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Helping to Resolve Taxonomic Conflicts within the Genus Amblyomma (Acari:Ixodidae) from a Molecular Perspective

Paula Lado

HELPING TO RESOLVE TAXONOMIC CONFLICTS WITHIN THE GENUS *AMBLYOMMA* (ACARI: IXODIDAE) FROM A MOLECULAR PERSPECTIVE

by

PAULA LADO
(Under the Direction of Lorenza Beati)

ABSTRACT

This work sought to reassess the taxonomic status of *Amblyomma parvum* Aragao, 1908 and of the *A. maculatum* group of ticks Camicas, 1998. By using different molecular markers, 12SrDNA, 16SrDNA, DL, COI, COII (mitochondrial) and ITS2 (nuclear), I analyzed the systematic relationships between these taxa and their closest relatives. Phylogenetic analyses by maximum parsimony, maximum likelihood, and Bayesian analysis were performed in order to determine relationships among species and populations, and to determine the evolutionary history of these ixodids. The data obtained supported the hypothesis of cryptic speciation occurring within *A. parvum,* with the northern populations of Central America being a different species from the one occurring in the southern latitudes, mainly in Brazil and Argentina. As for the *A. maculatum* group of species, the results strongly suggest that *A. triste* should be synonymized with *A. maculatum,* while *A. tigrinum* is maintained as a separated taxon until further biological evidence is gathered. In conclusion, the analyses presented herein successfully resolved some of the taxonomic issues within this large genus of hard ticks, while raising additional questions for future investigations.

INDEX WORDS: Georgia Southern University, Thesis, Ticks, *Amblyomma*, Taxonomy, Molecular Systematics.

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PAULA LADO

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PAULA LADO

Major Professor: Lorenza Beati
Committee: Lorenza Beati
Lance Durden
Laura Regassa

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DEDICATION

I would like to dedicate this work to every person who believe in itself and who works hard to reach personal and academic goals. To everyone who is willing to fall, learn, and continue regardless the obstacles.

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Chapter 1

Is *Amblyomma parvum* a complex of species?

Introduction

The hard tick *Amblyomma parvum* (Acari: Ixodidae) Aragao 1908 has a broad geographic distribution, ranging from Mexico to Argentina (Hoffman, 1962, Guglielmone and Hadani, 1980; 1982; Guglielmone et al. 1990; 2003; Nava et al., 2008a). Throughout its geographical distribution, this species has a predilection for drier areas of Central America, the Nothern coast of South America, and the dry diagonal of South America spanning from the Chaco and Pampa in Argentina, to the Cerrado and Caatinga in Brazil (Fairchild, 1966; Guglielmone and Hadani, 1980; Morrone, 2006; Nava et al., 2008a). Basically, with the exception of an isolated population in Roraima (Amazonia), *A. parvum* has a disjunt distribution with two main clusters (northern and southern) separated by the Amazon basin.

As for host range, adults commonly parasitize 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 (Labruna et al., 2005; Nava, 2006; 2008b; Saraiva et al., 2012).

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

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 on the festoons, not realizing that in his 1911

publication, Aragão 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 them were almost exclusively found on a wild rodent, *Dolychotis salinicola*. The taxonomic status of this group of species was resolved by Guglielmone et al. (1990) through an in depth analysis of the different morphological types. The authors redescribed the adults and described de immatures of *A. parvum. Amblyomma parvum carenatus* was synonymized with *A. parvum* because it corresponded to 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 correspond to a complex of species (Nava et al., 2008a). Increasing evidence shows that tick species with a wide geographic distribution can 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).

The 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 to the authors the possible occurrence of two species (Nava et al., 2008a).

Nevertheless, divergence values are relative numbers, that cannot be used as such to define species, particularly when based on the analysis of a single gene. Multiple sources of evidence, morphological, molecular, and biological, should be combined in order to reliably delimit species. Morphological data and cross breeding experiments between populations of *A. parvum* from Argentina and Brazil contradicted the 16SrDNA results: they did not detect significant morphological differences between populations nor did they reveal reproductive incompatibility (Nava et al., unpublished data). Although these ticks

might be geographically separated and prevented from interbreeding, their isolation has not yet resulted in speciation (Nava et al., unpublished data).

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, *Amblyomma cajennense*, with a similar geographical distribution and also associated with relatively drier areas, was found to be constituted by six different species, one of them with a Mexican and Central American distribution (*Amblyomma mixtum*) and five confined to continental South America (Beati et al., 2013; Nava et al., 2014)

Material and Methods

Sampling

Our samples included 90 adult specimens identified as *A. parvum* from the following countries: Argentina, Brazil, Paraguay, Costa Rica, El Salvador, Panama and Mexico (Fig. 1.1). In the case of 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 16 localities, corresponding to 7 countries across the geographic distribution of *A. parvum,* thus covering a wide range of latitudes (Table 1.1). The collection sites are shown in Fig. 1.1 and designated as follows: Argentina, Arg; (Cordoba, CB, Santiago del Estero, SDE; Catamarca, CA; La Rioja, LR), Brazil, Bra; (Piaui, PI; Mato Grosso do Sul, MGS; Minas Gerais, MG; Pantanal, PA, Goias, GO), Costa Rica, CR; (Palo Verde, PV; Santa Rosa, SR), El Salvador, ES; Mexico (Yucatan, YU), Panama, PM; (Panama, PM; Los Santos, LS), Paraguay, Par; (Boqueron, BO). In addition, specimens of *A. pseudoparvum* included in the analyses were from Salta (SA), Argentina.

DNA extraction, PCR, and sequencing

Tick DNA was extracted and the exoskeletons were preserved for further morphological analysis following previously published protocols (Beati and Keirans, 2001; Beati et al., 2012). For that purpose a small portion of the postero-lateral idiosoma of each tick was removed by using a disposable scalpel and the tick 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 exoskeletons were stored in 70% ethanol and kept as voucher specimens. 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 (Cythochrome oxydase subunit I), COII (Cythochrome oxydase subunit II), and d-loop (DL, control region) were amplified employing previously reported sets of primers (Beati and Keirans, 2001; Beati et al., 2012; Barret and Hebert, 2005; 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 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 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 manually 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) and DL (Zhang and Hewitt 1997). Codon organization was taken into account when aligning the COII data set. Each data set was analyzed by maximum parsimony (MP) and maximum likelihood (ML) using PAUP (Swofford, 2000), and through 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, with PHYML (100 replica) (Guindon and Gascuel 2003) in Phylogeny.fr (Dereeper et al., 2008) for ML, and by posterior probability with MrBayes. 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 (in DL, COII and ITS2 analyses) or as a missing character (12srDNA, 16SrDNA, COI and both concatenated datasets). ML heuristic searches were run after the nucleotide substitution model best fitting the data was selected by Modeltest v3.7 (Posada and Crandall 1998). Pairwise sequence distances were calculated based on the ML model by using PAUP. For ML searches, the MP tree with the best ML score was used as the starting tree. Two runs, 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, and the number of generations was increased, if needed, to avoid discarding more than 25% of the trees. The 50% majority-rule consensus tree of the remaining trees was inferred and posterior probabilities were recorded for each branch. Data sets were combined for total evidence analyses. One concatenated data set including both mitochondrial and nuclear sequences (n+mtDNA), were analyzed following the same procedure outlined for the separate analyses. The sequences were concatenated using MacClade. The outgroup used for all the phylogenetic analyses included the following species: *A.*

cajennense, A. sculptum, A. mixtum and *A. interandinum. A. pseudoparvum* was included in the analyses because it corresponds to the closest morphological relative of *A. parvum*.

Results

Sequence diversity

Due to variations in the amplification success, it was not possible to obtain sequences for all the genes for each sample. However, we obtained sequences for all the geographic regions and localities (Table 1.1).

In terms of sequence diversity, the 39 12SrDNA (343 bp) sequences were represented by 12 unique haplotypes; the 65 16SrDNA (406 bp) sequences by 21 haplotypes; the 36 COI (604 bp) sequences by 20 haplotypes; the 15 COII (602 bp) sequences by 9 haplotypes; the 33 DL (455 bp) sequences by 29 haplotypes and the 8 sequences from the ITS2 (1161 bp) by 7 genotypes (Table 1.2). The 455 bp DL dataset was reduced to 389 bp after eliminating the hypervariable region, which could not be aligned with sufficient confidence. There were no shared haplotypes between the countries or regions, according to the designation in Table 1.2.

Individual gene markers

12SrDNA

The MP analysis for the 12SrDNA sequences detected a total of 73 parsimony-informative characters, and the heuristic search found 3 equally parsimonious trees with relatively little homoplasy: length = 163; consistency index (CI) = 0.812; retention index (RI) = 0.859; homoplasy index (HI) = 0.188. The ML model that best fitted the data according to the Akaike Information Criterion was TVM+G with base frequencies of A = 0.42; C = 0.09; G = 0.13 and T = 0.36 and proportion of invariable sites (PI) = 0 and gamma distribution shape parameter (G) = 0.2406.

The MP, ML and Bayesian analyses (Figs. 1.2, 1.3 and 1.4 respectively) all revealed that the ingroup is not monophyletic due to the presence of *A. pseudoparvum* embedded within it. MP, ML 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 the Argentinian (A) lineages were clustered in a monophyletic group, and the Central American (CA) clade was resolved and separated from the other ones. 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 6.5 to 8.0%, while between A or B and *A. pseudoparvum* they varied from 8.3 to 9.1%. *A. pseudoparvum* differed from CA by 8.6-8.9%. The distance separating the ingroup from the outgroup ranged from 14.2 to 19.6%, and the distance within species of the outgroup varied from 7.9 to 14.3% (Table 1.3).

16rDNA

The MP analysis of the 16SrDNA gene sequences detected a total of 90 parsimony-informative sites, and the search found 3 equally parsimonious trees. The length of the trees was 194 (CI = 0.778; RI = 0.885; HI = 0.222). The ML model that best fitted the data according to the Akaike Information Criterion was TVM+G with base frequencies of A=0.43; C=0.08; G=0.13 and T=0.36; PI = 0; G = 0.1556.

The MP, ML and BA trees were totally congruent (Figs. 1.5-1.7). *A. pseudoparvum* constituted a basal lineage within a monophyletic group, and all *A. parvum* clustered in a well-resolved separated lineage. The first node within the ingroup divided the sequences in two resolved clades, the basal 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). *Amblyomma pseudoparvum* was more closely related to the CA clade than to the rest of the *A. parvum* subgroups.

Divergence values within the clades (A, B, and CA) were always below 1.3%. Distance between either A, B or Paraguayan, and CA samples ranged from 6.5 to 7.9%. *A. pseudoparvum* differed from the A, B or Paraguayan sequences by 10.3 to 11.9%. The divergence values between CA and *A. pseudoparvum* were 8.5 - 10.1%. The distance separating the ingroup from the outgroup ranged from 11.6 to 17.7%, and from 7.2 to 12.2% within species of the outgroup (Table 1.3).

COI

The MP analysis of COI gene sequences detected 137 parsimony informative sites and the heuristic search led to 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 base frequencies of A=0.31; C=0.17; G=0.13 and T=0.39; PI = 0.5399 and G = 0.7578.

The MP, ML and BA results (Figs 1.8-1.10) revealed that the ingroup was paraphyletic because of the position of *A. pseudoparvum.* The MP analysis showed three lineages arising from a polytomy: *A. pseudoparvum,* CA (100%), and the A – B lineage (91%). The latter was further split into A and B (99 and 98% respectively). The topology of the ML reconstruction was identical. By using BA the CA lineage was basal to everything else, followed by the *A.pseudoparvum* branch, located between CA and the A – B cluster. A and B were monophyletic sister lineages. ML divergence values within B ranged from 0.2 to 0.5%, within A from 0.2 to 3.5%, and within CA from 0.3 to 1.3%. Clades A and B were separated from each other by 4.3 - 6.2%; and either one 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 by 14.7% to 18.5%. The variation within the outgroup ranged from 11.6 to 15.6% (Table 1.4).

COII

The MP analysis of the COII dataset identified a total of 120 parsimony-informative characters, with 8 best trees (length = 310; CI= 0.803; RI= 0.840; HI= 0.197). The ML model best fitting the data, according to the Akaike Information Criterion, was TrN+G with the following base frequencies: A=0.37; C=0.08; G=0.17 and T=0.38; PI = 0 and G = 0.1954.

The MP, ML and BA results (Figs. 1.11-1.13) were fully congruent. The ingroup was monophyletic and resolved into two clades: B - A and CA. 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 CA clade varied from 11.2 to 12.1%, while between the ingroup and the outgroup differed by 16.6 - 21.9%. Variation within outgroup species oscillated between 10.7 and 19.3% (Table 1.4).

DL

The MP analysis of DL sequence detected 72 parsimony-informative sites and the heuristic search led to 2000 equally parsimonious trees. The length of the trees was 174; CI = 0.753; RI = 0.860 and HI = 0.247. The ML model best fitting the data, according to the Akaike Information Criterion, was TVM+I+G with base frequencies of A=0.42; C=0.13; G=0.13 and T=0.32; PI = 0.444; G = 0.531.

The MP and ML were congruent. *Amblyomma pseudoparvum* was always embedded within the monophyletic *A. parvum* (Figs. 14 and 15). Two clades were well resolved, the CA - *A. pseudoparvum* and the A - B. In the former, the CA samples were monophyletic. In the second, only B was supported. For BA, the ingroup was also not monophyletic (Fig. 1.16). The monophyletic CA clustered with *A. pseudoparvum.* The Brazilian clade was monophyletic, while the Argentinian sequences did not form a well-supported clade.

ML pairwise distances within the clades were always below 1.1%. CA differed from A or B by 7.1 to 7.9%, and *A. pseudoparvum* differed from A or B by 8.6 to 9.5%. The divergence value between CA and *A. pseudoparvum* ranged

from 6.6 to 6.8%. The distances separating the ingroup from the outgroup ranged from 11.8 to 16.2%, and within the ougroup from 6.8 to 12.1% (Table 1.5).

ITS2

Unfortunately, we were unable to obtain an ITS2 sequence for *A. pseudoparvum*. The MP analysis of the remaining ITS2 gene sequences detected 244 parsimony-informative sites and the length of the 2 best trees was 383 (CI=0.924; RI=0.958; and HI=0.076). The ML model best fitting the data by the Akaike Information Criterion was GTR+G with base frequencies of A=0.17; C=0.36; G=0.28 and T=0.19; PI = 0; G=0.4616.

In both the MP and ML (Figs. 1.17 and 1.18) analyses the ingroup was monophyletic. The MP reconstruction identified two clades within the ingroup: CA and B - A. With this molecular marker, neither the Brazilian nor the Argentinian sequences clustered into monophyletic groups. The ML analysis was characterized by overall lower resolution, with only CA constituting a relatively weakly (70%) supported clade, sister group to the Brazilian samples from Goiás. The remaining Brazilian and Argentinian sequences were polytomic and basal to the other clades. The BA reconstruction resolved the monophyletic ingroup into two resolved clades: one (88%) included samples from Argentina (A) and Brazil (PA and MG), while the other (98%) clustered the sister lineages from Brazil (GO) and Central America (100%) (Fig. 1.19).

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 either Brazil from MG and PA 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 and 5.5%. Between the ingroup and the outgroup the distances oscillated from 13.5 to 17.4%, and within the outgroup from 2.1 to 7.1% (Table 1.6).

Concatenated datasets (mitochondrial gene sequences)

The concatenated matrix included sequences representative of the countries included in the study. In addition, a sequence of *A. pseudoparvum* was also included. Sequences of all the mitochondrial molecular markers were concatenated with the exception of COII, a gene for which we did not have a sufficient number of amplicons. The concatenated matrix included 84 sequences (1755 bp) corresponding to 16 unique haplotypes, 1 sequence of *A. pseudoparvum* and 4 outgroups.

The MP analysis of the concatenated mitochondrial dataset detected 350 parsimony-informative characters and the heuristic search found 2 equally parsimonious trees. The length of the trees was 885; CI = 0.738; RI = 0.820; and HI = 0.262. The ML model best fitting the data, according to the Akaike Information Criterion, was GTR+I+G with base frequencies of A=0.38; C=0.12; G=0.14 and T=0.36; PI = 0.5606; G = 1.0068.

The MP and ML analyses (Figs. 1.20 and 1.21) revealed a polytomy with *A. pseudoparvum* embedded within the ingroup. Two clades were well-supported, CA (100%) and B - A (100%). The latter was further resolved into two lineages, B and A, both with 100% bootstrap support. The BA resulted in a reconstruction in which the ingroup was monophyletic, although the support was low (70%). The ingroup was resolved in two clades: B - A (100%) and CA (100%). The Panama samples constituted a supported lineage within CA. The B - A clade was split in two monophyletic lineages, A and B (both with 100% support). Within B, the MG branch was basal to the sister lineages, GO and PA (Fig. 1.22).

ML pairwise distances within the clades were always below 1.6%. CA differed from A or B by 8.1 to 8.5%, and *A. pseudoparvum* differed from A or B by 10.5 to 11%. The divergence value between CA and *A. pseudoparvum* was between 9.5 and 9.8%. The distances separating the ingroup from the outgroup ranged from 13.9 to 16.3%, whereas the distance between *A. pseudoparvum* and

the outgroup varied from 15.3 to 17.5%. The distances between species belonging to the ougroup differed by 9.5 to 13.1% (Table 1.7).

Concatenated datasets (mitochondrial and nuclear gene sequences)

The concatenated matrix included 95 sequences (2630 bp) corresponding to 15 unique haplotypes and 4 outgroups. The MP analysis for the concatenated dataset detected a total of 607 parsimony-informative sites, and the parsimonious heuristic search found 2 trees equally parsimonious. The length of those trees was 1119 and the coefficients as follows: CI= 0.833; RI= 0.895; HI= 0.167. The ML model that best fitted the data according to the Akaike Information Criterion was GTR+I+G with base frequencies of A=0.30; C=0.21; G=0.19 and T=0.30; PI = 0.3601; G = 0.7092.

The MP, ML and BA analyses were fully congruent leading to topologically identical trees, in which the ingroup was monophyletic and two clades were resolved: CA and A - B. This clade was further resolved into two; B and A. In the three phylogenetic reconstructions a lineage inluding Brazilian samples from Goias was supported within the B clade (Figs. 1.23-1.25).

Divergence values within the CA and A clades were always below 1.1%. Overall variation within B clade ranged between 0 and 1.5%, being 0 within the GO lineage. The distance between A and Brazilian samples from MG and PA ranged from 2.5 to 2.9%, whereas Brazilian samples from GO differed from Argentinian samples by 3.8 to 4.1%. Divergence values between either A or B and CA ranged from 6.5-6.7% and the distance splitting the ingroup from the outgroup was 18.6-21.1%. Variation within the outgroup varied between 7.6 and 11% (Table 1.8).

Discussion

The overall structure of the trees obtained with the 6 genes was often different, particularly when dealing with weakly supported nodes. However, when *A. pseudoparvum* was included in the analyses, it became clear that *A. parvum* is paraphyletic, with two consistently distinct clades diverging at the basal level, the CA and the A – B clade. When the position of *A. pseudoparvum* was resolved, it appeared to be more closely related to CA than to A – B. ML pairwise distance values between A and B were compatible with these geographically distant lineages being conspecific, particularly when compared to distance values within the outgroup a cluster of recognized different species. Between CA and A – B, CA and *A. pseudoparvum*, and A - B and *A. pseudoparvum*, the distance values are similar to values recorded between outgroup species and between the outgourp and the ingroup. This strongly suggests that we are dealing with three taxonomic entities, *A. parvum* (from Brazil and Argentina), a distinct species from Central America which needs to be described and characterized, and *A. pseudoparvum.* In order to fully ascertain the taxonomic status of the Central American lineage, it would be useful to cross-breed colony ticks from CA, B and A and verify whether or not they are reproductively compatible. In addition, it would be important to include two other *Amblyomma* taxa in future analysis, *A. auricularium* and *A. pseudoparvum*, two species closely related to our ingroup (Nava et al., 2008a).

Within the A- B clade, some of the analyses reveal that the samples from Brazil – GO have further differentiated from other Brazilian lineages, which appear to be more closely related to the Argentinian ones. Nevertheless, the differentiation between BR-GO and the other A-B samples is relatively weak, and cross-breeding experiments between A and Br-GO ticks showed reproductive compatibility (Gerardi et al., 2013; Nava et al., unpublished data).

If 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, it is possible that the *A. parvum* group of taxa has an early-middle Miocene origin, as was the case for another *Amblyomma* species with a similar geographical distribution (Beati et al., 2013). During that period, caviomorph rodents had already started diversifying in South America (Poux et al., 2006), thus providing a specific group of hosts host for *A. pseudoparvum*, a lineage in a basal position and distinct from clades that specialized on larger mammals, cattle for *A. parvum* and wild ungulates (among others) for the CA clade (Nava et al., 2008).

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APPENDIX 1

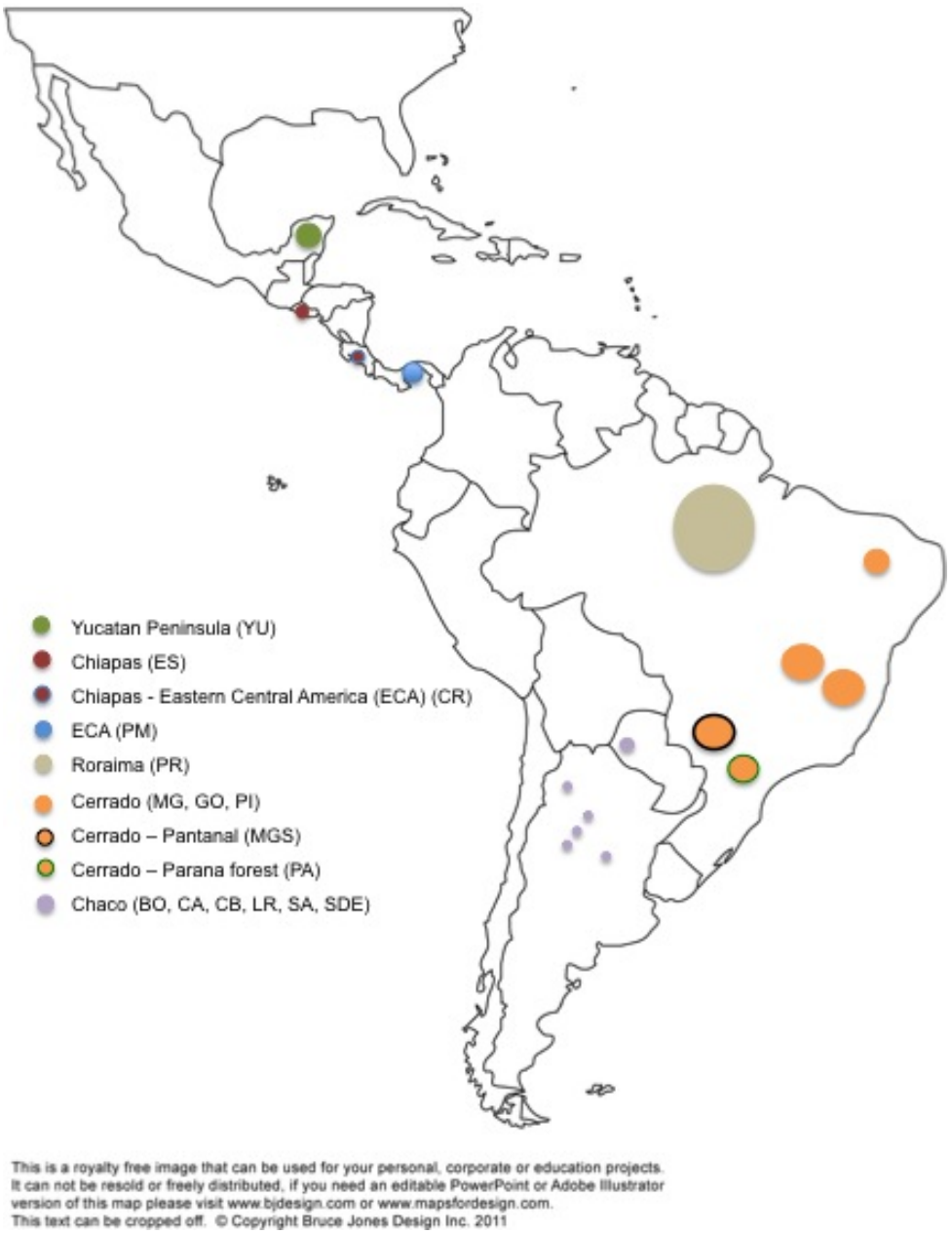

Figure 1.1. Political map of Central and South America showing the collection areas and the eco-regions to what they correspond. YU: Yucatan, Mexico; ES: El Salvador; CR: Costa Rica; PM: Panama; PR: Para, Brazil; MG: Minas Gerais, Brazil; GO: Goias, Brazil; PI: Piaui, Brazil; PA: Pantanal, Brazil; BO: Boqueron, Paraguay; CA: Catamarca, Argentina; CB: Cordoba, Argentina; LR: La Rioja, Argentina; SA: Salta, Argentina; SDE: Santiago del Estero, Argentina.

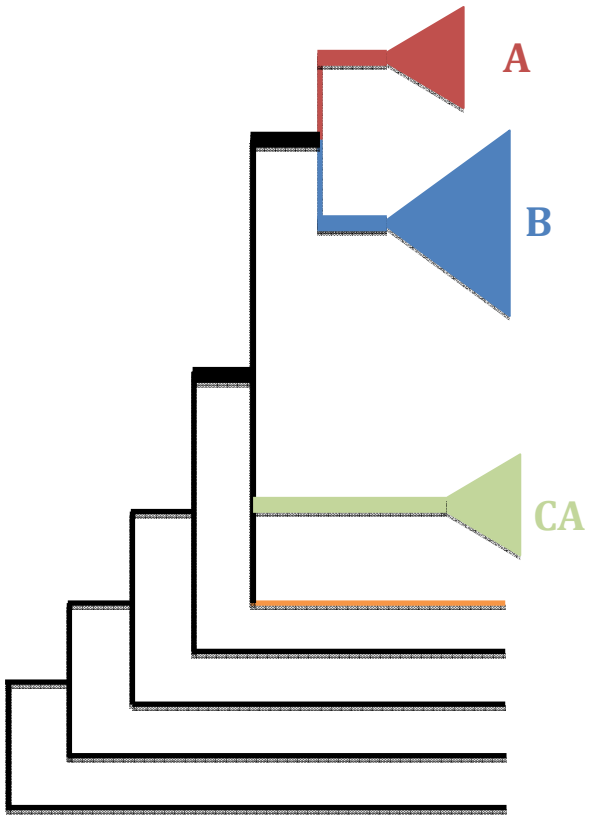

Fig. 1.2 *Amblyomma parvum* Maximum Parsimony (MP) reconstruction for 12S. Color codes: Black: outgroup species; Orange: *A. pseudoparvum;* Green: Central American clade of *A. parvum*; Blue: Brazilian clade of *A. parvum*; Red: Argentinian clade of *A. parvum*. Thicker lines represent branches with bootstrap support > 75%. Color codes and thickness of the branches is maintained throughout the document.

