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Exploring the diversity of leaf beetles (Coleoptera: Chrysomelidae) on the islands of Vietnam: A survey of Phu Quoc Island, south of Vietnam

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Research Article

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Abstract

Chrysomelidae on the islands of Vietnam has been poorly known. In this study, we investigated the diversity of Chrysomelidae on Phu Quoc Island in Kien Giang province, Southern Vietnam. Specimens were collected from Phu Quoc national park and the buffer zone forest. First, all specimens were ordered into a morph-species operational taxonomic unit (OTU). We collected 52 morphological OTUs of 31 genera and 5 subfamilies, 20 of which were identified as level species. Then, all morphological OTUs were extracted, amplified, and sequenced from the 658 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene. A total of 63 DNA barcode sequences of 13 species and 27 morphological OTUs were successfully sequenced and assigned to 32 Barcode Index Numbers (BIN) in Bold. In the comparison, between morphological OTUs and BINs, the number of OTUs was reduced to 20 species and 24 OTUs (a total of 44 OTUs). The number of species on Phu Quoc Island is estimated to be 1.38x –1. 96x greater. A total of 32 BINs were generated from this study, 30 of which were new to Vietnam and 28 of which were new to BOLD. The results of this study provide the first document of the leaf beetle fauna on Phu Quoc island and the first DNA Barcoding data for Chrysomelidae from this region in Vietnam, as well as additional documents of leaf beetles on islands from another world

Introduction

Chrysomelidae is one of the largest families of Coleoptera, with 35,000–60,000 species in the world (Schmitt 1996; Futuyma 2004; Splipnski et al. 2011; Jolivet 2015). In Vietnam, Chrysomelidae fauna have been studied only in the mainland area of North Vietnam (Tam Dao, Hoa Binh, Ha Nam, and Ninh Binh), several provinces in Middle Vietnam (Quang Binh, Quang Tri, and Thua Thien Hue provinces), and some provinces in the Central Highlands of Vietnam (Medvedev 1987, 2009 b; Dang and Medvedev 1982, 1983; Dang 1989; Tran and Dang 2005a, b; Tran et al. 2006, 2007, 2008). Recently, Nguyen and Gómez-Zurita (2016, 2017) used molecular biology tools to identify 155 species in Nui Chua National Park and described 13 new species from this region. Dcuments for the family Chrysomelidae on islands in Vietnam are poor. The first record of Chrysomelidae on the islands of Vietnam was reported by Medvedev (1992), with ten species from the Con Dao and Cham Islands. Delobel (2008) described two new species on the Phu Quoc island. Skomorokhov (2011) described two new species from the Con Dao and Phu Quoc islands, and he expected that there would be 25 leaf beetle species there, but a checklist was unpublished. Most recently, Nguyen & Bezděk (2021), and Nguyen et al. (2021) described two new species on Phu Quoc island.

To date, 661 DNA barcode sequences for Chrysomelidae in Vietnam have been recorded in BOLD. All the data from Nguyen and Gómez-Zurita (2016) submitted 520 DNA barcode sequences of 829 bp fragments of the COI gene for 155 species in Nui Chua National Park. Nguyen (2020) submitted 16 DNA barcode sequences of 658 bp fragments of the COI gene from 9 species of Chrysomelidae in Vietnam. In addition, several species of Vietnamese flea beetles have been found in Senthil and Srinivasan (2021).

Previous results indicate that the Chrysomelidae fauna on the islands of Vietnam are poorly known, and many Chrysomalid species in Vietnam lack publicly available DNA barcode sequences. Therefore, the objectives of this research were to (1) document the species richness of leaf beetles from the tropical forest on Phu Quoc island and (2) generate DNA barcode data for Chrysomelidae species. The results will be added to the database of Chrysomelidae fauna, including known and unknown species from Vietnam.

Materials And Methods

Study location and sampling strategy.

Specimens of the Chrysomelidae family were collected in July and November 2019, along the sampling paths in the forest Phu Quoc island in two regions: the buffer zone and Phu Quoc national park (along the Bien Phong road). Specimens were collected by three methods: caught directly by hand without collection tools; by sweeping trees by bug-catching net randomly along roads and by beating from low branches and vegetation, reaching as high as the arm's reach, and using sticks to catch beetles that have fallen from the threshing tray. The coordinates of the sampling sites are in Table 1 and Figure 1. The obtained specimens were immediately placed in vials containing absolute ethanol to preserve the DNA, and these vials were labeled with locality, temporal, and collector information for future research

Morphospecies identification: Specimens were sorted into morphological species and used to identify the species levels if possible (Beenen 2010; Bezděk 2009, 2012, 2017, 2019; Borowiec and Świętojańska 202; Hazmi 2012; Kimoto and Gressitt 1979, 1981, 1982; Kimoto 1989, 1998; Konstantinov et al. 2011; Medvedev 1998, 2000, 2003, 2009a, b, 2015; Moseyko 2020; Moseyko and Medvedev 2017; Romantsov 2018, 2020). The morphological species were compared with the identified BINs in BOLD. The taxonomic nomenclature at the family and subfamily levels follows Bourchard et al. (2011), the genus and species levels as in Seeno and Wilcox (1982)

Photographs of the species were taken with a Nikon Ds–Fi3 camera mounted on a Nikon SMZ800N stereo microscope and processed using the NIS–Element imaging software. Helicon Focus 7 software was used to combine the images, which are the same objects at different focal planes.

