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An *in silico* assessment of molecular phylogenetic affinities of *Laevicaulis alte* (Gastropoda: Systellommatophora) as determined by partial mitochondrial *COI* sequences

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Abstract
Invertebrate species contribute invisibly and precisely for the perpetual rejuvenation of habitats and hence it is of prime significance to appraise their phylogenetic affinities. The present analysis is emphasized on the molecular phylogenetic relationships of a tropical leather leaf slug *Laevicaulis alte* (Class: Gastropoda, Order: Systellommatophora) recovered from Visakhapatnam, Andhra Pradesh (India) using mitochondrial gene namely COI (Cytochrome oxidase subunit I) and inferred that the chosen species nested within the Veronicellidae taxa and also observed that *L. alte* is more closely related to its conspecifics across the Indian subcontinent. Our *in-silico* observation supported for the monophyly of Veronicellidae taxa.

Keywords: *Laevicaulis alte*, monophyly, veronicellidae, MEGA v6.0, RAxML v1.3

Introduction
Amidst gastropods, land snails and slugs comprise the leading group of species ranging in number between 30000-35000 (Wade *et al*., 2001) [19]. Espouse various lifestyle, habits, and habitats and mostly even in unpleasant seasonal droughts (Krupanidhi, 1984) [11]. Interestingly, *Laevicaulis alte* are pantropical, hermaphrodite and terrestrial slugs that have secondarily lost shell and developed pulmonary cavity (Barker, 2001) [1]. and grouped under Systellommatophora. Solem (1978) [17]. Categorized Veronicellidae within Systellommatophora. Importantly, slugs are one among the best bio-degraders in natural ecosystems (Barker, 1989) [2]. However, the systematic positions of Veronicellids have been inadequately characterized (Klussmann-Kolb *et al*., 2008) [12]. Gomes *et al*., (2010) [8]. Showed a few morphological characters, such as penial apparatus and a female pore on the right hyponotum. In addition to its habitat preferences, the divergence in cytochrome oxidase subunit I gene has been proven to be an adequate procedure to discriminate species of deeper taxonomic affinities between taxa (Remigio and Hebert, 2003) [14]. The nucleotide sequences of mitochondrial genomes are analyzed to unravel the taxonomical and phylogenetic relationship within the class Gastropoda at varying hierarchical levels (Wade *et al*., 2006 [20]. Klussmann-Kolb *et al*., 2008; [12]. Dayrat *et al*., 2011; [4]. Jena & Krupanidhi., 2017) [10]. Strikingly, Dayrat *et al*., (2011) [4]. has analyzed with an exceptional accentuation upon sampling of Onchidiids and Veronicellids and reported that they are strongly supported by monophyly of the clade Systellommatophora. However, Molecular studies concerning Veronicellids are rather rare (Wade *et al*., 2006) [20]. Therefore, in the present analysis an attempt is made using the partial sequence of COI in *Laevicaulis alte* to reinforce its phylogenetic affinities with the members of its clade Systelommatophora.

Materials and Methods
Sample collection and species identification
In the present evaluation, the slug, *Laevicaulis alte* (Figure 1) was used. *L. alte* was sampled from suburbs of Visakhapatnam, A.P., India, situated in Lat.17 41 'N and Long.83 13 'E during winter months i.e., November to December 2016. Soon after washing, slugs were kept in a plastic trough with edible herbs and fed *ad libitum* with *Hydrilla*, *Pistia*, amaranthus leaves, spinach, and lettuce and acclimatized for a week before used for experimental studies. The diagnostic characteristics and morphological markers to identify *L. alte* were as follows: the

body surface of slug was dim grey, practically black coloured with no shell, as a rule with a light yellow middle stripe, 7- 8 cm long. This slug had an exceptional and extremely narrow foot; juvenile specimens had a foot one mm wide and adult specimens had a foot that was merely 4-5 cm wide ventrally. Dorsal view of the slug comprised of with thickly organized and moderately coarse granules giving the surface a velvet-like appearance. The male genital pore was covered up close to the base of the right lower tentacle. The tentacles were small, 2- 3 mm long, the head stay covered up under the notum and the upper tentacles were visible. Slugs preferred to dwell in habitats rich in lowland forest and well-watered gardens.

Kingdom: Animalia
Phylum: Mollusca
Class: Gastropoda
Clade: Stylommatophora
Family: Veronicellidae
Genus: *Laevicaulis*
Species: *Laevicaulis alte*

Fig 1: Image of *Laevicaulis alte* and their taxonomic position

Tissue isolation and genomic DNA extraction

Isolated live slug muscle tissues were washed with distilled water. Total genomic DNA was extracted adopting the phenol-chloroform method described by Sokolov (2000) [15]. The yield of genomic DNA was found to be 2835 ng/µl (Nicklas and Buel 2003) [13].

