

1 **Molecular phylogeny of the dung beetle fauna (Coleoptera: Scarabaeidae) of the Western**
2 **Ghats biodiversity hotspot**

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24 Abstract

25 The tribal and generic level phylogeny of Scarabaeinae (Coleoptera: Scarabaeidae) dung beetles
26 have been often debated globally. However, fauna from India has not been a part of these
27 analyses due to lack of data in the databases. We used partial sequences of 16S mtDNA gene of
28 dung beetles collected from different parts of the Western Ghats to examine 1) the tribal
29 positions of Onthophagini, Onitini and Oniticellini and 2) the phylogenetic position of different
30 genera of Onthophagini. We found that Oniticellini nested within Onthophagini, suggesting
31 invalid position of Oniticellini. The non-*Onthophagus* genera of Onthophagini
32 – *Caccobius*, *Cleptocaccobius*, *Milichus* – nested within *Onthophagus*, suggesting that these
33 three genera might be invalid and could be subgenera of *Onthophagus*. Onitini formed a separate
34 clade in the phylogenetic tree. The results suggest for tribal level reclassification of dung beetles
35 as previous studies noted. This study might enrich the molecular data of the Indian dung beetles,
36 which is currently lacking.

37 Keywords: Onthophagini, Oniticellini, Onitini, 16S, phylogeny

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48 The dung beetles belonging to the sub-family Scarabaeinae are cosmopolitan, with 6775 species
49 distributed worldwide¹. They are important biodiversity indicator species with fascinating natural
50 history^{2,3,4}. On the global scale, there is only a little agreement on the biogeographic history of
51 the evolution of dung beetles. For the cosmopolitan distribution of dung beetles, the
52 biogeographic possibilities suggested are, an out-of-Africa hypothesis^{5,6,7} or Gondwanan
53 vicariance and African dispersal for the dung beetle evolution and dispersal^{8,9,10}. The nesting
54 behaviour was relied on for their tribal-level classification^{11,12}. Balthasar^{11,12} classified them into
55 12 tribes; out of these, six were rollers, and the other six were tunnellers. Until recently, 11 or 12
56 tribes have been considered valid^{13,14}. Recent molecular studies were pivotal for reclassifying the
57 dung beetle tribes. At present, sixteen tribes have been considered valid^{14,15,16,17}. They are
58 Ateuchini, Byrrhidiini, Coprini, Deltochilini, Dichotomiini, Endroedyolini, Eucraniini,
59 Gymnopleurini, Odontolomini, Oniticellini, Onitini, Onthophagini, Parachoriini, Phanaeini,
60 Scarabaeini, and Sisyphini^{14,15,16}.

61 Onthophagini is the largest lineage that includes the tunnellers¹⁸. It is believed to be a
62 modern tribe with a recent origin^{18,19}. Oniticellini and Onitini are closely related tribes to
63 Onthophagini^{20,21}. The cosmopolitan genus *Onthophagus* falls under the tribe
64 Onthophagini. *Onthophagus* is considered to be diversified around 23–33 million years ago in
65 the Cenozoic age along with the diversification of mammals^{7,22,23}. However, fossil records and
66 molecular dating studies suggested that they were of Mesozoic origin^{9,21,23}. It is suggested
67 that *Onthophagus* have a possible Afro-tropical origin^{3,22,24,25} and subsequent range expansions
68 into other biogeographical zones^{8,18}. The molecular phylogeny studies, however, suggested
69 multiple dispersal events for *Onthophagus*^{3,18}.

70 Previous studies have suggested polyphyly for Onthophagini^{3,20,21,26}. Emlen *et al.*²²,
71 Philips²⁴, Philips *et al.*²⁷ and Vaz-de-Mello²⁸ have suggested monophyly for this tribe. However,
72 the species in Oniticellini and Onitini used by Monaghan *et al.*³ and Wirta *et al.*²¹ were not used
73 by Emlen *et al.*²² in their study. Tarasov and Solodovnikov²⁵ performed a morphology-based
74 phylogenetic analysis of the ‘*Serrophorus* complex’ of Onthophagini. They have used 91
75 morphological characters of 52 species and several genera of Onthophagini and related tribes.
76 The results were congruent with that of Emlen *et al.*²² and incongruent with that of Monaghan *et*
77 *al.*³ and Wirta *et al.*²¹. Monaghan *et al.*³ has suggested that some basal lineages of the
78 Onthophagini may belong to Onitini or Oniticellini. Breeschoten *et al.*¹⁸ studied the tribal
79 phylogeny by exclusively focusing on *Onthophagus* and found that all the New World
80 Onthophagini formed a monophyletic group.

