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Intrageneric Phylogenetics based on Available Three Mitochondrial Genes and One Nuclear Gene Variation among Thirteen Species of *Coranus* Curtis and Two Ecotypes (Hemiptera: Reduviidae: Harpactorinae)

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Abstract

The sequence of three mitochondrial genes viz., 16S rRNA, Cyt b, COI and one nuclear gene, 28S rRNA of *Coranus* Curtis species downloaded from the GenBank were subjected to phylogenetic analyses to understand the intrageneric and intraspecific variations and the role of geographical isolation on speciation using CLUSTAL W in MEGA version 5.6. This analysis includes thirteen species of *Coranus* Curtis and probably two ecotypes of *Coranus callosus* Stål from four countries viz., Australia, Brunei, China and Nigeria of three continents viz., Africa, Asia and Australia. The pairwise genetic distances were calculated and phylograms were constructed using maximum likelihood, neighbor-joining, minimum evolution, UPGMA and maximum parsimony methods. These preliminary analyses not only demarcated the thirteen species of *Coranus* and the two ecotypes of *C. callosus* but also revealed phylogenetic relationships and the role of geographical isolation on speciation.

Keywords: *Coranus*; 16S rRNA; Cyt b; COI and 28S rRNA; Harpactorinae; Biocontrol Agents; Intrageneric Molecular Biosystematics; Speciation; Ecotypes; Geographical Isolation;

Introduction

Some assassin bugs have different morphs, biotypes, and ecotypes with various colors and shapes which often mislead a museum entomologist in recognizing the morphs and ecotypes of a particular species.

Hence, classifications of Reduviidae based on morphological characters may at times become insufficient, and there is an urgent need for a cohesive meaningful classification of Reduviidae based on ecological, morphological, behavioural, cytological, and biochemical data [1,2,3,4,5]. Moreover, a multidisciplinary biosystematics is imperative to accurately identify reduviids and employ them against a particular insect pest [4,5,6,7]. Though the literature available on multi-disciplinary biosystematics of Reduviidae including molecular tools is available at family or species level it is very meager [5,8,9,10,11,12,13,14].

Curtis established the genus *Coranus* with *Cimex subapterus* De Geer as the type species. *Coranus* is one of the largest genera of subfamily Harpactorinae in the family Reduviidae with 100 known species worldwide [2,3,4,15]. The members of *Coranus* are widely distributed and occur throughout the Eastern Hemisphere with 30 Palaearctic, 21 Oriental, 41 Ethiopian and 17 Australian species [11]. Malipatil revised the Australian *Coranus* with redescription of seven species, description of eight new species and formulated key to identify the fifteen species [16]. Liu . inferred the phylogenetic relationship of the harpactorine genus *Velinoides* Matsmura with *Coranus* Curtis based on three mitochondrial genes (cyt b, CoI and 16S rRNA) and one nuclear (28S rRNA) gene [11]. Since they found molecular affinity between these two genera supported with morphological and cytogenetic characteristics they validated the status of genus *Velinoides* and its phylogenetic affinity with the genus *Coranus*. They suggested that these two genera could be two subgenera of the genus *Coranus*. They further reported that the 28S rDNA gene alone might not be an optimal marker for the phylogeny of the genus *Coranus*. Though twelve species of *Coranus* have been recorded from India none of its gene sequence is available. Except the work of Liu *et al*. no work on the molecular phylogenetics of the genus *Coranus* is available [4,11].

Hence, this study was undertaken based on the sequences of three mitochondrial genes, 16S rRNA, Cyt b, COI and one nuclear gene, 28S rRNA of thirteen species of *Coranus* Curtis and probably two ecotypes of *Coranus callosus* Stål from four countries viz., Australia, Brunei, China and Nigeria of three continents viz., Africa, Asia and Australia and probably two ecotypes of *C. callosus* Stål from western Australia downloaded from the GenBank (Table 1). The inclusion of *Coranus* species from four countries of three continents further enhances the scope of the work at the intraspecific level and the understanding on the role of geographical isolation in biosystematics.

Materials and Methods

Taxon sampling

To understand the intrageneric biosystematics and phylogenetics the sequences of three mitochondrial genes, 16S rRNA, Cyt b, Cyt c oxidase subunit I gene and one nuclear gene, 28S rRNA of thirteen species of *Coranus* Curtis and probably two

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ecotypes of *C. callosus* Stål (Tables 1,2) downloaded from the GenBank were subjected to phylogenetic analysis.

Table 1: Thirteen species of *Coranus* and its two ecotypes subjected to phylogenetic analyses with their locality.

Species	Locality
Coranus sp.$_1$	Brunei
Coranus sp.$_2$	Nigeria: Ondo
Coranus sp.$_3$	Australia : South Australia
Coranus lativentris Jakovlev	China: Xiaowutai Mt., Hebei
Coranus hammarstroemi Reuter	China: Lvliang Mt., Shanxi
Coranus dilatatus (Matsumura)	China: Lvliang Mt., Shanxi
Coranus marginatus Hsiao	China: Yingjiang, Dehong, Yunnan
Coranus emodicus Kiritschenko	China: Yingjiang, Dehong, Yunnan
Coranus fuscipennis Reuter	China: Yunji Mt., Xinfeng, Guangdong
Coranus sichuensis Hsiao & Ren	China: Tengchong, Baoshan, Yunnan
Coranus spiniscutis Reuter	China: Jinghong, Xishuangbanna,Yunnan
Coranus subapterus (De Geer)	China: Tianchi, Urumchi, Xinjiang
Coranus callosus Stål*	Australia: Western
Coranus callosus Stål**	Australia: Western

* Ecotype 1, ** Ecotype 2

Phylogenetic analysis

The gene sequences were subjected into pairwise distance analysis and the phylogenetic trees were constructed based on maximum likelihood and neighbor-joining, maximum evolution, UPGMA and maximum parsimony methods with MEGA 5 software [17]. The five different methods were used to understand the utility of each method in the biosystematics.

Pairwise alignment

Pairwise distances were carried out with gap opening penalty 15 and gap extension penalty 6.66 (Clustal W) [18].

