New tools for diet analysis: nanopore sequencing of metagenomic DNA from rat stomach contents to quantify diet

Nikki E. Freed, William S. Pearman, Adam N. H. Smith, Georgia Breckell, James Dale, Olin K. Silander

Addresses of all authors: School of Natural and Computational Sciences, Massey University, Auckland 0745, New Zealand

4. Corresponding authors: Olin K. Silander, School of Natural and Computational Sciences, Massey University, Auckland 0745, New Zealand, olinsilander@gmail.com, +64 9 213 6618; Nikki E. Freed, School of Natural and Computational Sciences, Massey University, Auckland 0745, New Zealand, freednikki@gmail.com, +64 9 213 6639

Abstract

Accurate determination of animal diets is difficult. Methods such as molecular barcoding or metagenomics offer a promising approach, allowing quantitative and sensitive detection of different taxa. Here we show that rapid and inexpensive diet quantification is possible through metagenomic sequencing with the portable Oxford Nanopore Technologies (ONT) MinION. Using an amplification-free approach, we profiled the stomach contents from 24 wild-caught rats. We conservatively identified diet items from over 50 taxonomic orders, ranging across nine phyla, including plants, vertebrates, invertebrates, and fungi. This highlights the wide range of taxa that can be identified using this simple approach. We calibrated the accuracy of this method by comparing the characteristics of reads matching the ground-truth host genome (rat) to those matching diet items, and show that at the family-level, taxon assignments are approximately 97.5% accurate. Some inaccuracies may arise from database biases; we suggest a way to mitigate for database biases when using metagenomic approaches. Finally, we implemented a constrained ordination analysis and show that we can identify the sampling location of an individual rat within tens of kilometres based on diet content alone. This work establishes proof-of-principle for long-read metagenomic methods in quantitative diet analysis. We show that diet content can be quantified even with limited expertise, using a simple, amplification free workflow and a relatively inexpensive and accessible next generation sequencing method. Continued increases in the accuracy and throughput of ONT sequencing, along with improved genomic databases, suggests that a metagenomic approach for quantification of animal diets will become an important method in the future.

Keywords: Metagenomics, nanopore, diet analysis, rat, next generation sequencing

Background

Accurate quantification of animal diets can yield critical insights into ecosystem and food web dynamics. However, unbiased and sensitive assessment of diet content is difficult to achieve. This is largely due to the limited accuracy of many current methods. Such methods include visual inspection of gut contents (1,2), which presents bias against items are most easily degraded (for example, soft-bodied species); stable isotope analysis (3,4), which yields only broad information on diet, such as whether diet items are terrestrial or marine in origin (5,6); and time-lapse video (7,8), for which species identification is difficult for small prey items or in low-light conditions.

To circumvent these issues, DNA-based methods (9,10) have become popular. Perhaps the most widely applied DNA-based method is metabarcoding. This approach relies on PCR amplification and sequencing of conserved regions from nuclear, mitochondrial, or plastid genomes (9). With adequate primer selection, this method can detect a wide range of species, and does not require specific expertise, which is often necessary for other methods.

However, DNA metabarcoding is not free from bias: PCR primers must be specifically tailored to particular sets of taxa or species (11). Although “universal” PCR primer pairs have been developed (for example targeting all bilaterians or even all eukaryotes (12), all primer sets exhibit bias towards certain taxa. Five-fold differences in fungal operational taxonomic units (OTU) estimates have been found when using different sets of fungal-specific PCR primer pairs (13). It has also been shown that published universal primer pairs are capable of amplifying only between 57% and 91% of tested metazoan species, with as few as 33% of species in some phyla being amplified at all (e.g. cnidarians)(14). Different genomic loci from the same species can exhibit up to 2,000-fold differences in DNA concentration, as inferred using qPCR (15). The choice of polymerase can also bias diversity metrics when using metabarcoding (16). For these reasons, an approach that circumvents PCR and thus avoids these biases is desirable.

Metagenomic sequencing aims to directly sequence all of the DNA in a sample without introducing bias. Although there are still biases with this approach, for example due to nucleotide content affecting the likelihood of a molecule being sequenced, these are inherently less than those introduced by metabarcoding. Metagenomic approaches have most frequently been used to yield insights into microbial diversity and function (17–24), while metagenomic applications aimed at eukaryotic taxa identification are less common. Several metagenomic diet studies have implemented filtering steps to select only mitogenomic sequence or metabarcode regions, or have used abridged databases before data analysis in order to mitigate database biases (25–27). However, to our knowledge, very few studies have used unfiltered metagenomic sequence analysis to infer diet (30,31).

Here, we establish a proof-of-principle methodology to accurately classify metagenomic sequences from eukaryotic taxa and determine diet content using low-accuracy, long-read sequencing, Oxford Nanopore (ONT). Toward this aim, we quantified rat diets from several locations in the North Island of New Zealand using stomach samples. Using these samples and methodology provides three distinct advantages.

First, rats are extremely omnivorous. As such, they serve as an excellent means to quantify the breadth of taxa that can be detected using a metagenomic long read approach.

Second, the use of stomach samples means that a significant number of reads will be host reads. This allows us to assess the characteristics of true positive sequence reads (rat-derived reads that match rat database sequences), as well as false positive reads (rat-derived reads that match non-rat database sequences). We can then determine whether reads matching diet items have similar characteristics to known true positive reads. This use of host reads is exactly analogous to feeding the rats a diet of known content (i.e. rat) and testing whether the contents of the known diet can be accurately identified.

Third, quantifying rat diets has important ecological implications. It is well-established that the relatively recent introduction of mammalian predators to New Zealand has had significant negative effects on many of the native animal populations. This ranges from insects (34), to reptiles (35), to molluscs (36), to birds (37,38), with downstream effects on terrestrial and aquatic ecosystems (39). To counteract the effects of mammalian predators, an ambitious plan is currently being put into place that aims for the eradication of all mammalian predators from New Zealand (including possums, rats, stoats, and hedgehogs), by 2050 (http://www.doc.govt.nz/predator-free-2050; (40). A useful step toward this goal would be to prioritise the management of predators and establish in which locations native species experience the highest levels of predation. To do so requires establishing the diet content of local mammalian predators.

Importantly, using DNA sequencing methods to profile diet does not require high depth to accurately profile the breadth of diet items consumed by an animal. Here we aim to quantify all diet items present in the diet at 1% or more. At this fraction, if we assume that read counts are Poisson distributed, with only 2,000 reads, 99% of the time we will quantify such items within 2-fold of their true amount. Thus, diet quantification presents a clear example of a situation in which sequencing depth is not a critical factor. This contrasts with microbiome profiling, in which there may be very rare taxa (e.g. present at 0.01% frequency) that nevertheless have considerable effects on phenotype of community function.

Results

DNA sequencing

We selected eight rats from each of three locations near Auckland, New Zealand for diet quantification. Each location comprised a different type of habitat: undisturbed inland native forest (Waitakere Regional Parklands, WP); native bush surrounding an estuary (Okura Bush Walkway, OB); and restored coastal wetland (Long Bay Regional Park, LB). We isolated DNA from whole homogenised stomach contents from each rat (see Methods). We sequenced these DNA samples on two dates by multiplexing the samples, obtaining a total of 82,977 reads (January 2017) and 96,150 reads (March 2017). These numbers are not far below expectations given the flow cell and kit chemistry and MinKNOW software versions available at that time (47). However, these read numbers are considerably below those expected for current ONT flow cells and software, which has improved per flow cell output more than 100-fold above these numbers.

After de-multiplexing the reads, we found large variation in the numbers of reads per multiplex barcode: approximately 10-fold for the January samples, and up to 40-fold in March (**Fig. 1A** and **1B**). We hypothesise that this is due to the highly variable quality of DNA in each sample. This did not appear to have strong effects on read accuracy, as the median quality scores per read ranged from 7-12 (0.80 - 0.94 accuracy) for both runs. This quality is theoretically sufficient for accurate inference of taxa at the Family or even Genus level (32); we investigate this accuracy in analyses below.

The degradation of the DNA during digestion in the stomach, as well as fragmentation during DNA isolation (48) and sequencing library preparation led to relatively short median read lengths of 606 bp and 527 bp for the January and March datasets, respectively (**Fig. 1C**). However, there was wide variation in length, with almost 10% of all reads being longer than 1200 bp. Notably, reads of this length can allow more precise taxonomic identification than accurate shorter more accurate reads (e.g. Illumina) (32).

Assignment of reads to taxa

To quantify diet contents we first BLASTed all sequences against a combined database of the NCBI nt database (the partially non-redundant nucleotide sequences from all traditional divisions of GenBank excluding genome survey sequence, EST, high-throughput genome, and whole genome shotgun (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)) and the NCBI other_genomic database (RefSeq chromosome records for non-human organisms (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)). We used BLAST as it is generally viewed as the gold standard method in metagenomic analyses (50). Of the 133,022 barcoded reads, 30,535 (23%) hit a sequence in the combined nt and other_genomic database at an e-value cut-off of 1e-2.