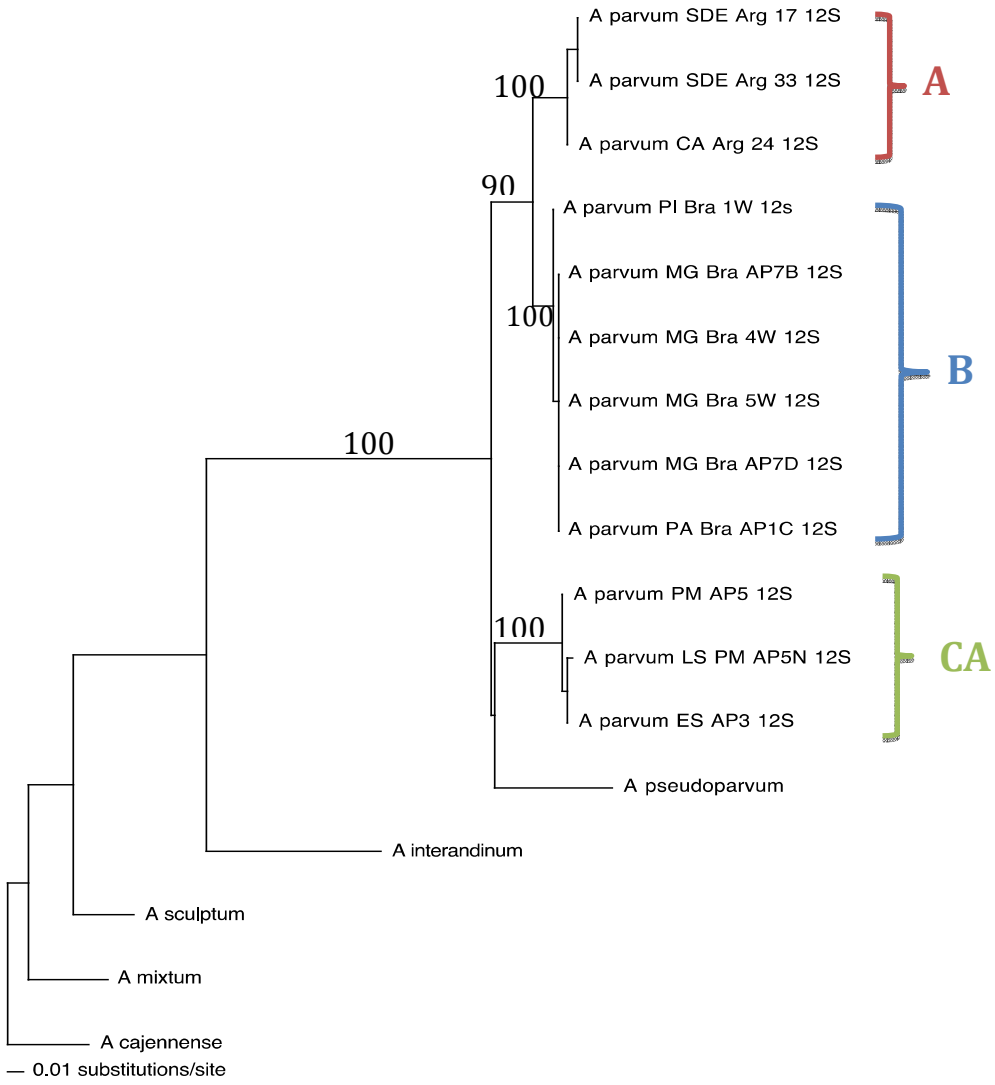

Fig. 1.3 Maximum likelihood tree for 12S.

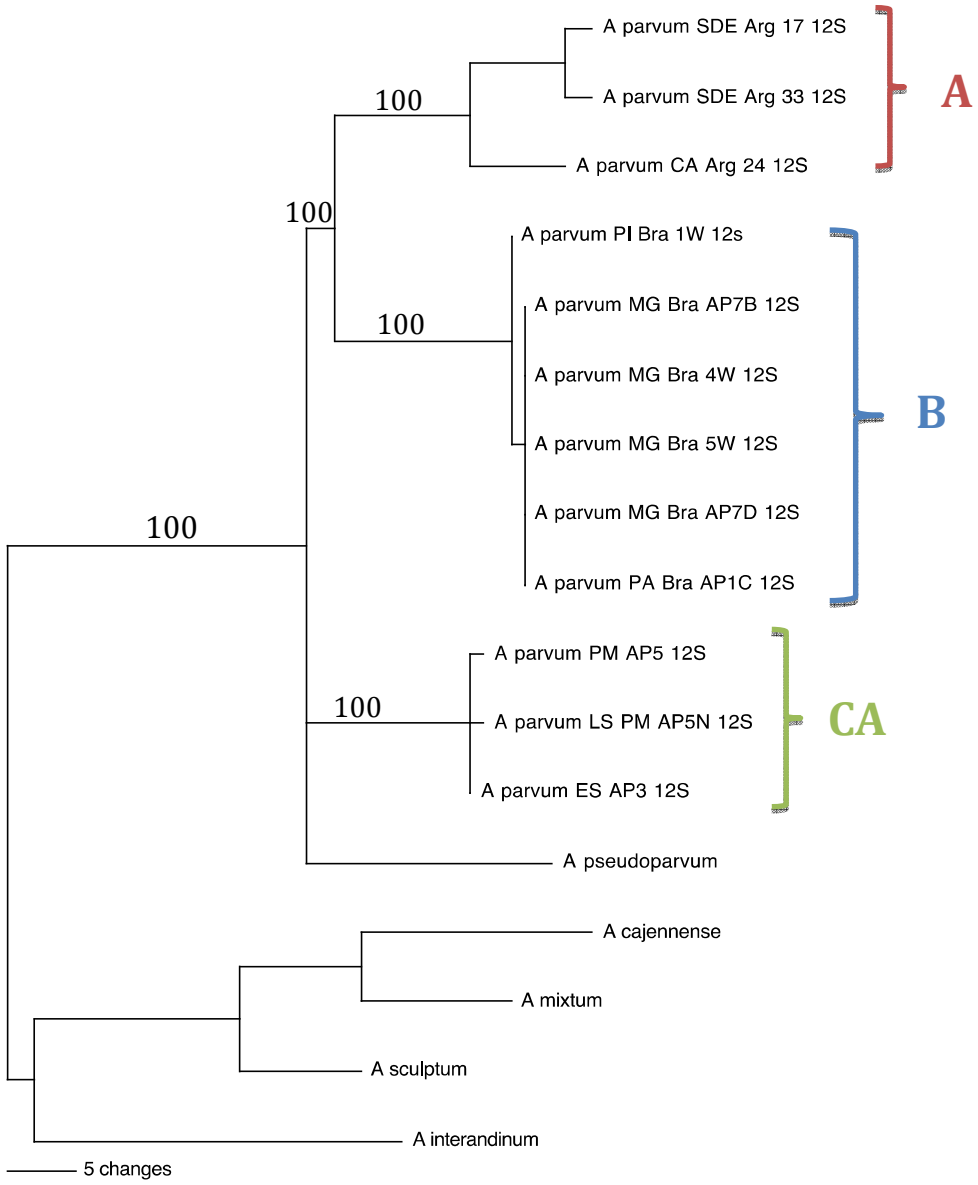

Fig. 1.4 Bayesian analysis for 12S.

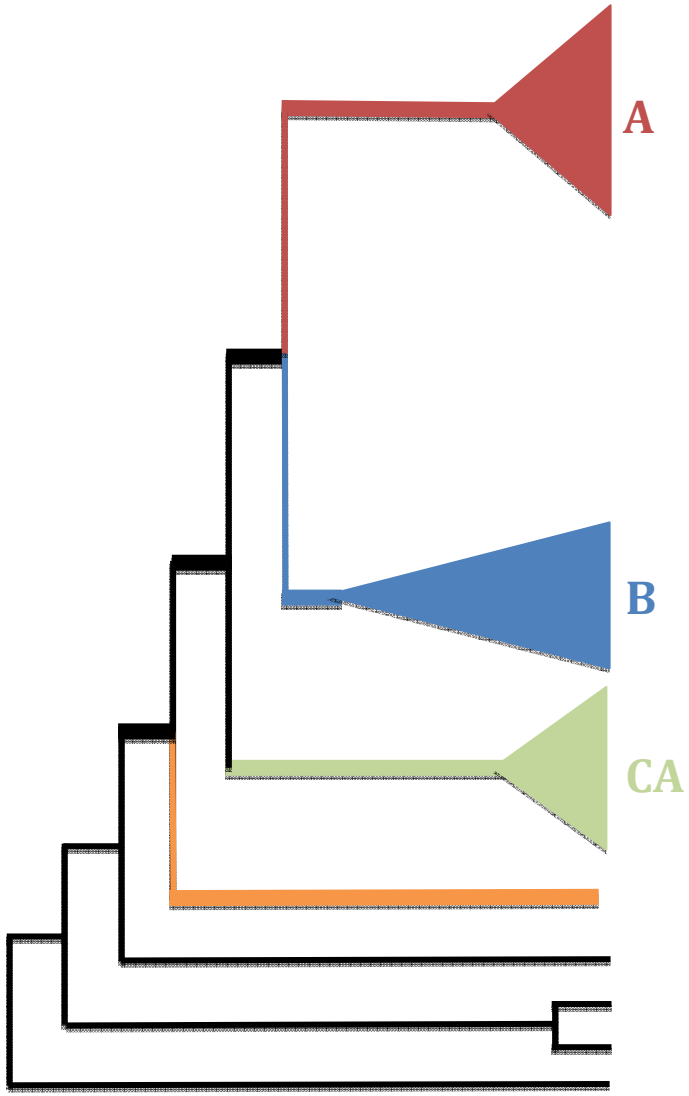

Fig. 1.5 Maximum parsimony reconstruction for 16S.

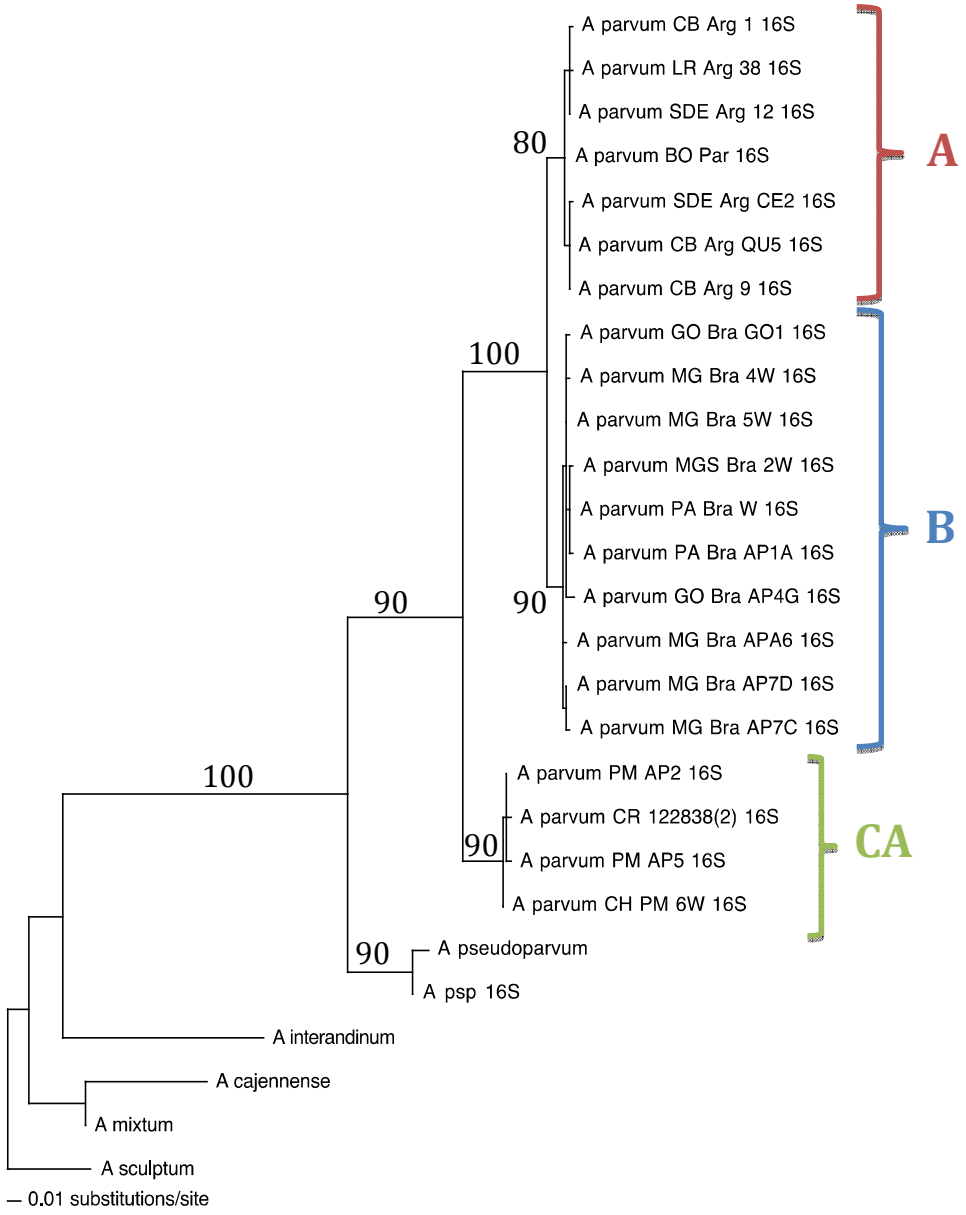

Fig. 1.6 Maximum likelihood tree for 16S.

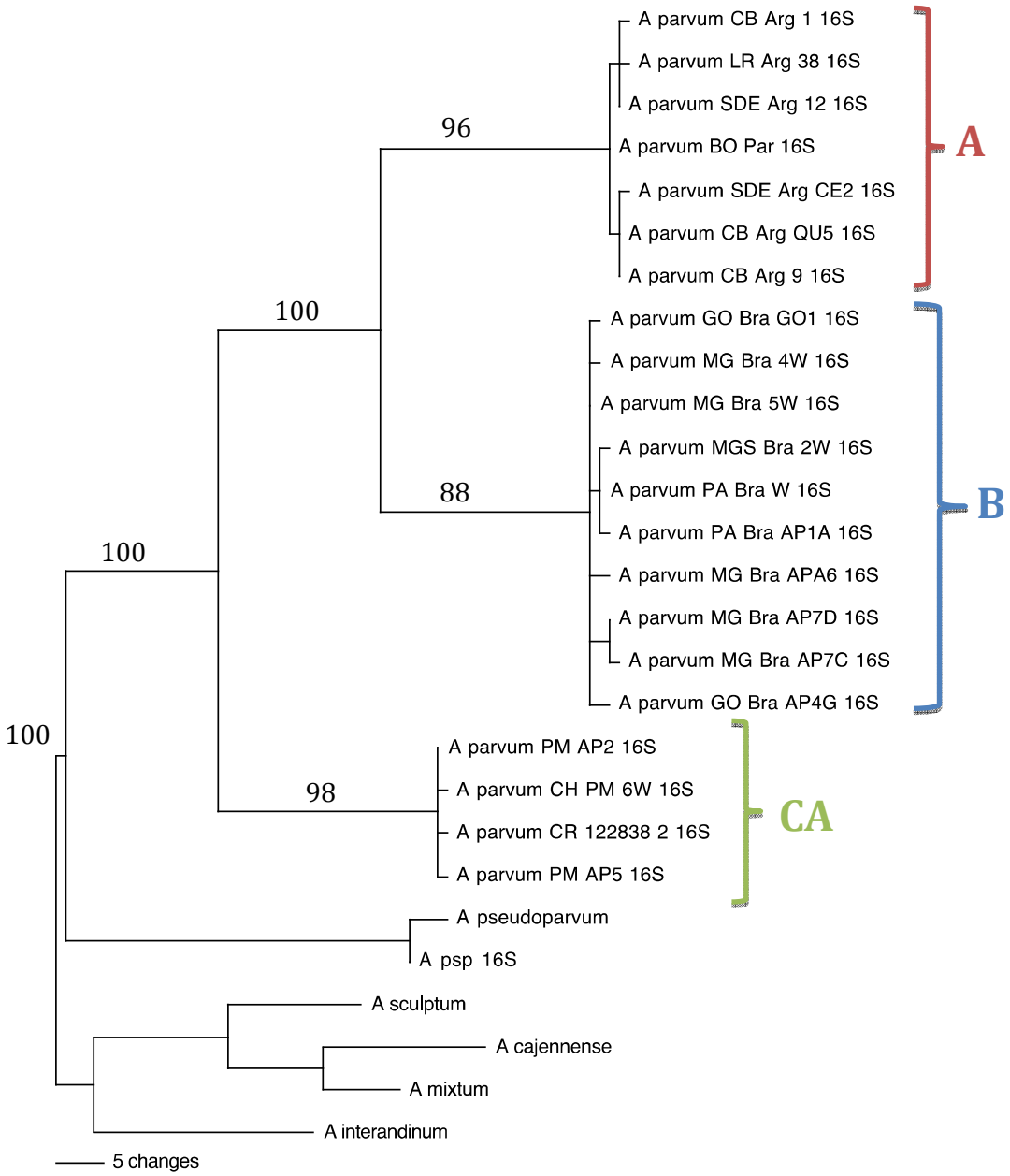

Fig. 1.7 Bayesian analysis reconstruction of 16S.

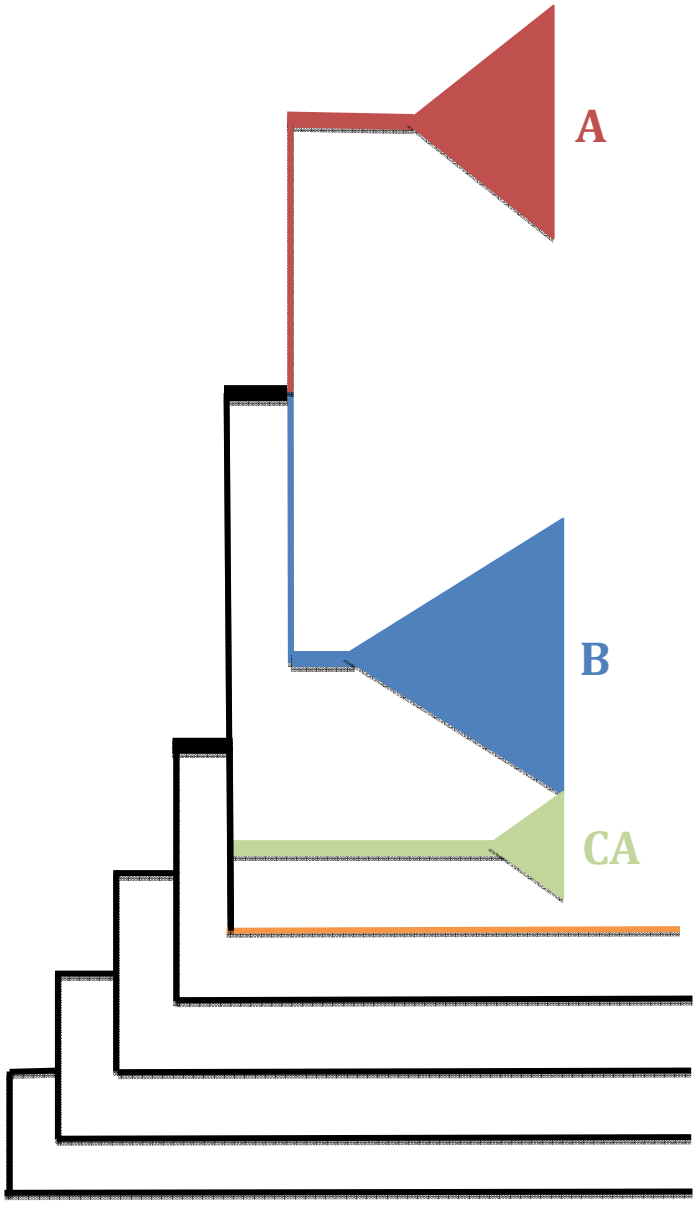

Fig. 1.8 Maximum parsimony reconstruction for COI.

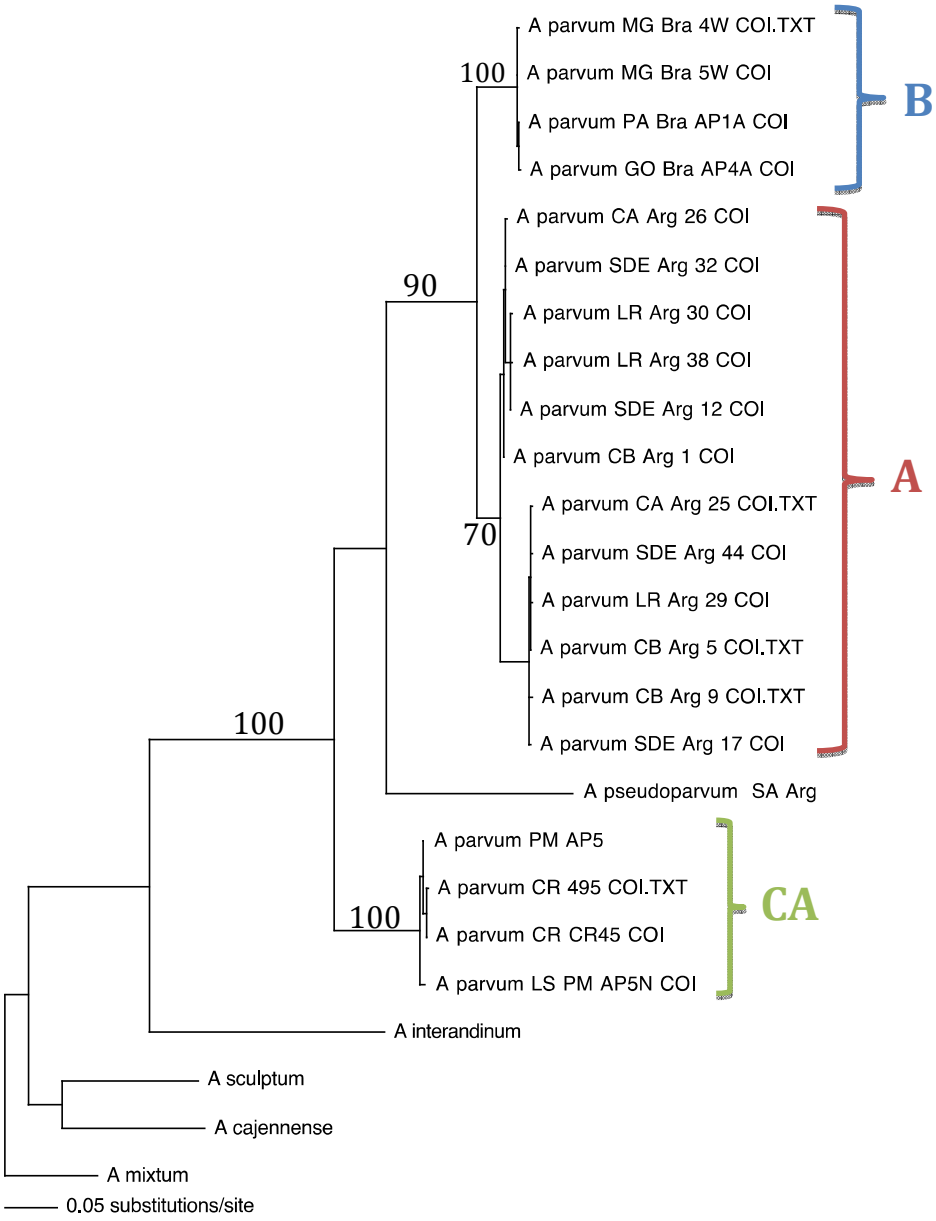

Fig. 1.9 Maximum likelihood tree for COI.

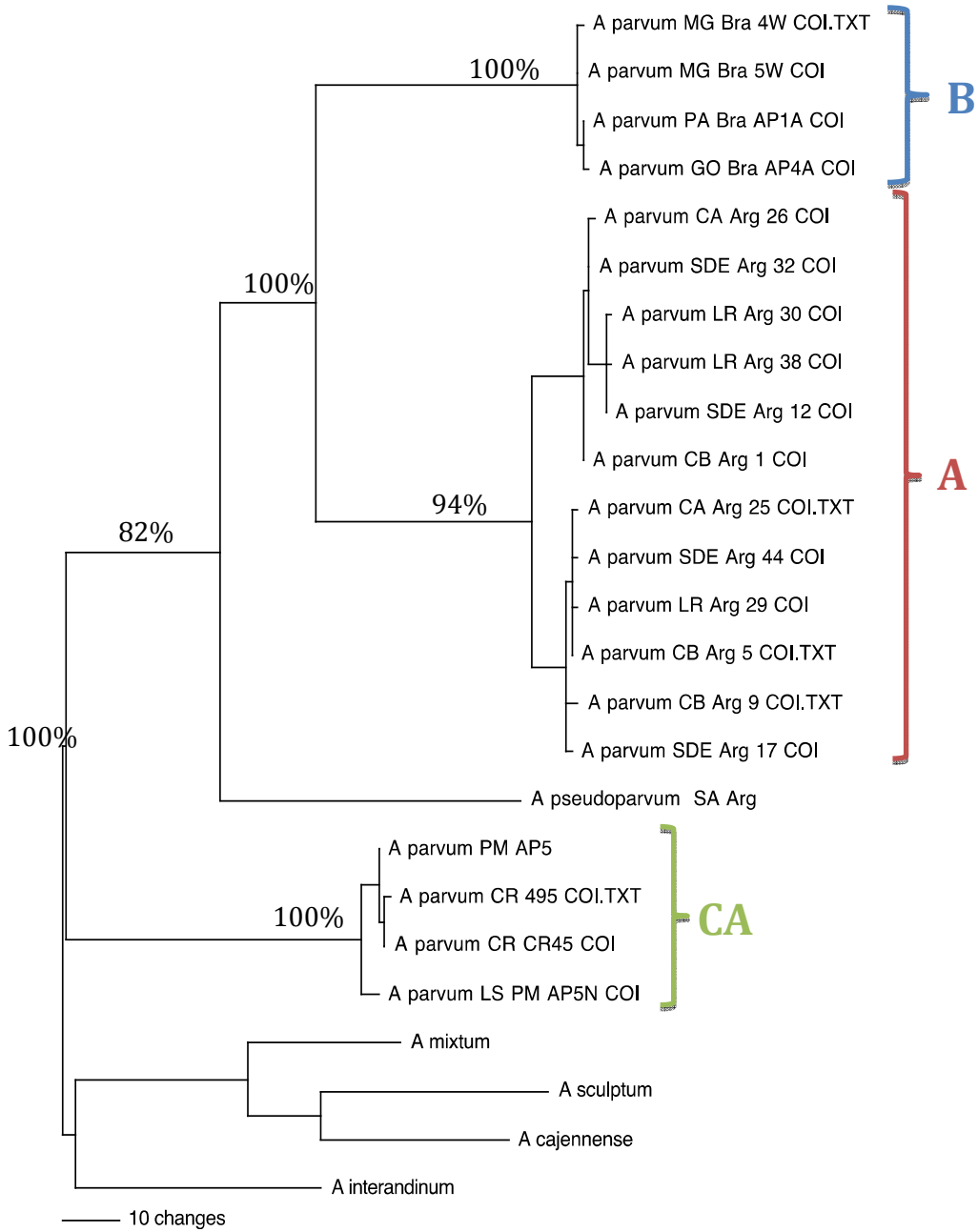

Fig. 1.10 BA for COI.

