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The Tick Genus Amblyomma in Africa: Phylogeny and Mutilocus DNA Barcoding

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The tick genus *Amblyomma* in Africa: phylogeny and multilocus DNA barcoding.

by

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(Under the Direction of Lorenza Beati and Lance A. Durden)

ABSTRACT

The tick genus *Amblyomma* includes approximately 130 species, 28 of which are found on the African continent and/ or in Madagascar. In order to understand the evolutionary phylogeography of the genus, it is necessary to gain a better understanding of the relationships between African taxa. Therefore, the main goals of this work were to, (1) reconstruct the phylogenetic relationships of the African *Amblyomma* available to us and (2) test markers for their usefulness as barcoding tools to link unknown immature specimens to their corresponding adults.

The mitochondrial gene markers used in this study (12SrDNA and COI) did not resolve the phylogeny of the studied taxa at all hierarchical levels. Nevertheless, they were informative in resolving recent diverging events between closely related species and at the intraspecific level. They also proved to be promising in terms of "DNA barcoding" and allowed us to identify a number of previously non-identified immature specimens. Combined datasets showed that the

former genus *Aponomma* is basal to *Amblyomma.* Also, reptile or bird feeding species were basal in the tree, intermediate lineages were associated with typical Afro-Asian mammals (elephant, rhinoceros and pangolin), and the most recently evolving taxa were associated with wild and domestic ungulates which are thought to have reached Africa more recently. *Amblyomma sparsum* and *A. nuttalli* were consistently grouped as sister taxa as were *A. cohaerens* and *A. splendidum*.

This information is critical in that it helps us to have a better understanding of the phylogeographical patterns associated with a genus that has a typical Gondwana distribution.

It is also beneficial to develop adequate epidemiological prevention strategies as it can influence the distribution of African *Amblyomma* tick species within and outside of Africa. It can also influence the rates and control of *Amblyomma* transmitted diseases in animals and humans.

Index words: Africa, *Amblyomma*, Gondwana, phylogeny, DNA barcoding, epidemiology

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Dedication

I dedicate this foremost to the Almighty God, to my wonderful parents; Mr and Mrs E.A Kushimo, and also to my siblings; Oluwatoyin, Omowunmi, Oluwadamilola, Toluwani, Olamilekan and David**.**

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INTRODUCTION

Ticks are obligate ectoparasites of terrestrial vertebrates found on all continents of the world. They are second to only mosquitoes in their importance as disease vectors (Sonenshine et al. 2002). The phylogenetic tree proposed by Hoogstraal and Aeschlimann (1982) for the evolution of tick families, subfamilies and genera has been tested using morphological and or molecular data and subsequent changes have been made to the tree (Klompen et al. 1997, 2000, 2002, Black et al. 1997, Dobson and Barker 1999, Beati and Keirans 2001, Murrell et al. 2000, Murrell et al. 2001, Beati et al. 2012). The genus *Amblyomma* is one of the largest hard-tick genera (family Ixodidae) with about 130 valid taxa (Camicas et al. 1998), some of which are of medical and veterinary importance because they can transmit pathogens (*Rickettsia africae*, *Rickettsia rickettsii*, *Ehrlichia ruminantium* among others) (Petney et al. 1977, Kelly et al. 1992, Berrada et al. 2011). The genus has a typical Gondwana geographical distribution, with over 90% of the species occurring only in the southern hemisphere (Fig. 1) (Camicas et al. 1998, Guglielmone et al. 2011). Molecular analyses have indicated that the two traditionally recognized genera within the Amblyomminae, *Amblyomma* and *Aponomma*, are not monophyletic. The genera *Aponomma* and *Amblyomma* were found to be paraphylectic based on their highly conserved 18SrDNA gene sequences (Dobson et al., 1999). As a result of molecular studies, taxa within the genus *Aponomma* were either synonymized with *Amblyomma* or assigned to a newly defined genus, the genus *Bothriocroton* (Klompen et al. 2002). Although African *Amblyomma* constitute about 20% of the known *Amblyomma* species (Table 1), their representation in published phylogenetic analyses is minimal with at best 4 African species

represented in most molecular analyses (Black et al. 1997, Dobson et al. 1999, Beati and Keirans 2001, Burger et al. 2012). A better understanding of the systematic relationships between African *Amblyomma* is not only needed for a reassessment of the evolutionary history of the genus in Africa and of the relationships between former *Aponomma* and *Amblyomma* taxa, but also for a better understanding of the phylogeographical patterns associated with a genus that has a typical Gondwana distribution.

