Fieldwork to sample microsnails for diet and microbiome studies along the Kinabatangan River, Sabah, Malaysian Borneo

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Introduction

During the past 30 years land snails in snail communities on limestone outcrops in the Kinabatangan River floodplain in Sabah, Malaysian Borneo have attracted the attention from several malacologists, most notably Jaap J. Vermeulen, Menno Schilthuizen, and more recently, Liew Thor-Seng. Much of their research has focussed on taxonomy and systematics (Vermeulen, 1991; Vermeulen, Liew, & Schilthuizen, 2015), evolution and sexual selection (Schilthuizen, Cabanban, & Haase, 2005; Schilthuizen et al., 2006), and biogeography (Schilthuizen, Rosli, et al., 2003; Schilthuizen et al., 2006). Based on published community data (Schilthuizen, 2011; Schilthuizen, Chai, Kimsin, & Vermeulen, 2003) R.S.E. and M.S. hypothesized that these snail communities, with the ecology of the different species seemingly highly similar, might assemble following simple neutral community assembly rules. Such neutral community assembly was described and modelled by Hubbell (2001), where "neutrality" was defined as "ecological similarity in species" from the same

trophic level and living sympatrically. This is thus in contrast to niche-based assembly, where assembly rules are dictated by complex species-specific interactions between individuals (Hutchinson, 1961). It is useful to know if communities indeed follow neutral assembly rules, because neutral theory can make useful, powerful predictions, such as on relative abundances, species-area-relationships, and beta-diversity (Rosindell, Hubbell, & Etienne, 2011).

In 2014 a PhD project was started to study the evolution and ecology of Bornean microsnails, carried out by K.P., and supervised by R.S.E. and M.S. While part of the PhD project is based on empirical community data (snail shells sampled from standardized plots as a proxy for the community abundances) and theoretical analyses of these data, we also set up a project to study possible influences of traits on the community assembly, where K.B. became involved. More specifically, we have started to apply modern metabarcoding techniques that allow the reconstruction of snail diet and gut microbiome based on genetic data (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). With these data we try to answer the question of whether the snail diet and/or microbiome influence the assembly of the species into communities. Or, are these traits that dictate assembly and sustain communities?

Fieldwork & Laboratory Procedures

With the financial aid of an MSL Early Career Research Grant (2017) and two others grants, K.B. and K.P. visited the Kinabatangan River floodplain in November 2017, together with another PhD student (F.J.R.M.) and three master students (J.C.K., H.H.K., and A.E.A.L.). Our goal was to resample plots from which K.P. had gathered community data (shells) during visits in 2015 and 2016, so that we could correlate newly gathered diet and microbiome data to previously gathered community data. A homestay in the small village of Sukau was our base for two weeks. From there, we visited seven different limestone outcrops (figure 1) along the river, travelling by car, boat, and on foot. From each outcrop, samples were taken from three plots (four in a single outcrop, Keruak) along the base of the outcrop, with a between-plot distance of 50 m (sometimes more if not possible otherwise due to too dense vegetation). Plots measured two by two metres (figure 2). We focussed on three target species of unrelated gastropod:

Plectostoma concinnum (Fulton, 1901), *Georissa similis* E. A. Smith, 1893 s.l.*, and *Alycaeus jagori* Von Martens, 1859. Studies using standardised plots along a transect that spans both limestone and non-limestone substrate have shown that the prosobranch microsnail genera *Plectostoma* and *Georissa* tend to occur nearly strictly on limestone (Schilthuizen, Chai, et al., 2003), while *Alycaeus* was found also away from limestone, but in very low numbers (personal observations). We aimed to collect 40 individuals per target species per plot, with a minimum of 20, evenly distributed over the plot. To this end, we subdivided each plot into four quadrants of one by one metre (figure 2), each quadrant sampled for 30 minutes. In addition, we aimed to collect each individual snail from at least 10 cm distance from previous ones of the same species (not always possible for *G. similis* s.l. due to a combination of low numbers and our target number). All other snail species encountered within the plot were collected as well, with a maximum of 20 individuals per species per plot. We conserved samples in 96% ethanol and froze them directly in a styrofoam box filled with ice after the 30-minute search session had ended.