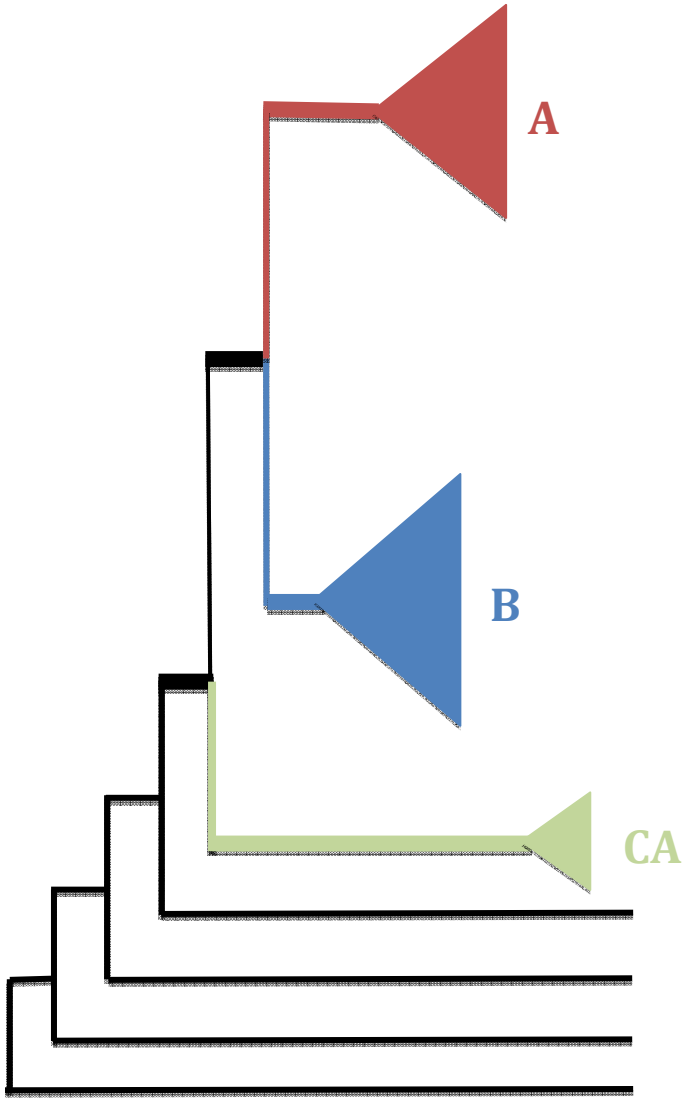

Fig. 1.11 Maximum parsimony tree for COII.

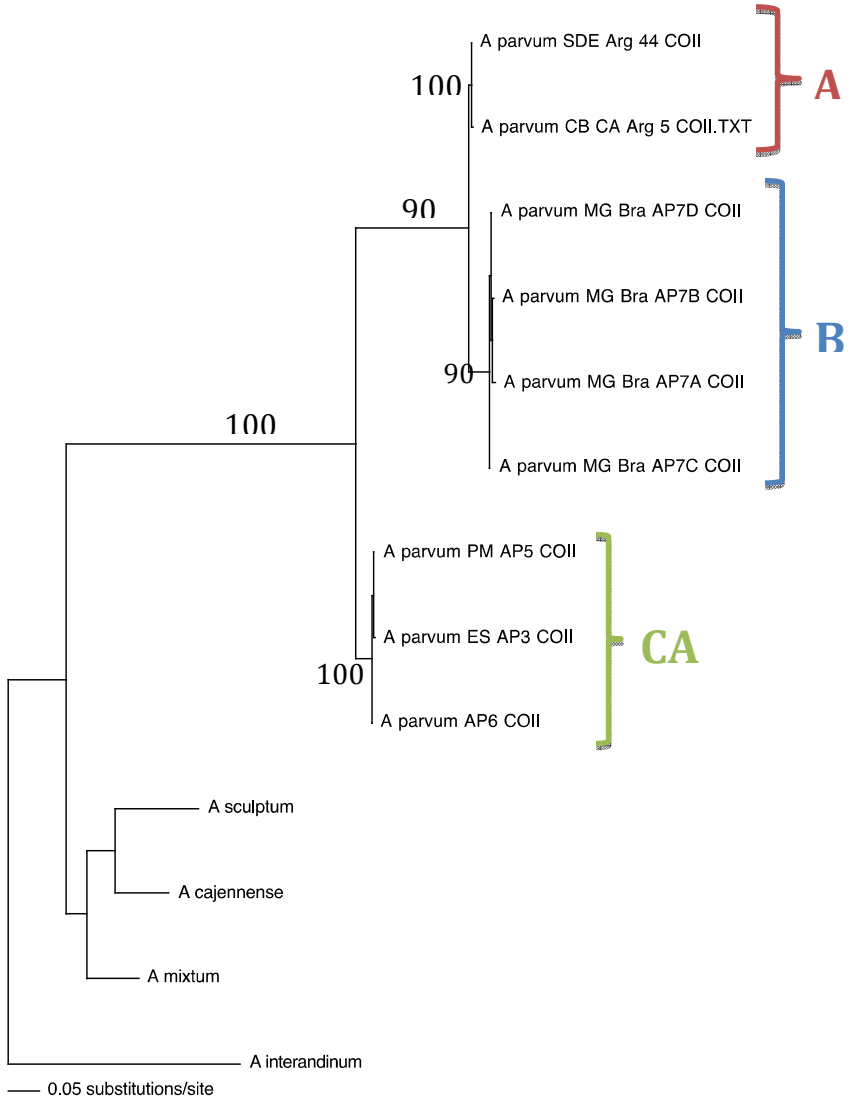

Fig. 1.12 Maximum likelihood tree for COII.

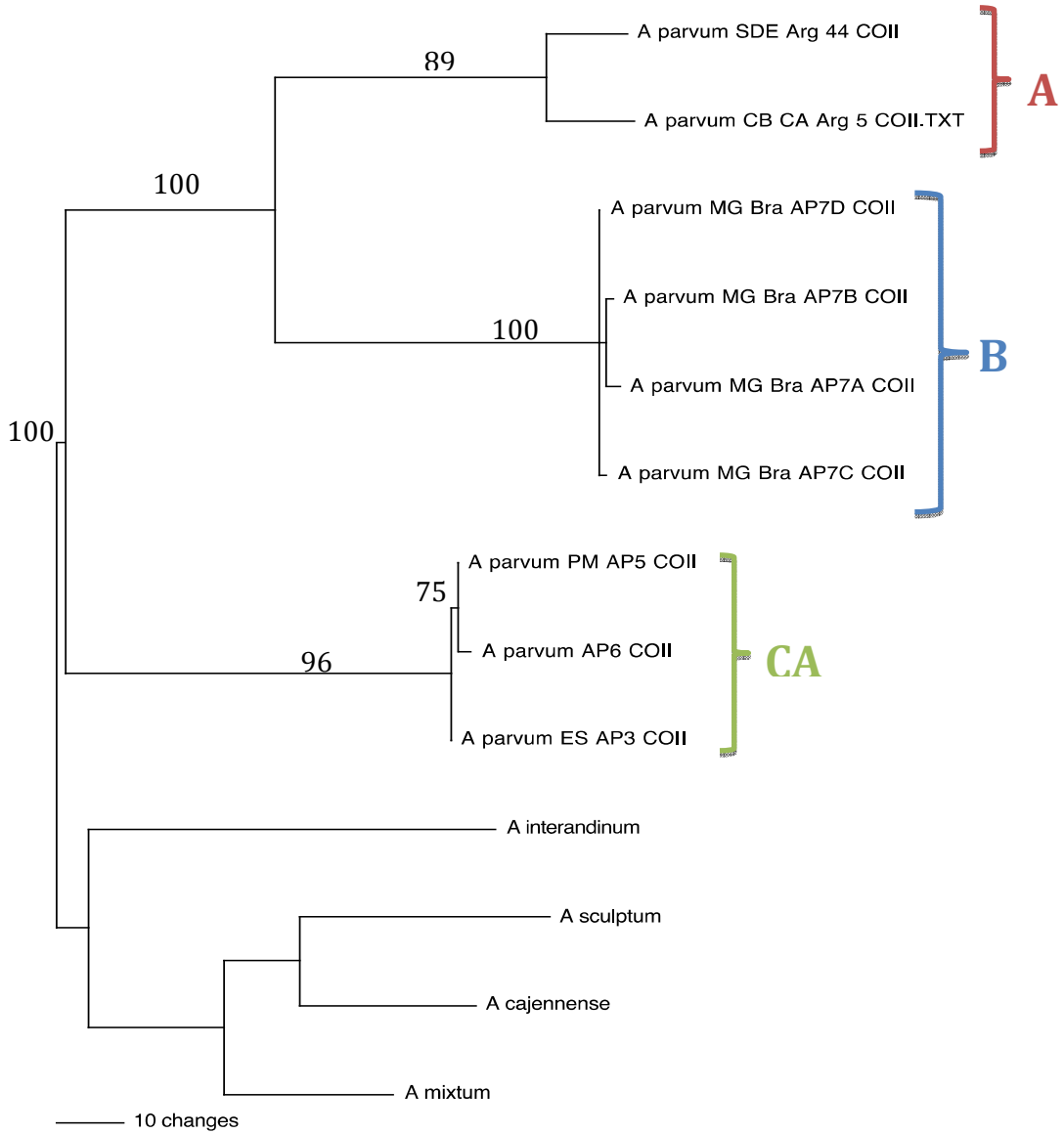

Fig. 1.13 BA for COII

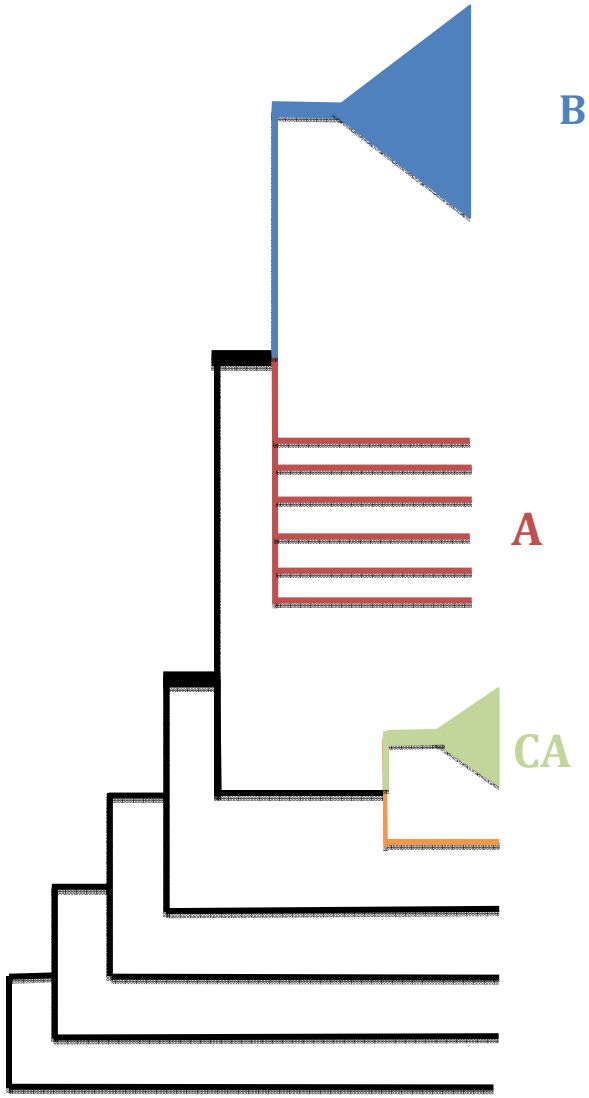

Fig. 1.14 Maximum parsimony reconstruction for DL.

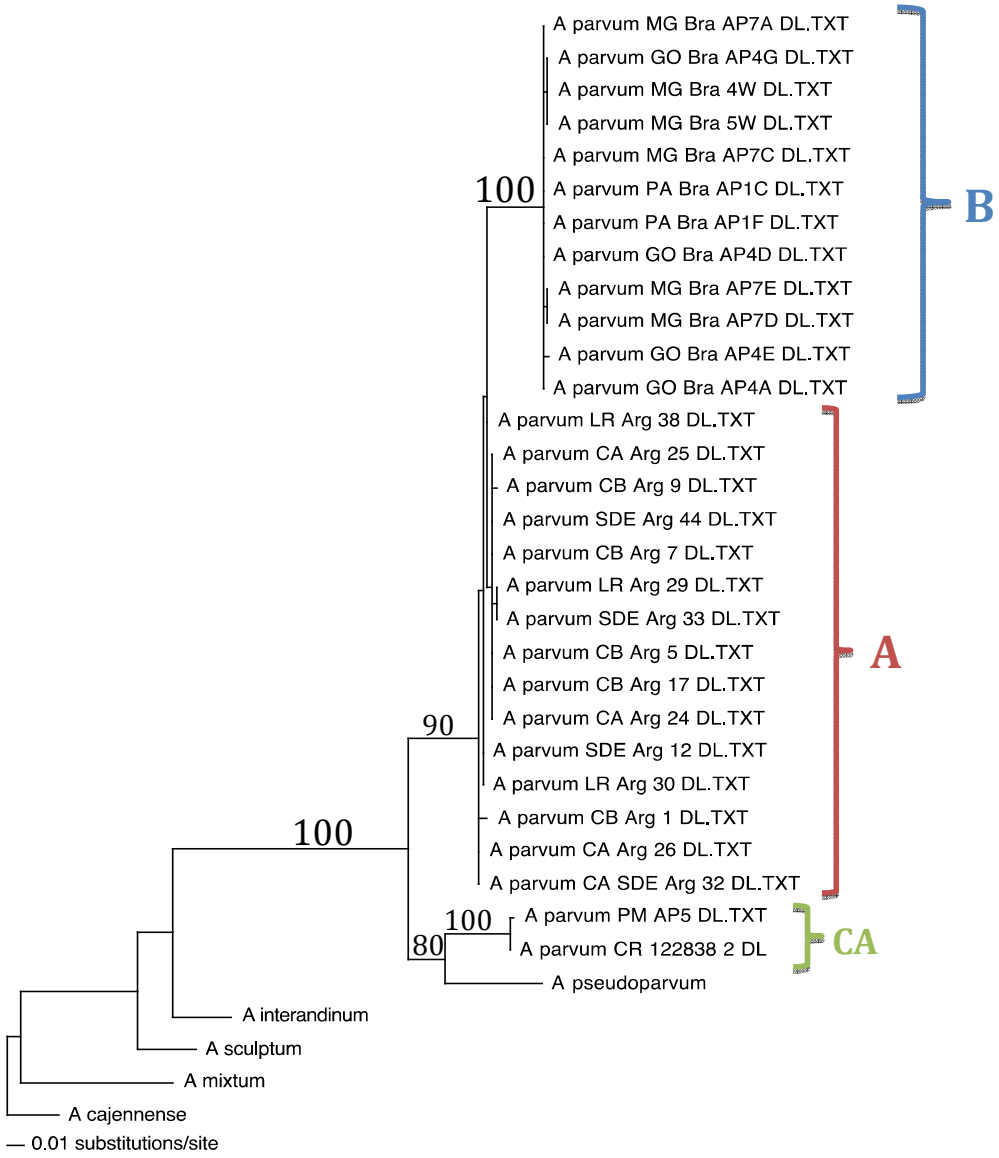

Fig. 1.15 Maximum likelihood tree for DL.

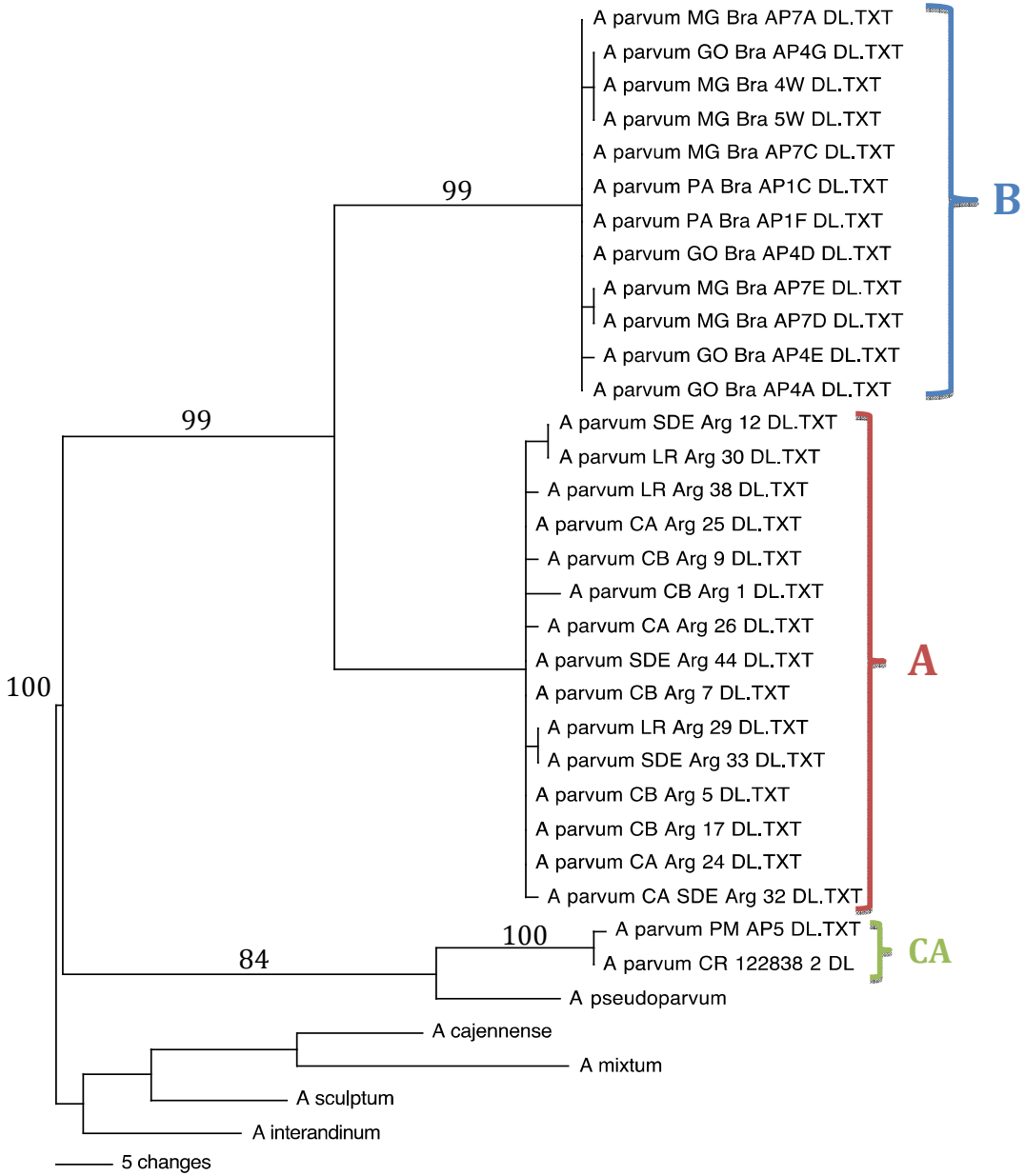

Fig. 1.16 BA for DL

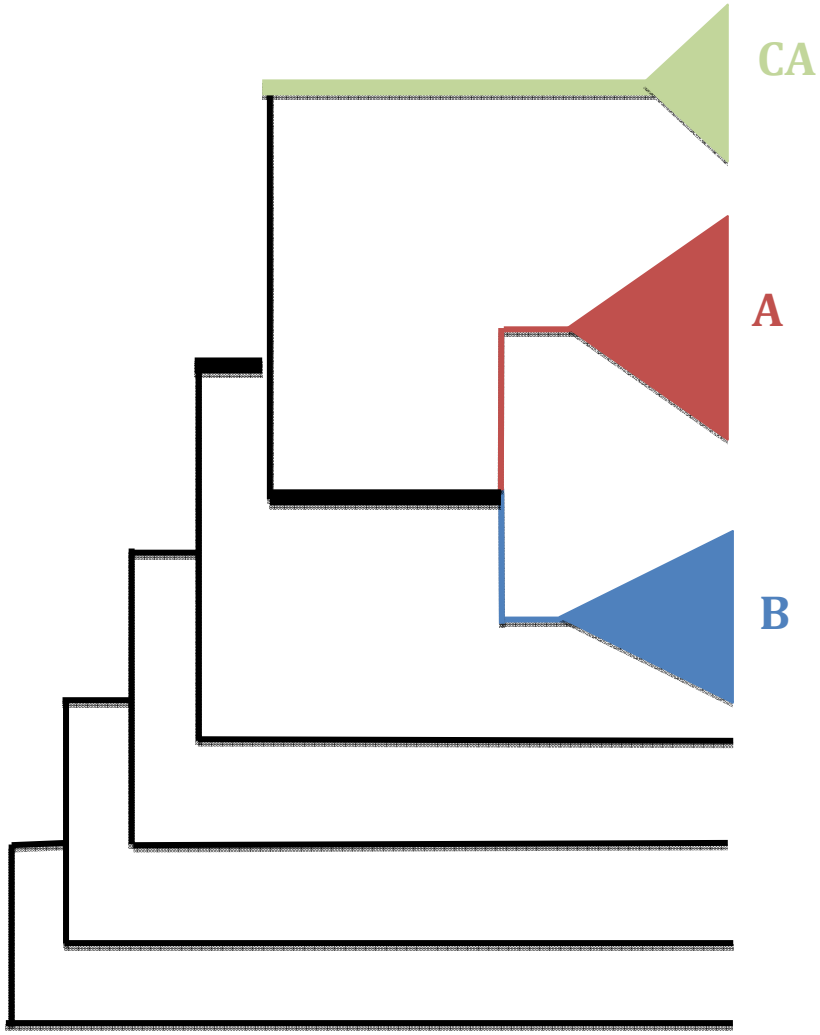

Fig. 1.17 Maximum parsimony reconstruction for ITS2.

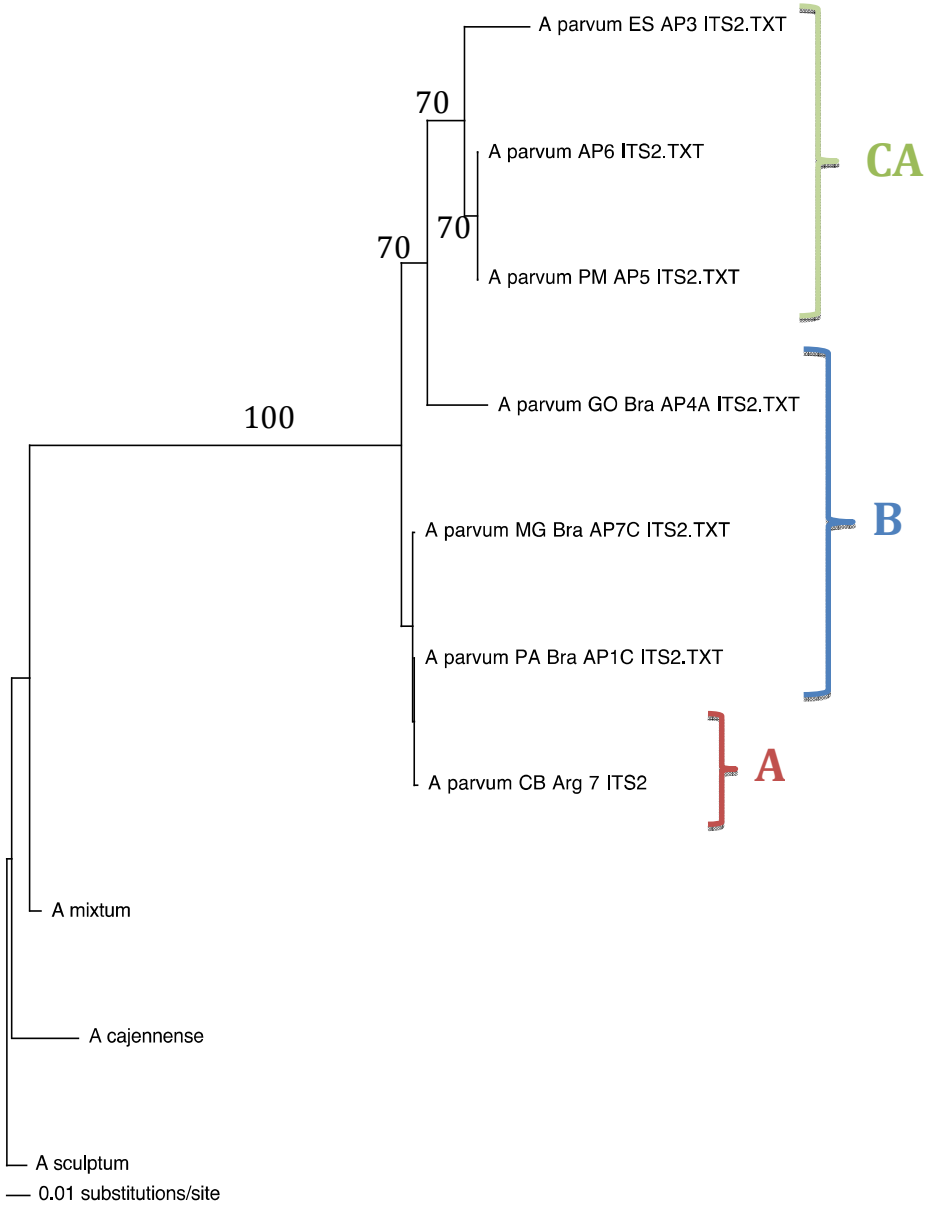

Fig. 1.18 Maximum likelihood tree for ITS2.