81 Most of the dung beetle phylogeny studies, either based on morphology or molecular
82 traits, have lacked representative species from the Indian tropics. The lack of sequences from
83 India in the publicly available databases might be a reason for taxonomic impediment. We aim to
84 fill this critical gap through the present study. We used partial sequences of 16S (*rrnL*) gene of
85 dung beetles collected from the Western Ghats biodiversity hotspot to understand the phylogeny
86 of dung beetle tribes. We specifically examined 1) the tribal positions of Onthophagini, Onitini
87 and Oniticellini of the Western Ghats biodiversity hotspot and 2) the position of different genera
88 of Onthophagini. Mitochondrial genes are commonly used as reliable molecular markers to study
89 the phylogeny and biogeography of closely and distantly related taxa among insects^{29,30}.

90 **Methods**

91 Dung beetles were sampled from various sites of Western Ghats in Indian peninsula –
92 Kodagu (12°0.489'N 76°2.279'E and 12°16.108'N 75°38.592'E), Kasaragod (12° 17.14'N 75°

93 15.1'E and 12°8.200'N 75°9.384'E) and Thiruvananthapuram (8°17.512'N 77°6.468'E and
94 8°47.686'N 76°45.902'E). We used cow dung pads for sampling beetles. The collected dung
95 beetles were transferred to 99% ethanol immediately. They were identified to species level using
96 Arrow³¹ and Balthasar^{11,12}. Voucher specimens were maintained and deposited in the
97 Entomology collection of the Central University of Kerala, India.

98 To ensure comprehensive geographical representation, sequences from various
99 geographical regions, namely Afrotropical, Neotropical, Oriental and Palearctic regions, were
100 included in the analysis. They were downloaded from NCBI Genbank (www.ncbi.nlm.nih.gov).
101 Representative species from Onitini and Oniticellini were also included in the
102 analysis. *Aphodius* spp. of the tribe Aphodiini were designated as the outgroup for the study. A
103 total of 79 species were included in the analysis as ingroup, which were seven Onitini, 13
104 Oniticellini and 55 Onthophagini. Four species of *Aphodius* were used for rooting the tree as the
105 outgroup.

106 A total of 38 species from the Western Ghats were included in the analysis: one Onitini,
107 three Oniticellini, 28 Onthophagini and one Aphodiini. The genus *Onthophagus* included a
108 maximum number (47) of species. The detailed list of species included in the analysis, collection
109 data and their Genbank accession number are given in Table 1.

110 Genomic DNA was isolated from the thorax of small species and the hind leg of large
111 species using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Germany) following the
112 instructions provided along with the kit with the following modifications. Tissues were incubated
113 overnight at 56°C in 180 µL of ATL buffer and 20 µL of Proteinase K to completely lyse the
114 tissue. Isolated DNA was quantified, and the quality was checked using a nanodrop

115 spectrophotometer. It was further checked using the 100 bp Invitrogen ladder as control by
116 agarose gel electrophoresis.

117 Approximately 520 bp of the 3' end of 16S ribosomal RNA (*rrnL*) was amplified using
118 the forward primer 16Sar 5'CGCCTGTTTAACAAAAACAT3³² and reverse primer 16SB2
119 5'CTCCGGTTTGAAGTCAATCA3³³. Whenever amplification was not possible with 16SB2,
120 another primer, 16Sb2 5'TTTAATCCAACATCGAGG3³³, was used. The PCR reaction mixture
121 was set up for 27µl with the following components: 12.5µl PROMEGA master mix (2X), 10
122 pmol forward primer, 10 pmol reverse primer, 4µl template DNA and 6.5µl nuclease-free water.
123 The following PCR conditions were applied: Initial denaturation for 3min at 95°C followed by
124 35 cycles of denaturation at 94°C for 30 s, annealing at 43.2°C– 44.3°C for 40 s, and extension at
125 72°C for 1 min followed by a final extension at 72°C for 5 min. DNA was amplified in the
126 Eppendorf master cycle Pro-S. The PCR products were analyzed for their quality and quantity by
127 running 2% agarose gel electrophoresis and using Nanodrop. The amplified PCR products were
128 then purified using Invitrogen's PureLink PCR Purification Kit as per the manufacturer's
129 instructions. Sequencing was performed on the ABI 3730 system using Big Dye Terminator v3.1
130 kit.