Maximum Parsimony

The maximum parsimony analyses were analysed with MEGA5 [17]. Bootstrap method was used with 100 replications and gap/missing data treatment by complete selection and substitution based on nucleotide sequences [19]. The maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 (Table 1) [20].

Maximum Likelihood

The evolutionary history was inferred based on the Tamura-Nei model [21]. Initial tree for the heuristic search was obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value (Table 2).

Neighbor-Joining

The evolutionary history was inferred using the neighbor-joining method [22]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was used [19]. The evolutionary distances were computed using the Tajima-Nei method [23].

Table 2: Mitochondrial and nuclear partial gene sequences of thirteen *Coranus* species and two ecotypes of *Coranus callosus* with their GenBank accession number

Mitochondrial and nuclear genes	Species	Genbank accession number
16S ribosomal RNA	*Coranus* sp.$_1$	JQ888411.1
	Coranus sp.$_2$	JQ888412.1
	Coranus sp.$_3$	JQ888413.1
	Coranus lativentris Jakovlev	EU128688.1
	Coranus hammarstroemi Reuter	EU128689.1
	Coranus dilatatus (Matsumura)	EU128690.1
	Coranus marginatus Hsiao	EU128691.1
	Coranus emodicus Kiritschenko	EU128692.1
	Coranus fuscipennis Reuter	EU128693.1
	Coranus sichuensis Hsiao & Ren	EU128694.1
	Coranus spiniscutis Reuter	EU128695.1
	Coranus subapterus (De Geer)	EU128696.1
	Coranus callosus Stål*	FJ230433.1

Cytochrome b (cyt b)	*Coranus lativentris* Jakovlev	EU128710.1
	Coranus hammarstroemi Reuter	EU128711.1
	Coranus dilatatus (Matsumura)	EU128712.1
	Coranus marginatus Hsiao	EU128713.1
	Coranus fuscipennis Reuter	EU128714.1
	Coranus sichuensis Hsiao & Ren	EU128715.1
	Coranus spiniscutis Reuter	EU128716.1
	Coranus subapterus (De Geer)	EU128717.1
Cytochrome c oxidase subunit I	*Coranus* sp.$_1$	JQ888572.1
	Coranus sp.$_2$	JQ888573.1
	Coranus sp.$_3$	JQ888574.1
	Coranus callosus Stål*	JQ888571.1
	Coranus callosus Stål**	JQ942321.1
28S ribosomal RNA	*Coranus* sp.$_1$	JQ888911.1
	Coranus sp.$_2$	JQ888756.1
	Coranus lativentris Jakovlev	EU128677.1
	Coranus hammarstroemi Reuter	EU128678.1
	Coranus dilatatus (Matsumura)	EU128679.1
	Coranus marginatus Hsiao	EU128680.1
	Coranus emodicus Kiritschenko	EU128681.1
	Coranus fuscipennis Reuter	EU128682.1
	Coranus sichuensis Hsiao & Ren	EU128683.1
	*Coranus sp*iniscutis Reuter	EU128684.1
	Coranus subapterus (De Geer)	EU128685.1
	Coranus callosus Stål*	FJ230594.1

Minimum evolution

The evolutionary history was inferred using the minimum evolution method [24]. The optimal tree with the sum of branch length = 8.45674115 is shown. The confidence probability (multiplied by 100) was estimated using the bootstrap test [24,25].

UPGMA

The evolutionary history was inferred using the UPGMA method [26]. The optimal tree with the sum of branch length = 8.42786450 is shown.

The substitution type based nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding and all the positions containing gaps and missing data were eliminated in all the five methods. Five phylograms were thus constructed based on maximum likelihood (ML), neighbor-joining (N-J), maximum evolution (ME), UPGMA and maximum parsimony (MP) methods for three mitochondrial genes, 16S rRNA, Cyt b and Cyt c oxidase subunit I and one nuclear gene, 28S rRNA. The trees were analyzed based on the arrangement of each species in the tree.

Results and Discussion

16S rRNA

The ML tree constructed for the 16s rRNA gene of three undetermined and ten determined *Coranus* species has two major clusters (Figure 1). The first major cluster divides into two subclusters; with *Coranus hammarstroemi* Reuter that evolved as a separate lineage; the other species in this subcluster further divides into two minor clusters. One such minor cluster has two species viz., *Coranus subapterus* (De Geer) and *Coranus emodicus* Kiritschenko. The minor cluster further diversified into two clusters one with *Coranus spiniscutis* Reuter and *Coranus lativentris* Jakovlev and another with *Coranus dilatatus* (Matsumura) as a separate lineage and was distant to all other *Coranus* species [11]. Another cluster forms with two species viz., *Coranus fuscipennis* Reuter and *Coranus marginatus* Hsiao while maintaining its affinity with *C. dilatatus* on one hand and with the minor cluster of *C. subapterus* and *C. emodicus* on the other hand [11]. The second major cluster diversified into *Coranus sichuensis* Hsiao & Ren as a separate lineage as *C. hammarstroemi* of the first major cluster and further diversified into two subclusters as observed by Liu *et al.* [11]. The first cluster has only *Coranus callosus* Stål and second cluster has two undetermined

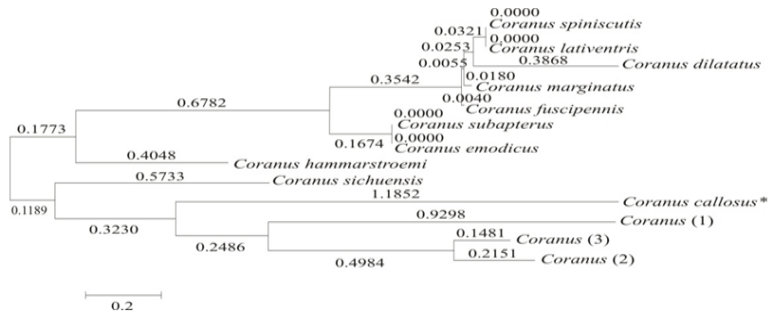

Figure 1: ML tree based on 16S gene variations showing the relationships of thirteen *Coranus* species.

species *Coranus* sp.$_1$ and a further diversified cluster with two undetermined species *Coranus* sp.$_3$ and *Coranus* sp.$_2$.