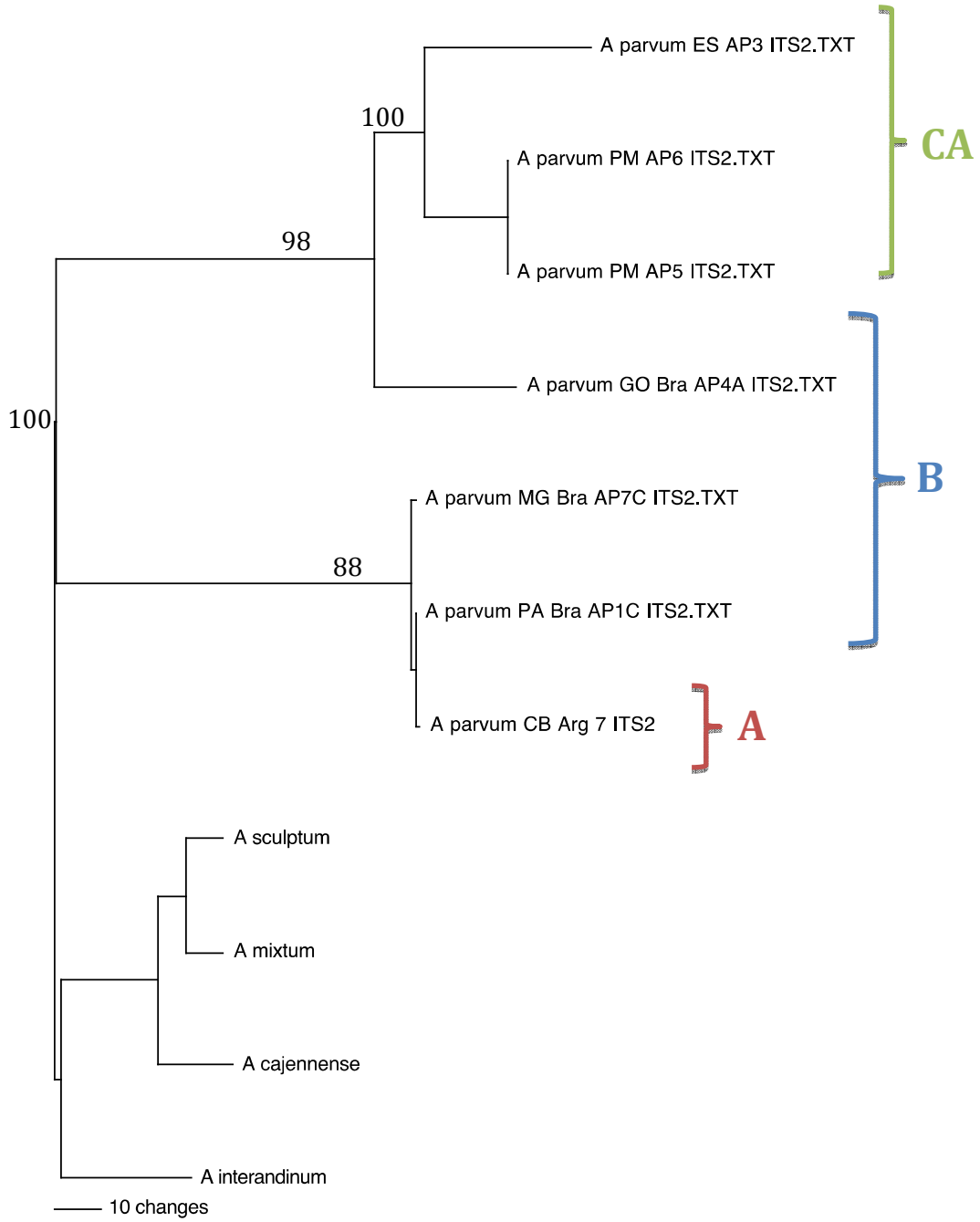

Fig. 1.19 BA for ITS2.

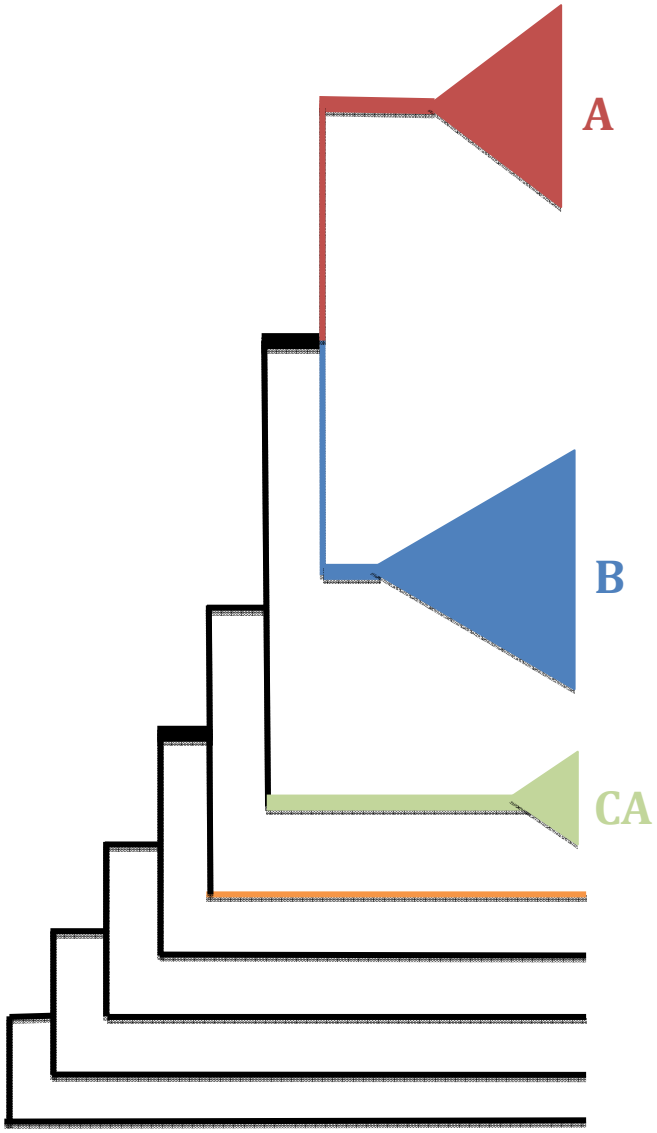

Fig. 1.20 Maximum parsimony reconstruction for the mitochondrial dataset (12S-16S-COI-DL).

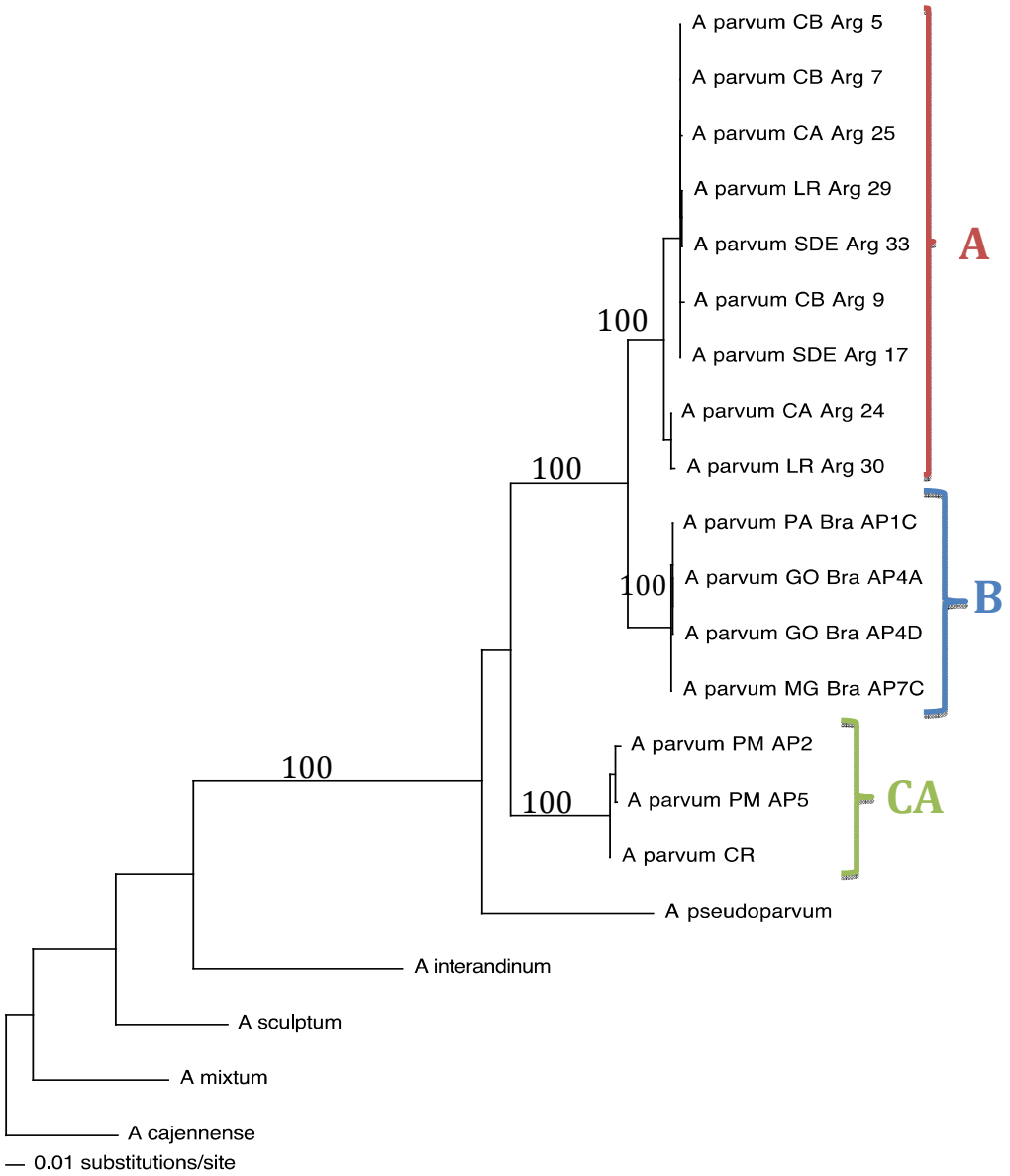

Fig. 1.21 Maximum likelihood tree for mitochondrial concatenated dataset.

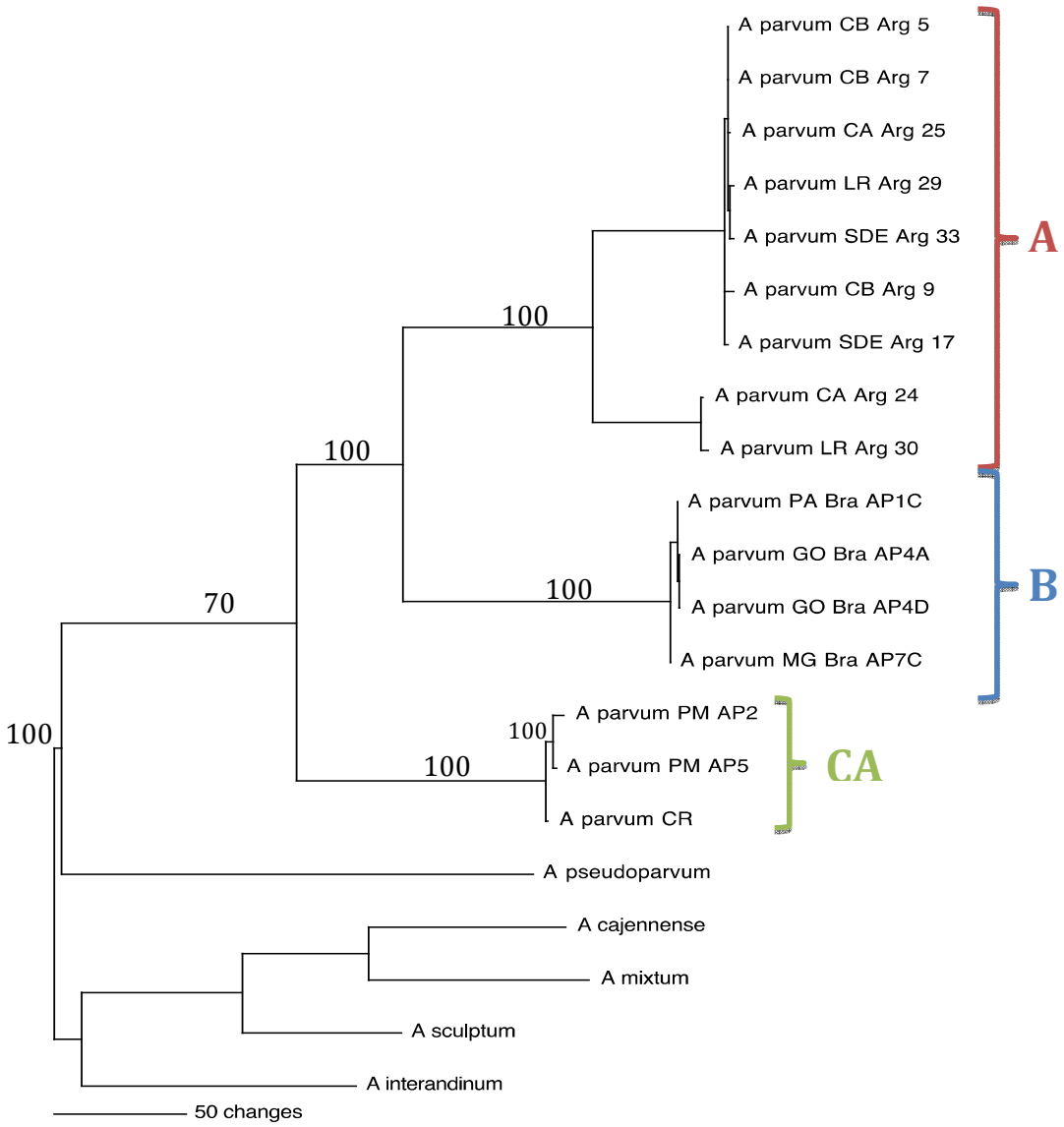

Fig. 1.22 BA for the mitochondrial concatenated dataset.

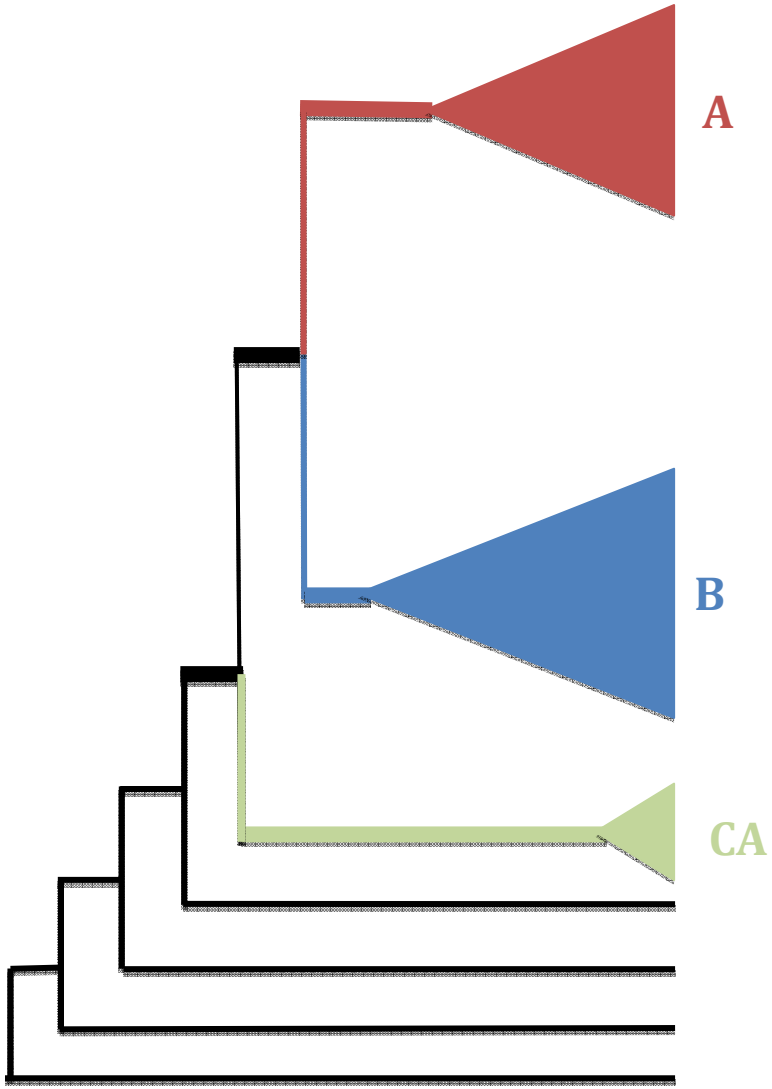

Fig. 1.23 Maximum parsimony reconstruction for the mitochondrial and nuclear concatenated dataset (12S-16S-COI-DL-ITS2).

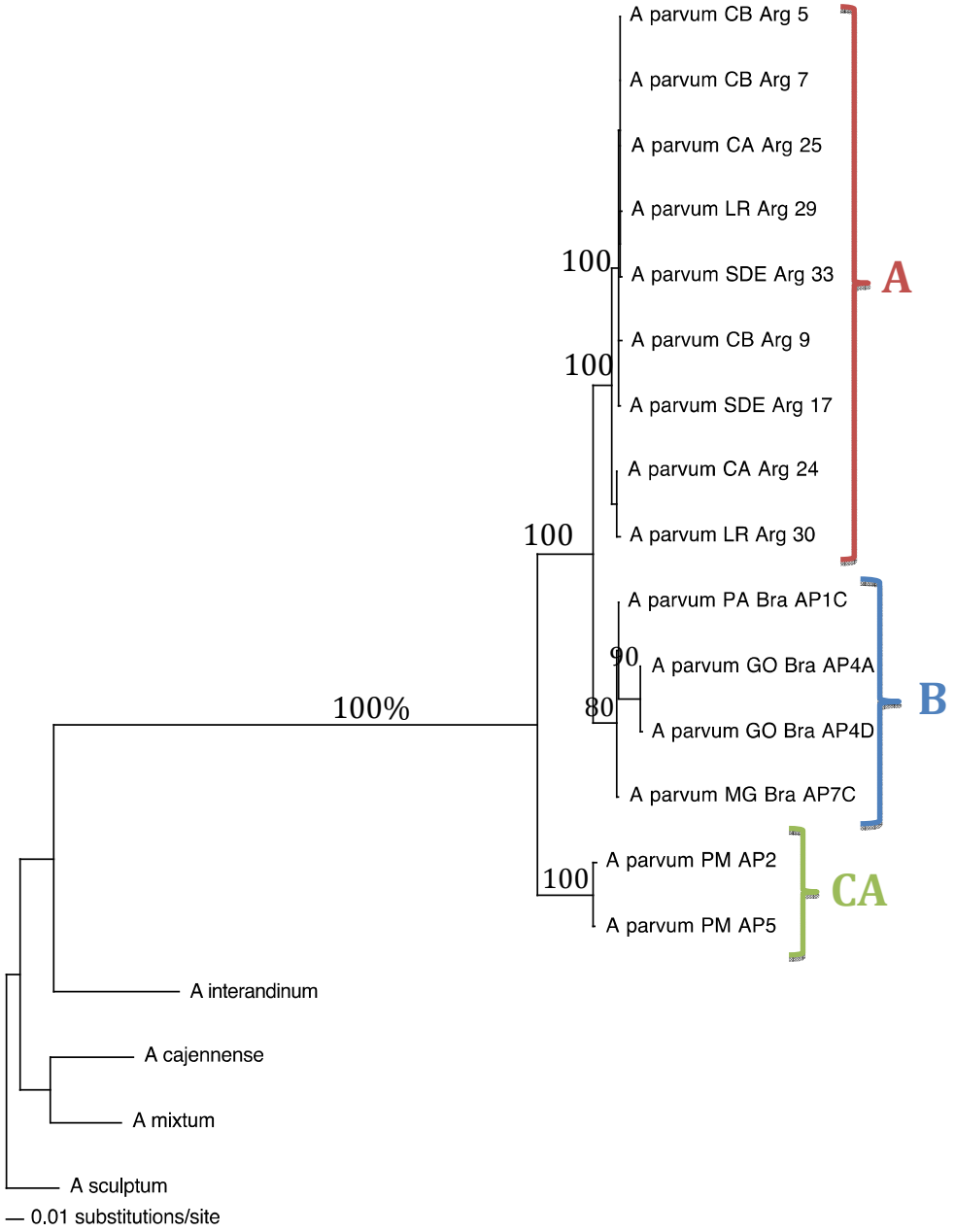

Fig. 1.24 Maximum likelihood tree for the nuclear mitochondrial concatenated dataset.

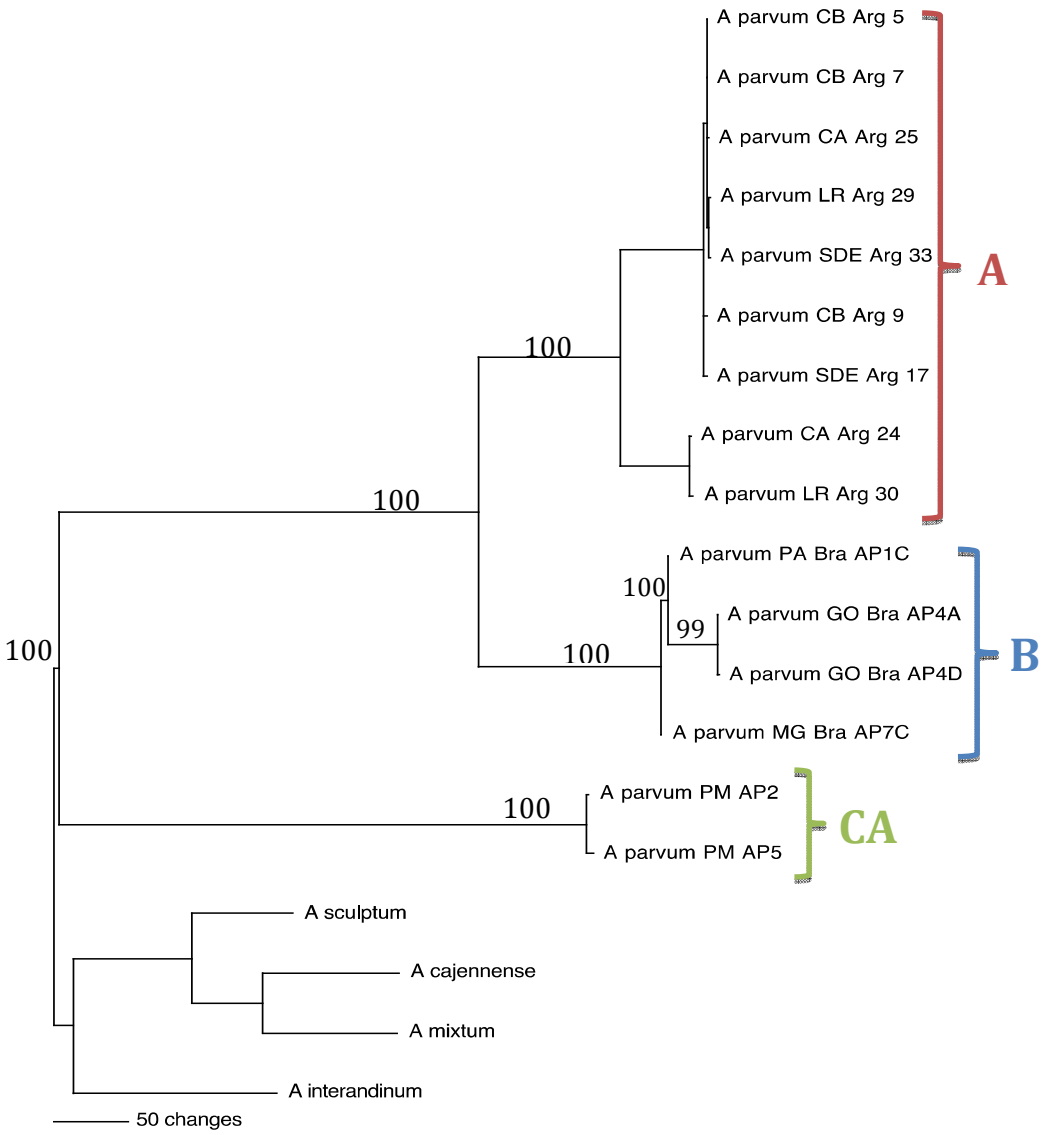

Fig. 1.25 BA for the nuclear and mitochondrial concatenated dataset.

Table 1.1. Localities and PCR amplification success for each of the samples, and each of the individual gene markers.