131 Data analysis

132 The sequences were compared with the sequences available in the NCBI GenBank by
133 BLAST search (<https://blast.ncbi.nlm.nih.gov>) to confirm the morphological identification.
134 Species identity could be confirmed only up to the genus level for certain species since the
135 sequences of the species used were not available in the database. The sequences' quality was
136 checked using Sequence Scanner Software 2 v2.0 (Applied Biosystems, USA). Only good
137 quality sequences were selected for the analysis. They were edited and aligned using BioEdit

138 sequence alignment editor, version 7.2.6³³, and ClustalW implemented in MEGA v6.06³⁴. The
139 downloaded 16S sequences of dung beetles from other regions available in NCBI and our
140 sequences were used to construct the phylogeny tree. The final dataset included sequences of 411
141 base pairs. We selected the best-fit substitution model according to BIC using the model finder in
142 IQTREE 1.6.12³⁵. The Tree searches were conducted with IQ-TREE 1.6.12³⁶. Maximum
143 Likelihood trees with 1000 ultrafast bootstrap replicates and SH-aLRT test were performed using
144 GTR+F+I+G4 model. The phylogenetic trees were edited using Figtree v1.3.1³⁷.

145 **Results and Discussion**

146 Sequences of 130 samples representing 26 *Onthophagus* species, two *Caccobius*, one
147 *Tiniocellus*, one *Oniticellus*, one *Liatongus*, one *Onitis* and one *Aphodius* species were generated
148 for 16S gene. Nine subgenera of *Onthophagus* were included in the analysis. We found that the
149 tribe Oniticellini nested within the tribe Onthophagini in the phylogenetic trees (Fig.1). The non-
150 *Onthophagus* genera within Onthophagini – *Caccobius*, *Cleptocaccobius*, *Milichus* – also nested
151 within *Onthophagus*. However, *Digitonthophagus* and *Phalops* – the other two non-
152 *Onthophagus* genera in Onthophagini, were distantly placed in a clade. Onitini, with all its
153 genera included in the analysis: *Bubas*, *Cheironitis*, *Heteronitis* and *Onitis*, were distantly placed
154 from Onthophagini+Oniticellini as a separate clade (Fig. 1).

155 In this study, we inquired whether the tribal positions of Onthophagini, Onitini, and
156 Oniticellini on the phylogeny tree remain the same as observed¹⁸ when the species of India – an
157 underrepresented, but important biogeographical region – was included the analysis. To the best
158 of our knowledge, this is the first phylogeny of dung beetles of India.

159 Balthasar^{11,12} classified dung beetles into two distinct subfamilies: Coprinae and
160 Scarabaeinae. The former subfamily included the tribes Coprini, Dichotomini, Phanaeini,

161 Oniticellini, Onitini, and Onthophagini, whereas the latter subfamily included the tribes
162 Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini, and Sisyphini. Our study does
163 not support this morphology-based tribal classification of Oniticellini and Onthophagini as
164 genera of Oniticellini nested within Onthophagini rather than grouping into two branches in the
165 tree. The close relationship of Onitini to Onthophagini and Oniticellini was also supported by
166 several past studies^{3,20,26,27,28}. Meanwhile, a study based on the morphological characters by
167 Tarasov and Génier⁸ showed that Onitini is distantly related to the other two. We obtained a
168 phylogenetic tree in which Onitini formed a separate clade, yet confirming the close relationship
169 to Onthophagini and Oniticellini.