The NJ (Figure 2) and ME trees (Figure 3) replicate the second major cluster as in ML tree. However, in the first major cluster, the positions of *C. dilatatus* and *C. hammarstroemi* vary. Though almost a similar kind of phylogeny is observed for UPGMA (Figure 4) and MP methods (Figure 5) slight deviations were found in relation to *C. dilatatus* in UPGMA and *C. hammarstroemi* and *C. callosus* in MP tree.

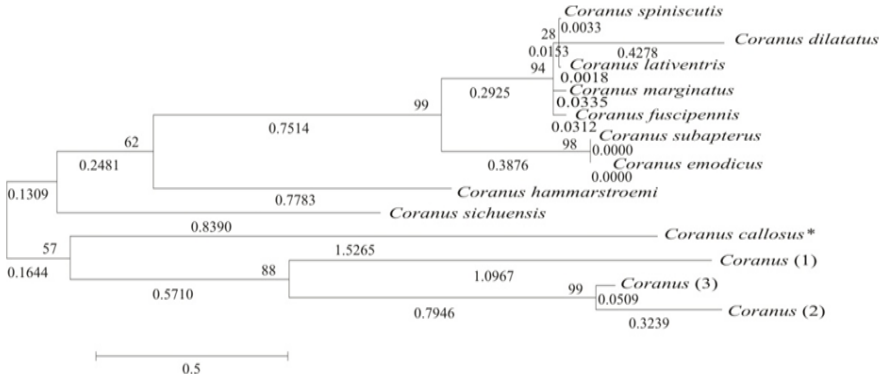

Figure 2: NJ tree based on 16S gene variations showing the relationships of thirteen *Coranus* species

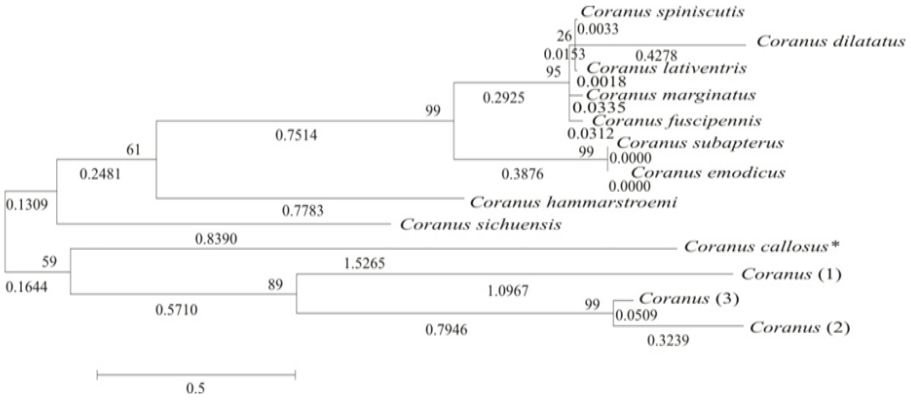

Figure 3: ME tree based on 16S gene variations showing the relationships of thirteen *Coranus* species.

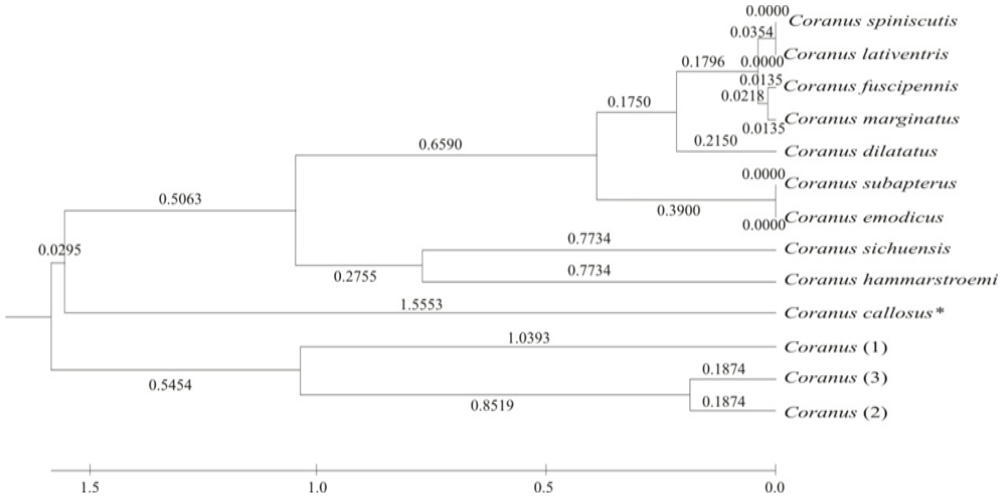

Figure 4: UPGMA method tree based on 16S gene variations showing the relationships of thirteen *Coranus* species.

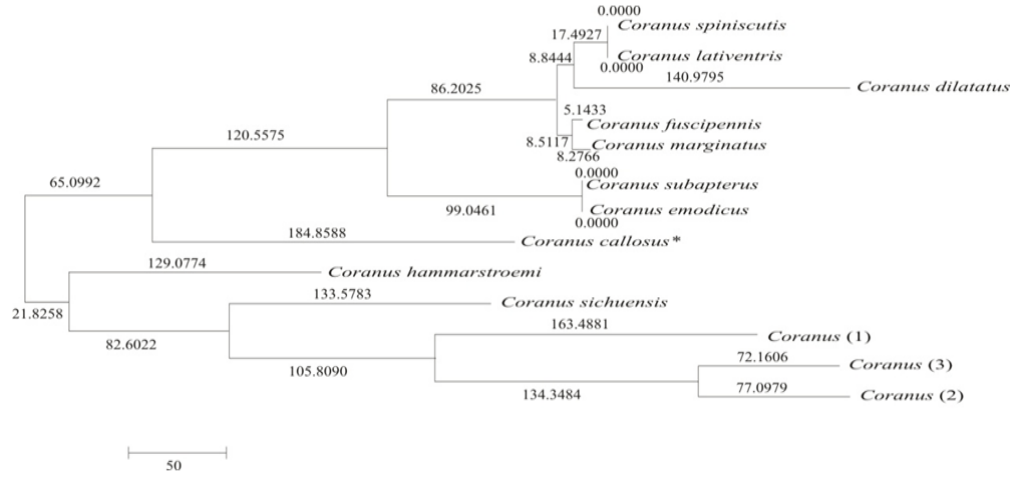

Figure 5: MP method tree based on 16S gene variations showing the relationships of thirteen *Coranus* species