Lab ID	Species	Locality	Code	Country	12S	16S	DL	ITS2	COI	COII
1W	A. parvum	Piaui	PI	Brazil	X					
2W	A. parvum	Mato Grosso do Sul	MGS	Brazil		X				
3W	A. parvum	Mato Grosso do Sul	MGS	Brazil		X				
4W	A. parvum	Minas Gerais	MG	Brazil	X	X	X		X	
5W	A. parvum	Minas Gerais	MG	Brazil	X	X	X		X	
6W	A. parvum	Chiriqui	PM	Panamá		X				
1	A. parvum	Córdoba	CB	Argentina		X	X		X	
5	A. parvum	Córdoba	CB	Argentina	X	X	X		X	X
7	A. parvum	Córdoba	CB	Argentina		X	X	X	X	X
9	A. parvum	Córdoba	CB	Argentina	X	X	X		X	X
12	A. parvum	Santiago del Estero	SDE	Argentina		X	X		X	
17	A. parvum	Santiago del Estero	SDE	Argentina	X	X	X		X	X
24	A. parvum	Catamarca	CA	Argentina	X	X	X		X	
25	A. parvum	Catamarca	CA	Argentina	X	X	X		X	X
26	A. parvum	Catamarca	CA	Argentina	X	X	X		X	
29	A. parvum	La Rioja	LR	Argentina	X	X	X		X	X
30	A. parvum	La Rioja	LR	Argentina	X	X	X		X	
32	A. parvum	Santiago del Estero	SDE	Argentina	X	X	X		X	
33	A. parvum	Santiago del Estero	SDE	Argentina	X	X	X		X	
38	A. parvum	La Rioja	LR	Argentina		X	X		X	
44	A. parvum	Santiago del Estero	SDE	Argentina		X	X		X	X
AP2	A. parvum	Puerto Limon	PM	Panamá	X	X	X			X

AP3	A. parvum	El Salvador	ES	El Salvador	X	X		X		X
AP4A	A. parvum	Goias	GO	Brazil	X		X	X	X	
AP4B	A. parvum	Goias	GO	Brazil	X		X	X	X	
AP4C	A. parvum	Goias	GO	Brazil	X	X	X		X	
AP4D	A. parvum	Goias	GO	Brazil	X	X	X		X	
AP4E	A. parvum	Goias	GO	Brazil	X	X	X			
AP4F	A. parvum	Goias	GO	Brazil	X	X	X			
AP4G	A. parvum	Goias	GO	Brazil	X	X	X			
AP5	A. parvum	Pan	PM	Panamá	X	X	X	X	X	X
AP6	A. parvum	Chiriqui	PM	Panamá	X			X		X
AP7A	A. parvum	Formoso (Minas Gerais)	MG	Brazil	X		X			X
AP7B	A. parvum	Formoso (Minas Gerais)	MG	Brazil	X		X			X
AP7C	A. parvum	Formoso (Minas Gerais)	MG	Brazil	X	X	X	X		X
AP7D	A. parvum	Formoso (Minas Gerais)	MG	Brazil	X	X	X			X
AP7E	A. parvum	Formoso (Minas Gerais)	MG	Brazil	X	X	X			
AP7F	A. parvum	Formoso (Minas Gerais)	MG	Brazil						
AP1A	A. parvum	Pantanal	PA	Brazil	X	X			X	
AP1B	A. parvum	Pantanal	PA	Brazil	X	X			X	
AP1C	A. parvum	Pantanal	PA	Brazil	X	X	X	X	X	
AP1D	A. parvum	Pantanal	PA	Brazil					X	
AP1E	A. parvum	Pantanal	PA	Brazil					X	
AP1F	A. parvum	Pantanal	PA	Brazil			X		X	

AP1G	A. parvum	Pantanal	PA	Brazil					X	
AP1H	A. parvum	Pantanal	PA	Brazil					X	
AP1I	A. parvum	Pantanal	PA	Brazil					X	
AP1J	A. parvum	Pantanal	PA	Brazil					X	
APP1	A. pseudoparvum	Salta	SA	Argentina					X	
AP5N	A. parvum	Los Santos	LS	Panama	X				X	
AP7N	A. parvum	Cartago	PV	Costa Rica						
AP8	A. parvum	Cartago	PV	Costa Rica	X					
APCA	A. parvum	Yucatan	YU	Mexico	X					
AP-FOR	A. parvum	Minas Gerais	MG	Brazil						
122838(1)	A. parvum	Santa Rosa	CR	Costa Rica	X					
122838(2)	A. parvum	Santa Rosa	CR	Costa Rica	X	X	X			
A parvum 12S CR	A. parvum	Santa Rosa	CR	Costa Rica	X					
123529	A. parvum	Santa Rosa	CR	Costa Rica	X					
CR45	A. parvum		CR	Costa Rica					X	
CR495	A. parvum		CR	Costa Rica					X	
OJ4	A. parvum	Santiago del Estero	SDE	Argentina		X				
CH4	A. parvum	La Rioja	LR	Argentina		X				
PAR	A. parvum	BOQUERON	BO	PARAGUAY		X				
GO1	A. parvum	Goias	GO	Brazil		X				
GO2	A. parvum	Goias	GO	Brazil		X				
GO3	A. parvum	Goias	GO	Brazil		X				
GO4	A. parvum	Goias	GO	Brazil		X				
GO5	A. parvum	Goias	GO	Brazil		X				

W	A. parvum	Pantanal	PA	Brazil		X				
APA6	A. parvum	Minas Gerais	MG	Brazil		X				
APA4_CR	A. parvum	Cartago	PV	Costa Rica		X				
APA2	A. parvum	Yucatan	YU	Mexico		X				
APA1	A. parvum	COLON	PM	Panama		X				
APA3	A. parvum	El Salvador	ES	El Salvador		X				
UR2	A. parvum	Santiago del Estero	SDE	Argentina		X				
YU1	A. parvum	Salta	SA	Argentina		X				
YU2	A. parvum	Salta	SA	Argentina		X				
YU3	A. parvum	Salta	SA	Argentina		X				
QU7	A. parvum	Córdoba	CB	Argentina		X				
SA2	A. parvum	Catamarca	CA	Argentina		X				
SA3	A. parvum	Catamarca	CA	Argentina		X				
ES3	A. parvum	Catamarca	CA	Argentina		X				
SA4	A. parvum	Catamarca	CA	Argentina		X				
QU5	A. parvum	Córdoba	CB	Argentina		X				
QU6	A. parvum	Córdoba	CB	Argentina		X				
CE2	A. parvum	Santiago del Estero	SDE	Argentina		X				
CE3	A. parvum	Santiago del Estero	SDE	Argentina		X				
CE4	A. parvum	Santiago del Estero	SDE	Argentina		X				
A44	A. parvum	Catamarca	CA	Argentina		X				
APA5	A. parvum	Palo Verde	PV	Costa Rica		X				

Total per gene					39	65	33	8	36	15
Total # samples					90					

Table 1.2. Individual gene haplotypes by country/region. The haplotypes are named with letters and combined with a number for each of the genes: 1, 12S; 2, 16S; 3, DL; 4, COI; 5, COII; 6, ITS2. The numbers in each cell correspond to the number of sequences that have that specific haplotype.

12S

Haplotype by Country or Region	1A	1B	1C	1D	1E	1F	1G	1H	1I	1J	1K	1L
Argentina	4	1	5	0	0	0	0	0	0	0	0	0
Brazil	0	0	0	1	1	1	8	7	1	0	0	0
C. America	0	0	0	0	0	0	0	0	0	5	3	1
Mexico	0	0	0	0	0	0	0	0	0	0	1	0

16S

Haplotype by Country or Region	2A	2B	2C	2D	2E	2F	2G	2H	2I	2J	2K	2L	2M	2N	2O	2P	2Q	2R	2S	2T	2U
Argentina	1	1	13	0	1	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paraguay	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brazil	0	0	0	0	0	0	0	5	1	1	1	2	1	7	1	1	0	0	2	0	0
C. America	0	0	0	0	0	0	0	0	0	5	3	1	0	0	0	0	2	5	0	1	1
Mexico	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0

DL

Haplotype by Country or Region	3A	3B	3C	3D	3E	3F	3G	3H	3I	3J	3K	3L	3M	3N	3O	3P	3Q	3R	3S	3T	3U
Argentina	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Brazil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
C. America	0	0	0	0	0	0	0	0	0	5	3	1	0	0	0	0	2	6	0	1	1

3V	3W	3X	3Y	3Z	3AA	3BB	3CC
0	0	0	0	0	0	0	0
1	2	3	1	1	1	0	0
0	0	0	0	0	0	2	1

COI

Haplotype by Country or Region	4A	4B	4C	4D	4E	4F	4G	4H	4I	4J	4K	4L	4M	4N	4O	4P	4Q	4R	4S	4T
Argentina	0	0	1	3	1	2	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Brazil	1	1	0	0	0	0	0	0	0	0	0	0	0	0	10	4	0	0	0	0
C. America	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1

COII

Haplotype by Country or Region	5A	5B	5C	5D	5E	5F	5G	5H	5I
Argentina	1	6	0	0	0	0	0	0	0
Brazil	0	0	1	1	1	1	0	0	0
C. America	0	0	0	0	0	0	2	1	1

ITS2

Haplotype by Country or Region	6A	6B	6C	6D	6E	6F	6G
Argentina	0	0	0	0	0	0	1
Brazil	0	0	0	1	1	2	0
C. America	1	1	1	0	0	0	0

Table 1.3. Maximum likelihood pairwise distances for 12S (bold) and 16S (italicts). Values expressed as percentage.

12S/*16S*	**Arg**	**Bra**	**Par**	**CA**	**Psp**	**Out**
Arg	**0.0-0.6**/*0.0-1.0*	**2.5**/*3.5*	*0.2-0.5*	*6.5-7.5*	*10.9-11.9*	*14.8-17.7*
Bra	**2.9-3.6**	**0**/*0.2-1.2*	*2.5-3.0*	*6.7-7.9*	*10.3-11.8*	*14.1-17.6*
Par	**n/a**	**n/a**	**n/a**	*6.7-7.2*	*10.9-11.4*	*14.8-17.1*
CA	**6.8-8.0**	**6.5-7.2**	**n/a**	**0.3-0.6**/*0.2-0.5*	*8.5-10.1*	*11.6-15.3*
Psp	**8.3-8.9**	**8.9-9.1**	**n/a**	**8.6-8.9**	**n/a**/*1.0*	*12.9-15.8*
Out	**14.9-18.1**	**15.4-19.1**	**n/a**	**14.2-19.6**	**16.4-21.0**	**7.9-14.3**/*7.2-12.2*

Table 1.4. Maximum likelihood pairwise distances for COI (bold) and COII (italicts). Values expressed as percentage.

COI/*COII*	**Arg**	**Bra**	**CA**	**Psp**	**Out**
Arg	**0.2-3.5**/*0.2*	*3.3-4.2*	*11.2-11.8*	*n/a*	*17.5-21.7*
Bra	**4.3-6.2**	**0.2-0.5**/*0.2-0.7*	*11.4-12.1*	*n/a*	*17.0-21.9*
CA	**9.8-11.2**	**10.3-11**	**0.3-1.3**/*0.2-0.5*	*n/a*	*16.6-20.2*
Psp	**11.9-13.0**	**12.0-12.3**	**11.6-12.3**	*n/a*	*n/a*
Out	**15.2-18.5**	**14.8-17.0**	**14.7-17.0**	**16.8-19.1**	**11.6-15.6**/*10.7-19.3*

Table 1.5. Maximum likelihood pairwise distances for DL. Values expressed as percentage.

DL	**Arg**	**Bra**	**CA**	**Psp**	**Out**
Arg	*0.0-1.0*	*2.4-3.7*	*7.3-7.9*	*8.9-9.5*	*11.8-16.0*
Bra		*0.0-0.5*	*7.1-7.9*	*8.6-9.1*	*12.4-16.2*
CA			*0.3*	*6.6-6.8*	*13.4-15.3*
Psp				*n/a*	*13.1-16.8*
Out					*6.8-12.1*

Table 1.6. Maximum likelihood pairwise distances for ITS2. Values expressed as percentage.

ITS2	**Arg**	**Bra**	**Bra-GO**	**CA**	**Out**
Arg	n/a				
Bra	0.1-0.4	0.2			
Bra-GO	3.9	3.8-4.1	n/a		
CA	3.6-5.5	3.3-5.6	4.3-5.9	0-3.2	
Out	13.8-15.0	13.5-16.8	15.5-16.8	14.3-17.4	2.1-7.1

Table 1.7. Maximum likelihood pairwise distances for the mitochondrial concatenated dataset. Values expressed as percentage.

mit	Arg	Bra	CA	Apsp	Out
Arg	0.0-1.5				
Bra	3.7-4.2	0.0-0.2			
CA	8.1-8.5	8.1-8.4	0.3-0.5		
Apsp	10.5-11.0	10.5-10.6	9.5-9.8	n/a	
Out	14.3-16.3	14.1-16.1	13.6-16.0	15.3-17.5	9.5-13.1

Table 1.8. Maximum likelihood pairwise distances for the nuclear and mitochondrial dataset. Values expressed as percentage.

mit+nuclear	Arg	Bra	Bra-GO	CA	Out
Arg	0.0-1.0				
Bra	2.5-2.9	0.2			
Bra-GO	3.8-4.1	1.3-1.5	0		
CA	6.6-6.7	6.5-6.6	6.8-6.9	0.2	
Out	19.4-21.1	19.4-20.9	19.4-21.1	18.6-20.6	7.6-11.0

Chapter 2

Molecular systematics of the *Amblyomma maculatum* group of species.

Introduction

The *Amblyomma maculatum* group includes the following species: *A. maculatum* Koch, 1844; *Amblyomma neumanni* Ribaga, 1902; *Amblyomma parvitarsum* Neumann, 1901; *Aamblyomma tigrinum* Koch, 1844 and *Amblyomma triste* Koch, 1844 (Camicas et al., 1998). Together with the *Amblyomma ovale* group, that encompasses *A. ovale* and *Amblyomma aureolatum,* they have been clustered by Camicas et al. (1998) in the revised version of subgenus *Anastosiella*, originally erected by Santos Dias (1963).

Within the *A. maculatum* group, *A. neumanni* and *A. parvitarsum* are morphologically easily distinguishable from the rest of the group species. Unlike the other taxa, they are both characterized by incomplete marginal grooves in males, and *A. parvitarsum* has beady and orbited eyes (Estrada-Peña et al., 2005). In females, all species are glabrous with the exception of *A. neumanni*. *A. parvitarsum* also has beady and orbited eyes. Other diagnostic differences are listed in Estrada- Peña et al. (2005), who suggested that *A. neummanni* and *A. parvitarsum* should be grouped with the *A. ovale* group in a yet to be determined subgenus, while *A. maculatum*, *A. triste*, and *A. tigrinum* would be the only remaining members of the subgenus *Anastosiella.*

In contrast with *A. parvitarsum* and *A. neumanni, A. maculatum, A. tigrinum* and *A. triste* are morphologically very similar. Koch (1844) briefly described the three taxa based on males of *A. maculatum* and *A. tigrinum*, and a female of *A. triste.* He completed his description in 1850 (Koch, 1850) and essentially reported differences in punctation and ornamentation. Neumann (1899) synonymyzed *A. tigrinum* and *A. triste* with *A. maculatum* after failing to

observe differences in the number of spines (modified setae) on tibiae II to IV (called tarsi by Neumann (1899), protarsi by Robinson (1926), and metatarsi by Kohls [1956]). Kohls (1956) reestablished *A. tigrinum* and *A. triste* as valid species and completely redescribed the three taxa. Since then, although considered to be separate species, the identification of these ticks has been challenging, in particular the distinction between *A. maculatum* and *A. triste* (Mendoza Uribe and Chavez Chorocco, 2004; Estrada-Pena et al., 2005; Mertins et al., 2010; Guglielmone et al., 2013) which has led to frequent misidentifications (Tagle y Alvarez, 1957, 1959; Aragao and Fonseca, 1961). Taxonomic conflicts are not limited to adult stages, as immatures, for which taxonomic keys are nevertheless available, are even more difficult to differentiate (Estrada-Pena et. al., 2002, 2005; Mertins et al., 2010).

The distribution of *A. maculatum* is confined to the southern United States, Central America and some areas of Colombia, Venezuela, Perú and Ecuador, whereas *A. tigrinum* is reported to occur only in South American countries (Kohls, 1956; Jones et al., 1972; Guglielmone et al., 1982, 2003). *Amblyomma triste* was considered to be exclusively South American until recently, when it was reported from Mexico and the U.S. (Guzman-Cornejo et al., 2006; Mertins et al., 2010), thus joining the group of ticks with a Neotropical and Neartic distribution (Guglielmone et al., 2013).

Notwithstanding the increasing number of publications dealing with the systematics of this group of taxa, the taxonomic status of the *A. maculatum* group of species remains controversial. Its reassessment is essential not only for systematic reasons, but also because *A. maculatum, A. triste* and *A. tigrinum* are involved in the transmission of different pathogens of public health and animal health importance, such as *Rickettsia parkeri* (Nava et al., 2008; Paddock et al., 2004; 2010; Romer et al., 2011; 2014; Ferrari et al., 2012; Lado et al., 2014; 2015; Venzal et al., 2004), and *Hepatozoon americanum* (Ewing and Panciera, 2003).

Molecular techniques used to infer phylogenetic relationships and evaluate the taxonomic status of the different species of the *A. maculatum* group

have so far not been applied in a comprehensive manner. However, preliminary reports based on the analysis of 16S rDNA sequences confirmed that *A. maculatum, A. triste* and *A. tigrinum* were closely related to each other, while *A. neumanni* and *A. parvitarsum* were distinct from each other, from the rest of the *A. maculatum* group of taxa, and also did not cluster with the *A. ovale* group of species (Estrada-Pena et al., 2005).

The main goal of this study is to reassess the taxonomic status of the *A. maculatum* group of species through the phylogenetic analysis of six (five mitochondrial and one nuclear) molecular markers.

Materials and Methods

Sampling

Our sample included a total of 95 adult specimens morphologically identified as *A. maculatum, A. triste, A. tigrinum,* and 2 specimens identified as *A. parvitarsum* and *A. neumanni. Amblyomma maculatum* ticks were from the U.S., Perú, and Colombia; *A. triste* from Argentina, Brazil, and Perú; *A. tigrinum* from Argentina and Brazil; and *A. parvitarsum* and *A. neumanni* from Argentina. When available, specimens from several localities were included in order to consider variation between and within different eco-regions (Table 2.1). Ticks were obtained from 12 localities and 5 countries, and coded as follows: Argentina, Arg; (Buenos Aires, BA; Corrientes, CR; Formosa, FO; Santiago del Estero, SDE), Brazil, Bra; (Goias, GO; Mato Grosso do Sul, MGS; Sao Paulo, SP), Colombia, CO; (Santander, SR), Perú, PU; (Ica; Tumbes, TU), and the United States, U.S.; (Florida, FL; Georgia, GA) (Fig. 2.1).

DNA extraction, PCR, and sequencing

Tick DNA was extracted and the 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 removed by using a disposable scalpel and the tick 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 exoskeleton was stored in 70% ethanol and kept as a voucher specimen. The lysed tissues were further processed as previously described (Beati and Keirans, 2001; Beati et al., 2012). Five mitochondrial gene sequences, 12SrDNA (small subunit ribosomal RNA), 16SrDNA (small subunit ribosomal RNA), COI (Cythochrome oxydase subunit I), COII (Cythochrome oxydase subunit II), and the control region or d-loop (DL) were amplified with previously reported sets of primers (Beati and Keirans, 2001; Beati et al., 2012; Barret and Hebert, 2005; Mangold et al., 1998). In addition, a portion of the nuclear ribosomal Internal Transcribed Spacer 2 (ITS2) was also amplified by slightly modifying a previously published protocol to include 35 instead of 27 annealing cycles (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 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 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 manually 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) and DL (Zhang and Hewitt 1997). Codon organization was taken into account when aligning the COII data set. Each data set was analyzed by maximum parsimony (MP) with PAUP (Swofford, 2000). Bayesian analysis (BA) was performed using MrBayes 3.2.4

(Huelsenbeck and Ronquist 2001, Ronquist, 2011). Branch support was assessed by bootstrap analysis (1000 replica) 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. Gaps were treated either as a 5th (16SrDNA, concatenated datasets and ITS2 analyses) or as a missing character (12srDNA, DL, COI and COII). Maximum likelihood distances were calculated after the nucleotide substitution model best fitting the data was selected by Modeltest v3.7 (Posada and Crandall 1998). Two runs, 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. When necessary, the number of generations was increased so that the number of discarded samples would not exceed 25% of the total sampled trees. The 50% majority-rule consensus tree of the remaining trees was inferred and posterior probabilities recorded for each branch. Congruent data sets were combined for total evidence analyses. One concatenated data set including both mitochondrial a nuclear sequences (n+mtDNA) were analyzed following the same procedure outlined for the separate analyses.

Amblyomma parvitarsum and *A. neumanni* were used as outgroups in our analyses. Additional species were also considered as possible outgroups and preliminary analyses were performed with the following: *A. aureolatum, A. coelebs, A. dubitatum, A. oblongoguttatum,* and *A. ovale.*

Results

Sequences and haplotype diversity

The alignment of the 75 12SrDNA gene sequences (16 unique haplotypes) resulted in a 338 bp data matrix. The 16S rDNA dataset was 411 bp long and included 79 sequences (31 unique haplotypes), that of the COI gene was 603 bp long and included 67 sequences (38 unique haplotypes). A total of 73 sequences

(374 bp) were obtained for the DL marker (40 unique haplotypes). Only 11 sequences were generated for the COII gene (608 bp), 8 of which were unique haplotypes. The nuclear ITS2 (1002 bp) gene sequence was sequenced from 50 ticks, with 13 unique genotypes (Table 2.2).

Phylogenetic Analyses

Phylogenetic analyses were first performed with several different sets of outgroups. They all proved to be too distantly related to our ingroup for their inclusion to result in good ingroup resolution, with the exception of *A. neumanni* and *A. parvitarsum* that were, therefore, used as outgroups in all analyses. For clarity, we are referring to *A. triste*, *A. maculatum,* and *A. tigrinum* collectively as the ingroup.

12SrDNA

The MP analysis detected 62 informative characters, and found 2 equally parsimonious trees (length=92; CI=0.913; RI=0.946 and HI=0.087). The ML model that better fitted the data using Modeltest was K81uf+I, with base frequencies of A=0.37, C=0.12, G=0.09 and T=0.42. The proportion of invariable sites (PI) = 0.6872.

The MP analysis identified a single monophyletic cluster (100% bootstrap) consisting of the whole ingroup, an unresolved polytomic lineage (Fig 2.2). In the BA tree, the polytomic Peruvian lineages were basal, to a supported clade (90%). In this cluster, *A. maculatum* from the U.S. was basal and paraphyletic. The remaining samples from Brazil and Argentina, including, *A. tigrinum*, grouped in a monophyletic clade (Fig. 2.3).

Intraspecific divergence values in the ingroup taxa ranged from 0 to 2.1%. Distance between *A. triste* and *A. maculatum* was 0.6-2.1%. The distance separating *A. tigrinum* from either *A. maculatum* or *A. triste* ranged from 1.8 to

2.7%. Divergence between the ingroup and the outgroup ranged from 11.9 to 16.2%, while distance within the outgroup species was 11-11.7% (Table 2.3).

16SrDNA

The MP analysis identified a total of 43 parsimony-informative sites and found 129 equally parsimonious trees (length=137; CI=0.766; RI=0.831 and HI=0.234). The selected ML model was GTR+I+G with base frequencies of: A=0.42, C=0.07, G=0.13 and T=0.38; PI = 0.5844; G = 0.6450.

The MP analysis revealed a monophyletic ingroup which was split in two monophyletic sister clades: *A. tigrinum* and *A. maculatum - A. triste*. In the MP reconstruction, three lineages were supported within the otherwise polytomic *A. triste - A. maculatum* clade: two sequences of *A. triste* (MGS, Brazil); two sequences of *A. maculatum* (GA and FL, U.S.); and two sequences of *A. maculatum* (GA, U.S. and SR, Colombia) (Fig. 2.4).

In the BA the ingroup was polytomic. Nevertheless, it included five resolved clusters: the *A. tigrinum* (99%) branch, two Brazilian *A. triste* lineages (84 and 98% respectively), one including *A. maculatum* sequences from the U.S. and Colombia, and finally one U.S. cluster (Fig. 2.5).

Intraspecific divergence values in the ingroup ranged from 0 to 2.7%. Distance between *A. triste* and *A. maculatum* varied from 0.5 to 2.7%. The distance separating *A. tigrinum* from either *A. maculatum* or *A. triste* ranged from 3.2 to 4.6%, and that between the ingroup and the outgroup varied from 10.8 to 12.6%. Divergence within the outgroup species was of 10.1% (Table 2.3).

DL

The MP analysis detected 51 parsimony-informative characters and 18 equally parsimonious trees were found (length=189; CI=0.862; RI=0.874 and HI=0.138). The model that best fitted the data was TVM+I+G with base frequencies of: A=0.42, C=0.13, G=0.13 and T=0.32; PI = 0.4423; G = 1.0222.

Both the MP and the BA separated the ingroup into two main clades: *A. tigrinum* (100%, 100%) and *A. triste* - *A. maculatum* (98%, 90%). The ingroup, however, was not monophyletic (Fig. 2.6). In the BA the *A. triste – A.maculatum* clade included several unsupported branches corresponding to sequences of *A. triste* from Brazil and Argentina, in addition to a supported Peruvian *A. triste* - *A. maculatum* lineage (99%) and a monophyletic cluster of *A. triste* from Brazil and Argentina (96%) (Fig. 2.7).

Intraspecific distances within *A. triste* and *A. tigrinum* varied from 0.3 to 2.4%, while within *A. maculatum* they reached 3.5%. Distances between *A. maculatum* and *A. triste* ranged from 0.3 to 4.6%, and those separating *A. tigrinum* from *A. triste* or *A. maculatum* varied between 9.4 and 11.3%. Between the ingroup and the outgroup, divergences ranged from 27.6 to 30.1% (Table 2.4).

ITS2

The MP analysis detected 188 informative sites and 5 equally parsimonious trees (length=369; CI=0.989; RI=0.981; and HI=0.011). The model that better fitted the data was GTR with base frequencies of: A=0.20, C=0.28, G=0.36 and T=0.16, PI = 0.

The MP analysis resulted in a poorly resolved ingroup, with only *A. tigrinum* separating itself from other supported lineages, that did not appear to correspond to any taxonomic or geographical pattern (Fig. 2.8). The BA resolved three lineages arising from a polytomy: *A. tigrinum* (100%) and two lineages of heterogeneous geographical origins. The remaining Argentinian and Brazilian samples of *A. triste* stemmed directly from the polytomy (Fig. 2.9).

Intraspecific divergences within the three ingroup taxa were never above 1.1%. Distance between *A. maculatum* and *A. triste* ranged from 0.3 to 1.3%. The distance separating *A. tigrinum* from either *A. triste* or *A. maculatum* varied between 1 and 1.7%. The divergence value separating the ingroup and the outgroup ranged from 17.2 to 21.8%, and variation within the outgroup was of 7.9% (Table 2.4).

COI

A total of 90 parsimony-informative characters were detected in the MP analysis and 2000 (Max trees sat to 2000) equally parsimonious trees were found (length=247; CI=0.810; RI=0.894 and HI=0.190). The model that better fitted the data was GTR+G with base frequencies of: A=0.31, C=0.16, G=0.13 and T=0.40; PI = 0.5791; G = 0.8554.

The MP analysis evidenced the monophyly of the ingroup and fully resolved two clades: *A. tigrinum* (99%) and *A. triste - A. maculatum* (100%). This was further subdivided in one supported clade that included the sample 21D of *A. maculatum* from Georgia, U.S., and the sample of *A. maculatum* from Colombia. The remaining sequences had no support except for a resolved lineage that corresponded to the Peruvian samples (71%), and one that clustered two Brazilian *A. triste* (91%) (Fig. 2.10).