After registration of samples and deposition into the Borneensis collection of Universiti Malaysia Sabah (UMS), Kota Kinabalu, Malaysia, samples

* *Georissa similis* E. A. Smith, 1893 was, until recently, considered a single species, endemic to the Kinabatangan River valley. Hendriks, Alciatore, Schilthuizen, & Etienne (in press), based on phylogeographic studies, suggested that the taxon could best be treated as a species-complex, characterized by high levels of endemism due to many long-distance colonization events. Recent taxonomic research by Khalik, Hendriks, Vermeulen, & Schilthuizen (2019), based on combined phylogenetic and conchological studies, also suggests that the taxon is in fact best treated as a complex of closely related species: *G. flavescens* (found along the Kinabatangan River at limestone outcrops Pangi, Keruak, Tomanggong Besar, and Tomanggong 2), *G. bangueyensis* (widely distributed over northern and eastern Sabah), *G. nephrostoma* (along the Kinabatangan River on outcrops Keruak and Tandu Batu), *G. xesta* (widely distributed over Sabah), and *G. similis* (widely distributed over eastern Sabah). The samples used in current study were identified as general "*G. similis*" only. Because the species complex is composed of closely related, genetically nested species, evolutionarily (and likely ecologically) widely different from all other species in the region (Khalik et al., 2019), we treat the taxon as a species complex here and refer to it simply as "*G. similis* s.l.".

were exported to the Netherlands as a long-term loan. We performed our laboratory work at Naturalis Biodiversity Center, Leiden, the Netherlands, in January and February 2018. In short, we (1) double-checked identities, (2) performed genomic DNA-extractions on the snail gut contents (see figure 3 for an example of how faecal pellets usually are visible when shells are translucent), (3) amplified and sequenced both plant and microbial DNA from the gut using metabarcoding, and (4) identified genetic read data by comparison to benchmark databases. We used general genetic markers that proved to work effectively in metabarcoding studies before: rbcL for the plant diet (Hofreiter et al., 2000) and 16S V3-4 region for the microbiome (Andersson et al., 2008; Liu, Lozupone, Hamady, Bushman, & Knight, 2007). Sequencing was performed on an Illumina MiSeq at BaseClear, Leiden, the Netherlands.

We filtered the resulting read data (removal of chimeras, too short reads, and reads with too low quality) and organized (grouping and counting of identical reads) using the software Qiime2 (Bolyen et al., 2018) in combination with the dada2 tool (Callahan et al., 2016). The identification of genetic read data, also performed in Qiime2, was based on the rbcL seed plant database by Bell, Loeffler, and Brosi (2017) and the 97% 16S GreenGenes database v13.8 (DeSantis et al., 2006). Subsequent analyses of the data are ongoing and performed in R v3.5.0 using package PhyloSeq v1.24.0 (McMurdie & Holmes, 2013).

Preliminary results

We obtained an enormous dataset of both diet and gut microbiome data, which we are still studying at the time of writing. We plan to publish these data, along with analyses in which we correlate these data with community data, within the next few months. Here, we offer a glimpse of what the full output is like.

We collected a total of 1,712 individual snails (excluding empty shells) belonging to 31 different gastropod species (table 1). 893 samples were *P. concinnum*, 267 *G. similis* s.l., and 359 *A. jagori*. *Plectostoma concinnum* was particularly abundant in all but two plots. *Alycaeus jagori*, a rather conspicuous species due to its relatively large size, was usually common, too, but sometimes

absent, such as from outcrop Batangan. *Georissa similis* s.l., at two millimetres the smallest of the three target species, was often difficult to locate, and in 11 out of 22 plots we found less than 10 individuals. It is clear that the three target species, as anticipated, were most abundant.

Sequence data for the diet, based on rbcL reads and after filtering, were obtained for 822 samples, covering 29 species. The full diet metabarcoding dataset consisted of a total of 6.55 million rbcL reads, with mean read number per sample 7,965 ± 8,129 (median 5,544). Sequence data for the gut microbiome, based on 16S V3-4 region reads and after filtering, were obtained for 823 samples, covering the same species as for the diet. The full microbiome metabarcoding dataset consisted of 7.47 million reads, with mean read number per sample 9,072 ± 4,613 (median 8,255). Most metabarcoding data could be classified down to at least the taxonomic level of the family (for plants) or phylum (for microbiome). Some preliminary results for randomly chosen specimens from six selected species, including our three target species, are given in figure 4. We see a diet consisting of multiple plant families for each individual, with a diet richness of up to six plant families for *A. jagori* (excluding the category "unassigned", which is a bin category for all rbcL reads that could be identified as seed plant, but not specifically to a plant family; causes may vary). Not all plant families were retrieved from all specimens, although families like Brassicaceae and Fabaceae were identified in most samples. The microbiome for each specimen usually contains a large proportion of Proteobacteria, while 13 more bacterial phyla were found from the six example specimens.