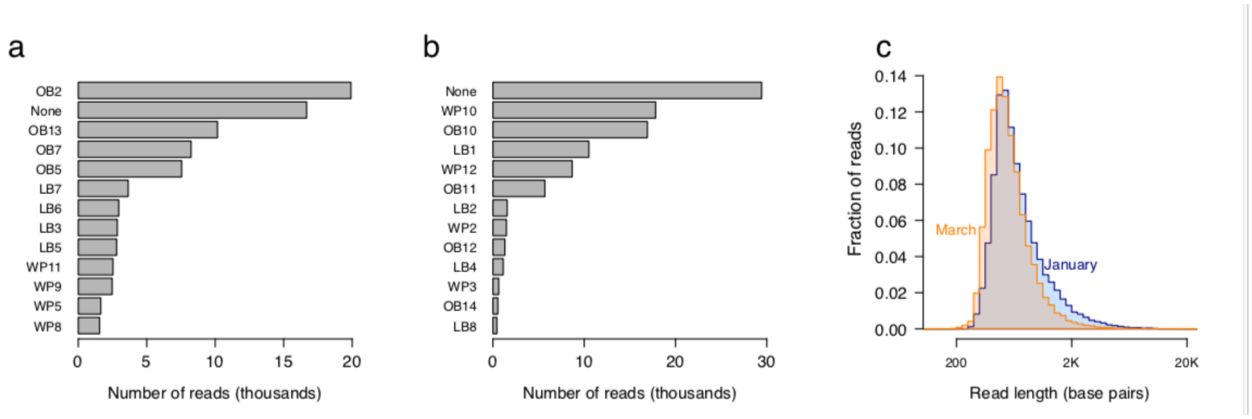


Fig. 1. Run statistics of nanopore metagenomic sequencing of rat stomach contents. (A) and (B) Barcode distributions for January and March runs, respectively. We multiplexed the samples on the flow cells, using 12 barcodes per flow cell. The distribution of read numbers across barcodes varied by up to 40-fold. 20% (January) and 30% (March) of all reads could not be assigned to a barcode ("None"). The inability to assign these reads to a barcode is due primarily to their lower quality. **(C) Read length distribution for January and March nanopore runs.** 90% of the reads were between 350 bp and 1,580 bp in length, with only 0.55% being longer than 4,000 bp.

We first aimed to assess the quality of these hits. We found a bimodal distribution of alignment lengths and e-values (**Fig. 2A**). We also noticed that mean read quality had substantial effects on the likelihood of a read yielding a BLAST hit, with almost 40% of high accuracy reads (greater than 92%) having hits in the March dataset, compared to

1% of low accuracy reads (less than 75% accuracy; **Fig. 2B**). We found the same pattern in the January dataset, although to a lesser extent.

We hypothesized that many of the short alignments with high e-values were clear false positives given the reported taxa. We thus first filtered the BLAST results, only retaining hits with e-values less than 1e-20 and alignments greater than 100 bp. To give some intuition for this length cut-off, a 100bp read with five 1 bp indels and seven mismatches (88% identity) would have an identical raw score and e-value (given the default match, mismatch, and gap parameters) to a single-end 70 bp Illumina read with a 100% identical database match. Similar or quality filters based on length and identity have been imposed previously (30). A total of 22,154 hits passed this e-value filter.

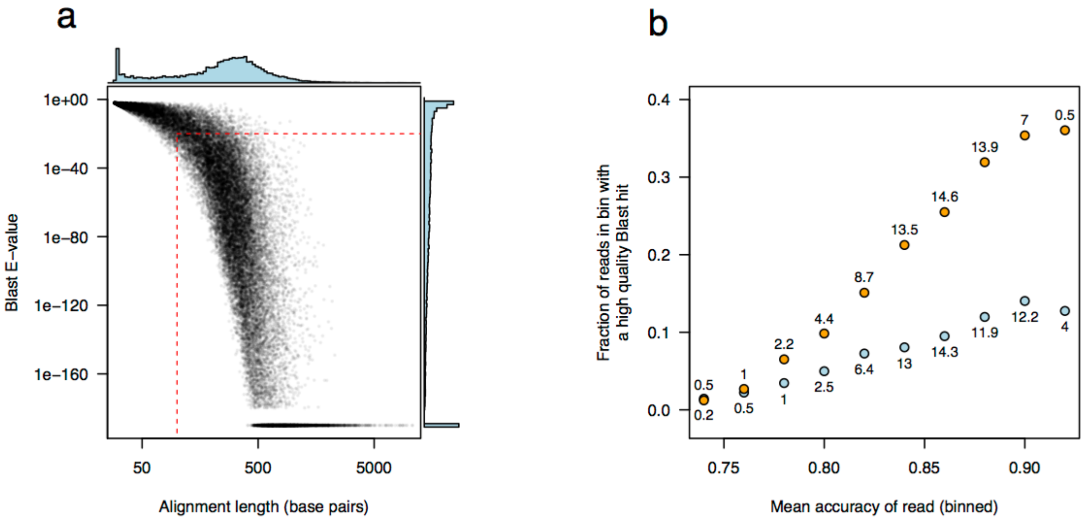


Fig. 2. BLAST hits of metagenomic reads. (a) Alignment lengths and e-values were bimodally distributed. The y-axis is plotted on a log scale, with zero e-values suppressed by adding a small number (1e-190) to each e-value. The horizontal red dotted line indicates the e-value cut-off we implemented and the vertical red dotted line indicates the length cut-off (e-value < 1e-20 and alignment length of 100, respectively) to decrease false positive hits. **(b)** The fraction of reads with high quality BLAST hits (e-value < 1e-20) increased as a function of read accuracy. We binned the data according to mean read accuracy (bin width = 0.02) and calculated the fraction of reads within

each bin that have a high quality BLAST hit (alignment length greater than 100bp and e-value less than 1e-20) for the January and March runs separately (blue and orange points, respectively). The number of reads in each bin is indicated above each point (in thousands). There is a clear positive correlation between mean accuracy and the likelihood of a high quality BLAST hit, reaching almost 40% for high accuracy reads (>92.5%) for the March dataset.

We next used MEGAN6 (41) to assign reads to specific taxa. MEGAN6 employs an LCA algorithm to assign reads to a taxon. For example, if a read has BLAST hits to five different species, three of which have bit scores within 20% of the best hit, the read will be assigned to the genus, family, order, or higher taxon level that is the LCA of those best-hit three species (51). If a read matches one species far better than any other, by definition, the LCA is that species.

16,820 reads (76%) were assigned to a taxon by MEGAN. Of these, 31% were assigned by MEGAN as being bacterial, and 55% of these were *Lactobacillus spp*. These results match previous studies on rat stomach microbiomes, which have found lactobacilli to be the dominant taxa (52–55). Plant-associated *Pseudomonas*, as well as *Lactococcus* taxa, were also common, at 7% and 6%, respectively.

MEGAN assigned reads to a wide range of eukaryotic taxa. To conservatively infer taxon presence, we first reclassified MEGAN species-level assignments to the level of genus. After this, several clear false positive taxon assignments remained (e.g. hippo and naked mole rat). These matches were generally short and of low identity. To reduce such false positive taxon inferences, we used information from reads assigned to the genera *Rattus* (rat) and *Mus* (mouse), using the following strategy.

We inferred that the reads assigned to *Rattus* (2,696 reads in total) were true positive genus-level assignments (deriving from DNA isolated from host stomach tissue), and that the reads assigned to *Mus* (2,798 reads in total) were false positive genus-level assignments (i.e. they were derived from Rattus host tissue and not *Mus*-derived). By using host reads, we can implement a ground-truth filtering strategy similar to that achieved by feeding a diet of known content (i.e. rat) and testing whether the contents of the known diet can be accurately identified.

First, it is critical to note that the reads assigned to *Mus* are false positive taxon assignments. If these were true positive *Mus* reads, then they would necessarily be due to mouse predation. Although rats are known to prey on mice (56), if this had occurred, we would expect that (assuming only a subset of rats had recently predated mice) if a rat had recently predated a mouse, (1) the ratio of mouse to rat reads would be higher than in rats that had not predated mice; (2) the percent identity of the reads assigned to *Mus* would be higher than in rats that had not predated mice. However, we found that the ratio of mouse to rat reads and percent identity of reads assigned to *Mus* was similar for all rats. This suggested either that all rats had predated mice very recently (which we view as unlikely), or that these *Mus* hits were indeed false positives. Thus, we use the *Mus* hits to delineate false positive and true positive genus-level assignment using the specific read identity and alignment length characteristics of each read set.

We first noted that the mean percent identity values of the best BLAST hits for *Rattus* and *Mus* reads differed, with reads matching *Rattus* having a median identity of 86.4%, and reads matching *Mus* having 81.0% (**Fig. 3A**). The mean percent identity for *Rattus* reads corresponds very well to that expected given the mean quality scores of the reads (86.4% identity corresponds to a mean quality score of 8.7, similar to what we observed; **Fig. S1A-C**).

