Santiago del Estero	Uratau	2	
	Santiago del Estero	Villa Ojo de Agua	4	**31**
Brazil	Goias	Araguapaz	12	
	Minas Gerais	Formoso	10	
	Mato Grosso do Sul	Nhecolandia	14	
	Piaui	Gilbues	1	**37**
Costa Rica	Cartago	Palo Verde	3	
	Guanacaste	Hacienda Orosi	5	**8**
El Salvador	Cabañas	Tejutepeque	2	**2**
Mexico	Yucatan	Chochola	2	**2**
Panama	Chiriqui	San Lorenzo	2	
	Colon	Santa Rosa	1	
	Herrera	Puerto Limon	1	
	Los Santos	Los Santos	1	
	Panama	Parque Summit	1	**6**
Paraguay	Boqueron	Filadelfia	1	**1**

A. pseudoparvum					
Argentina	Salta	Rivadavia Banda Sur	1	**1**	

Table 2. Sampling sites and amplification success for *A. parvum* and *A. pseudoparvum*. Geographical data along with amplification for the 6 molecular markers are presented for each sample. Crosses indicate successful amplification of the indicated gene.

Lab ID	*Species*	*Locality*	*State/Provence/Department*	*Code*	*Country*	*Coordinates*	*12S*	*16S*	*DL*	*ITS2*	*COI*	*COII*
1W	*A. parvum*	Gilbues	Piaui	PI	Brazil	9°50´S 45°21´W	X					
2W	*A. parvum*	Nhecolandia	Pantanal (MGS)	PA/MGS	Brazil	18°58°S 56°36´W		X				
3W	*A. parvum*	Nhecolandia	Pantanal (MGS)	PA/MGS	Brazil	18°58°S 56°36´W		X				
4W	*A. parvum*	Formoso	Minas Gerais	MG	Brazil	14°57´S 46°14´W	X	X	X		X	
5W	*A. parvum*	Formoso	Minas Gerais	MG	Brazil	14°57´S 46°14´W	X	X	X		X	
6W	*A. parvum*	San Lorenzo	Chiriqui	PM	Panamá	8°19´N 82°10´W		X				
1	*A. parvum*	San José de las Salinas	Córdoba	CB	Argentina	30°04´S 64°35´W		X	X		X	
5	*A. parvum*	Quilino	Córdoba	CB	Argentina	30°13´S 64°31´W	X	X	X		X	X
7	*A. parvum*	Quilino	Córdoba	CB	Argentina	30°13´S 64°31´W		X	X	X	X	X
9	*A. parvum*	San José de las Salinas	Córdoba	CB	Argentina	30°04´S 64°35´W	X	X	X		X	X
12	*A. parvum*	Urataú	Santiago del Estero	SDE	Argentina	25°42´S 63°02´W		X	X		X	
17	*A. parvum*	Cejolao	Santiago del Estero	SDE	Argentina	27°29´S 62°18´W	X	X	X		X	X
24	*A. parvum*	San Antonio	Catamarca	CA	Argentina	28°50´S 65°59´W	X	X	X		X	
25	*A. parvum*	Esquiú	Catamarca	CA	Argentina	29°22´S 65°17´W	X	X	X		X	X
26	*A. parvum*	Esquiú	Catamarca	CA	Argentina	29°22´S 65°17´W	X	X	X		X	
29	*A. parvum*	Chamical	La Rioja	LR	Argentina	30°26´S 66°12´W	X	X	X		X	X
30	*A. parvum*	Chamical	La Rioja	LR	Argentina	30°26´S 66°12´W	X	X	X		X	
32	*A. parvum*	Villa Ojo de Agua	Santiago del Estero	SDE	Argentina	29°29´S 63°41´W	X	X	X		X	
33	*A. parvum*	Villa Ojo de Agua	Santiago del Estero	SDE	Argentina	29°29´S 63°41´W	X	X	X		X	
38	*A. parvum*	Chamical	La Rioja	LR	Argentina	30°26´S 66°12´W		X	X		X	
44	*A. parvum*	Villa Ojo de Agua	Santiago del Estero	SDE	Argentina	29°29´S 63°41´W		X	X		X	X
AP2	*A. parvum*	Puerto Limon	Herrera	PL	Panamá	8°0´N 80°29´W	X	X	X			X
AP3	*A. parvum*	Tejutepeque	Cabañas	ES	El Salvador	13°50N 88°55´W	X	X		X		X

AP4A	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X		X	X	X	
AP4B	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X		X	X	X	
AP4C	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X	X	X		X	
AP4D	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X	X	X		X	
AP4E	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X	X	X			
AP4F	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X	X	X			
AP4G	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W	X	X	X			
AP5	*A. parvum*	Parque Summit	Panama	PM	Panamá	9°4´N 79°39´W	X	X	X	X	X	X
AP6	*A. parvum*	San Lorenzo	Chiriqui	PM	Panamá	8°19´N 82°10´W	X			X		X
AP7A	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W	X		X			X
AP7B	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W	X		X			X
AP7C	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W	X	X	X	X		X
AP7D	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W	X	X	X			X
AP7E	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W	X	X	X			
AP7F	*A. parvum*	Formoso	MG	MG	Brazil	14°57´S 46°14´W						
AP1A	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W	X	X			X	
AP1B	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W	X	X			X	
AP1C	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W	X	X	X	X	X	
AP1D	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
AP1E	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
AP1F	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W			X		X	
AP1G	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
AP1H	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
AP1I	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
AP1J	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W					X	
APP1	*A. pseudoparvum*	Rivadavia Banda Sur	Salta	SA	Argentina	24°11´S 62°53W					X	
AP5N	*A. parvum*	Los Santos	Los Santos	LS	Panama	7°50´N 80°20´W	X				X	
AP7N	*A. parvum*	Palo Verde	Cartago	PV	Costa Rica	10°23´N 85°19´W						