170 We were also interested to know the positions of all the genera of Onthophagini with
171 respect to the genus *Onthophagus* in the phylogenetic tree. The nesting of the genus *Caccobius*
172 within the genus *Onthophagus* questions the separate genus status for *Caccobius*¹⁸. Thus, our
173 study supports Breeschoten *et al.*¹⁸ and proposes a necessary change in the present classification
174 of *Caccobius*. They also found that *Caccobius*, *Cleptocaccobius* and *Milichus* have nested
175 within *Onthophagus* and suggested that Onthophagini might not be monophyletic. We also
176 obtained similar results in the phylogenetic tree. As suggested by Breeschoten *et al.*¹⁸, we
177 recommend that all these factors may be considered while redefining the tribal status. Halffter
178 and Matthews³⁸, Davis *et al.*³⁹ and Philips²⁴ have revised Onthophagini based on morphological
179 data and elevated the following subgenera of *Onthophagus*,
180 *Digitonthophagus* Balthasar, 1959, *Progoderus* Lansberge, *Strandius* Balthasar, *Diastellopalpus*
181 *Lansberge*, and *Euonthophagus* Balthasar^{24,38,39} into separate genera. Together with such
182 changes, the tribal level classification should also be supported by a global analysis with
183 sufficient sampling coverage and nodal support. Although our analysis is restricted to a

184 Maximum Likelihood tree, tracing the evolutionary origin can give more insight into the
185 evolution of dung beetles of the Western Ghats biodiversity hotspot. We caution that the results
186 discussed here are indicative since the study was based on a single mitochondrial marker gene.
187 For conclusiveness, we recommend further explorative studies based on both nuclear and
188 mitochondrial gene markers.

189 Overall, the present study agrees with the suggestions pointed out by earlier molecular
190 findings that the tribal level classification needs revision. This study will enrich the molecular
191 information on Indian dung beetles, which is currently lacking. It also calls for an in-depth
192 phylogeny with all the species reported so far from India.

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304 Table 1. Details of species used for phylogeny based on 16S (*rrnL*) in the study

GenBank					Collected	
accession	Tribe	Genus	Species	Author	from	Region
KU739500	Onthophagini	<i>Onthophagus</i>	<i>longimanus</i>	Bates, 1887	Belize	Neotropic
KU739499	Onthophagini	<i>Onthophagus</i>	<i>nitidior</i>	Bates, 1887	Belize	Neotropic
KU739498	Onthophagini	<i>Onthophagus</i>	<i>rhinolophus</i>	Harold, 1869	Belize	Neotropic
KU739497	Onthophagini	<i>Digitonthophagus</i>	<i>gazella</i>	Frey, 1971	Madagascar	African
KU739491	Oniticellini	<i>Drepanocerus</i>	<i>kirbyi</i>	Kirby, 1828	South Africa	African
KU739490	Oniticellini	<i>Euoniticellus</i>	<i>intermedius</i>	Reiche, 1850	South Africa	African
KU739489	Oniticellini	<i>Helictopleurus</i>	<i>quadripunctatus</i>	Olivier, 1789	Madagascar	African
				Laporte de Castelnau,		
KU739488	Oniticellini	<i>Liatongus</i>	<i>militaris</i>	1840	South Africa	Australian
KU739487	Oniticellini	<i>Oniticellus</i>	<i>egregius</i>	Klug, 1855	South Africa	African
KU739486	Oniticellini	<i>Yvescambefortius</i>	<i>sarawacus</i>	Gillet, 1926	Indonesia	Oriental