A second characteristic we considered was the ratio of alignment length to read length. If a read fully aligns, this ratio is one. Generally, higher quality alignments should have higher ratios. Indeed, we found this ratio differed substantially between the *Rattus*- and *Mus*-assigned reads: the median ratio of alignment length to read length was longer for *Rattus* (0.57) than *Mus* (0.52; **Fig. 3B**).

We used these read characteristics to select cut-off values for assigning reads as being true positive or false positive genus-level taxon assignments. For genus-level assignments, we required at least 82.5% alignment identity, and an alignment length to read length ratio of at least 0.55. For any alignment of lower quality, we assign reads at the Family level. These cut-offs exclude 88% of the reads assigned to *Mus*, instead assigning them one taxon level higher, to the Family *Muridae* (which contains the Genus *Rattus*).

However, this rate of false positive assignments (88%) is still relatively high. We thus also quantified the rate of false positive assignment at the level of family. We first identified all reads classified as being from the Order *Rodentia.* Within this Order, we assume that reads assigned to the Family Muridae are true positive, and that reads assigned to any other Family are false positives. This assumption Is conservative when calculating accuracy. We then again implemented specific cut-offs, requiring reads assigned at the Family level to have database matches of at least 77.5% identity, an alignment length to read length ratio of at least 0.1, and a total alignment length of at least 150 bp. With these cut-offs, 97.3% of all reads assigned to the Order *Rodentia* were classified in the Family *Muridae* (which contains the genus *Rattus*). The remaining 2.7% were assigned to the family *Cricetidae* (voles and lemmings), except for four reads assigned to *Spalacidae* (mole-rats) (**Fig. S2**). All of these Family assignments are clear false positives, as it is highly unlikely that these families were predated. However, these results establish that by implementing specific cut-off values for

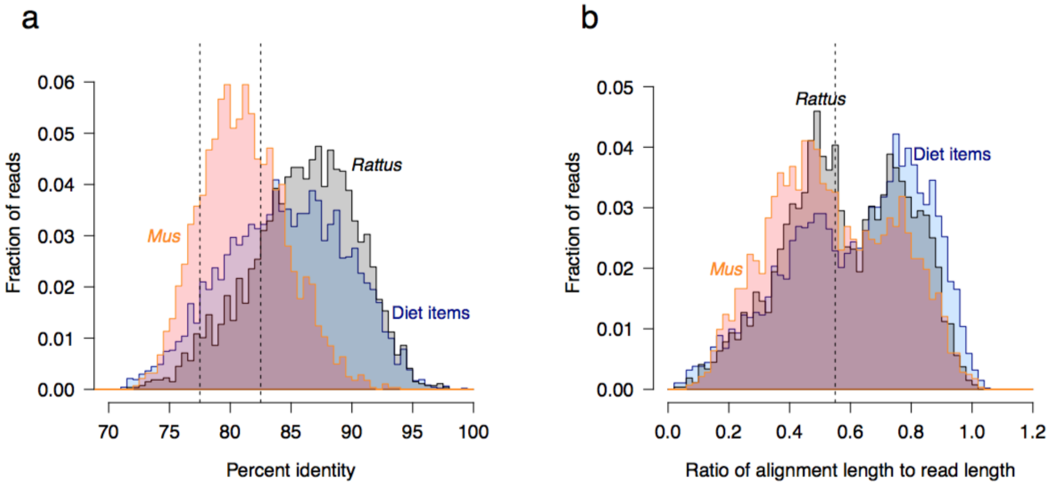


Fig. 3. Distributions of percent identity and length for alignments of reads matching *Rattus* (rat), *Mus* (mouse), and diet items. (a) The percent identity for alignments of rat (*Rattus*) and diet items is much higher than for mouse (*Mus*). Histograms of the percent identity of the alignment of the top BLAST hit with the read. *Mus* matches have substantially lower percent identity compared to both *Rattus* and diet items. The dotted lines indicate the cut-offs that we implemented for inferring reads as belonging to a specific genus (above 82.5% identity) or family (above 77.5% identity). **(b) Ratios of alignment lengths to read lengths of rat (*Rattus*) and diet items are higher than for mouse (*Mus*).** This plot is analogous to that in (a). The dotted line indicates the cut-off that we implemented for inferring reads as belonging to a specific genus (above 0.55).

alignments, we can ensure a low rate of false positive assignments at the Family level.

However, database bias may still play a role. For example, *Mus* and *Rattus* sequences are among the most common in the database. However, (as above), one expectation if reads are assigned to the wrong taxon is that these false positive assignments would have lower percent identities and low alignment lengths. We thus checked whether read alignments of all inferred diet items had percent identities and alignment lengths similar to the true positive *Rattus* alignments, or instead whether they were more similar to the false positive *Mus* alignments. We found that the majority of diet items had alignment percent identities that overlapped with the *Rattus* reads. Furthermore, the alignment length to read length ratios often exceed those for the *Rattus* reads. This suggests that the diet taxa assignments are often correct down to the level of genus (as the *Rattus*-assigned reads are correct to the level of genus) and are not false positive assignments. Despite this indication of Genus-level accuracy, here we conservatively report diet items at the level of Family.

For reads that did not pass the above cut-offs, we placed taxon assignments at the level of order, or used the taxon level assigned by MEGAN. Using these cut-offs, 16% of all reads were classified at the Genus level (although for the analyses below we consider these at the Family-level); 71% were classified at the family-level or below; 89% were classified at the order-level or below; and 98% were classified at the phylum-level or below.

There were few clear false positive taxon assignments after this analysis, and most had alignments lengths just above our cut-offs (**Table S4**). The exception to this were three reads from two rats matching *Buthidae* (scorpions), which had alignment lengths of 762 bp, 664 bp, and 298 bp with identities of 83%, 88%, and 79%, respectively. It is unlikely these are true positives, and instead we hypothesise that these rats predated harvestmen (*Opiliones*), a closely related sister taxon within *Arachnida*, but lacking significant amounts of genomic data. Despite the presence of these false positive taxa, we did not further increase the stringency of our filters, as the fraction was very small.

Diet quantification

Within each rat, we identified a wide variety of plant, animal, and fungal orders, ranging from two to 25 Orders per rat (mean 8.7; **Fig. 4**). In total, we identified taxa from 68 different Families, 55 different Orders, 15 different Classes, and eight different Phyla (**Fig. 5**). This is far beyond the range of diet items that could be identified using a straightforward metabarcoding approach.

Plants were the primary diet item, with four predominant Orders: *Poales* (grasses), *Fabales* (legumes), *Arecales* (palms), and *Araucariales* (specifically, *Podocarpaceae*, a common native New Zealand tree Family). The dominance of plant matter (fruits and seeds) in rat diets has been established previously (57,58). Animal taxa made up a smaller component of each rat's diet, with *Insecta* dominating: *Hymenoptera* (bees, wasps, and ants), *Coleoptera* (beetles), *Lepidoptera* (moths and butterflies), *Blattodea* (cockroaches), *Diptera* (flies), and *Phasmatodea* (stick insects). In addition, *Stylommatophora* (slugs and snails) were present in substantial numbers (**Fig. 5A** and **5B**). Fungi were only a small component of the rats' diet, although several orders were present: *Sclerotiniales* (commonly plant pathogens), *Saccharomycetales* (budding yeasts), *Mucorales* (pin molds), *Russulales* (brittlegills and milk-caps), and

Chytotheriales (black yeasts). Finally, for many rats, a substantial proportion of the stomach contents were parasitic worms (primarily *Spirurida* (nematodes) and *Hymenolepididae* (tapeworms)).

It is important to note that due to our metagenomic approach, the fraction of each element of the rats' diets may be distorted by biases in genomic databases: whole genome data exists for only a few taxa, while mtDNA, rDNA, metabarcode loci, and microsatellite sequence data are present in the database for many animal and plant genera. However, it is possible to mitigate this bias.

To quantify database-driven bias, for each taxon we determined the fraction of hits that mapped to mtDNA, rDNA, microsatellites, or EST libraries (we refer to this as *non-genomic*, as these data are not from genomic sequencing projects). We also determined the fraction of hits that mapped to DNA sequences arising from genome sequencing projects). We expect that for animals with sequenced genomes, these two fractions should be primarily determined by the relative amounts of mtDNA and nuclear DNA in a diet item, rather than database bias. If a diet item consists of cells that have large numbers of mitochondria (or if the animal has a small genome), we expect a large fraction of reads will map to mtDNA sequences. Alternatively, if a diet item consists of cells with few mtDNA, then most reads will map to genomic sequence. However, for animals without sequenced genomes, there should be considerably more hits to mtDNA, plastid, rDNA, and microsatellites (non-genomic sequence), and few (if any) genomic hits, regardless of the relative amounts of mtDNA and nuclear DNA in the diet item. By comparing these fractions for taxa that we know to have complete genome sequences in the database to taxa without complete genomes we aimed to assess and mitigate the effects of this bias. For this analysis, we consider Genus-level assignments.