Eight species of *Coranus* from China form the first major cluster where as the ninth species *C. sichuensis* either diversified separately or clustered with *Coranus* of Australia, Brunei and Nigeria. The results reveal monophyly though they belong to four countries and three continents as observed by Cui & Huang in Orthoptera, Liu *et al*. in *Coranus* species of China, Ambrose *et al*. in *Rhynocoris* Kolenati (Harpactorinae) and Lenin in *Acanthaspis* Amyot and Serville, *Edocla* Stål, *Empyrocoris* Miller and *Velitra* Stål (Reduviinae) and Manimuthu (Manimuthu *et al*.,) in *Ectomocoris* Mayr and *Catamiarus* (Serville) (Peiratinae) species of Reduviidae from India [5,11,12,13,14,27].

Coranus sp.$_2$ of Australia instead of clustering with *C. callosus* of Australia clustered with *Coranus* sp.$_3$ of Nigeria and *Coranus* sp.$_1$ of Brunei. These species exhibit affinity despite their geographical isolation as observed by Mahendran *et al*. in silk producing insects and Ambrose *et al*. in *R. fuscipes* (Fabricius) of India with *R. segmentarius* (Germar) of South Africa [5,28].

Cyt b. The five phylograms observed for eight *Coranus* species of China except *C. emodicus* formed into two major clusters (Figure 6,7,8,9 and 10). The first cluster had *C. spiniscutis, C. hammarstroemi, C. subapterus and C. sichuensis* and the second cluster had *C. dilatatus, C. lativentris, C. fuscipennis and C. marginatus* with slight modification in different phylograms, revealing monophyly as observed by Liu *et al*. in *Coranus* species of China, Baskar *et al.* and Ambrose *et al*. in *Rhynocoris* species of India [5,11,29].

Cyt c. The five phylograms (Figure 11,12,13,14 and 15) of Cyt c gene of three undetermined species of *Coranus* from Australia, Brunei and Nigeria *C. callosus* from western Australia, probably from two localities, i.e., two ecotypes revealed affinity between *Coranus* sp.$_1$ of Brunei with *Coranus* sp.$_2$ of Australia. *Coranus* sp.$_2$ of Australia thus instead of clustering with *C. callosus* of Australia aligns with that of *Coranus* sp.$_1$ from Brunei. Similarly two ecotypes of *Coranus* callosus of Western Australia instead of

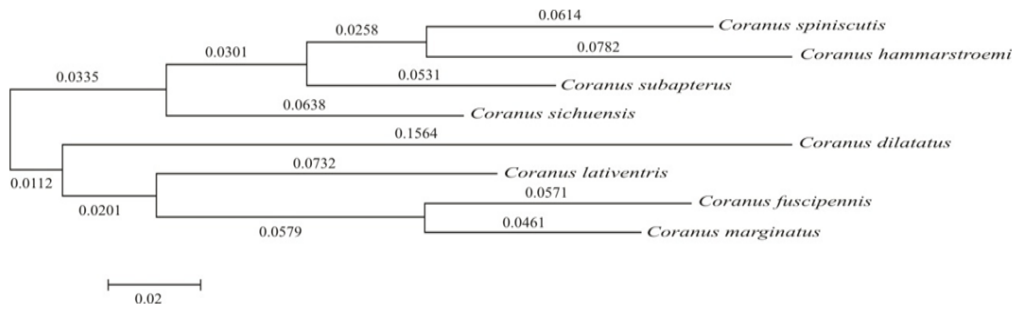

Figure 6: ML tree based on Cyt b gene variations showing the relationships of eight *Coranus* species.

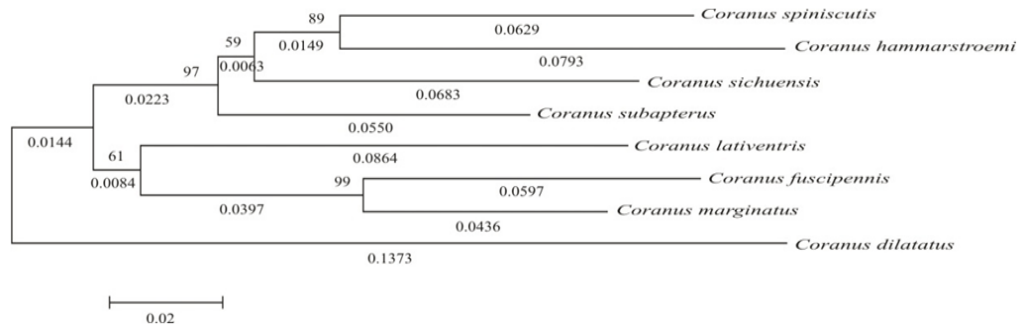

Figure 7: Neighbor-Joining tree based on Cyt b gene variations showing the relationships of eight *Coranus* species.