After being assigned to morphospecies the specimens were stored in 96% alcohol at 4^0 C until DNA extraction. Non-destructive DNA extraction was performed on whole specimens using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The mitochondrial cytochrome c oxidase subunit 1 (cox1) gene was amplified using the primers LepF1 (forward direction) (5'-ATTCAACCAATCATAAGAATTGG-3') and LepR1 (reverse direction) (5'- TAAACTTCTGGATGTCCAAAAAATCA-3') (Hebert et al. 2004) to amplify a 658 base pair (bp) fragment of the *COI* gene. Each PCR reaction mixture contained 2.5 µl of 10x reaction buffer (Evrogen, Russia), 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 1 µl of 25 mM Mg, 2 µl of template DNA, 0.2 µl of thermostable Taq DNA polymerase (Evrogen, Russia), and 17.8 µl deionized water. The PCR protocol used is as follows: initial denaturation at 94°C for 3 mins; 35 cycles of denaturation at 94°C for 30 s; annealing at 42°C for 40 s; elongation at 72°C for 60 s; and final elongation at 72°C for 5 mins. PCR products were visualized via electrophoresis using a 1.5% agarose gel and then purified using ammonium acetate and cold isopropanol. They were sequenced in both directions using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same PCR primers. The DNA-extracted specimens were mounted dry and labeled with a voucher number for future reference at the Institute of Ecology and Biological Resources (IEBR).

Forward and reverse Sanger sequences were assembled, edited, and aligned in Geneious Prime 2019.0.4 (https://www.geneious.com). Sequences were deposited in Genbank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers MW810795-MW810854 and BOLD v. 4 (www.barcodingfife.com, Ratnasingham and

Hebert 2007). Sequences were assigned automatically to Barcode Index Numbers (BINs) in BOLD (Ratnasingham and Hebert 2013). The inter-species and intra-species distances were computed in the MEGA-X software v. 10.2.4. using the p-distance (Kumar et al. 2018).

Species richness estimation: Our leaf beetle survey in Phu Quoc Island is the first in this locality, and there is no reference catalog for the total expected diversity of Chrysomelidae in this region of Southern Vietnam. To assess, even in exploratory terms, our degree of success in sampling local diversity, we applied nonparametric and rarefaction methods (factor 11x) based on incidence data to investigate expected species richness in different localities (in the national park and buffer zone) of the data. A range of species richness estimators was calculated using 100 sample order randomization in EstimateS 9.1 (Colwell 2013). The Chao2 indexes were estimated with bias correction, except when the estimated coefficient of variation in abundance and/or incidence distributions was above a certain threshold (CV > 0.5), and the classic Chao2 index was used instead (Chao, 1987).

Results

A total of 311 leaf beetle individuals were collected in this study, belonging to 44 OTUs (20 OTUs were identified to species level based on morphological characters) in 31 genera and five subfamilies after comparison with the DNA barcode. Of which 84 individuals of 19 OTUs were obtained in the buffer zone and 227 individuals of 33 OTUs were obtained in Phu QuocNational Park (Table 2, Figures 2–9). Most specimens belonged to the Chrysomelidae subfamilies dominant on Phu Quoc island, namely, Eumolpinae (177 specimens, 14 genera, 17 OTUs) and the assemblage of Galerucines (120 specimens, 10 genera, 20 OTUs). Other subfamilies were less frequent: Criocerinae (6 specimens, one genus, one OTU), Cassidinae (4 specimens, 3 genera, and 3 OTUs), Cryptocephalinae (4 specimens, 3 genera, 3 OUTs) (Table 3). In all collected genera, *Dercetina* and *Monolepta* have the highest number of OTU (four OTUs), *Cleoporus* with 3 OTUs; *Nonarthra*, *Pagria*, *Aulacophora*, *Hoplosaenidea*, and *Taumacera*, with two OTUs; other genera only recorded one OUT. Expected species diversity in Phu QuocIsland using the available samples and different partitions of data, with total species richness estimates ranging from 1.38x (ACE) to 1.96x (ICE and Chao2) higher than the number of obtained species (Table 4).

DNA Barcoding

A total of 52 morphological OTUs (20 species and 32 OTUs) were extracted DNA, but only 40 of these were successfully sequenced (13 species and 27 OTUs) and generated 62 barcode compliant sequences and one non-barcode compliant sequence. These 63 sequences were uploaded and assigned to 32 BINs (13 species and 19 of 27 OTUs) (in BOLD, (Table S1). The differences between the number of successfully sequenced OTUs and the number of BINs showed the polymorphic species, which was confirmed by the analysis of DNA Barcoding and reduced from 52 morphological OTUs to 44 OTUs. The number of OTUs agreed upon between the morphological identification and the DNA barcode method is 28 OTUs (63.6%).

Analysing the sequence composition in BOLD showed that the average percentage of the sequences G, C, A, and T was 16.47% (14.21%-18.28%), 18.13% (14.71%-23.2%), 29.78% (27.67%-32.53%), and 35.61% (30.72%-40.25%), respectively. The average percentage of GC content was 34.61% (29.97%–41.47%) and GC was biased at the first codon position with a mean GC content of 44.23% (38.99%–48.80%) (Table S2).

Intraspecific P-distances ranged from 0 to 0.02 with a mean of 0.011 and interspecific P-distances were arranged from 0.02–0.7 with a mean of 0.23 (Table S3 and Table 5).