African ticks began to be described in the literature over 200 years ago and have been extensively studied since (Robinson 1926, Hoogstraal 1956, Theiler and Salisbury 1959, Van der Borght-Elbl 1966, Yeoman and Walker 1967, Mathysse and Colbo 1987, Walker and Olwage 1987, Walker 1991). Nevertheless, the present classification and taxonomy is based only on the adult stages. Although several larvae &/or nymphs of African *Amblyomma* have been described (Van der Borght-Elbl 1977), the provided descriptions are not very informative. Knowledge of the immatures is generally restricted to the common taxa which are of known or potential medical or veterinary importance. This has resulted in the accumulations of unidentified immatures of *Amblyomma* spp. in several collections that are in need of identification. This is a significant problem considering that all instars of *Amblyomma* are obligate blood-feeding arthropods, but immatures often feed on different hosts than adults. Identification of immatures in this genus is required for a well defined understanding of the spectrum of host associations, geographic distribution, and vector competence.

In this study the systematic relationships between some of the African taxa were studied by comparing their mitochondrial small ribosomal subunit (12SrDNA) and cytochrome oxidase c subunit I (COI) gene sequences. The genes were also used to link immatures to known adult

specimens. The 12SrDNA gene was chosen based on previously published data showing that it may be evolving at a suitable mutation rate for relatively closely related taxa (Beati and Keirans 2001, Beati et al. 2012). The COI gene sequences are increasingly used as DNA barcoding tools to link adult and immature specimens in many arthropod taxa (Hebert et al. 2003) and it was, therefore, interesting to test them on *Amblyomma* species and to compare the findings to that of 12SrDNA.

MATERIALS AND METHODS

Sampling

Fresh tick specimens including immature ticks (numbers are in parentheses) were obtained from Yoko (3) and the Congo River (8) of the Democratic Republic of the Congo, Lushoto, Tanzania (3), Rumphi district of Malawi (4) and Burkina Faso (9) in Africa. Other study specimens were retrieved from the US National Tick Collection (USNTC) where they had been stored in 70% ethanol (Table 2).

DNA extraction, PCR and sequencing

DNA was extracted from each specimen by using the QIAamp DNA Micro kit (Qiagen, Valencia, CA). The first day extraction steps were performed by using a previously published protocol (Beati and Keirans 2001). Each tick was processed in a way that enables the preservation of the cuticle for further morphological identification and description. This involved the cutting off of a small slice of the posterior-lateral idiosoma of the adult and nymphs and poking a hole in the larvae with a sterile disposable scalpel. This was followed by an overnight incubation in 180 µl of Qiagen ATL lysis buffer and 40µl of 14.3mg/ml proteinase K (Roche

Applied Sciences, Indianapolis, IN). To facilitate the extraction of DNA from very old specimens, the incubated ticks were transferred into a -70 degree freezer for 24 hours, a procedure which is known to enhance cell wall destruction (H.S Klompen, personal communication). The frozen ticks were thawed, vortexed, and the cuticles were placed in 70% ethanol for further morphological examinations. The DNA extraction was completed as previously described (Beati et al. 2012).