KU739485	Oniticellini	<i>Tiniocellus</i>	<i>spinipes</i>	Roth, 1851	South Africa	African
KU739484	Onthophagini	<i>Caccobius</i>	<i>nigritulus</i>	Klug, 1855	South Africa	African
KU739436	Onthophagini	<i>Cleptocaccobius</i>	<i>convexifrons</i>	Raffray, 1877	South Africa	African
KU739481	Onthophagini	<i>Milichus</i>	<i>apicalis</i>	Fahraeus, 1857	South Africa	African
KU739480	Onthophagini	<i>Onthophagus</i>	Near <i>babirusa</i>		Indonesia	Oriental
KU739478	Onthophagini	<i>Onthophagus</i>	<i>haematopus</i>	Harold, 1875	Ecuador	Neotropic
KU739477	Onthophagini	<i>Onthophagus</i>	<i>obscurior</i>	Boucomont, 1914	Indonesia	Oriental
KU739476	Onthophagini	<i>Onthophagus</i>	<i>rorarius</i>	Harold, 1877	Indonesia	Oriental
KU739475	Onthophagini	<i>Onthophagus</i>	sp.	-	South Africa	African
KU739474	Onthophagini	<i>Onthophagus</i>	<i>vulpes</i>	Harold, 1877	Indonesia	Oriental
KU739473	Onthophagini	<i>Phalops</i>	<i>ardea</i>	Klug, 1855	South Africa	African
KU739472	Onthophagini	<i>Onthophagus</i>	<i>bicallosus</i>	Klug, 1855	South Africa	African
KU739471	Onthophagini	<i>Onthophagus</i>	<i>schwaneri</i>	Vollenhoven, 1864	Indonesia	Oriental
KU739469	Onitini	<i>Bubas</i>	<i>bubalus</i>	Olivier, 1811	Spain	Palaearctic
KU739468	Onitini	<i>Heteronitis</i>	<i>castelnaui</i>	Harold, 1862	South Africa	African
KU739467	Onitini	<i>Onitis</i>	<i>alexis</i>	Klug, 1835	South Africa	African
KU739466	Onitini	<i>Onitis</i>	<i>falcatus</i>	Wulfen, 1786	Hong Kong	Palaearctic
KU739431	Onitini	<i>Onitis</i>	<i>fulgidus</i>	Klug, 1855	South Africa	African
KU739430	Onthophagini	<i>Onthophagus</i>	<i>orientalis</i>	Harold, 1868	Cambodia	Oriental
KU739464	Onthophagini	<i>Onthophagus</i>	<i>baolocensis</i>	Ochi and Kon, 2015	Cambodia	Oriental
				Masumoto <i>et al.</i> ,		
KU739463	Onthophagini	<i>Onthophagus</i>	<i>yukae</i>	2002	Cambodia	Oriental
KU739462	Onthophagini	<i>Onthophagus</i>	c.f. <i>taurinus</i>	White, 1844	Cambodia	Oriental
KU739461	Onthophagini	<i>Onthophagus</i>	<i>gracilipes</i>	Boucomont, 1914	Laos	Oriental
KU739460	Oniticellini	<i>Scaptodera</i>	<i>rhadamistus</i>	Fabricius, 1775	Laos	Oriental
KU739459	Onthophagini	<i>Digitonthophagus</i>	<i>bonusus</i>	Fabricius, 1775	Laos	Oriental
KU739426	Onthophagini	<i>Onthophagus</i>	c.f. <i>tragus</i>	Fabricius, 1792	Laos	Oriental
KU739457	Onthophagini	<i>Phalops</i>	<i>barbicornis</i>	Lansberge, 1883	-	African