PCR Amplification

Primers for the partial COI gene designed by Folmer (1994) [5] [5]. were adopted in this study. PCR reaction was performed in Thermal Cycler (Agilent). The components of PCR mixture contained template genomic DNA, gene specific primers, dNTPs, *Taq* buffer, $MgCl_2$ buffer and *Taq* DNA polymerase. The reaction volume for all PCR reactions was set to 25 µl. All reagents required for PCR were purchased from HiMedia, Mumbai, India. The PCR was programmed for 30 runs followed by a final extension for 10 min at 72 ºC. The reaction mixture devoid of a template was run as a negative control. Amplified DNA fragment was separated by gel electrophoresis in 1.2 % agarose gel along with a 2-log DNA ladder as marker and images were taken using Gel Doc system (Mediccare™, Chennai, India).

Phylogenetic analyses

The DNA sequence alignment of selected genera was performed with default parameters in MUSCLE (Edgar, 2004b) using the program MEGA v6.0 (Tamura *et al*., 2013). The resulted aligned sequences in FASTA format were converted to PHYLIP format. The ML (maximum Likelihood) phylogenetic analysis involving the unpartitioned nucleotide sequences were analyzed through RAxML v1.3 software package (Silvestro and Michalak, 2012) [16]. in GTR+ Γ DNA substitution model. The constructed phylogenetic tree nodal support values were obtained by performing with 1000 bootstrap replications

Results and Discussion

The electrophoretic separation of amplified partial COI gene of *L. alte* was shown in figure 2. The eluted band of COI was sequenced and further annotated using ORF finder. The sequences obtained were submitted to NCBI and the given accession number was KY774830. In addition, 15 species/subspecies of Systellommatophoran were retrieved through BLAST based on the available closest sequences of COI obtained from GenBank. The representative species belonging to the superfamilies namely Veronicellidae and Onchidiidae were selected to derive phylogenetic affinities. ML phylogenetic analysis of 16 genera belonging to Systellommatophoran was shown in figure 3 and the derived tree using RAxML tool was rooted on the outgroup Cavoliniidae and Limacinidae (Colgan *et al*., 2000, Haszprunar and Huber, 1990) [3].

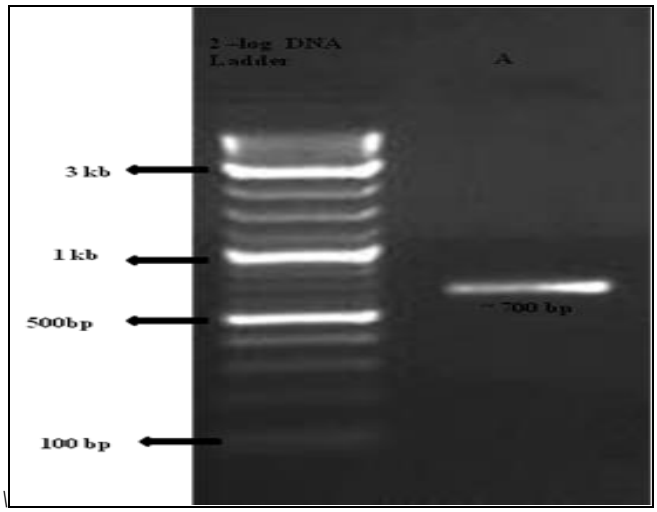

Fig 2: Amplified partial COI gene of *L. alte* (Lane A) shown along with DNA ladder run through 1.2% agarose gel.

The resolution shown by the results of our analyses was to clarify the phylogenetic affinities of the genus *Laevicaulis alte*. The monophyly of Systellmmatophoran at this juncture represented by Veronicellidae and Onchidiidae was strongly supported by RAxML v1.3 software. In furtherance, the nodes in the present cladogram showed low support (Figure 3), which was not surprising that the chosen ingroup taxa were highly divergent from each other. Importantly, the Indian representative genera namely *Laevicaulis alte* was nested within one clade having high bootstrap support with yet another *Laevicaulis* sp. (70%) and *Laevicaulis natalensis* (98%) and the second clade represented by Veronicella, Phyllocaulis, and Vaginulus was supported by a bootstrap value of 70% within Varonicellids. The deeper aspects of taxonomy in the clade Systellommatophoran needed to be explored for the understanding of their monophyly. The partial mitochondrial nucleotide sequence of COI gene of *Laevicaulis alte* confirmed its phylogenetic affinities with Veronicellids taxa and also corresponded to all the chosen

taxa (Figure 3) as described in the gastropodan classification (Guido and Sheila, 2006) [9].