Discussion

Our study demonstrates the potential of DNA metabarcoding techniques for the study of land snail community ecology and evolution. Direct observations of foraging snails, or a dissection of the gut and visual identification of its contents, are often hardly possible due to the small size of the many snail species in the communities we study. Visual identification of gut content is usually only possible down to broad categories. Instead, we reconstructed the diet using modern next generation sequencing techniques in combination with the latest genetic barcode reference databases for seed plants and bacteria. This allowed

us to collect plant dietary and gut microbial overviews (both from a single individual) for many hundreds of snails in just several months of research (including preparations, fieldwork, laboratory work, and computational analyses). A study of similar size based solely on field observations would have taken tens of years and would likely be far less detailed.

We have created an overview of plant diet for 29 species of land snail, but it is important to be aware of the diet components that our technique cannot pick up, *viz*. all materials without chloroplast genes. Although data are sparse, Barker & Efford (2004) give a diet overview for various pulmonate families. At least for several families also encountered in our study (Ariophantidae, Euconulidae, Rathouisiidae, and Trochomorphidae), it is known that the diet includes also fungi and algae. Hence, to obtain a more complete diet overview, it is suggested to, in future studies, include genetic markers for these groups, too.

One sample worth mentioning specifically is that of the single individual of the rathouisiid *Atopos* sp. (figure 4.1) we collected. This is a genus of carnivorous slugs, in the region mainly preying on *P. concinnum* (Liew & Schilthuizen, 2014). We found its diet to include at least some different plants, which conforms to the description by Van Benthem Jutting (1953), who mentions fungi and plant materials in the diet of *Atopos* (Barker & Efford, 2004). Based on our dataset, though, we cannot know if the plants we encountered in the gut derive directly from a herbivorous or omnivorous diet, or indirectly from eating other, herbivorous animals.

Our study yielded tons of exciting new data. With new knowledge come new questions. The data we present here are just a starting point for looking for geographical and taxonomic patterns, and studying possible correlations with community data.

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Table 1. Overview of collected species during our fieldwork along the Kinabatangan River, November 2017, with totals of non-empty shells per species (*N*). A total of 1,712 specimens were collected, with highest numbers for the three target species (in bold).

	N
NON-PULMONATE SNAILS	
Assimineidae	
Acmella cyrtoglyphe Vermeulen, Liew & Schilthuizen, 2015	5
Acmella striata Vermeulen, Liew & Schilthuizen, 2015	14
Cyclophoridae	
***Alycaeus jagori* Von Martens, 1859**	**359**
Chamalycaeus sp.	4
Japonia kinabaluensis (E.A. Smith, 1895)	3
Japonia sp.	5
Leptopoma pellucidum (Grateloup, 1840)	1
Leptopoma sericatum (Pfeiffer, 1851)	15
Pterocyclos / Opisthoporus sp.	8
Diplommatinidae	
Diplommatina asynaimos Vermeulen, 1993	1
Diplommatina calvula Vermeulen, 1993	3
Diplommatina gomantongensis (E. A. Smith, 1894)	5
Diplommatina rubicunda (Von Martens, 1864)	6
***Plectostoma concinnum* (Fulton, 1901)**	**893**
Plectostoma simplex (Fulton, 1901)	31
Helicinidae	
Sulfurina sp.	15
Hydrocenidae	
Georissa kinabatanganensis Khalik, Hendriks, Vermeulen & Schilthuizen, 2018	33
***Georissa similis* E. A. Smith, 1894 s.l.**	**267**
Georissa nephrostoma Vermeulen, Liew & Schilthuizen, 2015	5
PULMONATE SNAILS	
Ariophantidae	
Everettia sp.	4
Macrochlamys tersa (Issel, 1874)	6
Microcystina appendiculata (Von Moellendorff, 1893)	2
Euconulidae	
Kaliella accepta (Smith, 1895)	7
Kaliella barrakporensis (Pfeiffer, 1852)	2
Kaliella calculosa (Gould, 1852)	4
Kaliella scandens (Cox, 1872)	5
Rathousiidae	
Atopos sp.	1
Trochomorphidae	
Videna froggatti (Iredale, 1941)	1
Videna metcalfei (Pfeiffer, 1845)	4
Videna sp.	2
Valloniidae	
Ptychopatula orcula (Benson, 1850)	1
TOTAL	**1,712**