By BA, *A. tigrinum* (100%) was the sister group of everything else. The monophyletic *A. triste - A. maculatum* clade was further subdivided into Peruvian and non-Peruvian (75%) lineages. The non-Peruvian group included two well-supported groups, a North-American-Colombian (99%) and a Brazilian-Argentinian (84%) (Fig. 2.11).

Intraspecific divergence values within the three ingroup taxa were variable, ranging from 0 to 3.5%. The distance between *A. maculatum* and *A. triste* ranged from 0.0 to 4%, and that separating *A. tigrinum* from either *A. triste* or *A. maculatum* varied from 5.6 to 7.8%. The divergence between the ingroup and the outgroup ranged from 15.4 to 18.7%, and within the outgroup varied from 15.7 to 17.9% (Table 2.5).

COII

A total of 70 parsimony-informative characters were detected in the MP analysis and two trees were found (length=172; CI=0.924; RI=0.903 and HI=0.076). The model that better fitted the data was HKY+I with base frequencies of: A=0.38, C=0.09, G=0.15 and T=0.38; PI = 0.6889.

The MP analysis resolved two clades within the ingroup: *A. tigrinum* (100%) and *A. triste* - *A. maculatum* (99%). The latter was split in two lineages, one including *A. triste* from Argentina and Brazil in addition to *A. maculatum* from the U.S (75%) and the other represented by *A. maculatum* from Peru (Fig. 12). The BA revealed two well-supported clades within the ingroup: *A. tigrinum* (98%) and *A. triste* - *A. maculatum* (98%). No further resolution was achieved through this analysis (Fig. 2.13).

Intraspecific distances within the three ingroup species were never over 1.7%. Distance between *A. maculatum* and *A. triste* ranged from 0.8 to 1.7%. The distance separating *A. tigrinum* from either *A. triste* or *A. maculatum* varied between 5.8 and 6.6%. The ingroup and the outgroup differed by 11.7 - 16.8%, and the variation within the outgroup was 11.4% (Table 2.5).

Mitochondrial Concatenated dataset (12SrDNA+16SrDNA+COI)

The concatenated dataset represented a matrix of 1352 bp, 102 sequences including 32 unique haplotypes and two outgrups. The MP analysis identified a total of 200 parsimony-informative sites and found 11 equally parsimonious trees (length=531; CI=0.827; RI=0.869 and HI=0.173). The ML model chosen as more accurate for the data was GTR+I+G with base frequencies of: A=0.35, C=0.13, G=0.13 and T=0.39; PI = 0.5560; G = 0.7777.

The MP and the BA agreed on the monophyly of the ingroup and resolved two clades: *A. tigrinum* and *A. triste* - *A. maculatum.* The MP analysis further resolved the *A. triste* - *A. maculatum* clade in two lineages: *A. triste* from Peru (77%) and the remaining sequences (100%) which did further split in lineages with no obvious geographical meaning with the exception of the Peruvian sequences which were basal (Figs. 2.14 and 2.15).

In the BA phylogenetic reconstruction, the ingroup was resolved in two clades: *A. tigrinum* and *A. triste-A. maculatum.* Within the second, the Peruvian samples were basal and separated from a supported clade that included all the *A. maculatum* from the U.S., and all the *A. triste* from Brazil and Argentina. (Fig. 2.15).

Divergence values within each of the species of the ingroup were at or below 1.6%. The distance between *A. triste* and *A. maculatum* ranged from 0.5 to 1.6%. The distance separating *A. tigrinum* from either *A. triste* or *A. maculatum* varied between 4.3 and 5.1%. Divergence values between the ingroup and the outgroup were 13.5 – 15.7%, and within the outgroup was 12.7% (Table 2.6).

Mitochondrial - Nuclear concatenated dataset (12SrDNA+16SrDNA+COI+ITS2)

The concatenated dataset represented a matrix of 2328 bp, 81 sequences, corresponding to 25 unique haplotypes and two outgrups. The MP analysis identified a total of 324 parsimony-informative sites and found 41 equally parsimonious trees (length=892; CI=0.898; RI=0.834 and HI=0.102). The ML model chosen as more accurate for the data was GTR+I+G with base frequencies of: A=0.30, C=0.21, G=0.19 and T=0.30; PI = 0.3601; G = 0.7092.

The MP analysis evidenced a monophyletic ingroup (100%), with a basal *A. tigrinum* branch, followed by the Peruvian sample. All the *A. maculatum* samples from the U.S., together with the *A triste* from Brazil and Argentina were clustered in a monophyletic and polytomic lineage. (Fig. 2.16).
The BA had better resolution and also resulted in two separated lineages: *A. tigrinum,* basal, and the *A. triste - A. maculatum* (100%). Within this clade, a basal Peruvian *A. triste* lineage was separated from two sister clusters: a resolved North American *A. maculatum* clade and the *A. triste* from Brazil and Argentina (99%). Both the North American *A. maculatum* and the Brazilian-Argentinian sequences of *A. triste* constituted monophyletic lineages (100%) within that clade (Fig. 2.17).

Divergence values within each of the species of the ingroup were below 1%. The distance between *A. triste* and *A. maculatum* ranged from 0.8 to 1.1%. The distance separating *A. tigrinum* from either *A. triste* or *A. maculatum* varied between 3.1 and 3.4%. Divergence values between the ingroup and the outgroup taxa were of 15.7-18.0%, and within the outgroup was 11.4% (Table 2.7).

Discussion

Overall, the reconstructions obtained with different genes were not always congruent. Nevertheless, they agreed in several points. First, in most cases both, *A. maculatum* and *A. triste,* were paraphyletic. If there was clustering, it was determined by geography, rather than by taxonomic assignment. The Peruvian *A. maculatum* and *A. triste* often grouped together, as did the North-American-Colombian samples, or the southern samples from Brazil and Argentina. BA consistently showed that, within the mostly monophyletic *A. triste- A. maculatum* lineage, the branches were short. This would suggest a very recent and rapid divergence history with incomplete lineages sorting. Intra- and interspecific distance values obtained within and between *A. maculatum* and *A. triste* overlapped. Although divergence values in themselves cannot be used for species delimitation, with the support of the phylogenetic reconstructions, we can reliably propose that *A. maculatum* and *A. triste* should be considered conspecific. Therefore, *A. triste* should be returned to junior synonymy of *A. maculatum* (Koch, 1844). This would also result in questioning the taxonomic value of tibial spurs. As the so-called “spurs” are in fact simply modified robust setae, differences in length and thickness might merely be the result of local adaptation, without particular systematic meaning.

The question of whether or not *A. tigrinum* is distinct from *A. maculatum* is more difficult to answer. In most cases, the *A tigrinum* samples clustered in a distinct monophyletic clade. The geographical distribution of *A. tigrinum* in South America mostly overlaps that of the other two species (Estrada-Peña, 2005), although it reaches more southern latitudes than *A. maculatum – A. triste.* Although divergence values between *A. tigrinum* and *A. maculatum - A. triste* are moderately higher than intraspecific values, they remain much lower then the interspecific distances recorded between outgroup species, and between outgroup and ingroup taxa. Those values are also comparable to, or slightly higher than, intraspecific variation, but always lower than the interspecific

divergence values observed for other *Amblyomma* species analyzed by using some of the same gene markers (Beati et al., 2013). More importantly, the variable nuclear gene used in this study (ITS2) includes *A. tigrinum* in a polytomic ingroup and does not support a clear split between the taxa. The ITS2 is a gene marker that has successfully been used for taxonomic reassessments among South American *Amblyomma* species of similar geographical distribution (Marrelli et al., 2007; Beati et al. 2013). Therefore, according to the above information, either we can also consider *A. tigrinum* to be a synonym of *A. maculatum* which would conform to Neumann's opinion (Neumann, 1899), or we can temporarily maintain *A. tigrinum* as a separate taxonomic entity with a very short evolutionary history separating it from the sympatric *A. maculatum*. Only cross-breeding experiments are likely to determine whether the time elapsed since the divergence of the two lineages was sufficient for them to become different species, as morphological differences are minimal and mostly based on tibial spur arrangement which may not have taxonomic importance. Also shape and length of carena on festoons and ornamentation are known to sometimes be intraspecifically polymorphic (Nava et al., 2014). Our results can explain why the identification of these species throughout their distribution range has been so problematic. Furthermore, we can agree with Estrada-Peña et al. (2005) in considering *A. parvitarsum* and *A. neumanni* to be distant from the ingroup and not part of the same complex of species.

In conclusion, our data strongly support the synonymization of *A. triste* with *A. maculatum*, and suggest that this might be true also for *A. tigrinum.* Nevertheless, additional ecological and biological (cross-breeding) information should be gathered in order to establish whether or not *A. tigrinum* and *A. maculatum* are conspecific.

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APPENDIX 2

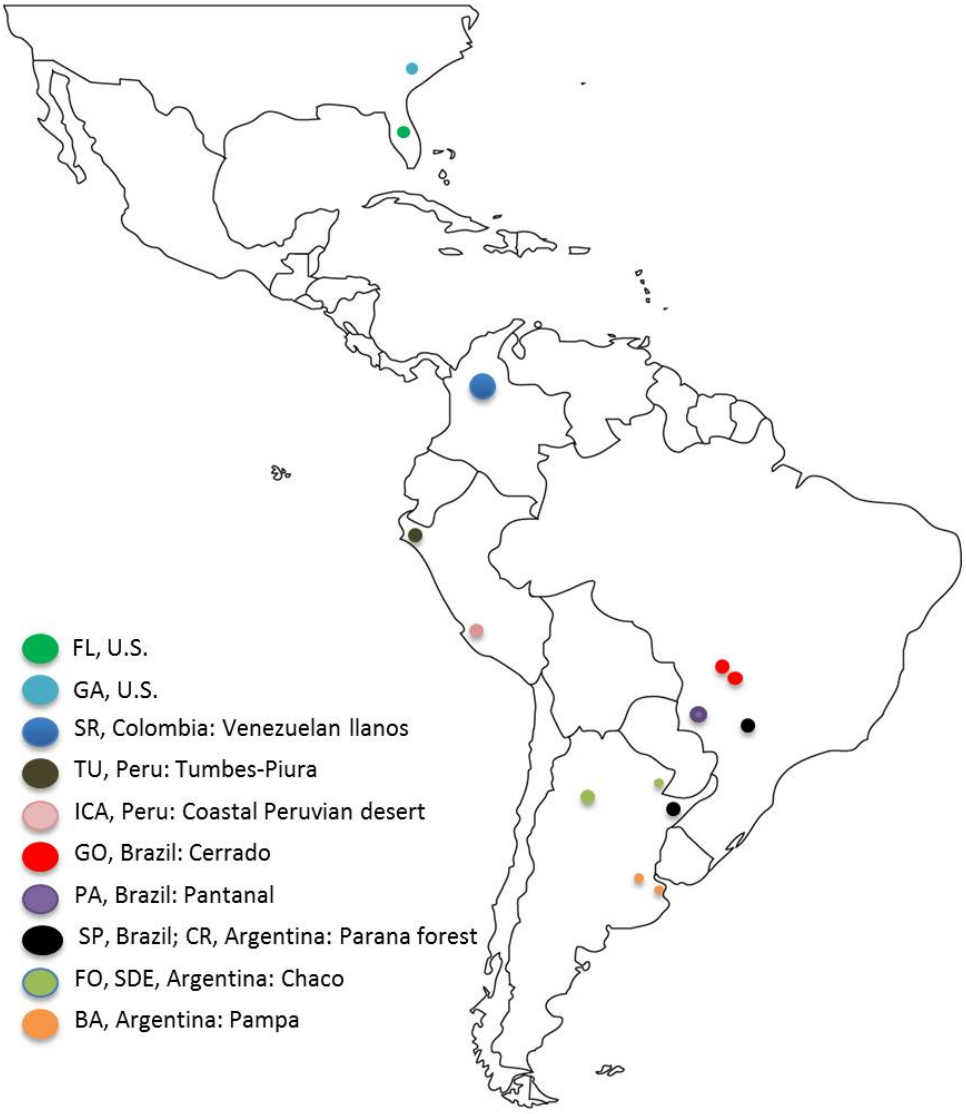

Figure 2.1. Political map of southern North America, Central and South America showing the collection areas and the eco-regions to what they correspond: GA, Georgia (U.S.); FL, Florida (U.S); SR, Santander (Colombia); TU, Tumbes (Peru); ICA (Peru); Brazil; GO, Goias (Brazil); PA, Pantanal (Brazil); SP, Sao Paulo (Brazil); CR, Corrientes (Argentina); FO, Formosa (Argentina); SDE, Santiago del Estero (Argentina); BA, Buenos Aires (Argentina).

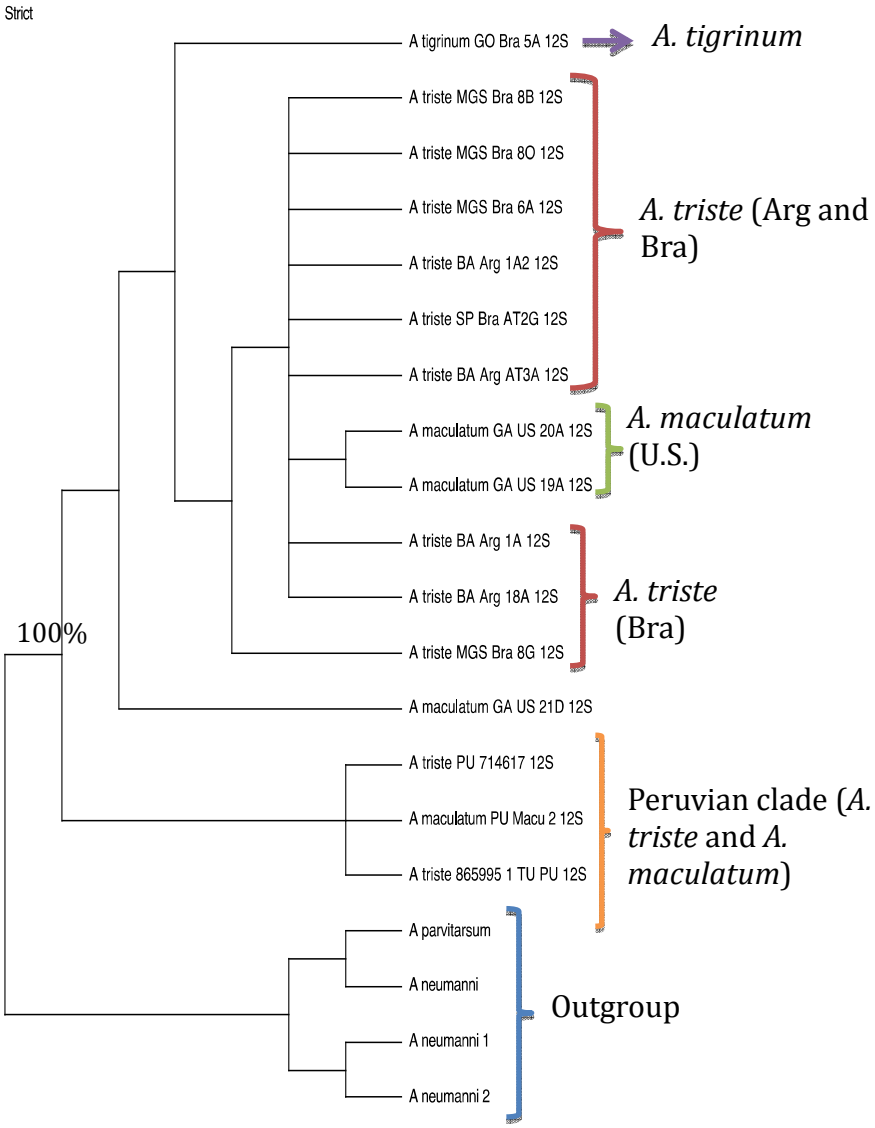

Fig. 2.2. Maximum parsimony (MP) for 12S.

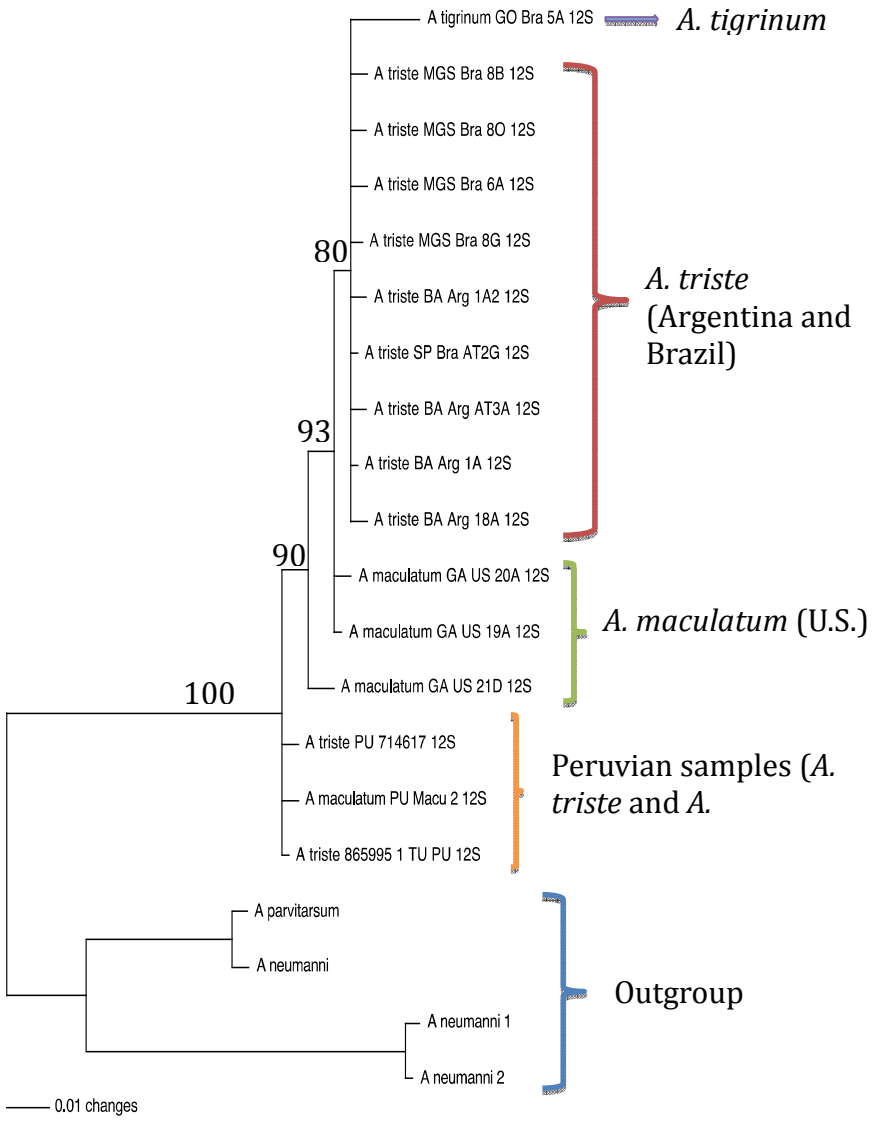

Fig. 2.3 Bayesian Analysis (BA) for 12S.

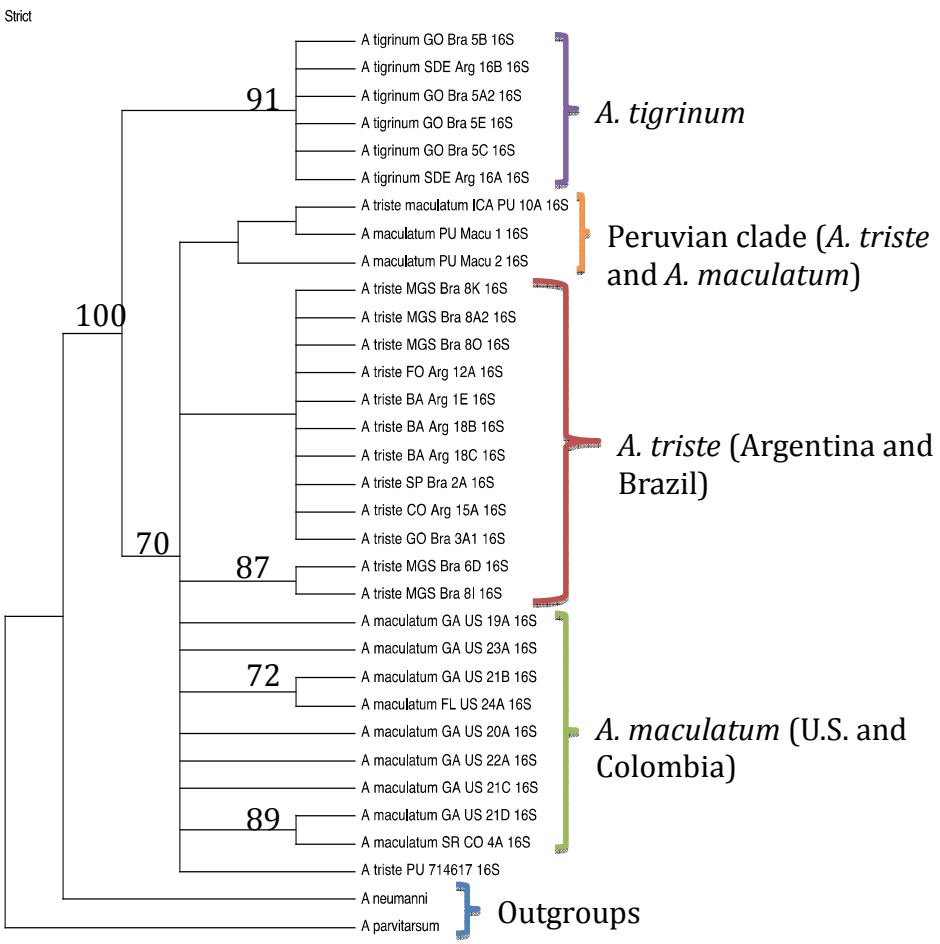

Fig. 2.4 MP analysis for 16S.

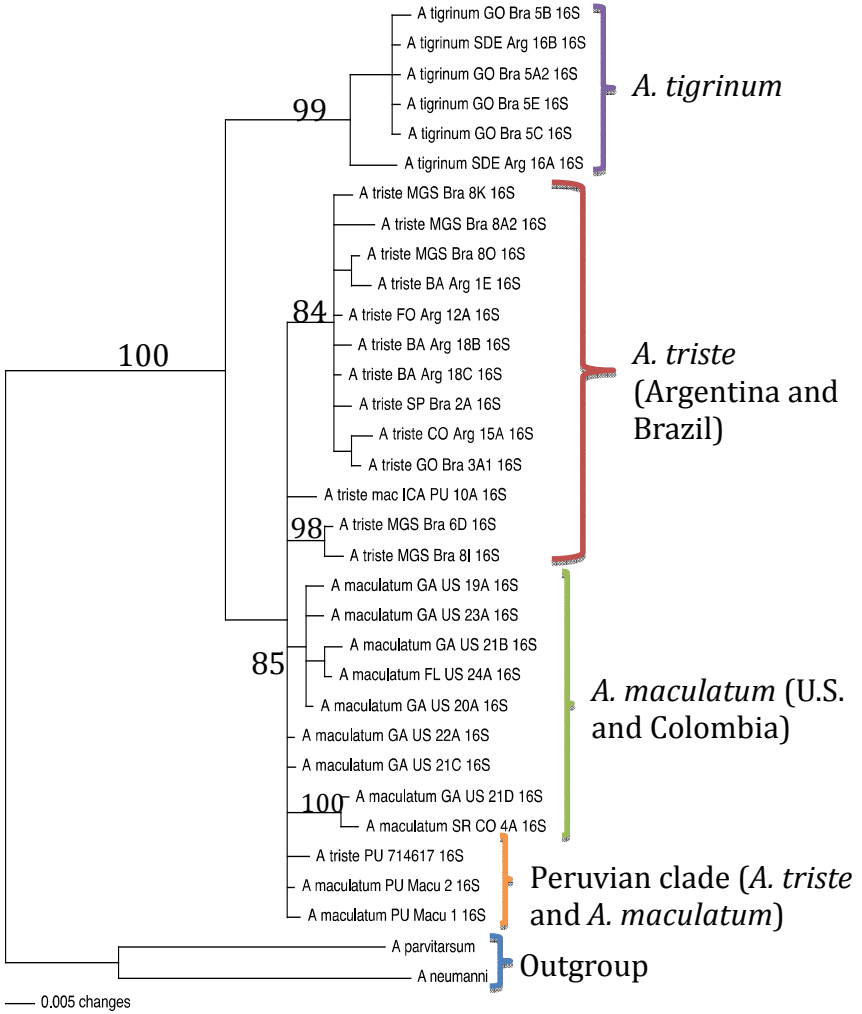

Fig. 2.5 BA for 16S.

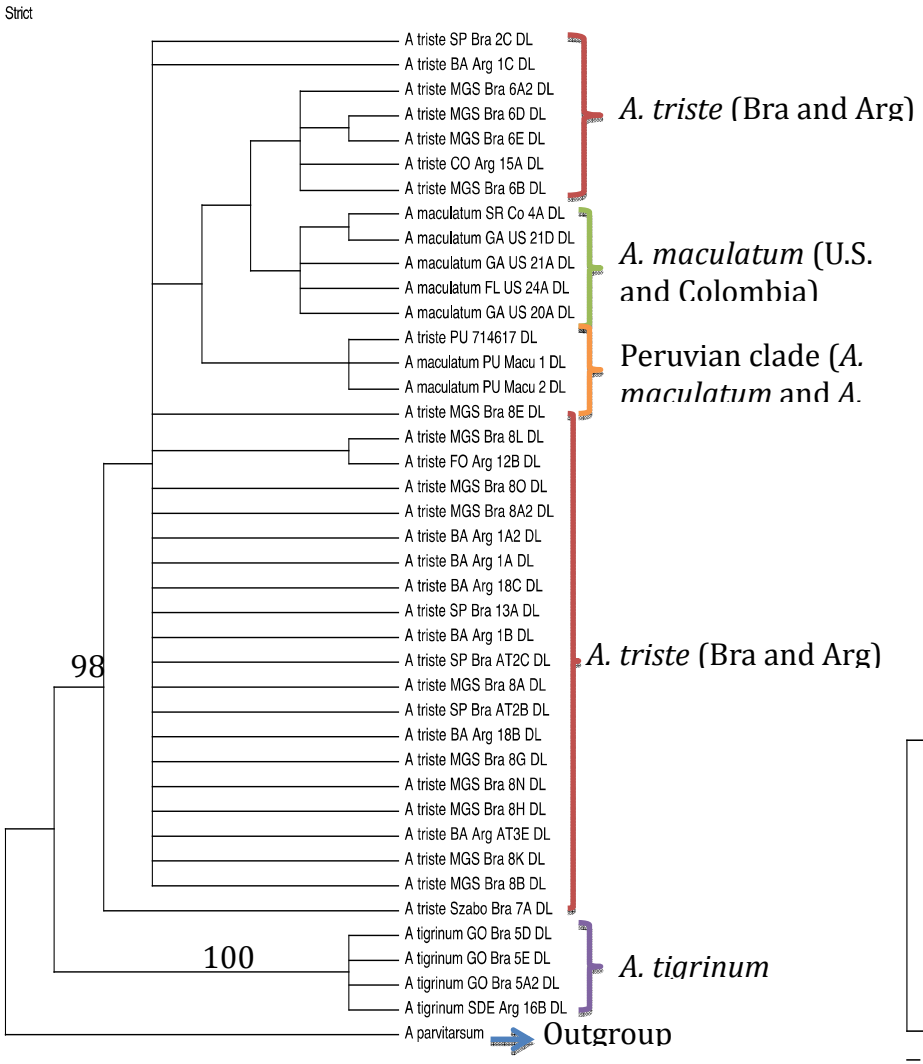

Fig. 2.6 MP for DL.