Primers T1B (5`-aaa cta gga tta gat acc ct-3`) and T2A (5`-aat gag agc gac ggg cga tgt-3`) were used to amplify approximately 360 base pairs of the 12srDNA gene sequence (Beati and Keirans 2001, Beati et al. 2012). An almost 600-base pair fragment of the CO1 sequence was amplified by primers F1 (5'-tac tct act aat cat aaa gac att gg-3') and R1 (5'-cct cct cct gaa ggg tca aaa aat ga - 3') (M. Montagna, unpublished data). The PCR solution included 10.3µl H_2O, 2.5µl of Taq buffer (10X), 5µl of Taq Master Enhancer (5X), 1.5µl of 25mM $Mg(OAc)_2$, 0.2µl of Taq polymerase, 1.25µl of a 10pmol/µl solution of each primer, 0.5 µl of a deoxynucleotide triphosphate mixture (10mM of each DNTP), and 3µl of the extracted DNA. The target DNA was amplified in an Eppendorf Master cycler, Model #5341 (Fisher scientific, Inc., Pittsburgh, PA). The 12SrDNA amplification program involved an initial denaturation step at 94°C for 5 min, a touch-down step (5 cycles with denaturation at 94°C for 20 seconds, annealing temperatures decreasing from 60 to 50 °C for 25 sec, and elongation temperatures decreasing from 72 to 68°C for 30 sec), 30 cycles with the annealing temperature at 49°C for 35 sec and an elongation temperature of 68°C for 30 sec. The CO1 thermal cycling program involved 5 min of denaturation at 94°C, denaturation at 94°C for 20 sec over 35 cycles, with annealing temperature at 54.5°C for 45 sec, and elongation at 72°C for 45 sec. The same primers used for the PCR were used to sequence the complementary strands at the High-Throughput Genomics Unit

(HTGU; University of Washington, Seattle, WA). The complementary strands were assembled with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic analyses

McClade 4.07 (Maddison and Maddison 2000) was used to manually align sequences according to secondary structure for 12SrDNA and coding reading frame for COI. Maximum parsimony (MP) and maximum likelihood (ML) analyses of the 12SrDNA and the COI datasets were first performed separately in PAUP v. 4.10b (Swofford, 2000). The datasets were further combined in a concatenated alignment for the specimens for which both gene sequences were available. The concatenated dataset was also analyzed by MP and ML. Bootstrap values for the MP reconstructions were obtained in Phylogenetic Analysis Using Parsimony (PAUP) (1000 replicates), whereas ML bootstrap values were obtained by using the online PHYML tool and 100 replicates (Dereeper et al. 2008). Sequences of *Amblyomma transversale* and *Amblyomma latum* (two species formerly assigned to *Aponomma*) were used as outgroups. The model best fitting the data for ML analysis was evaluated by using ModelTest (Posada and Crandall 1998).

Linking adults and immatures

According to the phylogenetic species definition, specimens belonging to the same species must share the closest common ancestor, and should not be para- or polyphyletic. The immature specimens analyzed in this study should, therefore, all cluster with their corresponding adult sequences and be more closely related to them than to other taxa.

Genetic distances were also compared between specimens to verify whether conspecific values are significantly lower than values between species.

Results

Sampling

For this study, we obtained 63 sequences from 13 species for the 12SrDNA and 52 sequences for 12 species for the COI gene. Sequences from additional African *Amblyomma* taxa, previously generated in our laboratory, were added to the dataset. After adding these sequences, the 12SrDNA dataset included 19 species, whereas the COI dataset included 16 species and the concatenated dataset included 14 species.

Phylogenetic analyses

The initial 12SrDNA alignment encompassed 72 sequences and was 361 bp long with 137 parsimony informative characters. After eliminating identical sequences, the dataset was reduced to 41 sequences. The MP search in PAUP generated 23 equally parsimonious trees (length 586) and the strict consensus of the trees is shown in Fig.2a. The model best fitting the data was found to be a general time reversible model with rate matrix, Rmat = (0.5584 8.2968 3.0589 3.2321 8.2968), rates = gamma, shape of gamma = 0.2964, and proportion of invariable sites = 0. The best ML tree (Fig. 2b) inferred by PAUP had a score of –lnL= 2575.45 which was better than the ML score of the best MP tree (2577.41). The 60 COI sequences were aligned in a 533 long matrix (218 informative characters), which was reduced to 37 unique sequences. The MP search found 24 equally parsimonious trees (length = 804, -lnL ML score = 4179.80) and their strict consensus is shown in Fig. 3a, whereas the best COI ML tree (-lnL = 4167. 15) is shown in Fig. 3b. The model selected by ModelTest was the general time reversible with Rmat =

(0.7115 10.9262 1.1451 0.0001 10.9262), rates= gamma, gamma shape = 0.7406, and proportion of invariable sites = 0.5406.