Discussion

Species-richness of Chrysomelidae on Phu Quoc Island

There have been no previous studies on the species richness of Chrysomelidae from the islands in Vietnam to compare with the current study, but this study can be compared with previous studies in Vietnam in the mainland forest, as Nguyen and Gómez – Zurita (2016) Report 155 species based on DNA barcode sequences in Nui Chua National Park by beating canopy trees; Tran and Dang (2005a) use sweeping method to collect 189 species in Tam Dao National Park; 96 species in Dakrong nature reserve (Tran and Dang 2005b); 115 species in the Muong Phang nature reserve, 86 species in Hang Kia – Pa Co nature reserve, and 45 species in Ba Be national park (Dang and Tran 2004). The low species richness obtained in Phu Quoc Island was due to the difference in environmental conditions and forest type on the island, sampling strategy, and sampling method compared with previous studies (Wagner 2000, Whittaker and Fernnández 2007). The results of this study can be compared with several studies on Islands from another world as 68 OTUs on the west coast Island of Sabah in Malaysia (Yeong et al. 2018) and 47 species on the island of St. Vincent (Peck 2010). The subfamilies Galerucinae and Eumolpinae are dominant on Phu Quoc Island, which is consistent with recent research in the Oriental region (Yeong et al. 2018, Nguyen and Gómez-Zurita 2016, Kimoto 1989). Two species, *Aulacophora indica*, and *Aulacophora lewisii* are agricultural pest species (pumpkin beetle) and were first recorded on the island of Vietnam.

Expected species richness estimators showed that we would have succeeded in sampling 51–72. 5% (depending on the estimator) of the total diversity in the tropical forest on Phu Quoc Island. This is in the same range as that achieved in similar studies of tropical leaf beetle communities, even those using more varied or systematic collection techniques (Niño-Maldonado et al. 2014; Nguyen and Gomez-Zurita 2016).

DNA Barcoding

This study generated 32 BINs in BOLD (19 BIN of unnamed species, 13 BIN of named species). One sequence was not long enough to be assigned to BIN (suppl. Materials 1). 18 BINs were unique, 28 BINs were new to BOLD, and 30 BINs were new to Vietnam (Table S1).

18 unique BINs of six species (*Charaea dinhcuongi* Nguyen (new species described from Vietnam in Nguyen et al. (2021)), *Nonarthra variabilis* Baly (distribution wide in Asia as Lee (2014)), *Tituboea laportei* Baly (distribution in Oriental region as Regalin (1997)), *Hemiplatys pascoei* Baly (distribution in Cambodia and Vietnam (Kimoto and Gressitt 1982)), *Laccoptera (Sindiola) vigintisexnotata* Boheman (distribution in Assam Indiae orientalis as Borowiec and Świętojańska (2021)) and *Mimastra submetallica* Jacoby (distribution in Southeast Asia as Bezděk (2009) and 12 OTUs.

Four species, *Lilioceris egena*, *Aulacophora indica*, *A. lewisii,* and *Aspidimorpha sp.*, were recorded in BOLD with a BIN of each species, but are new records in BOLD from Vietnam. Three species, *L. egena*, *A. indica, and A. lewisii,* were collected from the known geographic distribution. *Aspidimorpha s*p. was recorded in Myanmar.

A. lewisii and *A. indica* are agricultural pests of Cucurbitaceae and have a wide distribution in Asia, not in Bangladesh and Pakistan (Lee and Beenen 2015). Still, in BOLD they were recorded in Bangladesh and Pakistan, which are new localities for these species. *Lilioceris egena* has a wide distribution in Asia (Konstantinov et al. 2011) and species recorded in BOLD were collected from their known geographic distributions.

Four OTUs are polymorphism species: *Dercetina* sp. 1 has four morphological identified OTUs (figs. 9a-h) with mean intraspecific distances of 1.47%, *Hyperaxis* sp. has three morphological identified OTUs (figs. 4c - f) with mean intraspecific distances of 0.82%, *Pagria* sp. 1 and *Dercetina* sp. 2 have two morphological identified OTUs (figs. 6a-d and 9i-m, respectively) with mean intraspecific distances of 0% and 0.89% (respectively). All the intra-species distances are similar in the optimal threshold for molecular identification of Chrysomelidae, with genetic distances below 3% for the species level (Magoga et al., 2018; Papadopoulou et al., 2013). Polymorphism in Chrysomelidea is common and has been reported in previous documents (Flinte et al. 2010; Nie et al. 2012; Benkovskaya and Nikonorov 2016; Yeong 2018; Nahrung et al. 2020 and Lee 2022). The polymorphism in Chrysomelidae is a result of adaptation to the environment, such as plant host*, temperature, and humidity*, and may be under genetic control (De Jong and Nielsen, 1999; Nahrung and Allen, 2005; Strickland et al., 2019).

Conclusions

With 44 OTUs collected from Phu Quoc Island, 24 of which are unnamed species, this indicates that many species have not yet been discovered and that there is a need to expand the investigation diversity of Chrysomelidae on other islands in Vietnam. Of the 32 BINs generated from the study, 30 BINs were new to Vietnam and 28 BINs were new to BOLD, which indicates a severe lack of public sequence databases for Chrsomelidae species from Vietnam. The results of this study led to a better understanding of Chrysomelidae diversity on islands in Vietnam and contributed to the gradual building of a public reference database for Chrysomelidae fauna in Vietnam.