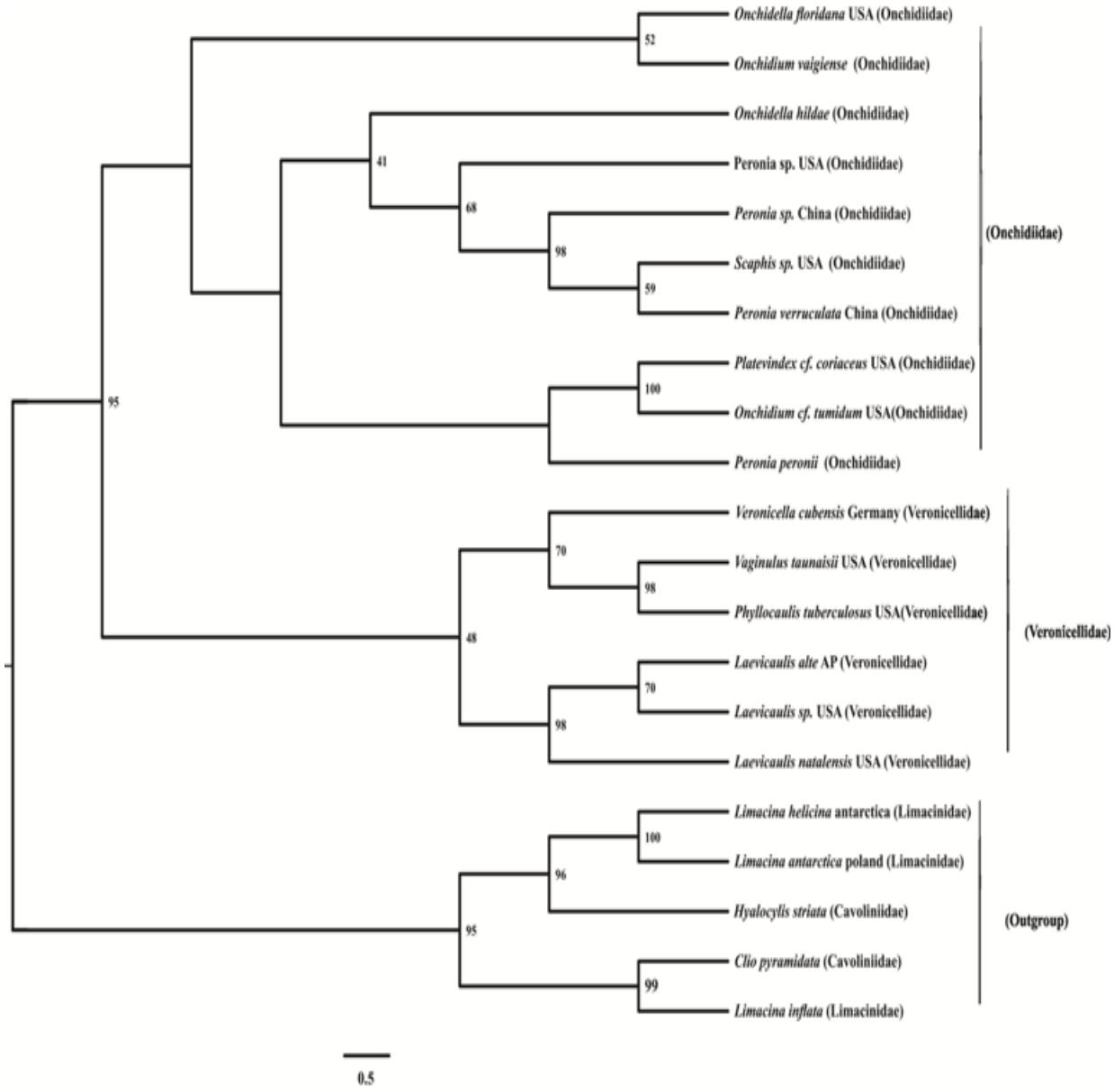

Fig 3: Maximum-likelihood cladogram based on RAxML analysis of the full conscatenated data set of partial gene *COI* of the chosen genera. The bootstrap values above 40% are shown.

Conclusion

This present study has revealed the utility of partial mitochondrial nucleotide sequence of COI gene of *Laevicaulis alte* in evaluating its conspecifics from the Indian subcontinent and the result has showed a strong support to the monophyletic of Varonicellids taxa along with our focal sample nested and branched with conspecifics form other regions of the Indian subcontinent.

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