The concatenated dataset was 1332 bp long with 322 parsimony informative characters. The MP search resulted in two best trees with a length of 1212 and a likelihood score of –lnL = 6217.03 and their consensus is shown in Fig. 4a. The best ML tree had a score of –lnL = 6211.51 and as its topology is fully congruent with the MP tree we only show the MP tree in Fig. 4 (with both the MP and ML bootstrap values indicated on the branches).

The overall support for all reconstructions was relatively low, in particular along the intermediate line of nodes between the earlier diverging events and the most recent diverging events. Nevertheless, the support for most of the crown clades is good (>70%). The MP and ML reconstructions were consistent in recognizing the same clades and also in having weak bootstrap support for the same lineages. Although the concatenated dataset included more sequence data, its analysis provided marginally better resolution, suggesting that the two gene sequences are informative at the same taxonomic hierarchical level. All analyses were congruent in finding former *Aponomma* taxa in the basal lineages. In general we found good support for clades containing specimens belonging to a single species, with some exceptions. In 12S, an *A. marmoreum* nymph clustered within the *A. sparsum* lineage and an *A. variegatum* nymph clustered within *A. gemma.* In addition, *A geochelone* and *A. loculosum* specimens were found to have the same sequence and to be undifferentiable from *A. chabaudi.* Overall, *A. sparsum* and *A. nuttalli* were consistently found to be sister taxa and closely related to other reptile (and bird) ticks. *A. cohaerens* and *A. splendidum* were sister taxa. In COI, the intraspecific groupings were also fairly well supported with the same exceptions mentioned for 12S and an additional issue appearing in the *A. cohaerens-A. splendidum* grouping, where *A. splendidum* was found

embedded in *A. cohaerens.* In the concatenated tree, only the relationships between the ungulate-associated ticks (*A. cohaerens, A. splendidum, A. gemma, A. hebraeum,* and *A. variegatum*) were characterized by better support. They were all included in a monophyletic clade, which was separated into two sister groups: the first included sister taxa *A. gemma* and *A. hebraeum,* whereas in the other lineage, *A. variegatum* was basal to the *A. cohaerens-A. splendidum* cluster.

Linking adults and immatures

The leaf labels of the phylogenetic 12SrDNA and COI trees (Figs 2-3) show the number of specimens found associated with each specific unique sequence. All immature specimens analyzed in this study could be linked to adults either because they shared an identical nucleotide sequence with the corresponding adults, or because they clustered in the same monophyletic group. The 12SrDNA divergence between clearly identified adult specimens of the same species was less than 2%, whereas it varied from 6 to 26% between species. The confirmed (adult) COI intraspecific genetic distances were always less than 1%, whereas the interspecific divergence levels varied from about 6 to 25%. Therefore, any immature specimen differing by less than 2% for 12SrDNA and 1% for COI from an identified adult specimen could confidently be considered to belong to the same species.

Discussion

The phylogenetic reconstructions generated in this study showed that 12SrDNA and COI sequences cannot resolve relationships between African *Amblyomma* at all hierarchical taxonomic levels. Genes evolving at a slower mutation rate, possibly the 28S or the 18SrDNA nuclear genes, could help to improve the support level of the weakly resolved intermediate

nodes. Only when combined in a single dataset, did the two genes succeed in resolving the diverging pattern of the most recently evolving lineages. Nevertheless, the tree obtained appears to indicate that all basal lineages in the tree presently feed on reptiles and/or birds. Tick taxa in the intermediate unresolved branches are either found on reptiles or on typical Afro-Asian species (elephant, rhinoceros, and pangolin). The most recently evolving taxa are associated with wild and domestic ungulates which are thought to have reached Africa only in the early Neogene (18-14 Mya) (Marshall and Sempere 1990), whereas reptiles and the ancestors of the elephants (Shoshani 1998) were present on the continent before the Neogene (Duellman 1990). Therefore, the overall diverging pattern in our trees appears to roughly match the sequential appearance of their corresponding ancestral vertebrate hosts in Africa.