KU739456	Onthophagini	<i>Onthophagus</i>	<i>ochreatus</i>	D'Orbigny, 1897	-	African
KU739424	Onthophagini	<i>Onthophagus</i>	<i>laticollis</i>	Klug, 1835	-	African
KU739454	Oniticellini	<i>Tragiscus</i>	<i>dimidiatus</i>	Klug, 1855	South Africa	African
KU739453	Oniticellini	<i>Euoniticellus</i>	<i>fulvus</i>	Goeze, 1777	Spain (?)	Palaearctic
KU739450	Onitini	<i>Cheironitis</i>	<i>hoplosternus</i>	Harold, 1868	South Africa	African
KU739452	Onthophagini	<i>Onthophagus</i>	<i>fmietarius</i>	Roth, 1851	South Africa	African
MT913390	Onitini	<i>Onitis</i>	sp.	-	India	Oriental
MT903415	Onthophagini	<i>Onthophagus</i>	<i>andrewesi</i>	Arrow, 1931	India	Oriental
MT903965	Onthophagini	<i>Onthophagus</i>	<i>bifasciatus</i>	Fabricius, 1781	India	Oriental
MT904010	Onthophagini	<i>Onthophagus</i>	<i>bronzeus</i>	Arrow, 1907	India	Oriental
MT904286	Onthophagini	<i>Onthophagus</i>	<i>castetsi</i>	Lansberge, 1887	India	Oriental
MT904662	Onthophagini	<i>Onthophagus</i>	<i>cervus</i>	Fabricius, 1798	India	Oriental
MT904883	Onthophagini	<i>Onthophagus</i>	<i>dama</i>	Fabricius, 1798	India	Oriental
MT905022	Onthophagini	<i>Onthophagus</i>	<i>duporti</i>	Boucomont, 1914	India	Oriental
MT905025	Onthophagini	<i>Onthophagus</i>	<i>fasciatus</i>	Boucomont, 1914	India	Oriental
MT905072	Onthophagini	<i>Onthophagus</i>	<i>favrei</i>	Boucomont, 1914	India	Oriental
MT905073	Onthophagini	<i>Onthophagus</i>	<i>fuscopunctatus</i>	Fabricius, 1798	India	Oriental
MT907290	Onthophagini	<i>Onthophagus</i>	<i>griseosetosus</i>	Arrow, 1931	India	Oriental
MT913524	Onthophagini	<i>Onthophagus</i>	<i>laevigatus</i>	Fabricius, 1798	India	Oriental
MT907292	Onthophagini	<i>Onthophagus</i>	<i>madoqua</i>	Arrow, 1931	India	Oriental
MT907293	Onthophagini	<i>Onthophagus</i>	<i>malabarensis</i>	Boucomont, 1919	India	Oriental
MT907467	Onthophagini	<i>Onthophagus</i>	<i>negligens</i>	Walker, 1858	India	Oriental
MT907468	Onthophagini	<i>Onthophagus</i>	<i>orientalis</i>	Harold, 1868	India	Oriental
MT907469	Onthophagini	<i>Onthophagus</i>	<i>parvulus</i>	Fabricius, 1798	India	Oriental
MT907472	Onthophagini	<i>Onthophagus</i>	<i>quadridentatus</i>	Fabricius, 1798	India	Oriental
MT907474	Onthophagini	<i>Onthophagus</i>	<i>rectecornutus</i>	Lansberge, 1883	India	Oriental
MT907499	Onthophagini	<i>Onthophagus</i>	<i>socialis</i>	Arrow, 1931	India	Oriental
MT908113	Onthophagini	<i>Onthophagus</i>	<i>spinifex</i>	Fabricius, 1781	India	Oriental

MT907514	Onthophagini	<i>Onthophagus</i>	<i>turbatus</i>	Walker, 1858	India	Oriental
MT908115	Onthophagini	<i>Onthophagus</i>	<i>unifasciatus</i>	Schaller, 1783	India	Oriental
MT908191	Onthophagini	<i>Onthophagus</i>	<i>usurpatus</i>	Balthasar, 1959	India	Oriental
MT908233	Onthophagini	<i>Onthophagus</i>	<i>vididus</i>	Arrow, 1907	India	Oriental
MT904654	Onthophagini	<i>Onthophagus</i>	<i>centricornis</i>	Fabricius, 1798	India	Oriental
MW362140	Aphodiini	<i>Aphodius</i>	sp.	-	India	Oriental
MW362138	Onthophagini	<i>Caccobius</i>	<i>aterrimus</i>	Fabricius, 1798	India	Oriental
MW348923	Oniticellini	<i>Tiniocellus</i>	<i>spinipes</i>	Roth, 1851	India	Oriental
MW348916	Oniticellini	<i>Oniticellus</i>	<i>cinctus</i>	Fabricius, 1775	India	Oriental
MW362139	Onthophagini	<i>Caccobius</i>	<i>meridionalis</i>	Boucomont, 1914	India	Oriental
EF656658	Aphodiini	<i>Aphodius</i>	sp.1	-	Madagascar	African
EF656659	Aphodiini	<i>Aphodius</i>	sp.2	-	Madagascar	African
EF656662	Aphodiini	<i>Aphodius</i>	sp.3	-	Madagascar	African
MW348573	Oniticellini	<i>Liatongus</i>	<i>indicus</i>	Arrow, 1908	India	Oriental

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306 Figure legend:

307 Fig. 1. Maximum Likelihood Phylogeny inferred by IQTREE. Numbers beside nodes are

308 IQTREE ultrafast bootstrap and SH-aLRT values. Species of Onthophagini is marked in red and

309 species of Oniticellini is marked in green colour. Species named without region name are

310 sampled from the Western Ghats.

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