Declarations

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Tables

Table 1. Sampling localities on Phu Quoc Island

Locality	Latitude	*Longitude*	Elevation (m)	Number of specimens
1	10.2396	103.970501	101	14
2	10.25105	103.96997	100	7
3	10.25313	103.950401	50	3
4	10.20949	103.96754	41	8
5	10.19024	104.00132	40	7
6	10.21635	104.01391	40	10
7	10.36534	103.99071	33	6
8	10.37155	103.988012	35	4
9	10.37697	103.982524	35	10
10	10.38655	103.978531	38	15
11	10.33399	103.973889	40	3
12	10.33229	103.97779	38	52
13	10.33267	103.98548	33	13
14	10.33123	103.98812	40	31
15	10.33003	103.99389	50	69
16	10.3266	104.011102	50	5
17	10.33032	104.023185	56	20
18	10.33572	104.04445	58	15
19	10.37222	104.04696	40	19

The localities from 1 to 10 are ecological restoration forest areas on Phu Quocisland (buffer zone), and the localities from 11 to 19 are strictly protected forest areas in Phu Quocnational park.

Table 2 is available in the Supplementary Files section.

Table 3: Summary of the numbers of the subfamilies, individuals, genera, species, and sequences of Chrysomelidae found on Phu Quoc Island

	Subfamily	The number of individuals	The number of genera	The number of species	The number of sequences
1	CASSIDINAE	4	3	3	2
2	CRIOCERINAE	6	1	1	1
3	CRYPTOCEPHALINAE	4	3	3	1
4	EUMOLPINAE	177	14	17	22
5	GALERUCINAE	120	10	20	37
	Total	**311**	**31**	**44**	**63**

Table 4: Abundance-based species richness estimators for Chrysomelidae in Phu Quoc Island

Data	N	Rarefaction (S)	ACE	ICE	Chao1	Chao2	Jack1	Jack2
Phu Quoc Island	44	62.61±7.87	60.69	86.04±0.15	62.22±12.19	86.29±24.16	67.68±6.46	84.13