Among the results that require further discussion, the finding of the sequences of an *A. variegatum* nymph embedded within *A. gemma* is not surprising. These two tick species feed on the same hosts (often domestic ungulates) and share a similar geographical distribution (Walker and Olwage 1987). Similarly, *A. marmoreum* and *A. sparsum* can also feed on the same hosts. As satisfactory keys for the identification of African immature *Amblyomma* are not available, often researchers consider immatures found with identified adults to belong to the same species as the adult. In this case, we deduce that an *A. gemma* nymph was accidentally included in an *A. variegatum* batch, and an *A. sparsum* nymph in an *A. marmoreum* batch. Once formal descriptions of African nymphs become available, the cuticles will need to be examined to confirm this suspicion. Nevertheless, this appears to indicate that both the COI and the 12SrDNA genes are good "barcoding" tools and can help discover incorrectly identified ticks.

The grouping of *Amblyomma geochelone*, *Amblyomma chabaudi* and *Amblyomma loculosum* into a clade with a bootstrap value of 100 is more disturbing. Two of these species are found in Madagascar and one of them (*A. loculosum*) on islands of the Indian Ocean. *A. geochelone* and *A. chabaudi* were described from Malagasy tortoises, whereas *A. loculosum* was described from birds and reptiles from the Seychelles. Neumann (1907) described the male and female of *Amblyomma loculosum* without providing any illustration. Robinson (1926) described the female of *A. loculosum* based on a specimen from Neumann's lot but did not draw the male. The male of *A. chabaudi* collected from an endemic terrestrial tortoise (*Pyxis arachnoides*) in the south of Madagascar was described by Rageau (1964) and the female of the species remains undescribed. Both the male and female of *A. geochelone* (Durden et al. 2002) were described more recently from the endangered Malagasy ploughshare tortoise (*Geochelone yniphora*). Our findings would require a thorough reanalysis of the type specimens of the three species in order to establish if, in fact, they belong to a single taxon. Also, the lack of adequate descriptions of *A. loculosum* and *A. chabaudi* may have resulted in males and females of different taxa to be assigned to the wrong taxonomic entity. Unfortunately we were unable to generate COI sequences for all the specimens involved and cannot confirm the 12SrDNA data. The discrepancies observed in the position of *A. splendidum* is also not too surprising as female *A. splendidum* cannot be differentiated from female *A. cohaerens.*

Both, the 12S and the COI gene sequences proved to be good markers usable for DNA barcoding of *Amblyomma* or *Aponomma* ticks, at least at the African scale. Overall, immature and corresponding adult sequences were either identical, or clustered closely together with sequence divergence lower than that observed between recognized species. The markers also allowed us to detect possibly incorrectly identified ticks. As the immature stages of some of

these species have never been described, particularly those of the reptile-related taxa, those data will provide the scientific community with new descriptions of *Amblyomma* immature stages. In addition, they will allow us to draw better maps of geographical distributions of these ticks, and to better understand their relationships with hosts and pathogens. Better informed prevention strategies against disease transmission can only be developed if the geography-vertebrate host-tick-pathogen interactions are fully understood.

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Table 1. List of African species of *Amblyomma* and the merged *Aponomma*

Species	Author (s)
Amblyomma astrion	Dönitz, 1909
Amblyomma chabaudi	Rageau, 1964
Amblyomma cohaerens	Dönitz, 1909
Amblyomma compressum	Macalister, 1872
Amblyomma eburneum	Gerstaecker, 1873
Amblyomma falsomarmoreum	Rondelli, 1935
Amblyomma gemma	Dönitz, 1909
Amblyomma geochelone	Durden, Keirans & Smith, 2002
Amblyomma hebraeum	Koch, 1844
Amblyomma lepidum	Dönitz, 1909
Amblyomma loculosum	Neumann, 1907
Amblyomma marmoreum	Koch, 1844
Amblyomma nuttalli	Dönitz, 1909
Amblyomma paulopunctatum	Neumann, 1899
Amblyomma personatum	Neumann, 1901
Amblyomma pomposum	Dönitz, 1909
Amblyomma rhinocerotis	DeGeer, 1778
Amblyomma sparsum	Neumann, 1899
Amblyomma splendidum	Giebel, 1877
Amblyomma superbum	Santos Dias, 1953
Amblyomma sylvaticum	DeGeer, 1778
Amblyomma tholloni	Neumann, 1899
Amblyomma variegatum	Fabricius, 1794
*Amblyomma exornatum**	Koch, 1844
*Amblyomma flavomaculatum**	Lucas, 1846

*Amblyomma inopinatum**	Santos Dias, 1989
*Amblyomma latum**	Koch, 1844
*Amblyomma transversale**	Lucas, 1878

Note- Asterisked species were formerly assigned to the genus *Aponomma*.