AP8	*A. parvum*	Palo Verde	Cartago	PV	Costa Rica	10°23´N 85°19´W	X		
APCA	*A. parvum*	Chochola	Yucatan	YU	Mexico	20°45´N 89°55´W	X		
AP-FOR	*A. parvum*	Formoso	Minas Gerais	MG	Brazil	14°57´S 46°14´W			
122838(1)	*A. parvum*	Hacienda Orosi	Guanacaste	HO	Costa Rica	10°58´N 85°32´W	X		
122838(2)	*A. parvum*	Hacienda Orosi	Guanacaste	HO	Costa Rica	10°58´N 85°32´W	X	X	X
A parvum 12S CR	*A. parvum*	Hacienda Orosi	Guanacaste	HO	Costa Rica	10°58´N 85°32´W	X		
123529	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W	X		
OJ4	*A. parvum*	Villa Ojo de Agua	Santiago del Estero	SDE	Argentina	29°29´S 63°41´W		X	
CH4	*A. parvum*	Chamical	La Rioja	LR	Argentina	30°26´S 66°12´W		X	
PAR	*A. parvum*	Filadelfia	Boqueron	BO	Paraguay	22°20´S 60°02´W		X	
GO1	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W		X	
GO2	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W		X	
GO3	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W		X	
GO4	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W		X	
GO5	*A. parvum*	Araguapaz	Goias	GO	Brazil	15°03S 50°05°W		X	
W	*A. parvum*	Nhecolandia	(Pantanal) MGS	PA/MGS	Brazil	18°58°S 56°36´W		X	
APA6	*A. parvum*	Formoso	Minas Gerais	MG	Brazil	14°57´S 46°14´W		X	
APA4_CR	*A. parvum*	Palo Verde	Cartago	PV	Costa Rica	10°23´N 85°19´W		X	
APA2	*A. parvum*	Chochola	Yucatan	YU	Mexico	20°45´N 89°55´W		X	
APA1	*A. parvum*	Santa Rosa	Colon	PM	Panama	9°11´N 79°39´W		X	
APA3	*A. parvum*	Tejutepeque	Cabañas	ES	El Salvador	13°50N 88°55´W		X	
UR2	*A. parvum*	Urataú	Santiago del Estero	SDE	Argentina	25°42´S 63°02´W		X	
YU1	*A. parvum*	Dragones	Salta	SA	Argentina	23°16´S 63°21´W		X	
YU2	*A. parvum*	Dragones	Salta	SA	Argentina	23°16´S 63°21´W		X	
YU3	*A. parvum*	Dragones	Salta	SA	Argentina	23°16´S 63°21´W		X	
QU5	*A. parvum*	Quilino	Cordoba	CB	Argentina	30°13´S 64°31´W		X	
QU6	*A. parvum*	Quilino	Cordoba	CB	Argentina	30°13´S 64°31´W		X	

QU7	*A. parvum*	Quilino	Cordoba	CB	Argentina	30°13´S 64°31´W	X	
SA2	*A. parvum*	San Antonio	Catamarca	CA	Argentina	28°50´S 65°5´W	X	
SA3	*A. parvum*	San Antonio	Catamarca	CA	Argentina	28°50´S 65°5´W	X	
SA4	*A. parvum*	San Antonio	Catamarca	CA	Argentina	28°50´S 65°5´W	X	
ES3	*A. parvum*	Esquiú	Catamarca	CA	Argentina	29°22´S 65°17´W	X	
CE2	*A. parvum*	Cejolao	Santiago del Estero	SDE	Argentina	27°29´S 62°18´W	X	
CE3	*A. parvum*	Cejolao	Santiago del Estero	SDE	Argentina	27°29´S 62°18´W	X	
CE4	*A. parvum*	Cejolao	Santiago del Estero	SDE	Argentina	27°29´S 62°18´W	X	
CR_45	*A. parvum*	Hacienda Orosi	Guanacaste	HO	Costa Rica	10°58´N 85°32´W		X
CR_495	*A. parvum*	Hacienda Orosi	Guanacaste	HO	Costa Rica	10°58´N 85°32´W		X

Supplementary material

File 1. Haplotype diversity.

File 2. Bayesian and Maximum parsimony trees for 12SrDNA.

File 3. Maximum likelihood pairwise distances.

File 4. Bayesian and Maximum parsimony trees for 16SrDNA.

File 5. Bayesian and Maximum parsimony trees for COI.

File 6. Bayesian and Maximum parsimony trees for COII.

File 7. Bayesian and Maximum parsimony trees for DL.