Figures

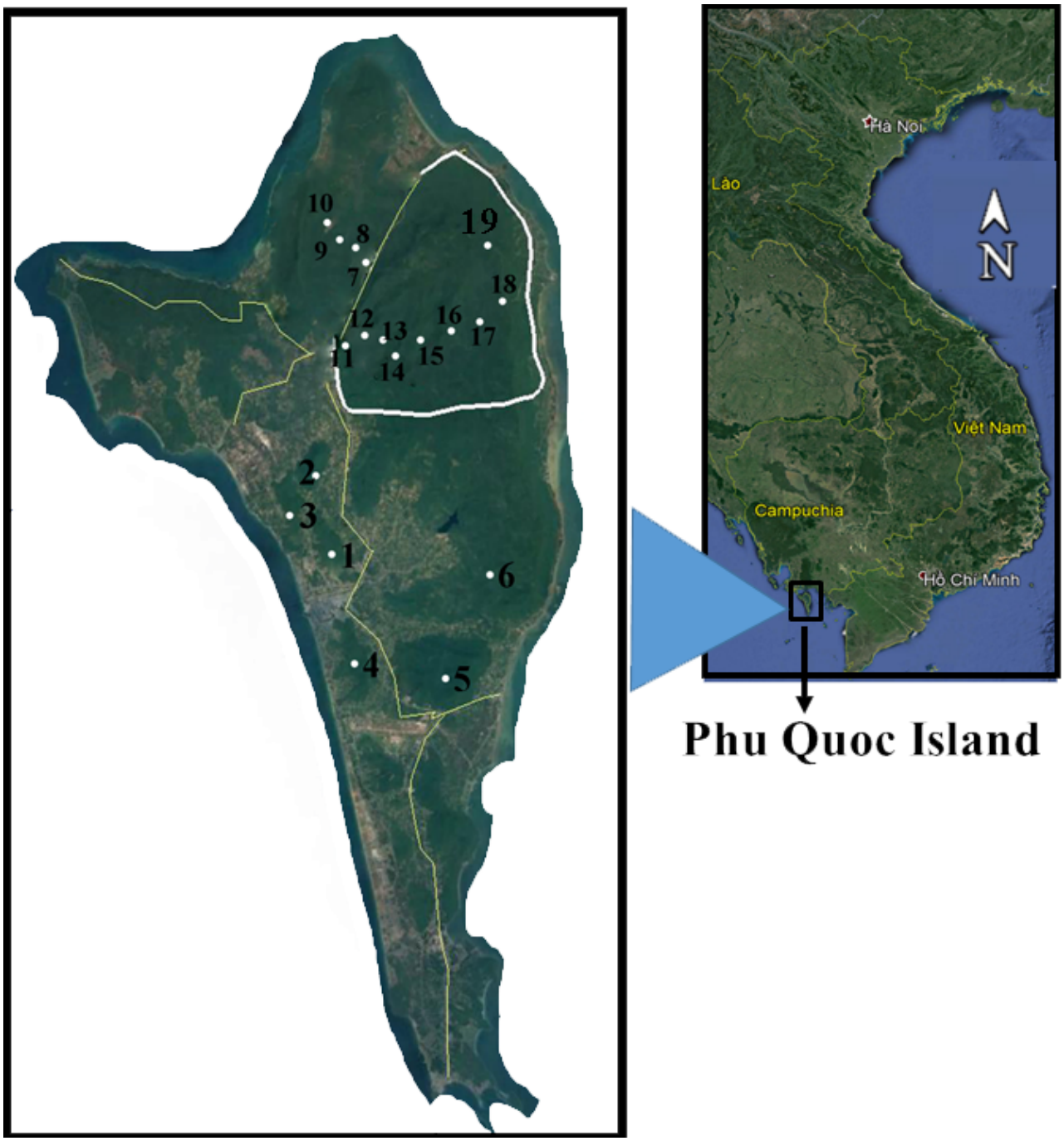

Figure 1

The sampling map of Chrysomelidae on Phu Quoc Island

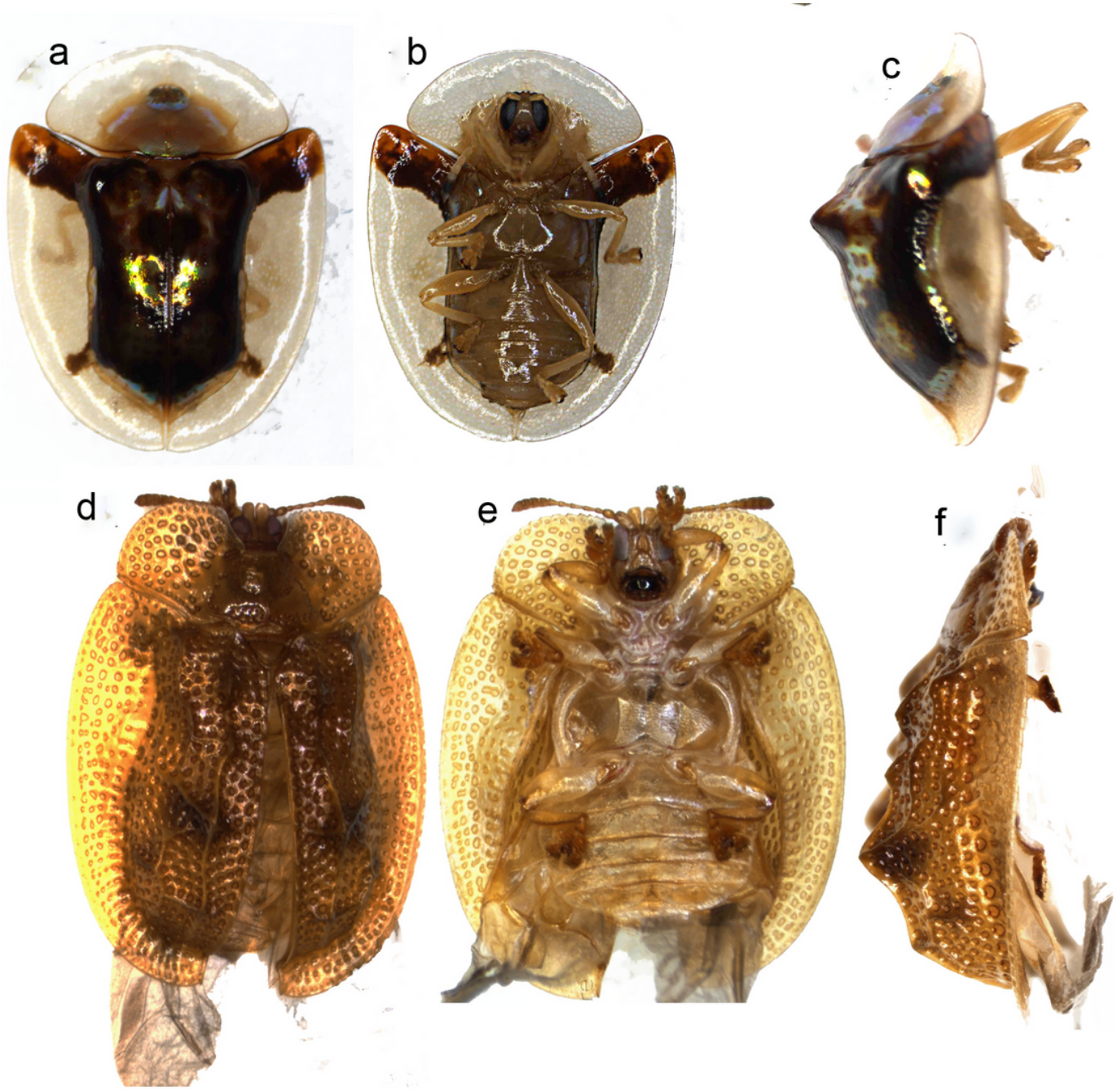

Figure 2

Dorsal and ventral habitus of leaf beetle species. (a-c) *Aspidimorpha* sp.; (d - f) *Notosacantha rufa*