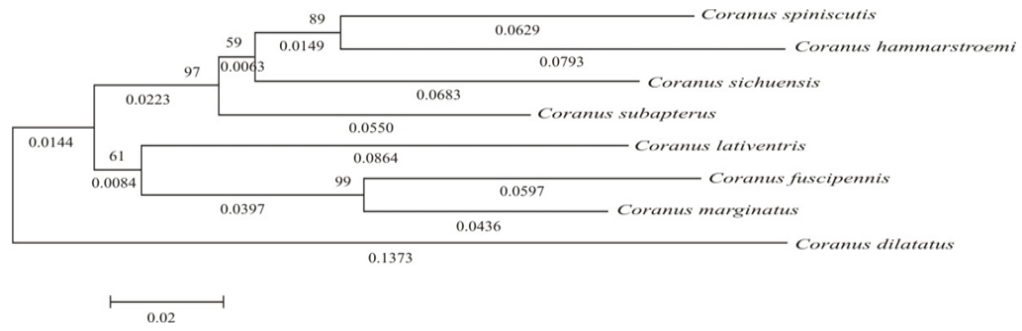

Figure 8: Minimum Evolution tree based on Cyt b gene variations showing the relationships of eight *Coranus* species.

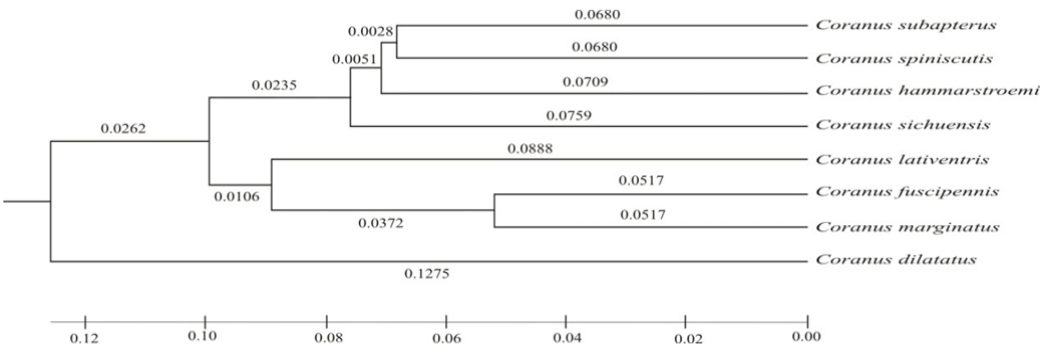

Figure 9: UPGMA tree based on Cyt b gene variations showing the relationships of eight *Coranus* species.

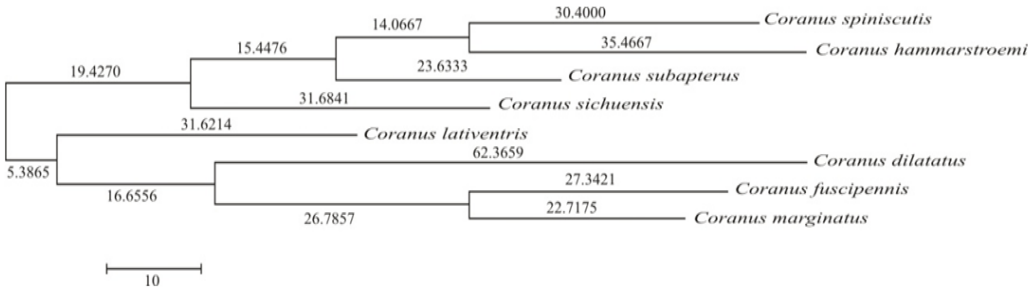

Figure 10: MP tree based on Cyt b gene variations showing the relationships of eight *Coranus* species.

aligning together, one aligns with *Coranus* sp.$_3$ of Nigeria while another with *Coranus* sp. of Brunei and *Coranus* sp.$_2$ of Australia. Thus, monophyly phylogenetic affinity is pronounced despite geographical isolation. However, we admit that it is premature to suggest the role of geographical isolation without knowing the molecular characteristics such as number of segregating sites, nucleotide diversity and haplotype diversity and the geographical genetic structure. Moreover, the quantity of sampling is too small. Baskar (2010) and Baskar *et al*. (2012, a, b, c) reported genetic diversity among the ecotypes of four Indian *Rhynocoris* species viz., *R. kumarii* Ambrose and Livingstone, *R. marginatus* (Fabricius), *R. longifrons* (Stål), and *R. fuscipes* (Fabricius) based on mitochondrial genes and correlated the affinity with the ecological diversity of semiarid, scrub jungle, and tropical rainforest habitats [29,30,31,32]. Ambrose *et al*. (2014) also reported a similar phenomenon in four ecotypes of *R. kumarii* [5]. The present results corroborates with the findings of Liu *et al*. (2009), Baskar (2010), Baskar *et al*. (2012 a, b, c) and Ambrose *et al.* (2014) suggesting the existence of genetic diversity, with low level of gene flow in *Coranus* species [5,11,29,30,31,32]. However, these observations are contrary to those of Giordano *et al.* (2005) in *Triatoma* infestans (Klug) [33]. This contradiction might be the result of the non-dispersal haematophagus feeding behaviour of Triatoma in contrast to the dispersal predatory behavior of *Coranus*. The findings further suggest that the Cyt b fragment is a useful marker to describe the genetic structure of ecotypes of

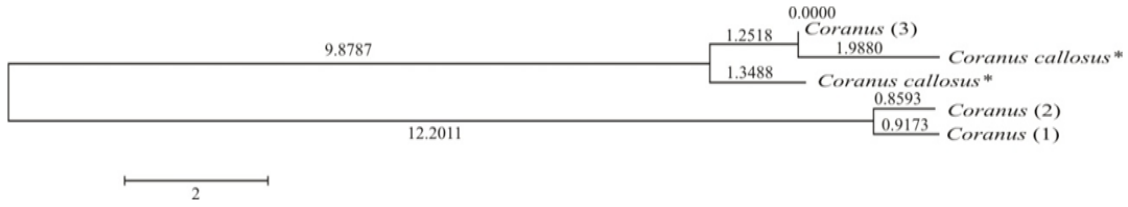

Figure 11: MLmethod tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Coranus* and two ecotypes of *Coranus callosus*.