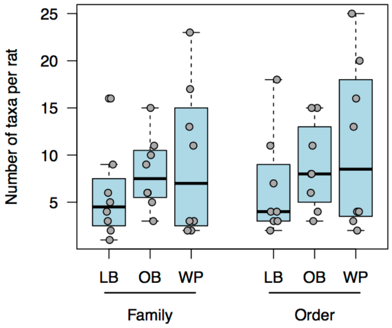
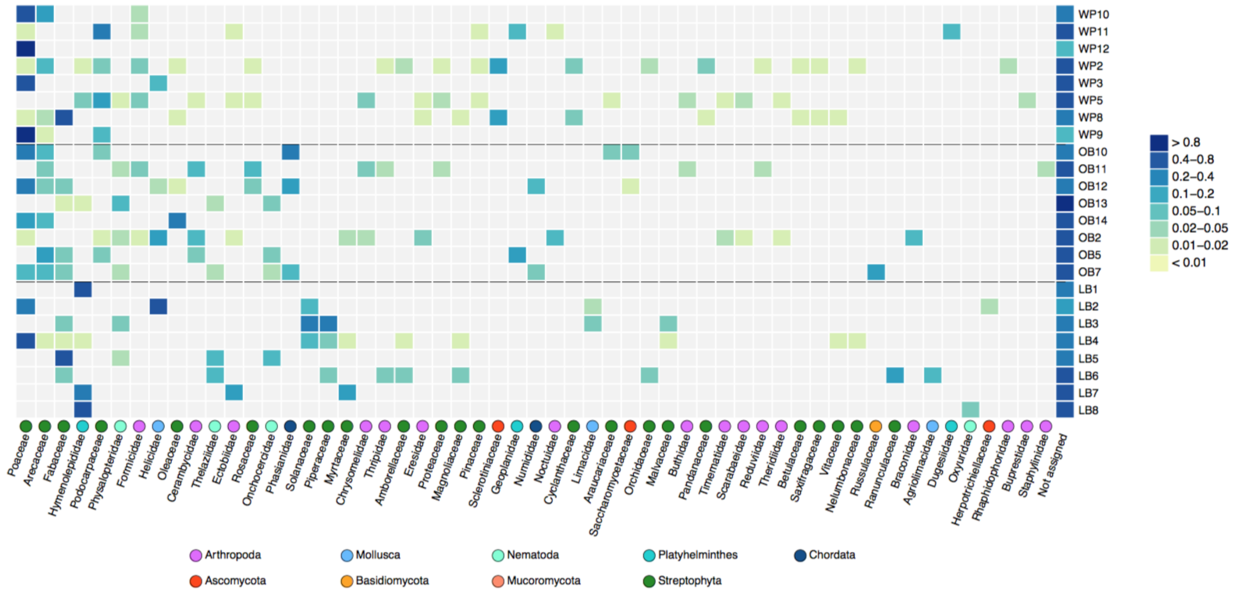


Fig. 4. Numbers of taxa in individual rats. Each boxplot indicates the range of families (left boxes) or orders (right boxes) consumed by each rat in each location (OB: Okura Bush, native bush; LB: Long Bay Park, restored wetland; WP: Waitakere Park, native forest). The numbers for individual rats (eight per location) are plotted in grey.



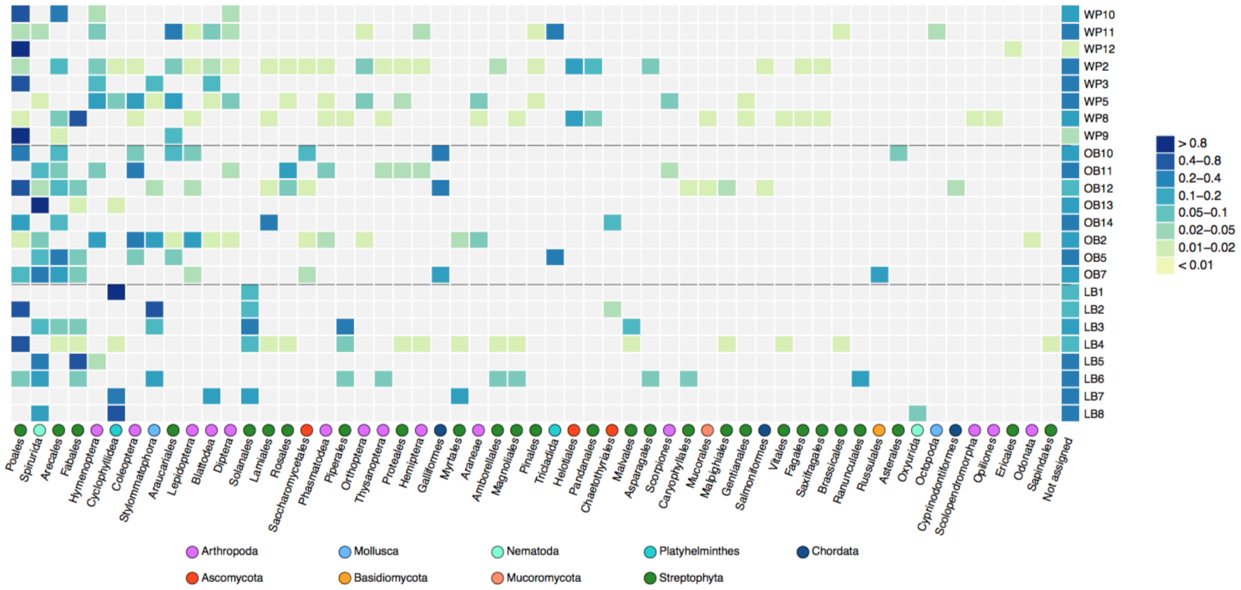


Fig. 5. Proportions of taxa in the diets of individual rats. (a) Reads assigned to taxa at the family and (b) order level. The rows correspond to a single rat, with the proportions of reads for that rat assigned to each family or order indicated in shades of blue and yellow. Reads that were not assigned to a specific family or order are indicated at the right side of the figure. The families and orders have been sorted so that the most common diet components appear on the left. Only the 55 most common families are shown. Note that the color gradations presented on the scale are not linear.

For animal Genera with at least of species that had sequenced genomes in the database, we found that the fraction of reads that mapped to non-genomic sequence (mtDNA, rDNA, microsatellite, plastid, and EST library) ranged from 0% (*Coturnix* (quail)) to 39% (*Gallus* (chicken)) (**Fig. 6**). This is a considerable range, and we hypothesise that variation in the fraction of non-genomic reads is due to the type of tissue sequenced (affecting mtDNA content). However, it is also possible that the results are biased by the relative frequency of specific types of studies, such as those generating microsatellite data. For *Rattus* (27% non-genomic), the sequenced tissue was primarily stomach muscle, which has a relatively high fraction of mtDNA, perhaps explaining the large fraction of reads mapping to non-genomic DNA.

For plants with sequenced genomes, the fraction of reads matching non-genomic sequence (mostly mtDNA, plastid, and rDNA) was generally lower: between 2% (*Cenchrus* buffelgrass)) and 12% (*Zea* (corn)). Thus, on average, for animals with sequenced genomes present in the database, approximately 30% of all reads mapped to non-genomic sequences; for plants, approximately 5% mapped to non-genomic sequences.

In contrast, for taxa with little or no genomic sequence in the database, the vast majority of matches were non-genomic (mtDNA, plastid, rDNA, or microsatellite loci): 90% of *Phoenix* (date palm) hits; all *Helix* (snail); and all *Rhaphidophorae* (endemic cave weta) hits. All *Arthurdendyus* (endemic New Zealand flatworm) hits were solely to rDNA loci. We note that these data indicate the accuracy of the read classifications, as several of these are endemic New Zealand species.

These ratios are in strong contrast to animals with sequenced genomes, for which ana average of only 30% of all reads should map to non-genomic sequence. This suggests that for animal taxa with little or no genomic sequence data, we have underestimated the actual number of sequences from that taxon by two- to three-fold. For plant taxa with little or no genomic sequence data, we have underestimated read abundance by approximately 20-fold. In terms of diet biomass, there is considerable uncertainty in both of these estimates.