Table 2. African species of *Amblyomma* included in the study

Species	Stages used &/ identified
Amblyomma chabaudi	Nymph, Female
Amblyomma cohaerens	Female
Amblyomma compressum	Male
Amblyomma gemma	Nymph, Male
Amblyomma geochelone	Adult (sex not recorded)
Amblyomma hebraeum	Nymph, Male
Amblyomma lepidum	Adult (sex not recorded)
Amblyomma loculosum	Nymph, Female
Amblyomma marmoreum	Nymph
Amblyomma nuttalli	Nymph
Amblyomma rhinocerotis	Adult (sex not recorded)
Amblyomma sparsum	Nymph, Male
Amblyomma splendidum	Male, Female
Amblyomma sylvaticum	Male
Amblyomma tholloni	Nymph
Amblyomma variegatum	Nymph
Amblyomma flavomaculatum	Nymph, Male, Female
Amblyomma latum	Nymph
Amblyomma transversale	Adult (sex not recorded)

Figure 1. Gondwanan distribution of the genus *Amblyomma*

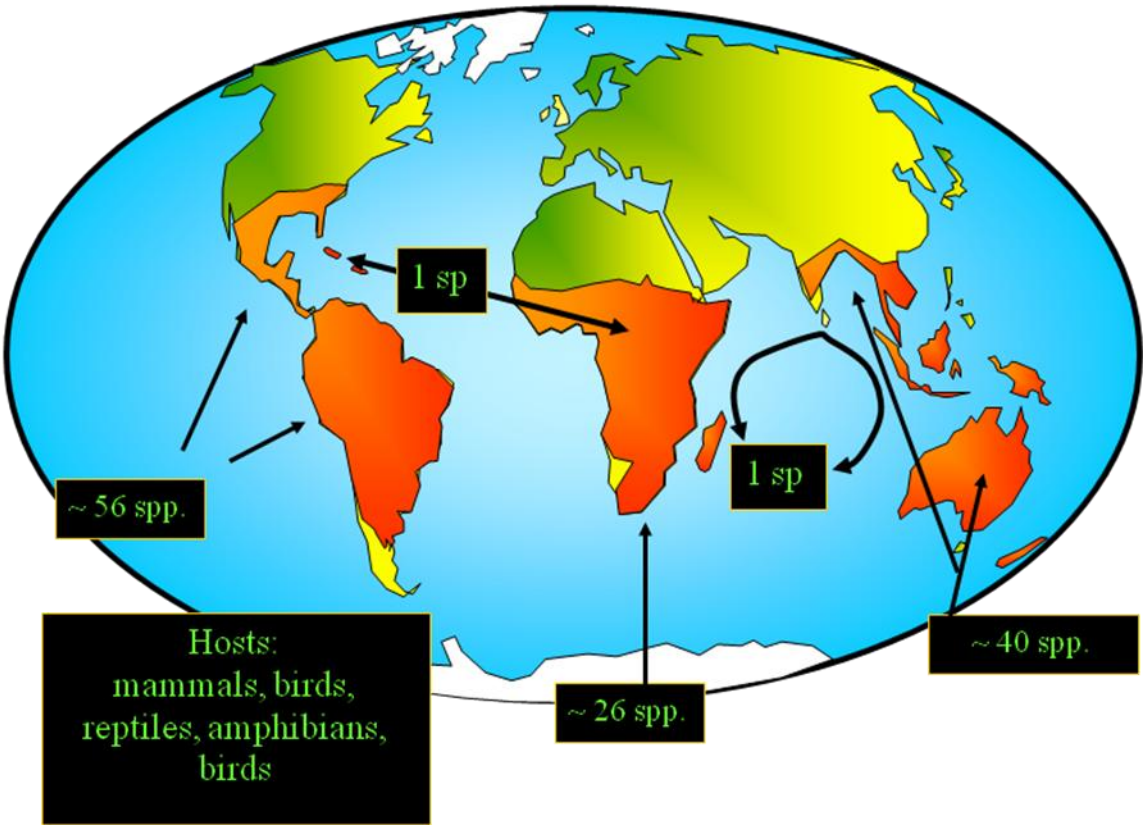

Figure 2a. Maximum parsimony tree (12S)