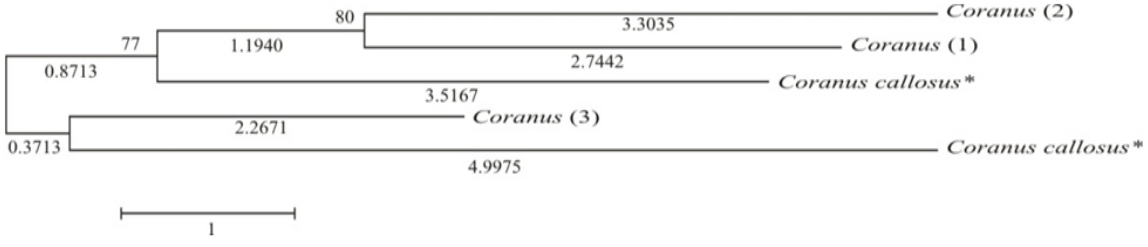

Figure 12: NJ tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Coranus* and two ecotypes of *Coranus callosus*.

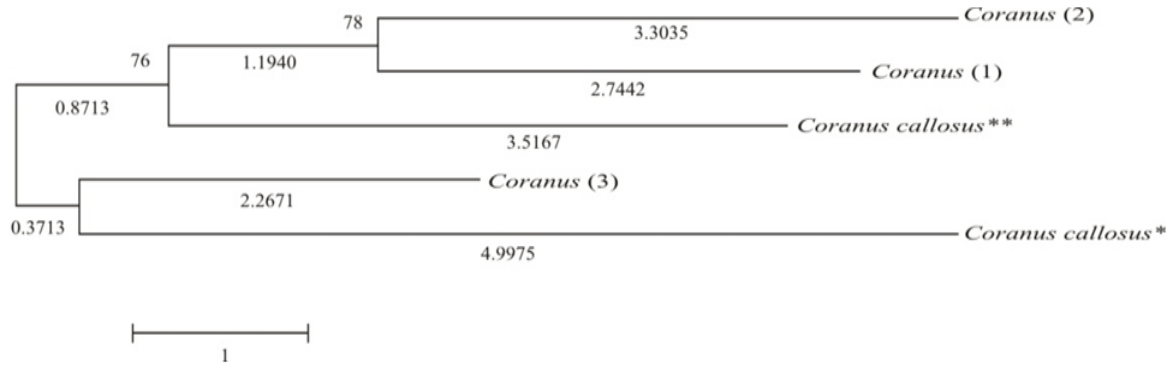

Figure 13: ME tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Coranus* and two ecotypes of *Coranus callosus*.

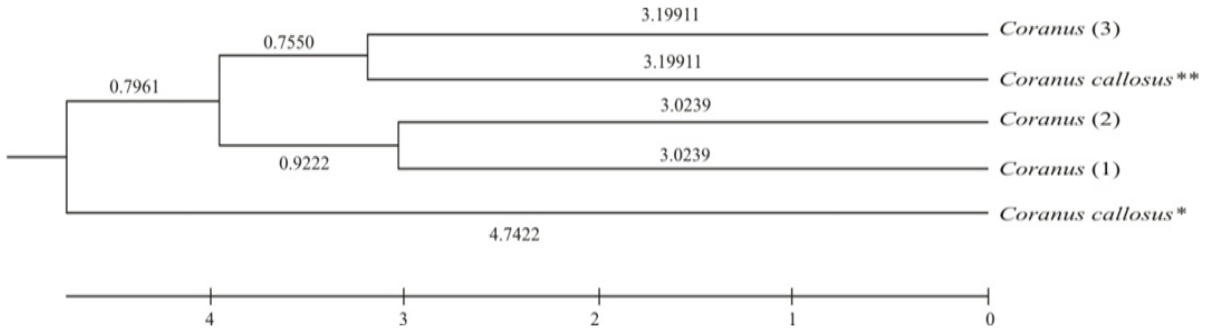

Figure 14: UPGMA method tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Coranus* and two ecotypes of *Coranus callosus.*

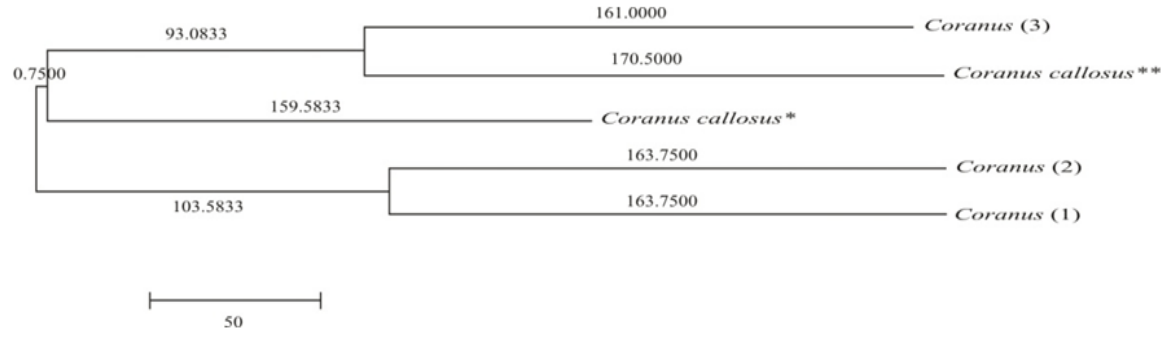

Figure 15: MP tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Coranus* and two ecotypes of *Coranus callosus*.

closely related habitats (Naranjo *et al.*, 2010) [34].

28S rRNA. Five phylograms (Figure16,17,18,19,20) were constructed for twelve species of *Coranus*, i.e., except *Coranus* sp.$_3$. In maximum likelihood method (Figure 16), all the nine *Coranus* species from China formed a major cluster. *Coranus* sp.$_1$ of Brunei diversified as a separate lineage. From this common node a sub cluster formed with two Australian species viz., *C. callosus* and *Coranus* sp.$_2$. An almost similar kind of phylogency is revealed by NJ, ME, UPGMA and MP methods (Figure 17,18,19,20). Thus, the affinity between the nine species of *Coranus* from China and that of two species from Australian is well pronounced. Although Liu *et al.* (2009) reported that 28S rRNA is a highly conserved gene and may not be an optimum molecular marker for Coranus, the present analysis contradicts their view and suggests its usefulness in phylogenetics [11].