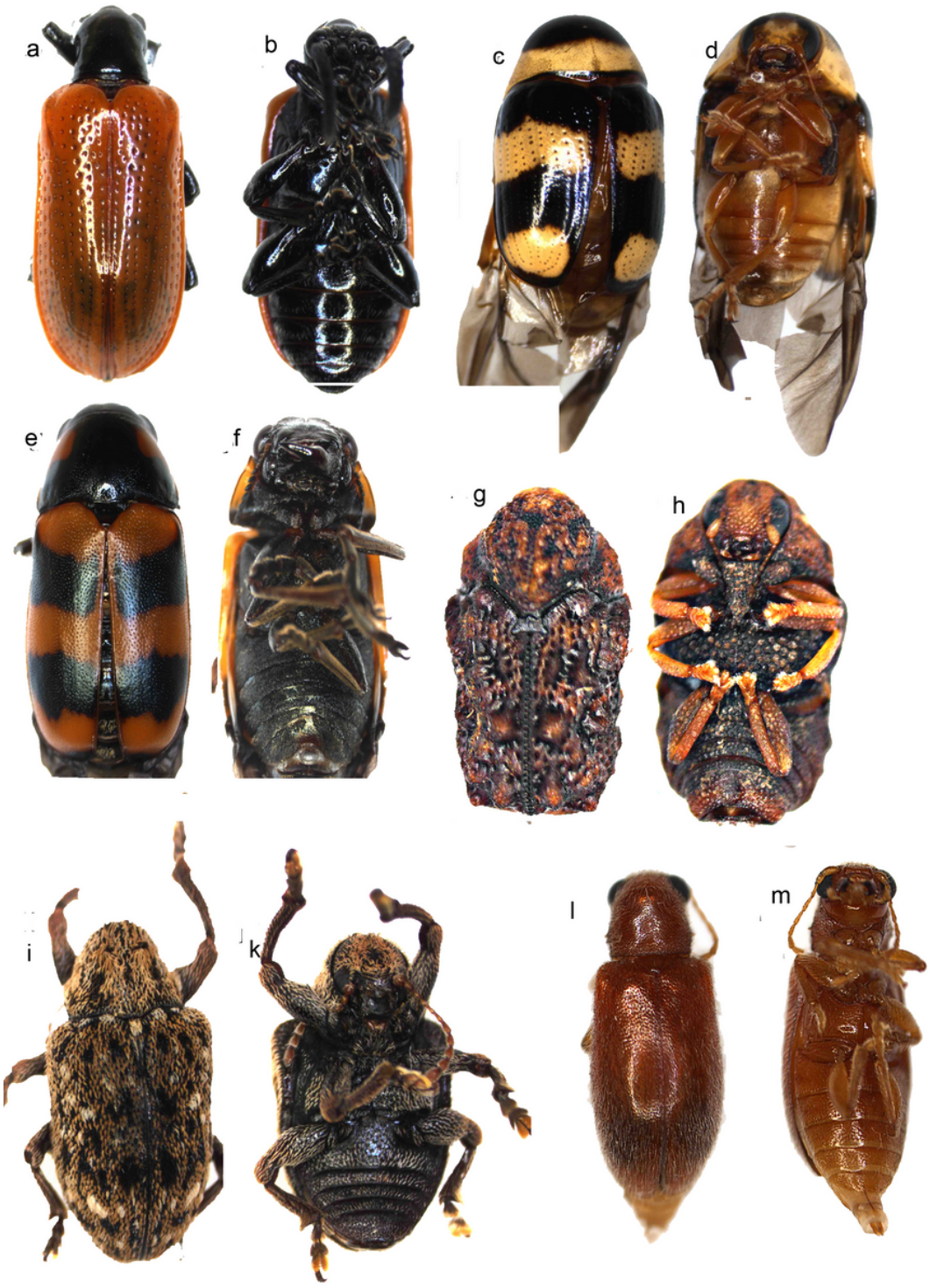

Figure 3

Dorsal and ventral habitus of leaf beetle species. (a, b) *Lilioceris egena*; (c, d) *Cryptocephalus yoshimotoi;* (e, f) *Tituboea laportei*; (g, h) *Chlamisus* sp; (I, k) *Aulacolepis mouhoti*; (l, m) *Aulexis unispinosa*

Figure 4

Dorsal and ventral habitus of leaf beetle species. (a, b) *Hemiplatys pascoei*; (c - f) *Hyperaxis* sp.; (g, h) *Pseudometaxis serraticolis*; (I, k) *Scelodonta granulosa*; (l, m) *Lepina* sp;