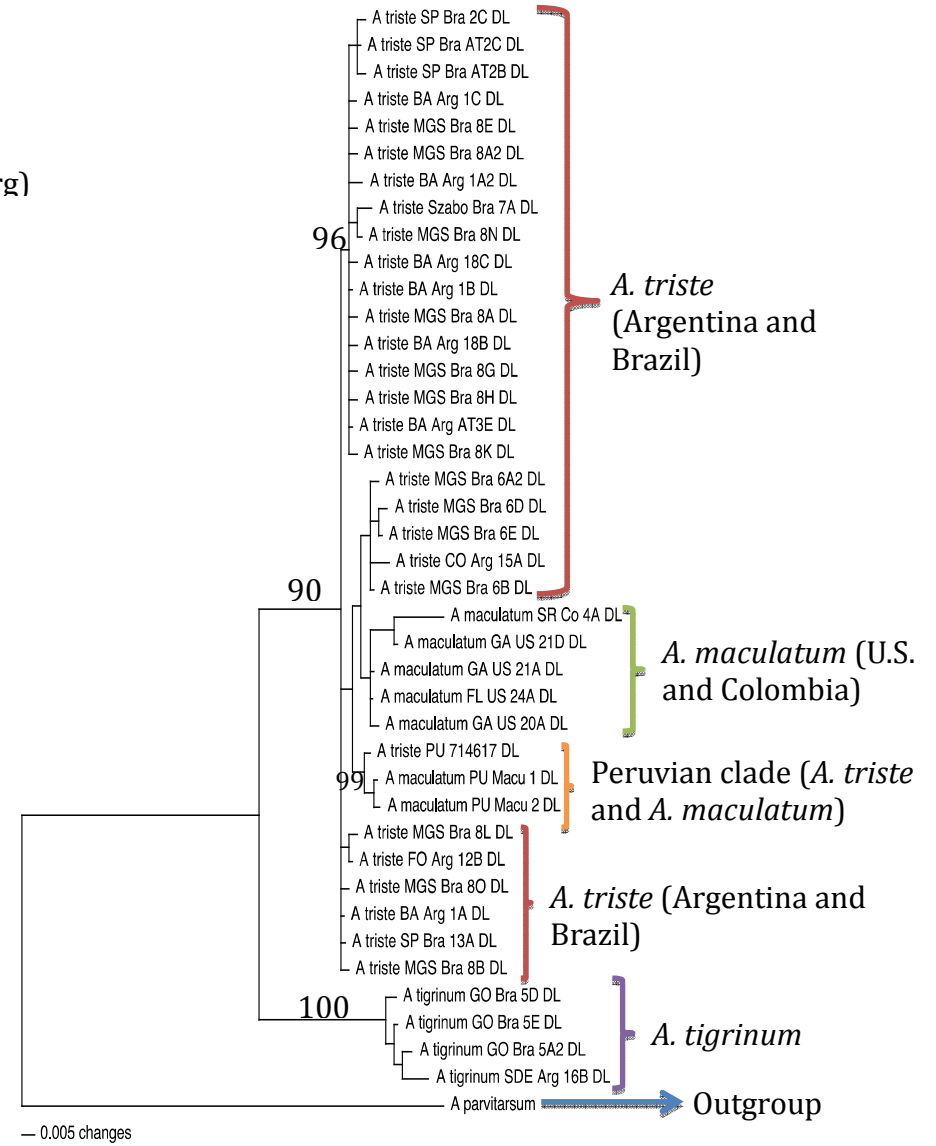

Fig. 2.7 BA for DL.

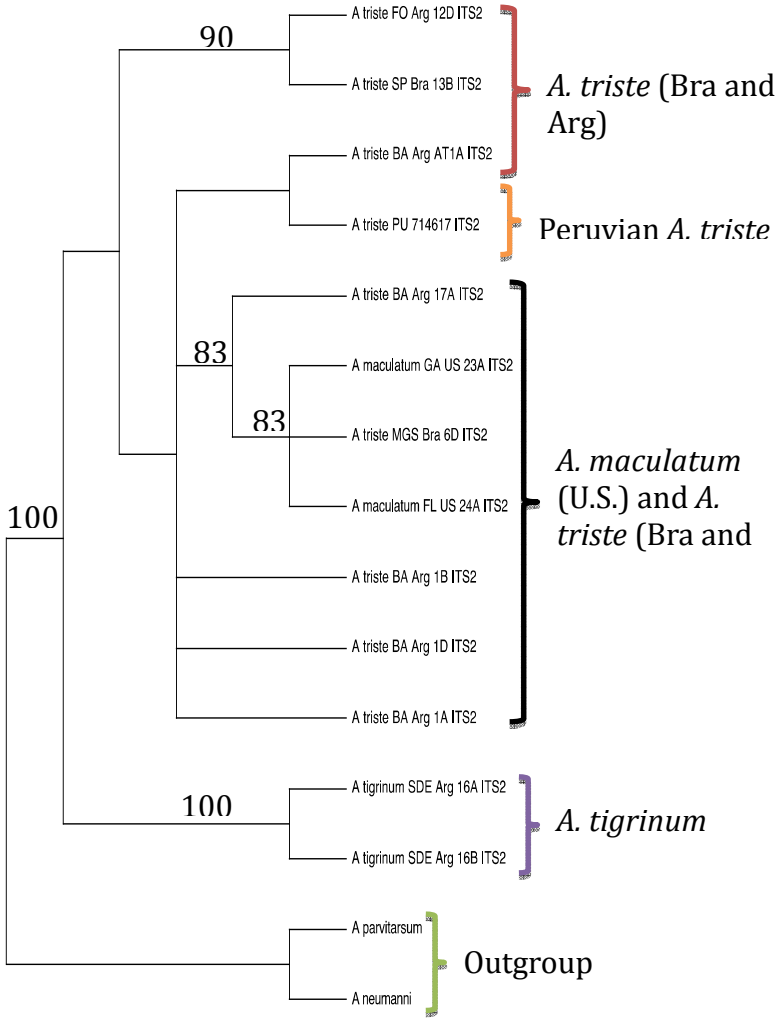

Fig. 2.8 MP tree for ITS2.

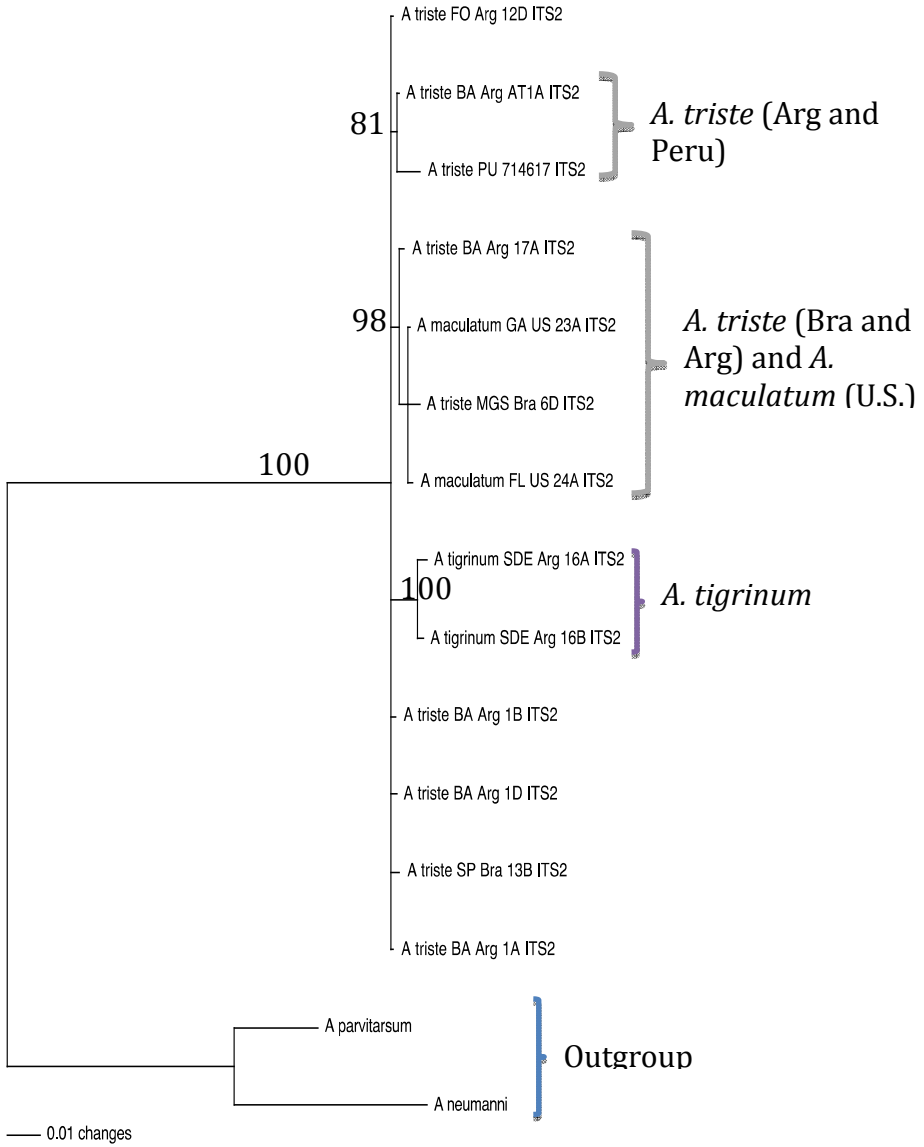

Fig. 2.9 BA for ITS2.

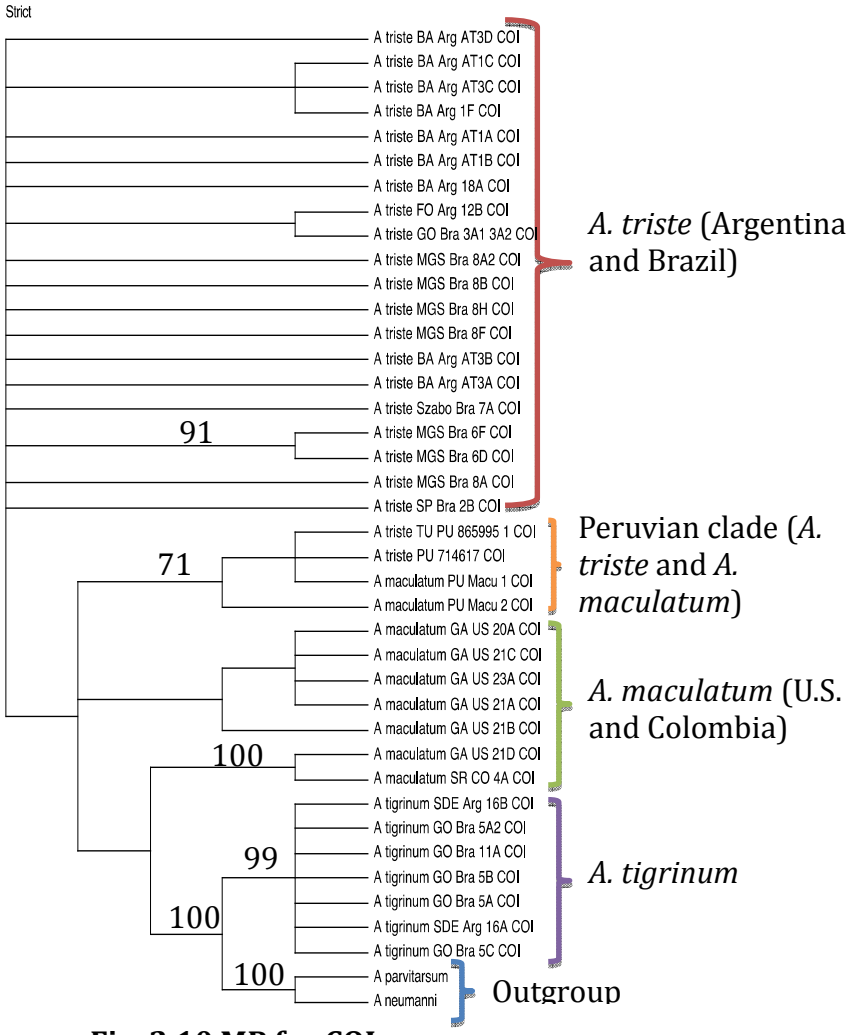

Fig. 2.10 MP for COI.

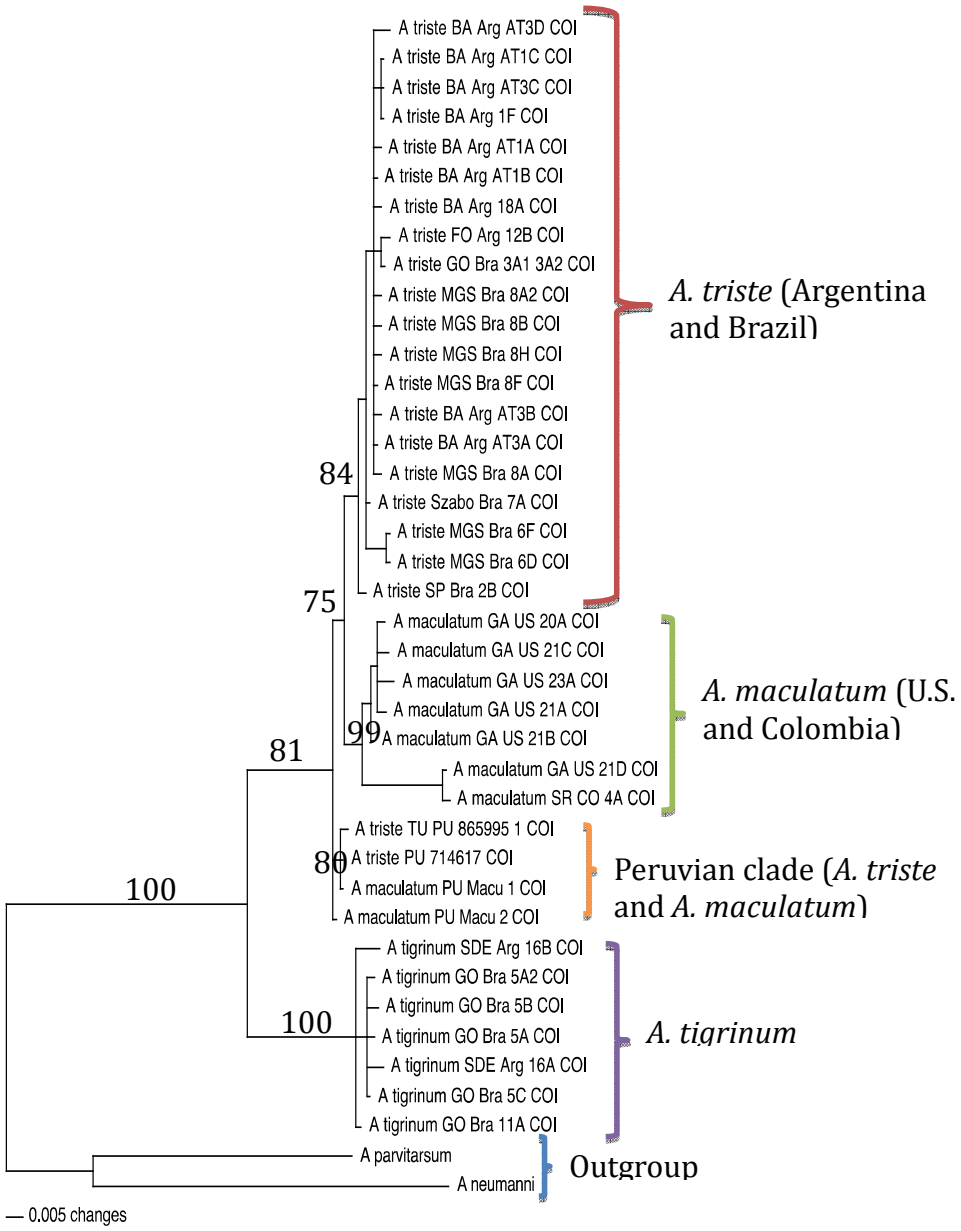

Fig. 2.11 BA for COI.

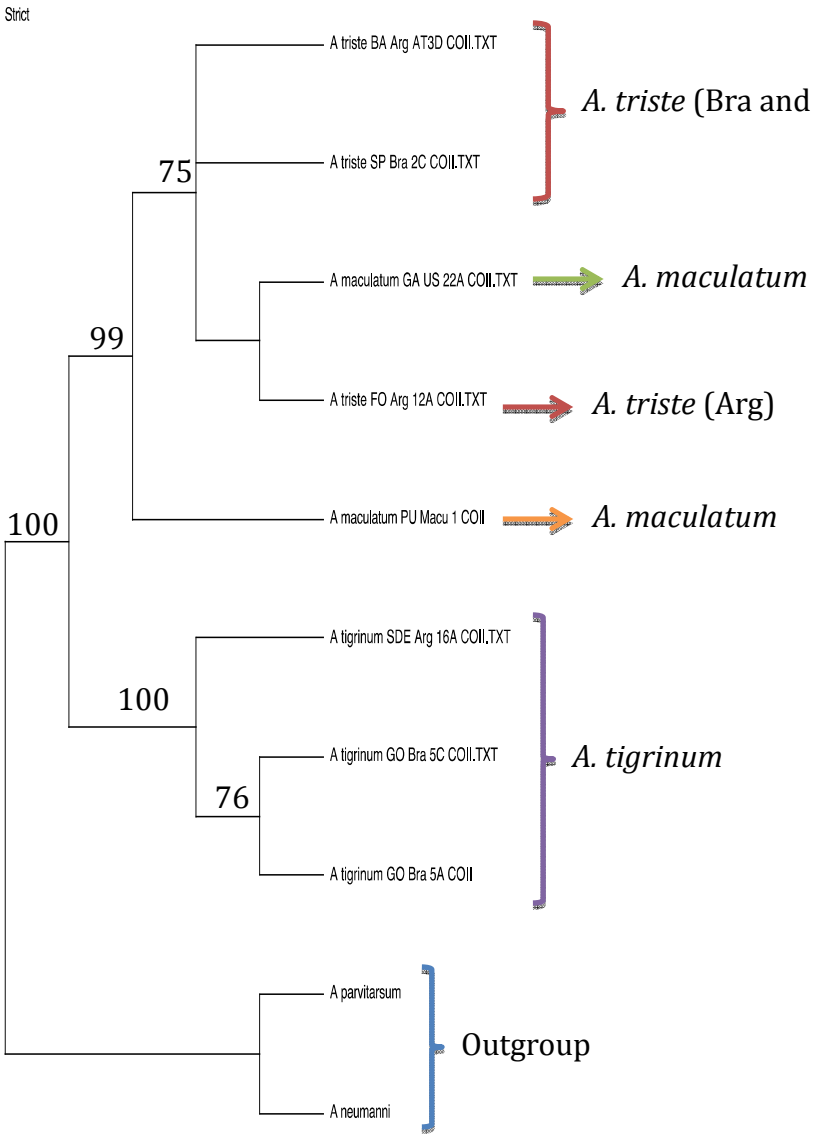

Fig. 2.12 MP for COII.

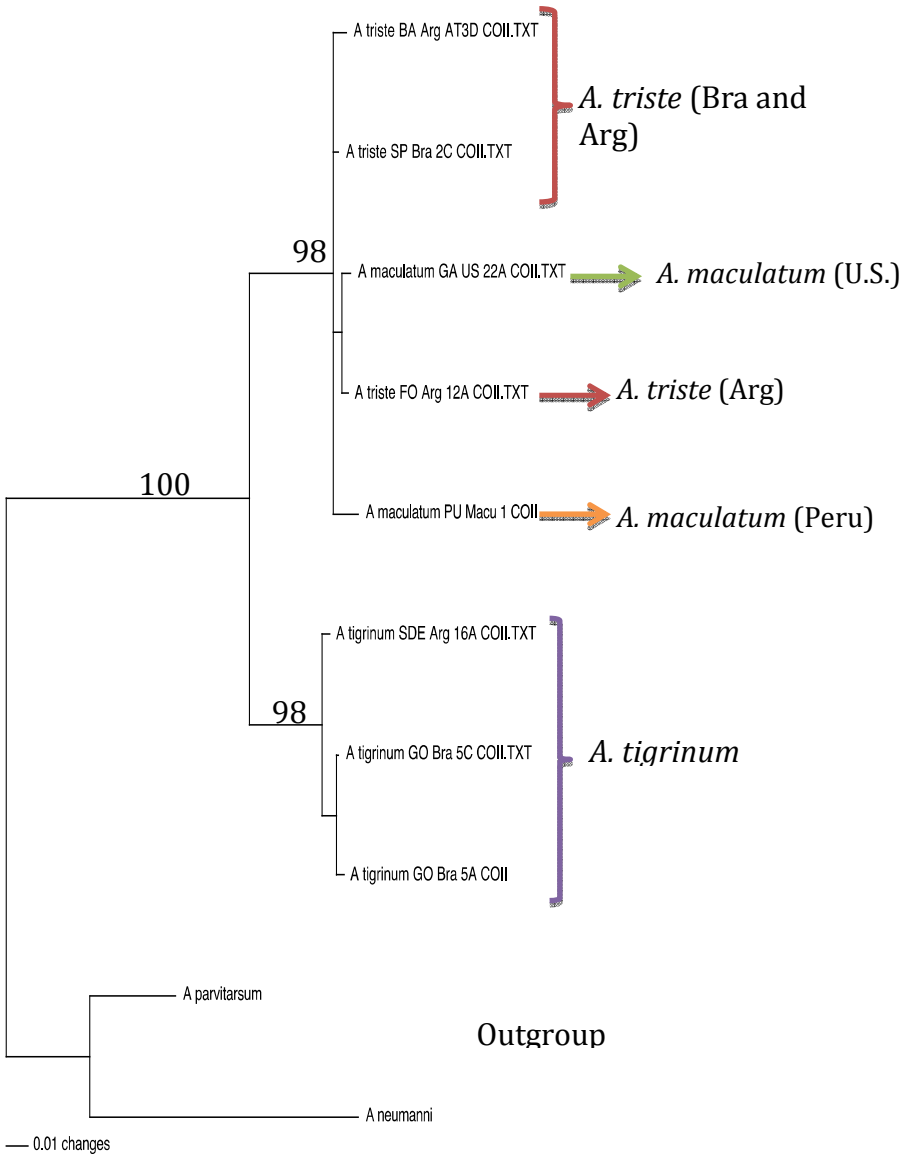

Fig. 2.13 BA for COII.

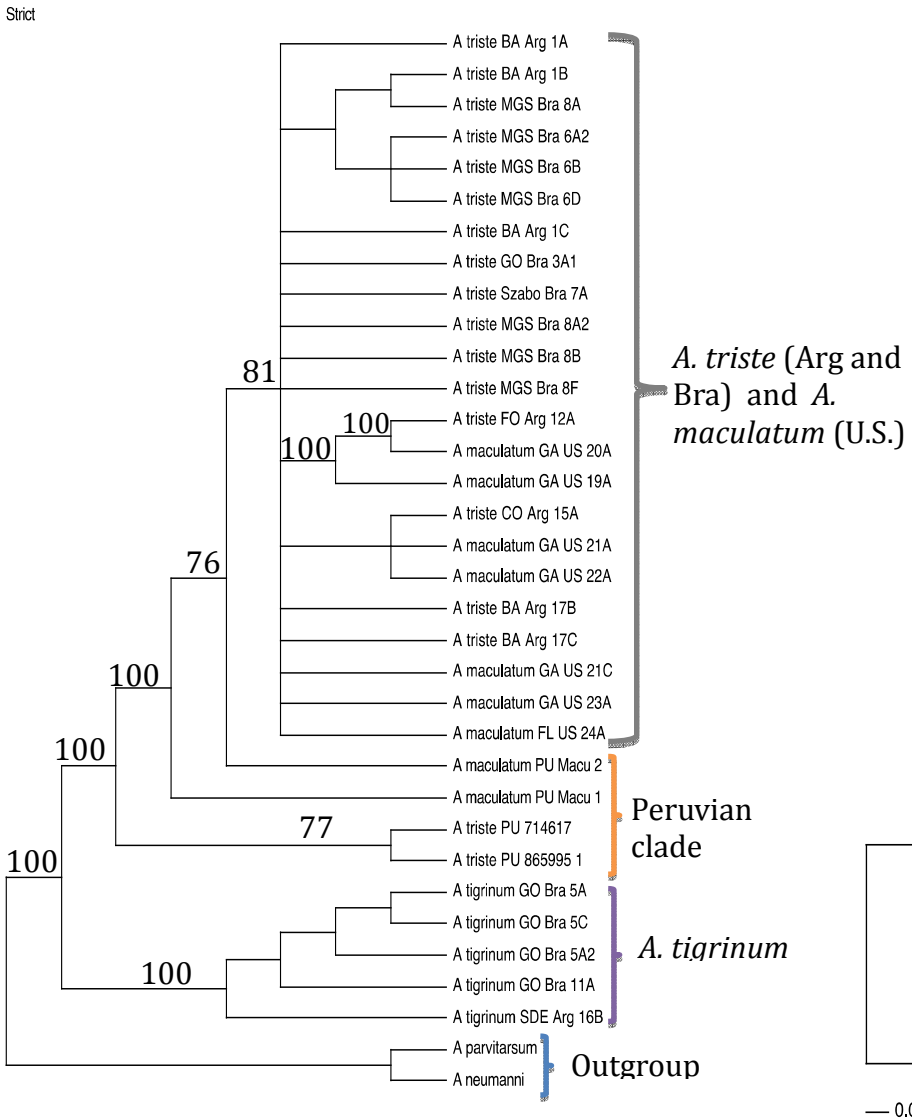

Fig. 2.14 MP for the mitochondrial concatenated dataset (12S-16S-COI).