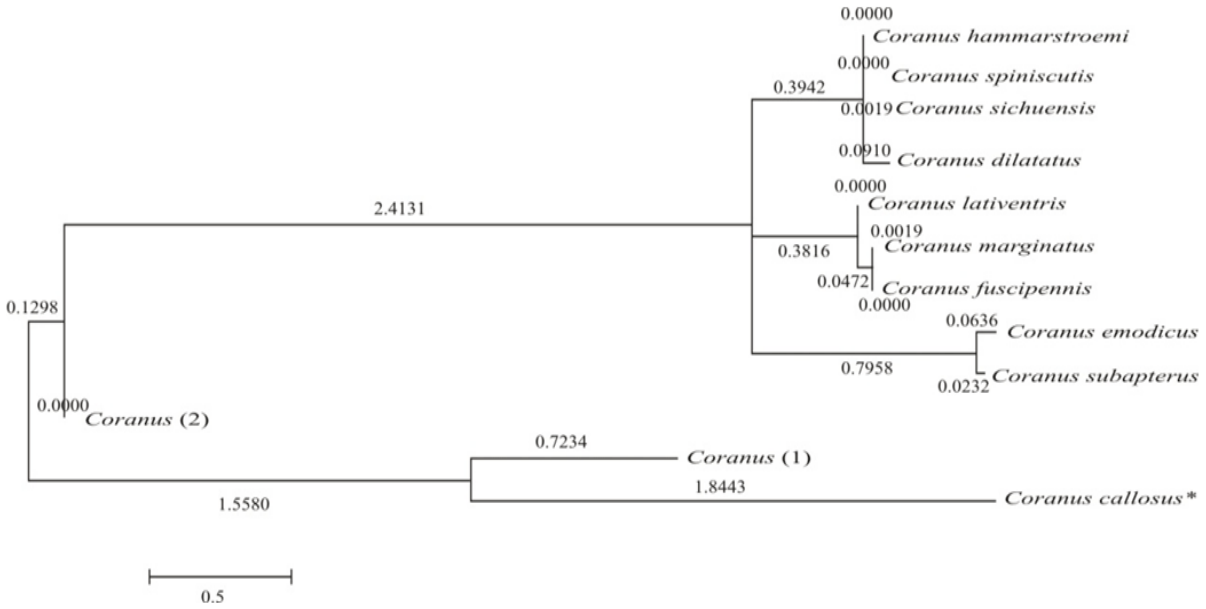

Figure 16: ML tree based on 28S gene variations showing the relationships of 12 *Coranus* species

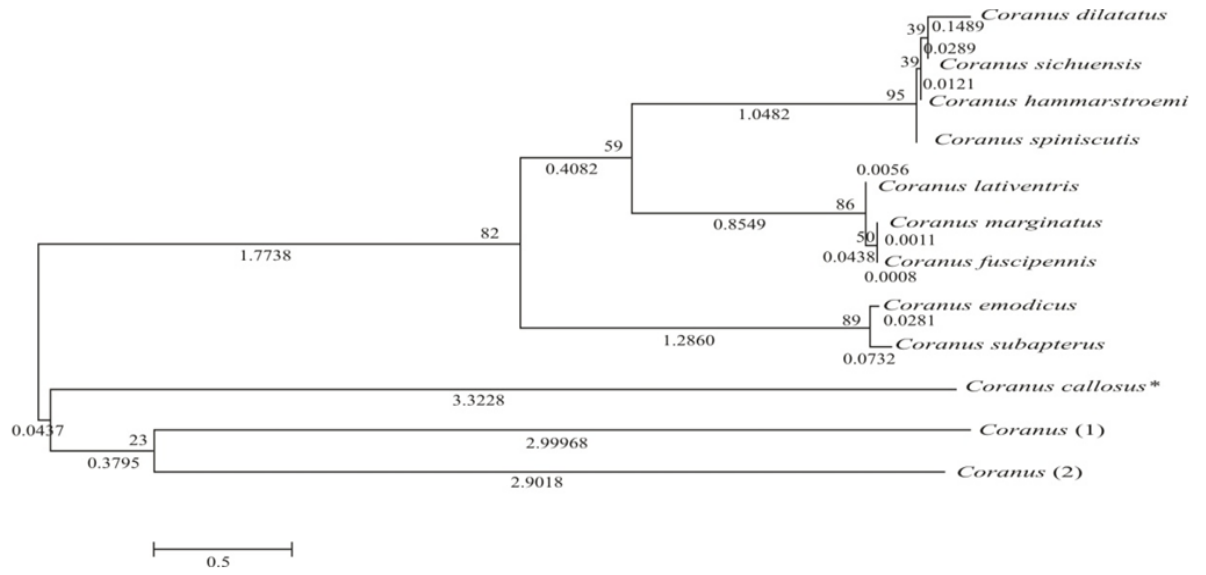

Figure 17: NJ tree based on 28S gene variations showing the relationships of 12 *Coranus* species

Conclusion

The results obtained not only have enriched our knowledge on *Coranus* biosystematics but also supplemented multidisciplinary data of the genus. The results further reveal the utility of mitochondrial gene 16S, Cyt b and Cyt c oxidase subunit I and nuclear gene 28S rRNA sequence in phylogenetic analysis in *Coranus*. The findings further suggest intraspecific and interspecific phylogenetic affinity of *Coranus* species from four countries and three continents. Moreover, the genetic diversity observed among probably two ecotypes of *C. callosus,* from western Australia suggest progression of speciation warranting further studies in this direction that could lead to meaningful revision, regrouping or replacement of species with new revelation through molecular analysis. The analysis further suggests the usefulness of Cyt b fragment as a useful marker to understand the phylogenetics of ecotypes of closely related habitats and that of 28S rRNA as an optimum molecular marker for *Coranus*. However, our sampling of only 13 species of the genus *Coranus* which has more than forty species emphasizes further studies with more species.