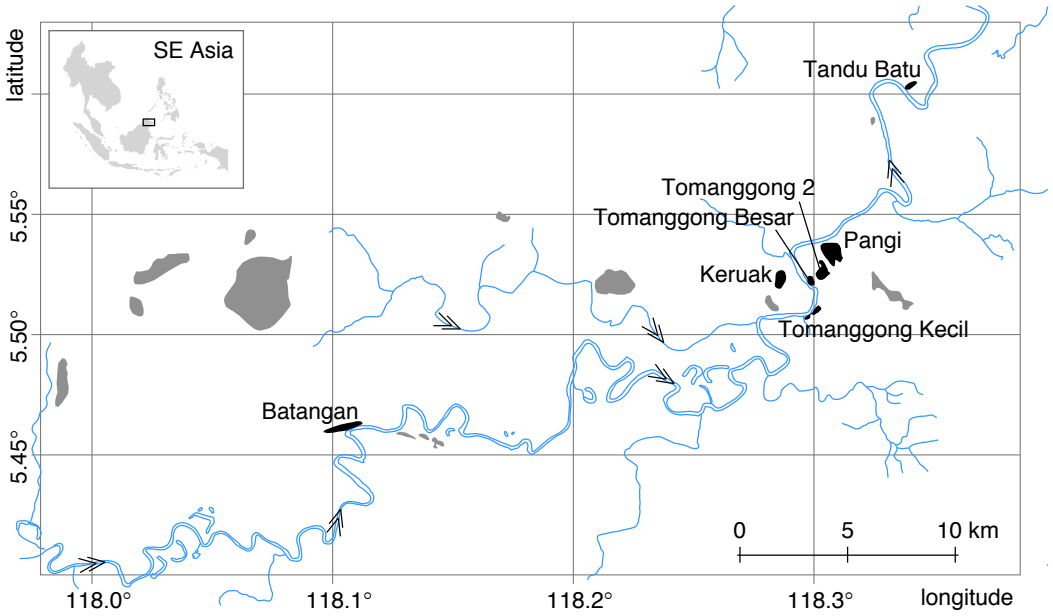
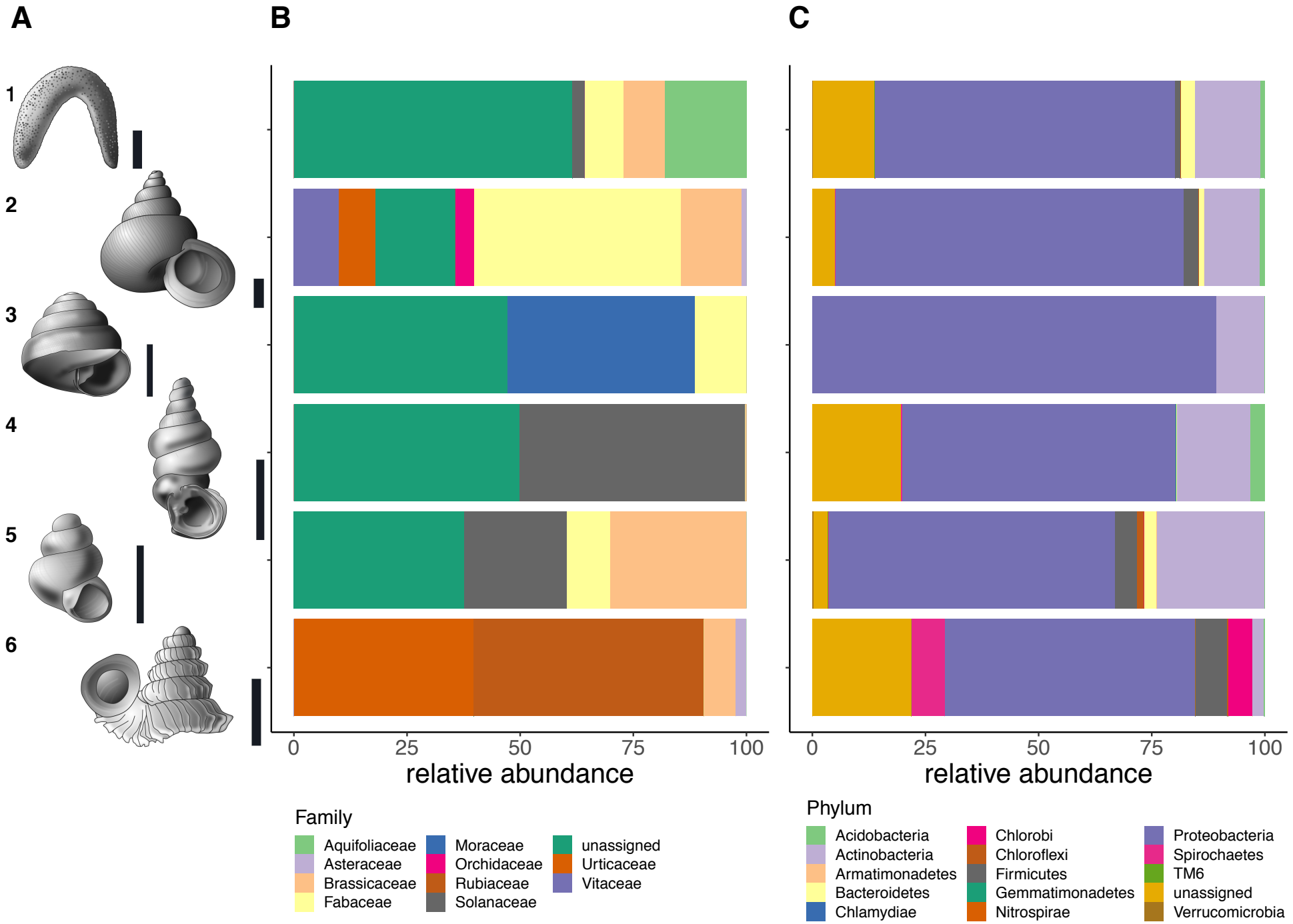