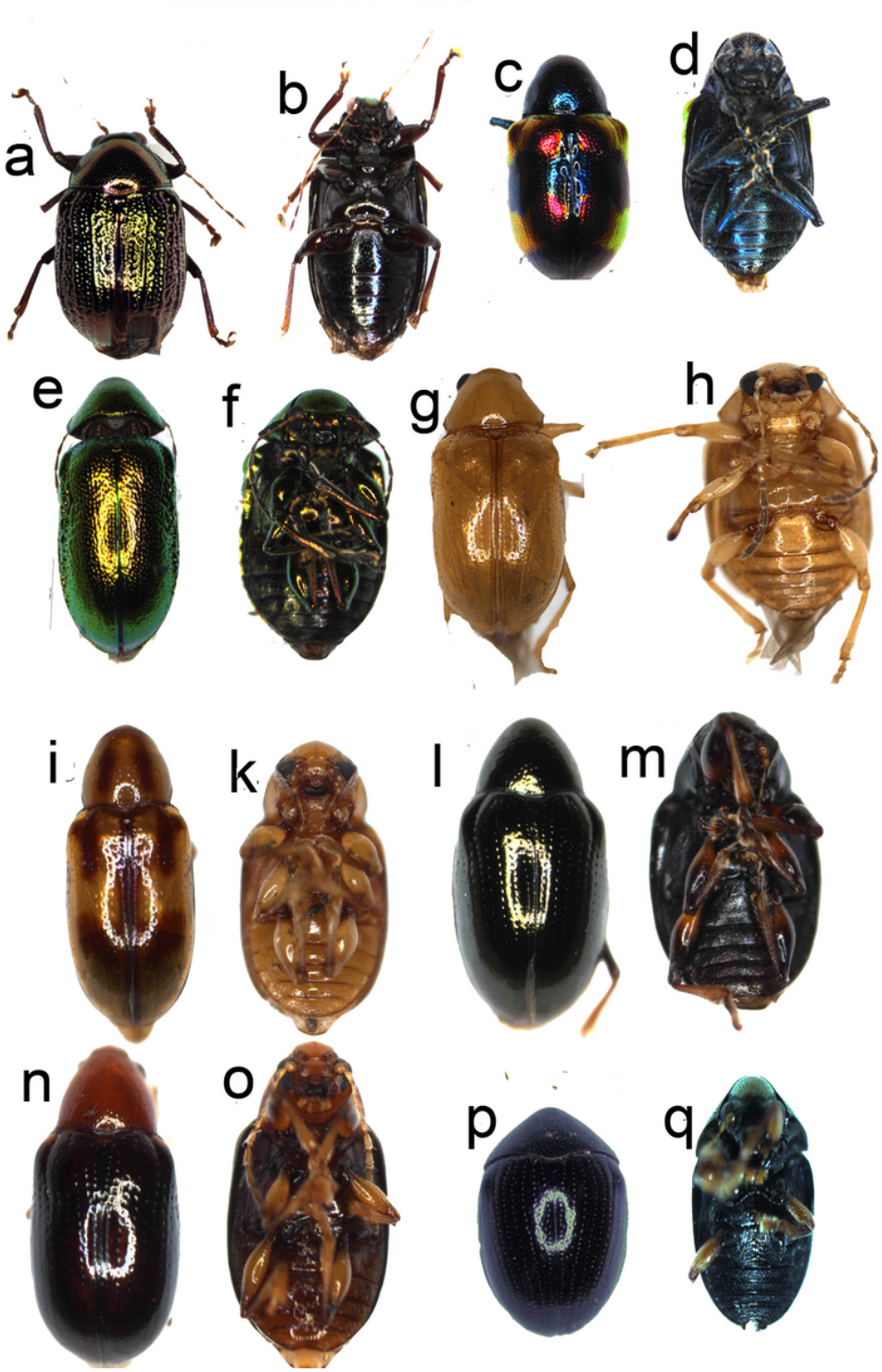

Figure 5

Dorsal and ventral habitus of leaf beetle species. (a, b) *Colaspoides rugulosus*; (c, d) *Platycorynus igneofasciatus*; (e, f) *Colasposoma*sp.; (g, h) *Basileptas*p.; (i, k) *Cleoporus*sp. 1; (l, m) *Cleoporus*sp. 2; (n, o) *Cleoporus*sp. 3; (p, q) *Nodina*sp.

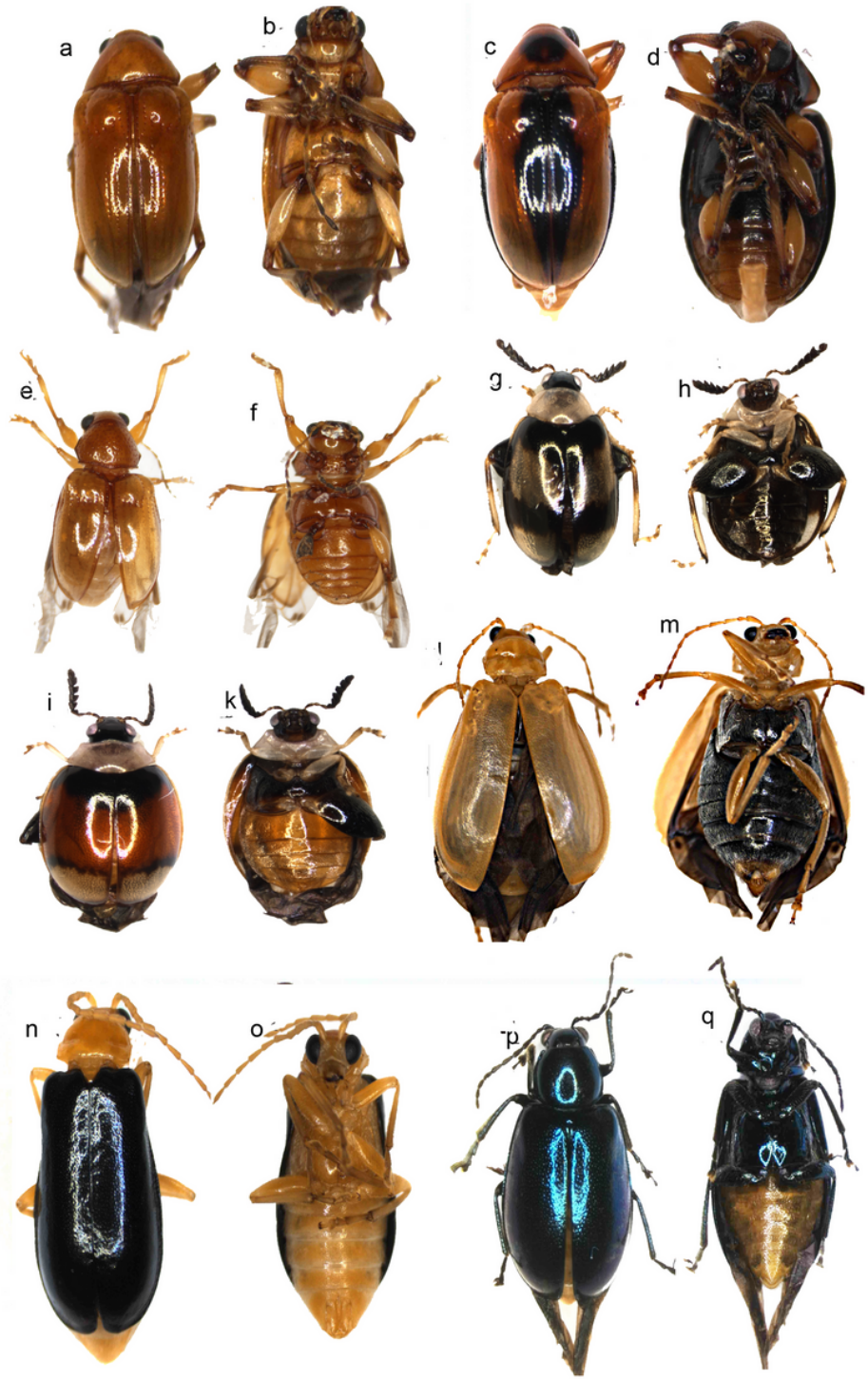

Figure 6

Dorsal and ventral habitus of leaf beetle species. (a - d) *Pagri*sp. 1; (e, f) *Pagria* sp. 2; (g, h) *Nonarthra variabilis;* (i, k) *Nonarthra sp.*; (l, m) *Aulacophora indica*; (n, o) *Aulacophora lewisii*; (p, g) *Charaea dinhcuongi*