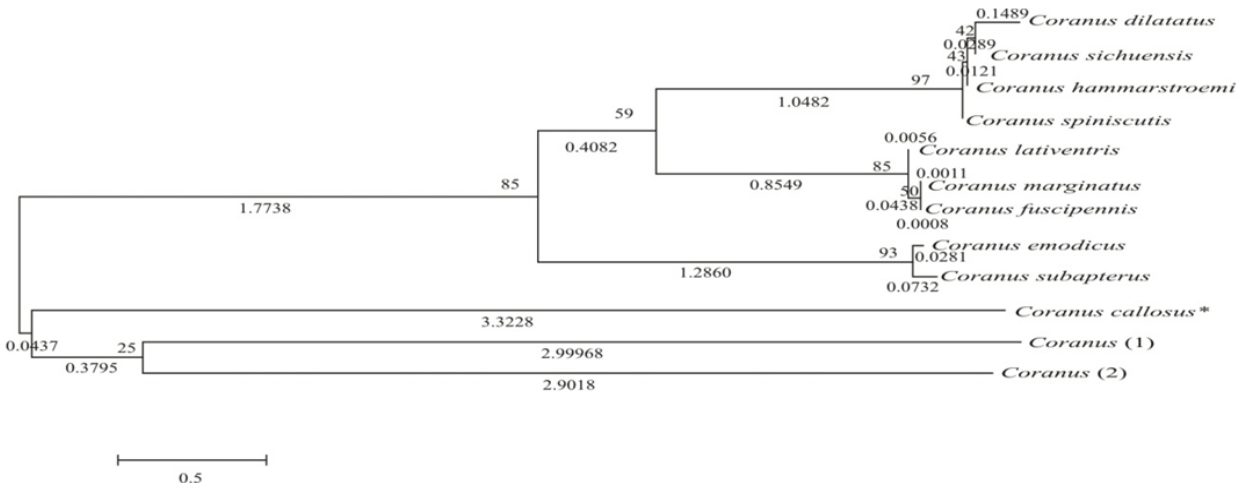

Figure 18: ME tree based on 28S gene variations showing the relationships of 12 *Coranus* species.

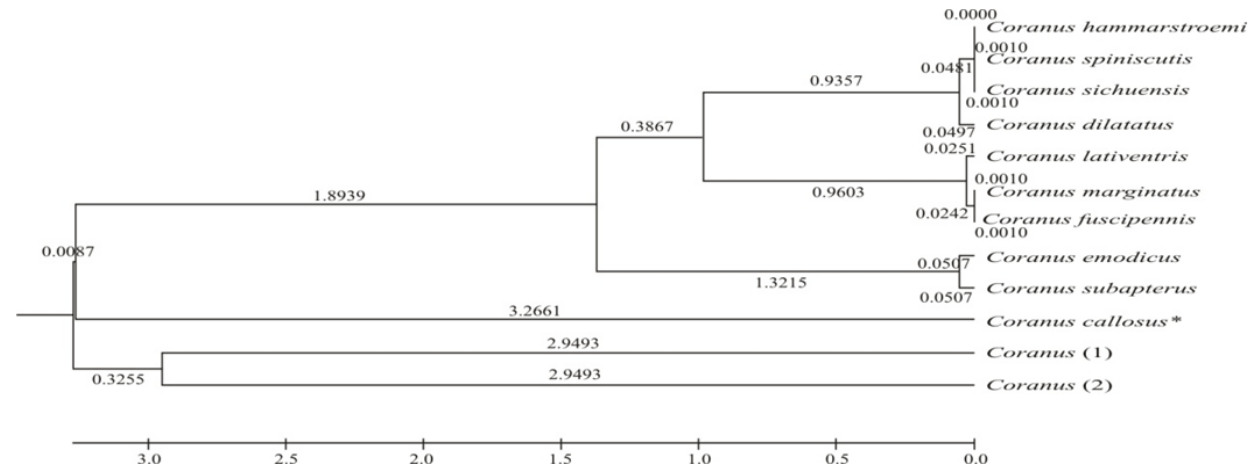

Figure 19: UPGMA tree based on 28S gene variations showing the relationships of 12 *Coranus* species.

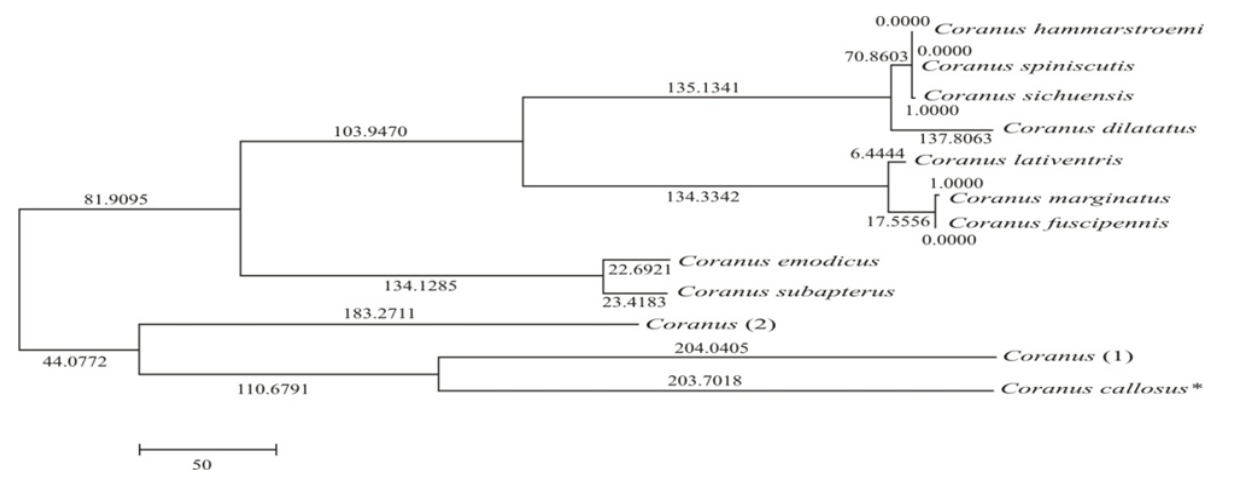

Figure 20: MP tree based on 28S gene variations showing the relationships of 12 *Coranus* species.

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