Figure 1. Sampling locations (in black and named) along the Kinabatangan River (in blue), Sabah, Malaysian Borneo. Other limestone outcrops in the region in grey. Each named outcrop was sampled from three different plots (four in Keruak). Inset map © Free Vector Maps.com.

Figure 2. Sampling microsnails from limestone. Plots were defined by a grid (made of rope) of two by two metres, subdivided into four quadrants of one by one metre (highlighted in yellow). Each quadrant was sampled for 30 minutes. Researchers (from left to right): K.B., J.C.K., A.E.A.L., and H.H.K.

Figure 3. Specimen of the euconulid snail *Kaliella calculosa* (Gould, 1852) with faecal pellets in the gut clearly visible through the translucent shell. Such pellets were extracted from the snail in a sterile environment and used for genomic DNA-extraction. The white scale bar equals 1 mm. This is specimen BORMOL13455.01.


A
B
C
1
2
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4
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6
0
25
50
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relative abundance
0
25
50
75
100
relative abundance
Family
Aquifoliaceae
Asteraceae
Brassicaceae
Fabaceae
Moraceae
Orchidaceae
Rubiaceae
Solanaceae
unassigned
Urticaceae
Vitaceae
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
Chlorobi
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospirae
Proteobacteria
Spirochaetes
TM6
unassigned
Verrucomicrobia

Figure 4. Preliminary results of diet and gut microbiome compositions for randomly chosen individuals from six different species. (A) Drawings of species' representatives of the six species: (1) *Atopos* sp. (data are for specimen BORMOL13662.01), (2) *Alycaeus jagori* Von Martens, 1859 (BORMOL13479.02), (3) *Kaliella accepta* (Smith, 1895) (BORMOL13455.01), (4) *Diplommatina calvula* Vermeulen, 1993 (BORMOL13425.01), (5) *Georissa similis* E. A. Smith, 1893 s.l. (BORMOL13410.01), and (6) *Plectostoma concinnum* (Fulton, 1901) (BORMOL13403.01). Drawings by Bas Blankevoort, Naturalis Biodiversity Center. Black scale bars equal 1 mm; (B) Relative distribution of seed plant families found from the diet, based on rbcL read numbers and normalized to 100%; (C) Relative distribution of microbial phyla found from the gut microbiome, based on 16S V3-4 region and normalized to 100%.

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