Figure 7

Dorsal and ventral habitus of leaf beetle species (a, b) *Haplosomoides curvipes*; (c, d) *Hoplosaenidea* sp. 1; (e, f) *Hoplosaenidea* sp. 2; (g, h) *Mimastra submetallica*

Figure 8

Dorsal and ventral habitus of leaf beetle species. (a, b) *Monolepta wilsoni*; (c, d) *Monolepta* sp. 1; (e, f) *Monolepta* sp. 2; (g, h) *Monolepta* sp. 3; (i, k) *Paleosepharia* sp.; (l, m) *Taumacera phuquoca*; (p, q) *Taumacera* sp.

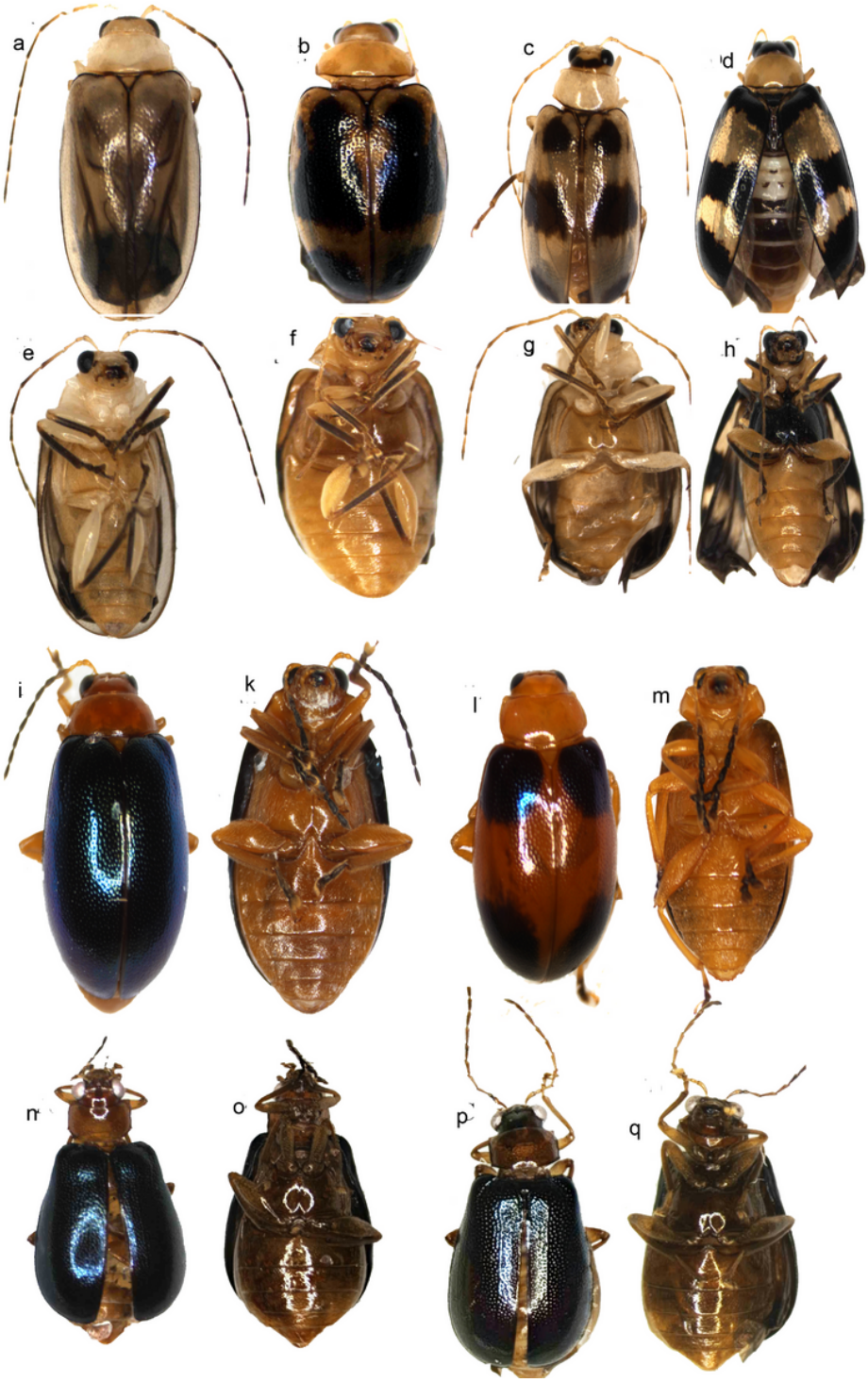

Figure 9

Dorsal and ventral habitus of leaf beetle species. (a- h) *Dercetina* sp. 1; (i - m) *Dercetina* sp. 2; (n, o) *Dercetina* sp. 3; (p, q) *Dercetina* sp. 4;

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.docx
- TableS1binreport.xls
- TableS2SequenceComposition.pdf
- TableS3Intraspecificpdistance.xlsx