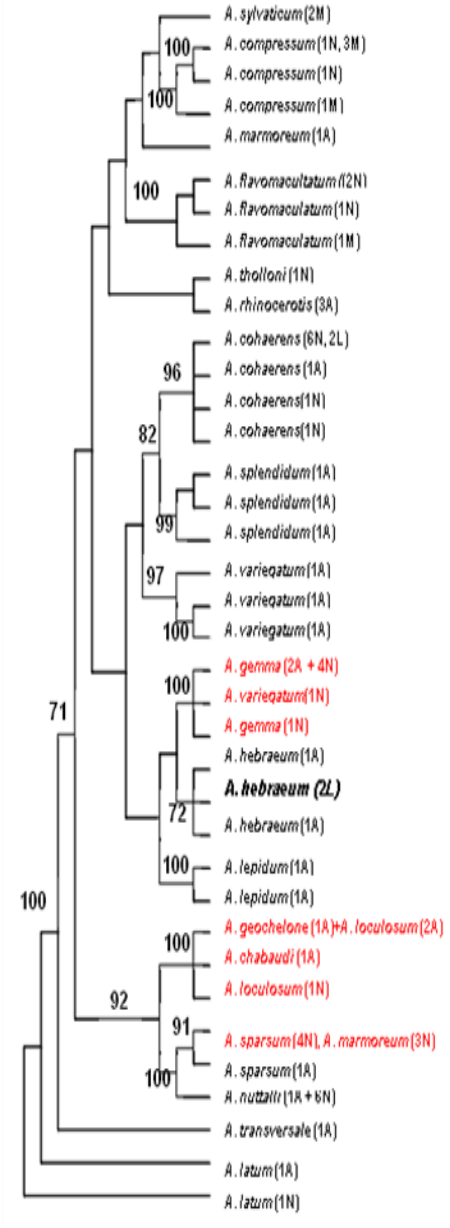

Figure 2b. Maximum likelihood tree (12S)

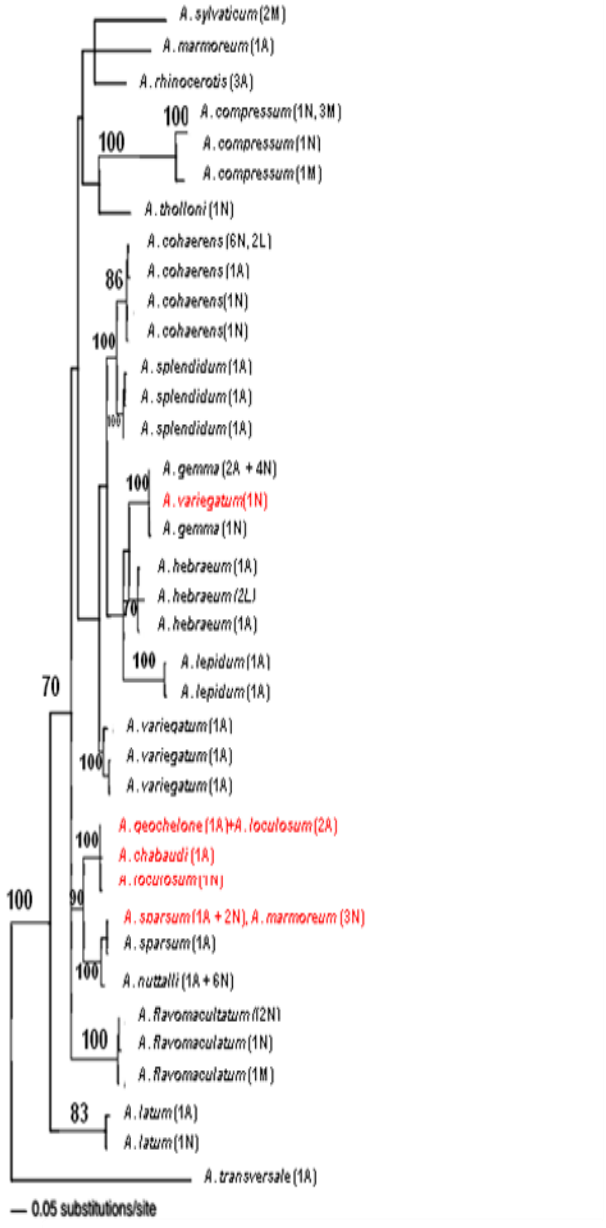

Figure 3a. Maximum parsimony tree (CO1)