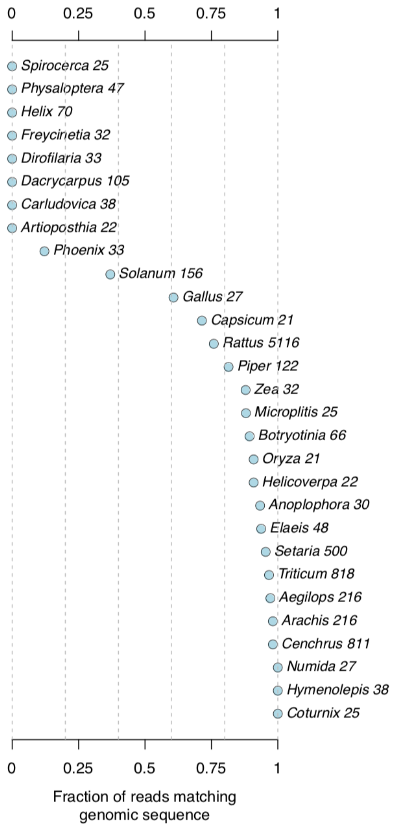


Figure 6. Fractions of reads matching genomic and non-genomic sequence for the best BLAST hit of each read. For the species with complete genomes, the fraction of reads matching genomic sequence ranges from 40% (*Solanum*) to 100%. This large range is likely due to the tissue from which the DNA was isolated. For example, muscle tissue has a higher fraction of mtDNA to nuclear DNA than egg. For species without fully sequenced genomes, this fraction ranges from 0% to 20% (*Phoenix*, which has a small amount of genomic data present in the database).

It was apparent after read classification, specific taxa were overrepresented in the diets of rats from particular locations. For example, six out of eight rats from the native estuarine bush habitat (OB) consumed *Arecaceae*, while only one in the restored wetland area (LB) did. All three rats that consumed *Phaseanidae* were from the native estuarine habitat (OB). All five rats that consumed *Solanales* were from the restored wetland area. These patterns suggested that it might be possible to use diet components alone to pinpoint the habitat from which each rat was sampled.

nMDS and CAP analysis by location

In order to determine if diet composition of the rats differed consistently between locations, we first performed an unconstrained analysis using non-metric multidimensional scaling (nMDS) on taxa assigned at the family level. The input for the nMDS was the dissimilarity matrix (Bray-Curtis distance of diet at the family level). NMDS uses rank-based distances to cluster samples that are most similar.

The family-level unconstrained ordination (nMDS) showed no obvious grouping of rats with respect to the locations (**Fig. 7A**), indicating that locations did not correspond to the predominant axes of variation among the diets. We next performed a constrained ordination method, Canonical Analysis of Principal coordinates (CAP, see Methods). CAP identifies axes of variation, if any, that distinguish the diets of rats from different locations (**Fig. 7B**). We found that the CAP axes correctly classified the locations of 19 out of 24 (79%) rats using a leave-one-out procedure. The families having the largest correlations with the first two principal coordinates, and thus most responsible for the separation between groups, were primarily plants: *Arecaceae*, *Podocarpaceae*, *Piperaceae*, and *Pinaceae*. In addition, insect groups (*Cerambycids* and *Formicids*) and birds (*Phaseanidae* and *Numididae*) played a role (**Fig. 7C**).

The families driving similarity within the three locations (i.e., those that had the greatest within-location SIMPER scores, see Methods) varied among locations. LB had average Bray-Curtis within-location similarity of 13%; mostly attributable to *Hymenolepidae* (accounting for 51% of the within-group similarity), *Solanaceae* (11%), and *Fabaceae* (11%). The average similarity for OB was 21%, with the greatest contributing taxa being *Arecaceae* (33%), *Poaceae* (23%), *Fabaceae* (9%), and *Phasianidae* (8%). The average similarity for WP was 24%, with the greatest contributing taxon being *Poaceae* (72%) (**Table S4**).

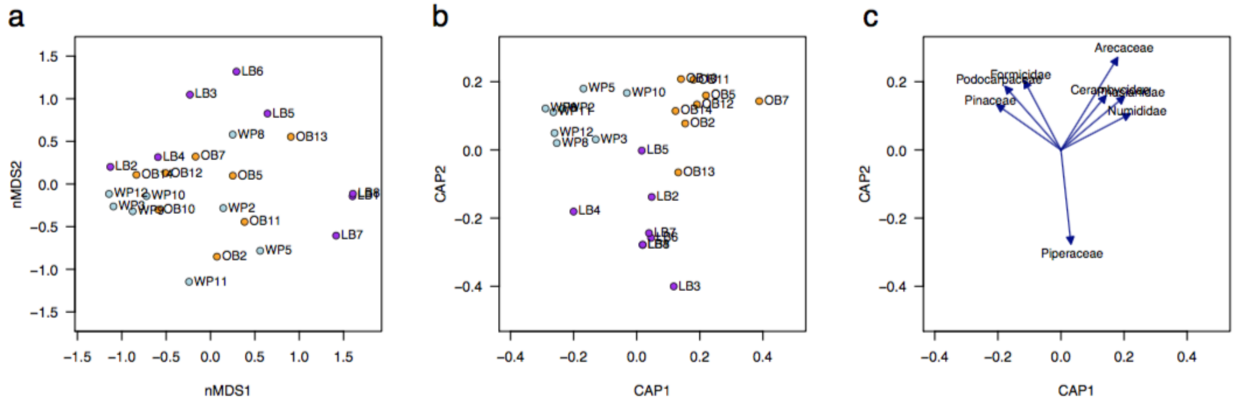


Fig. 7. Unconstrained nMDS (a) and constrained CAP (b) ordinations of the diets of rats from three locations. Both ordinations were based on Bray-Curtis dissimilarities of square root transformed proportions of reads attributed to each family. The locations were a native estuarine bush (OB, orange); a restored marine wetland (LB, purple); and a native forest (WP, light blue). The CAP ordination is repeated in panel **(c)** as a biplot with the rats omitted to show the Pearson correlations between families and the first two CAP axes. The eight families with the strongest correlations are shown, indicating the taxa associated with each location.

Discussion

Accuracy and sensitivity

Here we have shown that using a simple metagenomic approach with error-prone long reads allows rapid and accurate classification of rat diet components (approximately 2.7% error in taxon assignments at the family-level). We expect that this technique can be used to infer diet for a wide variety of animal and sample types, including samples that use less invasive collection methods, such as fecal matter. The accuracy of this approach will likely improve as the accuracy and yield of ONT sequencing continues to increase. The analysis here is based on fewer than 200,000 reads from two flow cells. Current yields for similar read length distributions are in excess of ten million reads per flow cell. As ONT modal sequencing accuracy is currently just above 96%, and continues to improve. This increase in read accuracy will clearly affect the accuracy of taxon assignment, illustrated by the fact that the fraction of reads yielding BLAST hits increases substantially for high accuracy reads, approaching 40% for high quality reads in our dataset (reads with greater than 92.5% accuracy, **Fig. 2B)**. With current ONT sequencing techniques, 92.5% is at the lower end of read accuracy.

Furthermore, as the species sampling of genomic databases increases (59), the taxon-level precision of this method will improve. Given the current rate of genomic sequencing, with careful sampling, the vast majority of multicellular plant and animal families (and even genera) will likely have at least one type species with a sequenced genome within the next decade. Continued advancement in sequence database search algorithms as compared to current methods (23,24,60) should considerably decrease the computational workload necessary to find matching sequences.

Methodological advantages

As genomic databases become more complete, metagenomic approaches will offer significant advantages due to decreased bias as compared to other methods. We found that rats consumed many soft-bodied species (e.g. mushrooms, flat worms, slugs, and lepidopterans) that would be difficult to identify using visual inspection of stomach contents. Achieving data on such a wide variety of taxa (across multiple phyla) would also be difficult to quantify using metabarcoding, as there are no universal 18S or COI universal primers capable of amplifying sequences in all these taxa. While it might be possible to use several different primer sets targeted at different phyla or orders, quantitatively comparing diet components across these using sequences amplified with different primer sets is extremely difficult due to differences in primer binding and PCR efficiency.

The ONT-based sequencing method has several unique advantages. Perhaps the most obvious is the accessibility of the platform. Compared to other high throughput sequencing technologies (e.g. Illumina, IonTorrent, or PacBio), there is no initial capital investment required. On a per-sample basis, data generation is inexpensive (assuming 12 multiplexed samples, approximately $150 USD per sample, and half this price if reagents are purchased in bulk). Library preparation and sequencing can be extremely rapid, going from DNA sample to sequence in less than two hours (61). Furthermore, the sequencing platform itself is highly portable. Given (1) that ONT-based methods are now similar in cost-per-read as the most accessible Illumina method (we estimate $650 for 10 million reads using ONT, versus $1300 for 20 million reads using MiSeq); and (2) that even marginal increases in read length are likely to significantly improve species identification, we expect that ONT-based methods should soon become useful for routine ecological monitoring of species (62).

Some modifications to our approach might further increase the precision of our ability to infer community composition. Any error-prone long read dataset (i.e. PacBio or ONT) has both short (e.g. 500 bp) and long (e.g. 5,000 bp) reads, as well as high quality (e.g. mean accuracy greater than 90%) and low quality (e.g. mean accuracy less than 80%) reads. When inferring community composition, a null expectation is that taxa should be equally represented by long, high quality reads as they are by short, low quality reads. If some taxa are represented only by short, low quality reads, this suggests that these taxa may be false positive inferences. Similarly, the difficulty in correctly mapping short inaccurate reads could be mitigated by weighting the probability of taxon mapping by the number of long, accurate reads that map to certain taxa. Thus, the fact that not all reads are extremely long and accurate does not mean that they cannot all be used to infer taxon presence in metagenomic analyses.