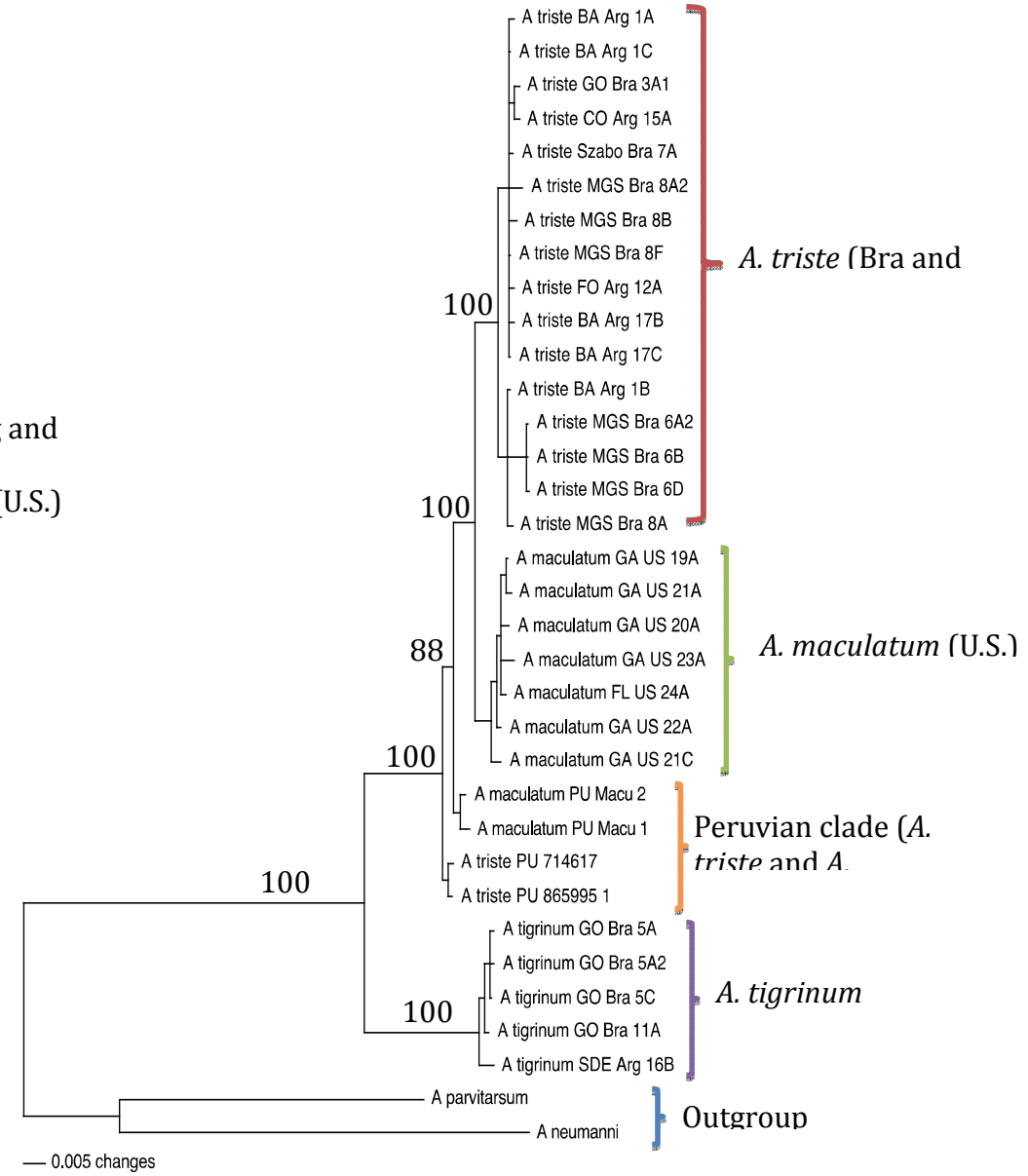

Fig. 2.15 BA for the mitochondrial concatenated dataset (12S-16S-COI).

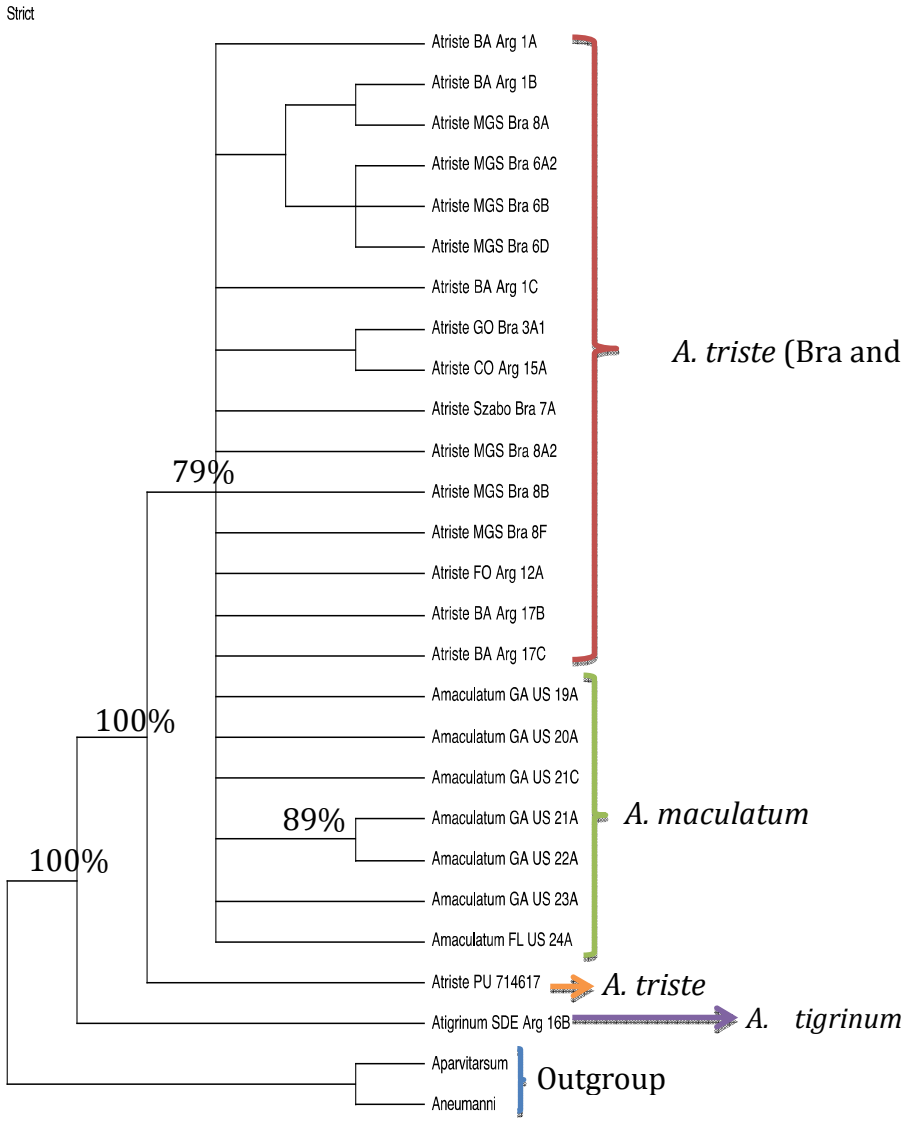

Fig. 2.16 MP for the mitochondrial + nuclear concatenated dataset (12S-16S-COI-ITS2).

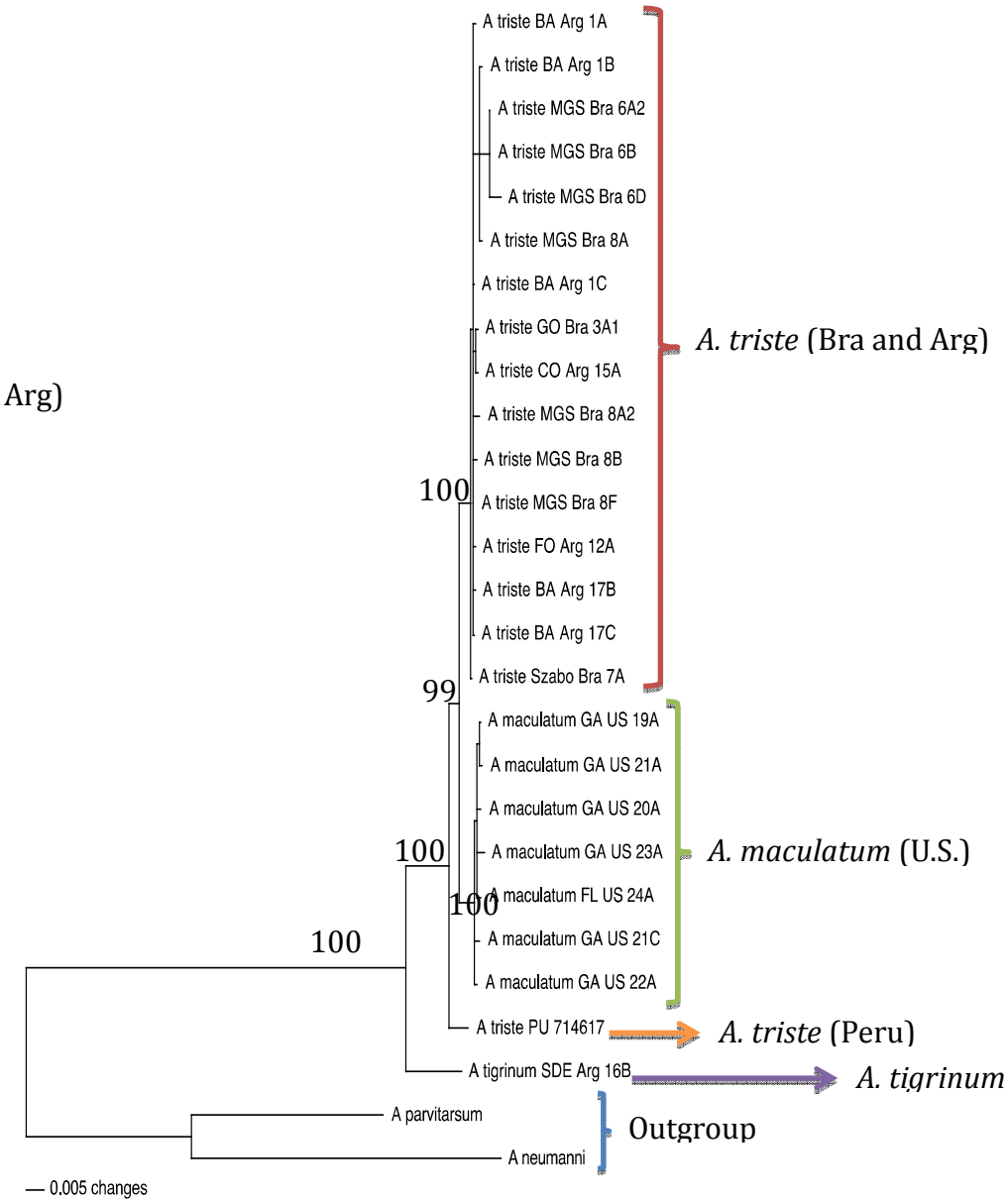

Fig. 2.17 BA for nuclear + mitochondrial concatenated data set (12S-16S-COI-ITS2).

Table 2.1. Localities and PCR amplification success for each of the samples, and each of the individual genes markers.

Lab ID	Species	Locality	State/Provence/Department	Code	Country	12S	16S	DL	ITS2	COI	COII
AT1A	*A. triste*	Zarate	Buenos Aires	BA	Argentina				X	X	
AT1B	*A. triste*	Zarate	Buenos Aires	BA	Argentina	X				X	
AT1C	*A. triste*	Zarate	Buenos Aires	BA	Argentina	X				X	
AT3A	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X				X	
AT3B	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina					X	
AT3C	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X				X	
AT3D	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina					X	X
AT3E	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina			X		X	
1A	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X	X	X	
1A2	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X		X	
1B	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X	X	X	
1C	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X	X	X	
1D	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina		X		X		
1E	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina		X		X	X	
12A	*A. triste*	Reserva El Bagual	Formosa	FO	Argentina	X	X	X	X	X	X
12B	*A. triste*	Reserva El Bagual	Formosa	FO	Argentina		X	X		X	
12C	*A. triste*	Reserva El Bagual	Formosa	FO	Argentina		X	X	X		
12D	*A. triste*	Reserva El Bagual	Formosa	FO	Argentina	X	X	X	X		
12E	*A. triste*	Reserva El Bagual	Formosa	FO	Argentina	X	X	X	X		
15A	*A. triste*	Colonia Pellegrini	Corrientes	CR	Argentina	X	X	X	X	X	
16A	*A. tigrinum*	Pozo Hondo	Santiago del Estero	SDE	Argentina	X	X		X	X	X
16B	*A. tigrinum*	Pozo Hondo	Santiago del Estero	SDE	Argentina	X	X	X	X	X	
17A	*A. triste*	Zarate	Buenos Aires	BA	Argentina	X	X		X	X	
17B	*A. triste*	Zarate	Buenos Aires	BA	Argentina	X	X	X	X	X	
17C	*A. triste*	Zarate	Buenos Aires	BA	Argentina	X	X	X	X	X	

18A	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X		X	
18B	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X	X		
18C	*A. triste*	Inta Delta	Buenos Aires	BA	Argentina	X	X	X	X		
13A	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X	X	X		X	
13B	*A. triste*	Promissão	Sao Paulo	SP	Brazil		X	X	X		
13C	*A. triste*	Promissão	Sao Paulo	SP	Brazil		X	X		X	
AT2A	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X				X	
AT2B	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X		X		X	
AT2C	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X		X			
AT2D	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X					
AT2E	*A. triste*	Promissão	Sao Paulo	SP	Brazil					X	
AT2F	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X		X			X
AT2G	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X				X	
2A	*A. triste*	Promissão	Sao Paulo	SP	Brazil		X				
2B	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X	X			X	
2C	*A. triste*	Promissão	Sao Paulo	SP	Brazil	X	X	X			X
3A1	*A. triste*	Mineros	Goias	SP	Brazil	X	X	X	X	X	
5A	*A. tigrinum*	Mineros	Goias	GO	Brazil	X	X	X		X	X
5A2	*A. tigrinum*	Mineros	Goias	GO	Brazil	X	X	X		X	
5B	*A. tigrinum*	Mineros	Goias	GO	Brazil	X	X			X	
5C	*A. tigrinum*	Mineros	Goias	GO	Brazil	X	X	X		X	X
5D	*A. tigrinum*	Mineros	Goias	GO	Brazil		X	X			
5E	*A. tigrinum*	Mineros	Goias	GO	Brazil	X	X	X			
6A	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X		X	X	
6A2	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
6B	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
6C	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	

6D	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
6E	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
6F	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil		X	X		X	
6G	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil		X	X		X	
7A	*A. triste*	Colonia Uberlandia			Brazil	X	X	X	X	X	
7B	*A. triste*	Colonia Uberlandia			Brazil	X	X	X	X	X	
7C	*A. triste*	Colonia Uberlandia			Brazil		X	X	X	X	
8A	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
8A2	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
8B	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
8C	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8D	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil		X		X	X	
8E	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil		X	X	X		
8F	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X	X	
8G	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X		X	
8H	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X		X	
8I	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8J	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8K	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8L	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X			
8M	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X			
8N	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8O	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X			
8P	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
8Q	*A. triste*	Pantanal	Mato Grosso do Sul	MGS	Brazil	X	X	X	X		
11A	*A. tigrinum*	Caldas Novas	Goias	GO	Brazil	X	X	X		X	
19A	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X	X	X	

19B	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X				X	
20A	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X	X	X	
21A	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X	X	X	
21B	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X		X	
21C	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X	X	X	
21D	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X		X	
22A	*A. maculatum*	Monroe Co.	Georgia	GA	USA	X	X	X	X	X	X
23A	*A. maculatum*	Bulloch Co.	Georgia	GA	USA	X	X	X	X	X	
24A	*A. maculatum*	Howard Co.	Florida	FL	USA	X	X	X	X	X	X
4A	*A. maculatum*	Poima	Santander	SR	Colombia		X	X		X	
Macu_1	*A. maculatum*	17 Pirua		PU	Peru	X	X	X		X	X
Macu_2	*A. maculatum*	17 Pirua		PU	Peru	X	X	X		X	X
865995_1	*A. triste*	Tumbes	TU	PU	Peru	X	X	X		X	
865995_2	*A. triste*	Tumbes	TU	PU	Peru	X	X	X			
714617	*A. triste*			PU	Peru	X	X	X	X	X	
10A	*A. triste*	Ica	Ica	PU	Perú		X				
Total per gene						75	79	73	50	67	11
Total # samples						95					

Table 2.2. Haplotypes by species and by region/country for each of the molecular markers analyzed. Distinct haplotypes are named with letters, and combined with a number for each gene marker as follows: 1, 12S; 2, 16S; 3, DL; 4, COI; 5, COII and 6, ITS2.

12S

Species by Country	1A	1B	1C	1D	1E	1F	1G	1H	1I	1J	1K	1L	1M	1N	1O	1P
***A. triste* Arg**	0	0	0	0	0	1	0	1	0	0	0	15	0	1	0	0
***A. triste* Bra**	0	1	1	6	2	0	1	0	0	0	0	23	0	0	0	0
***A. triste* Peru**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2
***A. tigrinum* Arg**	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Bra**	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* PU**	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
***A. maculatum* U.S.**	0	0	0	0	0	0	0	0	1	1	0	0	0	0	8	0

16S

Species by Country	2A	2B	2C	2D	2E	2F	2G	2H	2I	2J	2K	2L	2M	2N	2O	2P	2Q	2R	2S
***A. triste* Arg**	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	12	0	1
***A. triste* Bra**	1	0	1	3	2	0	0	0	0	2	1	12	1	0	0	0	15	3	0
***A. triste* Peru**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Arg**	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Bra**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* PU**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* U.S.**	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* CO**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2T	2U	2V	2W	2X	2Y	2Z	2AA	2BB	2CC	2DD	2EE
0	0	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	3	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	1	1
0	0	1	1	1	1	1	1	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0	0

DL

Species/Country	3A	3B	3C	3D	3E	3F	3G	3H	3I	3J	3K	3L	3M	3N	3O	3P	3Q	3R	3S
***A. triste* Arg**	0	1	0	0	0	0	0	1	1	0	1	1	1	1	0	0	0	1	0
***A. triste* Bra**	1	0	1	1	1	1	1	0	1	3	1	0	4	0	2	1	1	0	1
***A. triste* Peru**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Arg**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Bra**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* PU**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* U.S.**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* CO**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

3T	3U	3V	3W	3X	3Y	3Z	3AA	3BB	3CC	3DD	3EE	3FF	3GG	3HH	3II	3JJ	3KK	3LL
0	0	7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
1	2	3	0	0	0	1	0	0	0	0	2	3	2	1	0	0	0	0
0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4
0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	5	1	0	0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

3MM	3NN
0	0
0	0
0	0
0	1
1	0
0	0
0	0
0	0

COI

Species by Country	4A	4B	4C	4D	4E	4F	4G	4H	4I	4J	4K	4L	4M	4N	4O	4P	4Q	4R	4S	4T
***A. triste* Arg**	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	2	0	2	2
***A. triste* Bra**	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	3	0	0
***A. triste* Peru**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Arg**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. tigrinum* Bra**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* PU**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* U.S.**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A. maculatum* CO**	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4U	4V	4W	4X	4Y	4Z	4AA	4BB	4CC	4DD	4EE	4FF	4GG	4HH	4II	4JJ	4KK	4LL
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0
0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
0	3	1	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

COII

Species by Country	5A	5B	5C	5D	5E	5F	5G	5H
***A. triste* Arg**	1	0	0	0	1	0	0	0
***A. triste* Bra**	1	0	1	0	0	0	0	0
***A. tigrinum* Arg**	0	0	0	0	0	1	0	0
***A. tigrinum* Bra**	0	0	0	0	0	0	1	1
***A. maculatum* PU**	0	2	0	0	0	0	0	0
***A. maculatum* U.S.**	0	0	0	1	1	0	0	0

ITS2

Species by Country	6A	6B	6C	6D	6E	6F	6G	6H	6I	6J	6K	6L	6M
***A. triste* Arg**	2	1	1	0	0	0	0	0	1	1	0	0	10
***A. triste* Bra**	0	0	0	0	0	0	1	0	0	0	0	1	22
***A. triste* Peru**	0	0	0	0	0	0	0	1	0	0	0	0	0
***A. tigrinum* Arg**	0	0	0	0	1	1	0	0	0	0	0	0	0
***A. maculatum* U.S.**	0	0	0	6	0	0	0	0	0	0	1	0	0

Table 2.3. ML pairwise distances for 12S (bold) and 16S (italics). The values are expressed as percentage.

12S/*16S*	***A. triste***	***A. maculatum***	***A. tigrinum***	**Out**
A. triste	**0-2.1**/*0.2-2.7*	*0.5-2.7*	*3.4-4.6*	*10.8-12.6*
A. maculatum	**0.6-2.1**	**0.3-1.8**/*0.2-2.5*	*3.2-4.4*	*10.8-11.6*
A. tigrinum	**1.8-2.7**	**2.4-2.7**	**n/a**/0-1.7	*11.0-12.6*
Out	**11.9-16.2**	**11.9-16.2**	**12.2-16.2**	**11.0-11.7**/*10.1*

Table 2.4. ML pairwise distances for ITS2 (bold) and DL (italics). The values are expressed

ITS2/*DL*	***A. triste***	***A. maculatum***	***A. tigrinum***	**Out**
A. triste	**0-1.1**/*0.3-2.4*	*0.3-4.6*	*9.4-11.1*	27.6-28.9
A. maculatum	**0.3-1.3**	**0.1**/*0.3-3.5*	*9.5-11.3*	27.9-29.5
A. tigrinum	**1.0-1.7**	**1.2-1.4**	**0.3**/*0.5-1.6*	29-30.1
Out	**17.2-21.8**	**17.5-21.4**	**17.9-21.7**	**7.9/***n/a*

Table 2.5. ML pairwise distances for COI (bold) and COII (italics). The values are expressed as percentage.

COI/COII	***A. triste***	***A. maculatum***	***A. tigrinum***	**Out**
A. triste	**0-1.8**/0.7-1.0	0.8-1.7	5.9-6.6	12.7-16.3
A. maculatum	**0.0-4.0**	**0.2-3.5**/1.7	5.8-6.4	12.9-16.3
A. tigrinum	**6.0-7.5**	**5.6-7.8**	**0.2-1.2**/0.3-1.0	11.7-16.8
Out	**15.4-18.4**	**15.9-18.7**	**15.4-17.9**	**15.7-17.9**/11.4

Table 2.6. ML pairwise distances for the concatenated mitochondrial dataset. Abbreviations: PU, Peru; BA, Brazil and Argentina; US, the United States of America. The values are expressed as percentage.

Concat_mit	***A. triste*-PU**	***A. triste*-BA**	***A. maculatum-PU***	***A. maculatum*-US**	***A. tigrinum***	**Out**
***A. triste*-PU**	**0.1**					
***A. triste*-BA**	**1.2-1.6**	**0.0-1.0**				
A. maculatum-PU	**0.5**	**1.1-1.6**	**0.3**			
***A. maculatum*-US**	**1.2-1.5**	**1.0-1.6**	**1.1-1.5**	**0.1-0.5**		
A. tigrinum	**4.3-4.5**	**4.6-5.1**	**4.3-4.6**	**4.7-5.1**	**0.1-0.5**	
Out	**13.6-15.1**	**13.5-15.4**	**13.8-15.2**	**13.9-15.7**	**13.5-15.4**	**12.7**

Table 2.7. ML pairwise distances for the nuclear + mitochondrial concatenated dataset. The values are expressed as percentage.

Concat_mit+nuclear	*A. triste*	*A. maculatum*	*A. tigrinum*	Out
A. triste	**0.0-1.6**			
A. maculatum	**0.8-1.1**	**0.0-0.3**		
A. tigrinum	**3.1-3.4**	**3.2-3.4**	**n/a**	
Out	**15.7-17.9**	**15.7-18.0**	**15.8-17.9**	**11.4**

Chapter 3

Conclusions

The overall objective of this work was to resolve taxonomic controversial issues among some lineages within the genus *Amblyomma* (Acari: Ixodidae). We chose to study two groups of taxa with similar large geographical distribution ranges: *Amblyomma parvum* and its morphologically related taxa (Chapter 1), and the *A. maculatum* group of species (Chapter 2). Their systematics was reassessed by analyzing mitochondrial and nuclear gene markers.
Although these groups of taxa were set apart by dissimilar and independent taxonomic problems, the successful unravelling of their evolutionary history and their systematic relationships was achieved through the use of the same methodology and the same molecular gene markers (12SrDNA, 16SrDNA, DL, COI, COII and ITS2). The data obtained in the present study confirmed that these markers are phylogenetically informative at the specific and/or intra-specific level, as previously reported by Beati et al. (2013). The resolution obtained in most of the phylogenetic analyses was good, allowing us to determine the taxonomic relationships between and within the taxa, and therefore, accomplishing our main objectives. The analyses strongly supported the hypothesis of cryptic speciation occurring in *A. parvum*, with populations from northern latitudes (Central America) corresponding to a different and yet to be described species, whereas populations from southern latitudes (Argentina and Brazil) were conspecific, and corresponded to *A. parvum.* As for the *A. maculatum* group of species, the phylogenetic reconstructions together with ML pairwise distances values strongly suggested that *A. triste* should be synonymized with *A. maculatum.* In conclusion, although the two groups of taxa had, both, large distribution ranges, the molecular analysis of their genetic diversity revealed two opposite scenarios, one involving cryptic speciation (morphological similarities masking genetic differentiation), and the other involving intraspecific morphological polymorphism without corresponding

genetic divergence. In addition, this study has revealed additional taxonomic issues that should further be investigated.

Reference

Beati L, Nava S, Burkman EJ**,** Barros-Battesti DM, Labruna MB, Guglielmone AA, Cáceres AG, Guzmán-Cornejo CM, León R, Durden LA**,** Faccini JLH**.** 2013. *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae), the Cayenne tick: phylogeography and evidence for allopatric speciation. *BMC Evolutionary Biol*. 13:267.