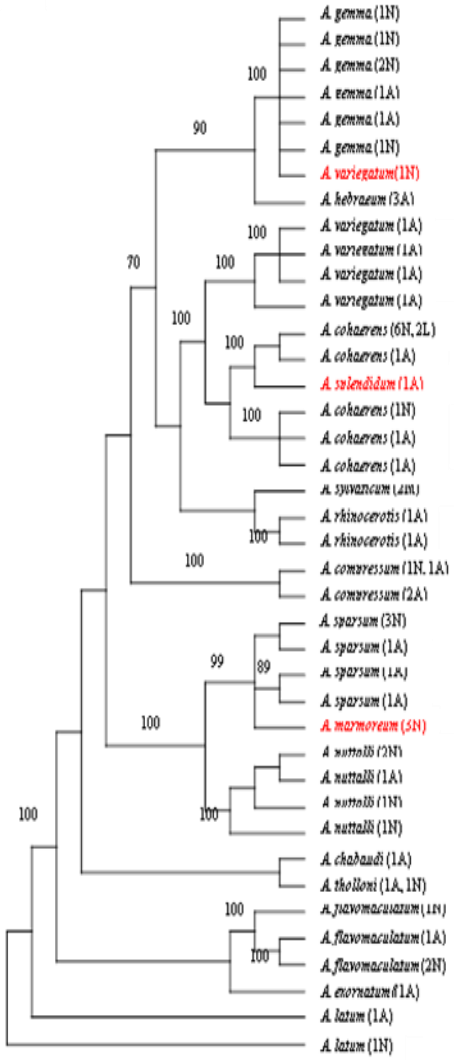

Figure 3b. Maximum likelihood tree (CO1)

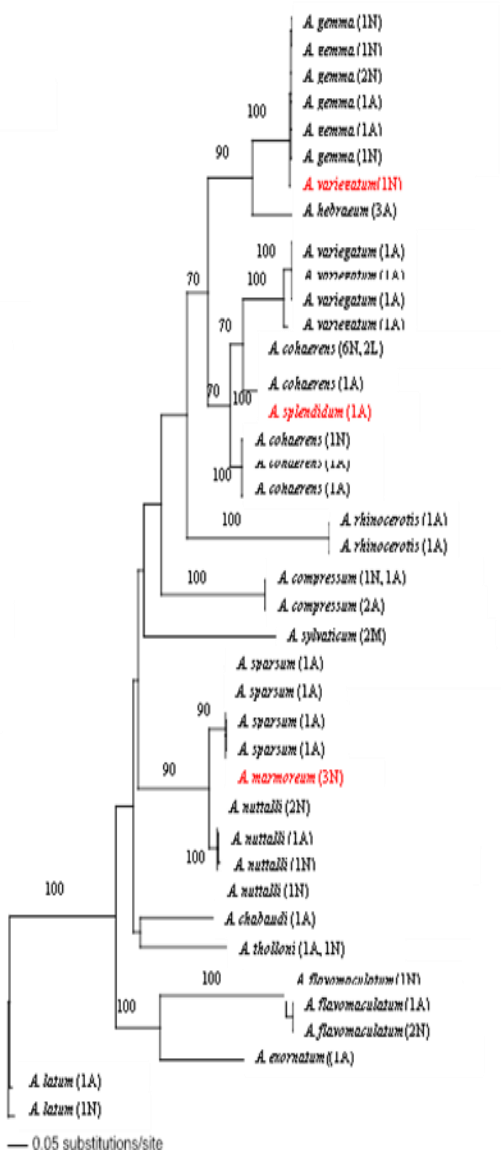

Figure 4. Maximum parsimony tree of concatenated dataset