Finally, it is critical to note that for many diet studies, the aim is to resolve biomass, nutritional content, or prey numbers. However, estimating these numbers is constrained by the fact that different tissues and different taxa have different amounts of DNA (both nuclear and mitochondrial) per gram of biomass. It is nearly to impossible to fully account for this variation using any DNA-based method. Regardless, there is considerable utility in using DNA-based approaches for diet assessment, not least because it is one of the few methods that allows the full breadth of the diet to be observed, as illustrated here by the number of different orders we find that rats predate.

Conclusions

Here we have shown that using a rapid error-prone long read metagenomic approach we can accurately characterise diet taxa at the Family level, and distinguish between the diets of rats according to the locations from which they were sourced. This information may be used to guide conservation efforts toward specific areas and habitats in which native species are most at risk from this highly destructive introduced predator.

Methods

Study Areas

We trapped rats from three locations near Auckland, New Zealand. Each location comprised a different type of habitat: undisturbed inland native forest (Waitakere Regional Parklands, WP); native bush surrounding an estuary (Okura Bush Walkway, OB); and restored coastal wetland (Long Bay Regional Park, LB) (**Fig. 8**). Snap traps in OB and LB were baited with peanut butter, apple, and cinnamon wax pellets; or bacon fat and flax pellets.

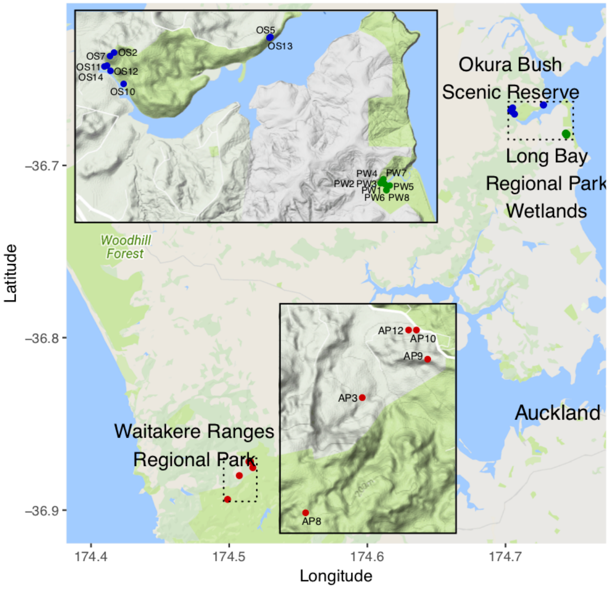


Fig. 8. Location of rat sampling sites in the greater Auckland area in the North Island of New Zealand. Each point indicates a trap where one rat was captured, with the colour of the points indicating the three broad locations: the native estuarine bush habitat of Okura Bush (OB), the restored wetland of Long Bay (LB), and the native forest of Waitakere Park (WP). The two insets show the three locations in higher resolution with topographical details. Green indicates park areas. Precise geographical coordinates were only available for five out of eight rats in WP.

Traps in WP were baited with chicken eggs, rabbit meat, or cinnamon scented poison pellets. From 16 November to 16 December 2016, traps were surveyed by established conservation groups at each site every 48 hours. A total of 36 rats were collected from these locations. Three of the rats from WP had poison in their stomachs. However, all three of these were killed by the snap traps, and as such are unlikely to have swallowed any bait. In addition, none of these rats were identified as having chicken or rabbit in their diet. The majority of rats collected (34/36) were determined to be male *Rattus rattus* by visual inspection. These 34 rats were selected for further analysis.

DNA Isolation

Within 48 hours of trapping, rats were stored at either -20°C or -80°C until dissection. We dissected out intact stomachs from each animal and removed the contents. After snap freezing in liquid nitrogen, we homogenised the stomach contents using a sterile mini blender to ensure sampling was representative of the entire stomach.

We purified DNA from 20 mg of homogenised stomach contents using the Promega Wizard Genomic DNA Purification Kit, with the following modifications to the Animal Tissue protocol: after protein precipitation, we transferred the supernatant to a new tube and centrifuged a second time to minimise protein carryover. The DNA pellet was washed twice with ethanol. These modifications were performed to improved DNA purity. We rehydrated precipitated DNA by incubating overnight in molecular biology grade water at 4°C and stored the DNA at -20°C. DNA quantity, purity, and quality was ascertained by nanodrop and agarose gel electrophoresis. The DNA samples were ranked according quantity and purity (based on A260/A280 and secondarily, A230/A280 ratios). The eight highest quality DNA samples from each of the three locations were selected for sequencing. We did no size selection on the purified DNA.

DNA Sequencing

Sequencing was performed on two different dates (24 January 2017 and 17 March 2017) using a MinION Mk1B device and R9.4 chemistry. For each sequencing run, DNA from each rat was barcoded using the 1D Native Barcoding Kit (Barcode expansion kit EXP-NBD103 with sequencing kit SQK-LSK108) following the manufacturer's instructions. This included an AMPure bead purification step to remove adaptors, which also likely removed very short reads (less than 200 bp; see **Fig. 1A**). Twelve samples were pooled and run on each flow cell, for a total of 24 individual rats. The flow cells had 1373 active pores (January 2017) and 1439 active pores (March 2017). Both runs were re-basecalled after data collection using Albacore 2.2.7 with demultiplexing performed in Albacore and filtering disable*d (optio*ns *--barcoding --disable_filtering*).

Sequence classification

All sequences were BLASTed (blastn v2.6.0+) against a locally compiled database consisting of the combined NCBI other_genomic and nt databases (downloaded on 13th June 2018 from NCBI). Default blastn parameters were used (match 2, mismatch -3, gapopen -5, gapextend -2). Due to the predominance of short indels present in nanopore sequence data, we used an initial set of basecalled data to test whether changing these default penalties affected the results (gapopen -1, gapextend -1). We found that these adjusted parameters did not qualitatively change our results.

We assigned sequence reads to specific taxon levels using MEGAN6 (v.6.11.7 June 2018) (41). We only used reads with BLAST hits having an e-value of $1x10^{-20}$ or lower (corresponding to a bit score of 115 or higher given the databases we used) and an alignment length of 100 base pairs or more. To assign reads to taxon levels, we considered all hits having bit scores within 20% of the bit score of the best hit (MEGAN parameter Top Percent).

Multivariate analyses

Multivariate analyses were done using the software PRIMER v7 (42). The data used in the multivariate analyses were in the form of a sample- (i.e. individual rat) by-family matrix of read counts. All bacteria, rodent, and primate families were removed as these are not diet content. The majority of the primate hits (32 in total) were assigned to Hominidae (19), which likely resulted from sample contamination (**Table S3**).

The read counts were converted to proportions per individual rat by dividing by the total count for each rat, to account for the fact that the number of reads varied substantially among rats (43). The proportions were then square-root transformed so that subsequent analyses were informed by the full range of taxa, rather than just the most abundant families (44). We then calculated a matrix of Bray-Curtis dissimilarities, which quantified the difference in the gut DNA of each pair of rats based on the square-root transformed proportions of read counts across families (43).

We used unconstrained ordination, non-metric multidimensional scaling (nMDS) applied to the dissimilarity matrix to examine the overall patterns in the diet composition among rats. To assess the degree to which the diet compositions of rats were distinguishable among the three locations, we applied canonical analysis of principal coordinates (CAP) (45) to the dissimilarity matrix. CAP is a constrained ordination which aims to find axes through multivariate data that best separates *a priori* groups of samples (in this case, the groups are the locations from which the rats were sampled); CAP is akin to linear discriminant analysis but it can be used with any resemblance matrix. The out-of-sample classification success was evaluated using a leave-one-out cross-validation procedure (45).

We used Similarity Percentage (SIMPER; (46)) to characterise and distinguish between the locations. This allowed us to identify the families with the greatest percentage contributions to (1) the Bray-Curtis similarities of diets within each location (**Table S5**) and (2) the Bray-Curtis dissimilarities between each pair of locations (**Table S6**).

Declarations

Ethics approval

Sample collection was performed under (Auckland Council Permit to Undertake Research WS1064).

Availability of data and materials

Sequence data are available in the SRA archive (accession number PRJEB27647).

Competing interests

WP received funding from Oxford Nanopore Technologies (1000$USD) to present this work at a conference (Ecological Society of Australia 2018).

Funding

This work was supported by a Massey University Research Fund to NF, a Marsden Fund Grant (15-MAU-136) to JD and Marsden Fund Grant (MAU1703) to OS.

Authors' contributions

WP, JD, NF, and OS conceived the project. WP performed the stomach dissections. WP and NF optimised the genomic DNA isolation and library preparation. NF performed the nanopore sequencing. GB and OS processed and performed quality control on the sequencing data. WP and OS performed the sequence classification. WP, AS, NF, and OS analysed the data. WP, NF, AS, and OS wrote the paper, with input from all authors.

Acknowledgements

Thanks to Friends of Okura Bush, Mary Stewart from Auckland Council, and Gillian Wadams and the volunteers at the Waitakere Ranges for collecting rat samples and aiding in rat species identification.

References

1. Daniel MJ. Seasonal Diet Of The Ship Rat (Rattus Rattus) In Lowland Forest In New Zealand. Proc. 1973;20:21–30.
2. Pierce GJ, Boyle. A review of methods for diet analysis in piscivorous marine mammals. Oceanogr Mar Biol Annu Rev. 1991;29:409–86.
3. Major HL, Jones IL, Charette MR, Diamond AW. Variations in the diet of introduced Norway rats (Rattus norvegicus) inferred using stable isotope analysis. J Zool. 2007 Apr 1;271(4):463–8.
4. Carreon-Martinez L, Heath DD. Revolution in food web analysis and trophic ecology: diet analysis by DNA and stable isotope analysis. Mol Ecol. 2010

Jan;19(1):25–7.

5. Hobson KA. Use of stable-carbon isotope analysis to estimate marine and terrestrial protein content in gull diets. Can J Zool. 1987 May 1;65(5):1210–3.

6. Basha WA, Chamberlain AT, Zaki ME, Kandeel WA, Fares NH. Diet reconstruction through stable isotope analysis of ancient mummified soft tissues from Kulubnarti (Sudanese Nubia). Journal of Archaeological Science: Reports. 2016 Feb 1;5:71–9.

7. Dunlap M, Pawlik JR. Video-monitored predation by Caribbean reef fishes on an array of mangrove and reef sponges. Mar Biol. 1996 Mar 1;126(1):117–23.

8. Brown KP, Moller H, Innes J, Jansen P. Identifying predators at nests of small birds in a New Zealand forest. Ibis . 2008;140(2):274–9.

9. King RA, Read DS, Traugott M, Symondson WOC. Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol Ecol. 2008 Feb;17(4):947–63.

10. Soininen EM, Valentini A, Coissac E, Miquel C, Gielly L, Brochmann C, et al. Analysing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. Front Zool. 2009 Aug 20;6:16.

11. Jarman SN, Gales NJ, Tierney M, Gill PC, Elliott NG. A DNA-based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. Mol Ecol. 2002 Dec;11(12):2679–90.

12. Jarman SN, Deagle BE, Gales NJ. Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. Mol Ecol. 2004 May;13(5):1313–22.

13. Tedersoo L, Anslan S, Bahram M, Põlme S, Riit T, Liiv I, et al. Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. MycoKeys. 2015;10:1.

14. Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, et al. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool. 2013 Jun 14;10:34.

15. Pawluczyk M, Weiss J, Links MG, Egaña Aranguren M, Wilkinson MD, Egea-Cortines M. Quantitative evaluation of bias in PCR amplification and next-generation sequencing derived from metabarcoding samples. Anal Bioanal Chem. 2015 Mar;407(7):1841–8.

16. Pereira RPA, Peplies J, Brettar I, Hoefle MG. Impact of DNA polymerase choice on

assessment of bacterial communities by a Legionella genus-specific next-generation sequencing approach [Internet]. bioRxiv. 2018 [cited 2018 Jan 31]. p. 247445. Available from: https://www.biorxiv.org/content/early/2018/01/12/247445.abstract

17. Hover BM, Kim S-H, Katz M, Charlop-Powers Z, Owen JG, Ternei MA, et al. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. Nat Microbiol. 2018 Apr;3(4):415–22.

18. Anantharaman K, Brown CT, Hug LA, Sharon I, Castelle CJ, Probst AJ, et al. Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. Nat Commun. 2016 Oct 24;7:13219.

19. Xu Z, Knight R. Dietary effects on human gut microbiome diversity. Br J Nutr. 2015 Jan;113 Suppl:S1–5.

20. Fierer N, Leff JW, Adams BJ, Nielsen UN, Bates ST, Lauber CL, et al. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. Proc Natl Acad Sci U S A. 2012 Dec 26;109(52):21390–5.

21. Breitwieser FP, Salzberg SL. KrakenHLL: Confident and fast metagenomics classification using unique k-mer counts. bioRxiv [Internet]. 2018; Available from: https://www.biorxiv.org/content/early/2018/06/06/262956.abstract

22. Huson DH, Mitra S, Ruscheweyh H-J, Weber N, Schuster SC. Integrative analysis of environmental sequences using MEGAN4. Genome Res. 2011 Sep;21(9):1552–60.

23. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: rapid and sensitive classification of metagenomic sequences. Genome Res. 2016 Dec;26(12):1721–9.

24. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014 Mar 3;15(3):R46.

25. Paula DP, Linard B, Crampton-Platt A, Srivathsan A, Timmermans MJTN, Sujii ER, et al. Uncovering Trophic Interactions in Arthropod Predators through DNA Shotgun-Sequencing of Gut Contents. PLoS One. 2016 Sep 13;11(9):e0161841.

26. Srivathsan A, Ang A, Vogler AP, Meier R. Fecal metagenomics for the simultaneous assessment of diet, parasites, and population genetics of an understudied primate. Front Zool. 2016 Apr 21;13:17.

27. Arribas P, Andújar C, Hopkins K, Shepherd M, Vogler AP. Metabarcoding and mitochondrial metagenomics of endogean arthropods to unveil the mesofauna of the soil. Yu D, editor. Methods Ecol Evol. 2016 Sep 7;7(9):1071–81.

28. Linard B, Crampton-Platt A, Gillett CPDT, Timmermans MJTN, Vogler AP. Metagenome Skimming of Insect Specimen Pools: Potential for Comparative Genomics. Genome Biol Evol. 2015 May 14;7(6):1474–89.

29. Meiser A, Otte J, Schmitt I, Grande FD. Sequencing genomes from mixed DNA samples - evaluating the metagenome skimming approach in lichenized fungi. Sci Rep. 2017 Nov 2;7(1):14881.

30. Srivathsan A, Sha JCM, Vogler AP, Meier R. Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (Pygathrix nemaeus). Mol Ecol Resour. 2015 Mar;15(2):250–61.

31. Søe MJ, Nejsum P, Seersholm FV, Fredensborg BL, Habraken R, Haase K, et al. Ancient DNA from latrines in Northern Europe and the Middle East (500 BC–1700 AD) reveals past parasites and diet. PLoS One. 2018 Apr 25;13(4):e0195481.

32. Pearman WS, Freed NE, Silander OK. Testing the advantages and disadvantages of short-and long-read eukaryotic metagenomics using simulated reads. BMC Bioinformatics 21, 1-15

33. Hillmann B, Al-Ghalith GA, Shields-Cutler RR, Zhu Q, Gohl DM, Beckman KB, et al. Evaluating the Information Content of Shallow Shotgun Metagenomics. mSystems [Internet]. 2018 Nov;3(6). Available from: http://dx.doi.org/10.1128/mSystems.00069-18

34. Gibbs GW. Why are some weta (Orthoptera: Stenopelmatidae) vulnerable yet others are common? J Insect Conserv. 1998 Dec 1;2(3-4):161–6.

35. Towns DR, Daugherty CH, Cree A. Raising the prospects for a forgotten fauna: a review of 10 years of conservation effort for New Zealand reptiles. Biol Conserv. 2001 May 1;99(1):3–16.

36. Stringer IAN, Bassett SM, McLean MJ, McCartney J, Parrish GR. Biology and conservation of the rare New Zealand land snail Paryphanta busbyi watti (Mollusca, Pulmonata). Invertebr Biol. 2003 Sep 1;122(3):241–51.

37. Diamond JM, Veitch CR. Extinctions and introductions in the new zealand avifauna: cause and effect? Science. 1981 Jan 30;211(4481):499–501.

38. Dowding JE, Murphy EC. The impact of predation by introduced mammals on endemic shorebirds in New Zealand: a conservation perspective. Biol Conserv. 2001 May 1;99(1):47–64.

39. Graham NAJ, Wilson SK, Carr P, Hoey AS, Jennings S, MacNeil MA. Seabirds enhance coral reef productivity and functioning in the absence of invasive rats. Nature. 2018 Jul;559(7713):250–3.

40. Russell JC, Innes JG, Brown PH, Byrom AE. Predator-Free New Zealand: Conservation Country. Bioscience. 2015 May 1;65(5):520–5.

41. Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, et al. MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. PLoS Comput Biol. 2016 Jun;12(6):e1004957.

42. Clarke KR, Gorley RN. PRIMER v7: User Manual/Tutorial. PRIMER-E, Plymouth; 2015 p. 296.

43. Clarke KR, Robert Clarke K, Somerfield PJ, Gee Chapman M. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. J Exp Mar Bio Ecol. 2006;330(1):55–80.

44. Clarke KR, Green RH. Statistical Design and Analysis for a “biological Effects” Study. Mar Ecol Prog Ser. 1988;46:213–26.

45. Anderson MJ, Willis TJ. Canonical Analysis Of Principal Coordinates: A Useful Method Of Constrained Ordination For Ecology. Ecology. 2003 Feb 1;84(2):511–25.

46. Clarke KR. Non-parametric multivariate analyses of changes in community structure. Austral Ecol. 1993;18(1):117–43.

47. Jain M, Koren S, Miga KH, Quick J, Rand AC, Sasani TA, et al. Nanopore sequencing and assembly of a human genome with ultra-long reads. Nat Biotechnol. 2018 Apr;36(4):338–45.

48. Deagle BE, Eveson JP, Jarman SN. Quantification of damage in DNA recovered from highly degraded samples--a case study on DNA in faeces. Front Zool. 2006 Aug 16;3:11.

49. Jain M, Olsen HE, Paten B, Akeson M. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol. 2016 Nov 25;17(1):239.

50. McIntyre ABR, Ounit R, Afshinnekoo E, Prill RJ, Hénaff E, Alexander N, et al. Comprehensive benchmarking and ensemble approaches for metagenomic classifiers. Genome Biol. 2017 Dec 21;18(1):182.

51. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007 Mar;17(3):377–86.

52. Maurice CF, Knowles SCL, Ladau J, Pollard KS, Fenton A, Pedersen AB, et al. Marked seasonal variation in the wild mouse gut microbiota. ISME J. 2015 Nov;9(11):2423–34.

53. Brownlee A, Moss W. The influence of diet on lactobacilli in the stomach of the rat. J Pathol. 1961 Oct 1;82(2):513–6.

54. Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z, et al. Microbial Biogeography and Core Microbiota of the Rat Digestive Tract. Sci Rep. 2017 Apr 4;8:45840.

55. Horáková Z, Zierdt CH, Beaven MA. Identification of lactobacillus as the source of bacterial histidine decarboxylase in rat stomach. Eur J Pharmacol. 1971 Sep;16(1):67–77.

56. Bridgman LJ, Innes J, Gillies C, Fitzgerald N, King CM. Do ship rats display predatory behaviour towards house mice? Anim Behav. 2013 Aug 1;86(2)):257–68.

57. Sweetapple PJ, Nugent G. Ship rat demography and diet following possum control in a mixed podocarp—hardwood forest. N Z J Ecol. 2007;31(2):186–201.

58. Riofrío-Lazo M, Páez-Rosas D. Feeding Habits of Introduced Black Rats, Rattus rattus, in Nesting Colonies of Galapagos Petrel on San Cristóbal Island, Galapagos. PLoS One. 2015 May 18;10(5):e0127901.

59. Lewin HA, Robinson GE, Kress WJ, Baker WJ, Coddington J, Crandall KA, et al. Earth BioGenome Project: Sequencing life for the future of life. Proc Natl Acad Sci U S A. 2018 Apr 24;115(17):4325–33.

60. Nasko DJ, Koren S, Phillippy AM, Treangen TJ. RefSeq database growth influences the accuracy of k-mer-based species identification. 2018;1–21.

61. Zaaijer S, Gordon A, Speyer D, Piccone R, Groen SC, Erlich Y. Rapid re-identification of human samples using portable DNA sequencing. Elife [Internet]. 2017 Nov 28;6. Available from: http://dx.doi.org/10.7554/eLife.27798

62. Kamenova S, Bartley TJ, Bohan DA, Boutain JR, Colautti RI, Domaizon I, et al. Chapter Three - Invasions Toolkit: Current Methods for Tracking the Spread and Impact of Invasive Species. In: Bohan DA, Dumbrell AJ, Massol F, editors. Advances in Ecological Research. Academic Press; 2017. p. 85–182.

Supplemental Tables

Table S1. Read numbers and total base pairs for each barcode in the January 2017 sequencing run.

Sample	Total reads	Total Mbp	Mean length
OB2	19907	14.62	734
WP11	10164	9.63	947
WP5	8237	6.78	823
LB7	7548	7.04	933
OB13	3644	3.63	995
WP9	2954	2.4	814
OB5	2850	2.06	721
WP8	2801	2.32	827
LB6	2531	1.6	632
OB7	2473	1.87	756
LB5	1641	1.16	705
LB3	1554	0.99	636
None	16673	13.01	781
Total	82977	67.1	809

Table S2. Read numbers and total base pairs for each barcode in the March 2017 sequencing run.

Sample	Total reads	Total Mbp	Mean length
LB1	17820	9.21	517
LB8	16923	13.13	776
WP2	10511	7.00	666
LB4	8684	4.92	567
OB11	5689	3.40	598
WP10	1563	0.99	633
OB12	1479	0.89	604
WP12	1309	0.78	596
LB2	1127	0.76	676
WP3	637	0.73	1141
OB14	541	0.37	683
OB10	435	0.24	555
None	29432	21.33	725
Total	96150	63.75	663

Table S3. Characteristics of alignments for reads assigned to the Primate family. Many reads are both long and have high identity, suggesting that they are not false positive assignments, but contamination.

Rat	Read length	Mean read accuracy	% ID	Alignment length	Genus
WP10	428	0.91	94.0	314	Homo
WP10	782	0.92	93.8	657	Homo
OB5	365	0.90	93.2	249	Homo
LB3	515	0.95	92.6	462	Homo
WP10	510	0.90	92.0	460	Homo
WP10	704	0.90	91.1	587	Homo
WP10	467	0.89	90.3	402	Homo
WP10	494	0.89	89.7	388	Homo
WP10	339	0.88	89.6	269	Homo
WP10	446	0.88	89.6	326	Homo
WP10	327	0.91	89.1	210	Homo
OB5	415	0.89	88.8	277	Homo
OB5	561	0.86	88.7	257	Homo
WP11	434	0.86	88.7	301	Homo
WP10	486	0.88	88.2	365	Homo
WP10	613	0.90	88.2	526	Homo
WP10	563	0.87	88.2	457	Homo
WP10	1373	0.91	87.7	1337	Homo
WP11	526	0.91	87.5	473	Homo
OB14	478	0.89	86.9	373	Homo
OB5	715	0.89	86.8	673	Homo
WP10	475	0.87	86.7	362	Homo
WP10	398	0.86	86.5	259	Homo
WP10	377	0.88	85.3	251	Homo
WP9	558	0.87	85.2	508	Homo
WP10	429	0.86	84.8	276	Homo
WP10	322	0.81	84.5	174	Homo
WP8	723	0.84	83.1	438	Homo
LB8	965	0.86	80.4	245	Rhinopithecus

LB5	3042	0.94	79.4	1018	Cebus
WP2	464	0.93	77.3	216	Homo
LB8	671	0.90	73.9	406	Aotus

Table S4. Characteristics of alignments for reads that are likely false positive assignments. Most are short long or have low identity, suggesting that they are false positive assignments. The exception are the reads matching Buthidae, which we hypothesize are due to the rats predation of the sister taxa, harvestmen. Octopodidae, Salmonidae, and Poeciliidae (guppies and similar aquaria fish) are possible but improbable prey items.

Rat	**Read length**	**Mean read accuracy**	**% ID**	**Alignment length**	**Genus**	**Megan family**
WP5	1285	0.90	82.9	298	Centruroides	Buthidae
OB11	1874	0.90	88.0	664	Centruroides	Buthidae
WP5	1711	0.93	79.3	762	Centruroides	Buthidae
WP11	859	0.93	86.1	151	Octopus	Octopodidae
WP2	516	0.86	81.4	172	Oncorhynchus	Salmonidae
OB12	424	0.90	85.7	140	Xiphophorus	Poeciliidae
OB12	643	0.84	89.3	177	Xiphophorus	Poeciliidae

Table S5. SIMPER analysis of family contributions to group similarities.

Family	**Average**	**Average**	**Similarity/SD**	**Percentage**	**Group**
Hymenolepididae	3.37	6.87	0.34	51.2	LB
Solanaceae	1.57	1.48	0.34	11.1	LB
Fabaceae	1.74	1.41	0.44	10.5	LB
Arecaceae	2.86	7.11	1	33.4	OB
Poaceae	2.87	4.82	0.55	22.7	OB
Fabaceae	1.17	1.98	0.51	9.3	OB
Phasianidae	1.79	1.67	0.34	7.9	OB
Poaceae	5.08	17.61	0.62	72.1	WP

Table S6. SIMPER analysis of family contributions to group dissimilarities.

Species	Av Abund Group1	Avg Abund Group2	Avg. Diss	Diss/SD	% contrib	Group 1	Group2
Poaceae	1.95	5.08	15.15	1.04	16.74	LB	WP
Poaceae	2.87	5.08	11.29	1.26	13.78	OB	WP
Hymenolepididae	3.37	0.48	10.8	0.73	11.93	LB	WP
Hymenolepididae	3.37	0.29	9.37	0.79	10.32	LB	OB
Poaceae	1.95	2.87	8.37	1.1	9.22	LB	OB
Arecaceae	0.05	2.86	6.99	1.41	7.7	LB	OB
Arecaceae	2.86	1.31	5.92	1.29	7.23	OB	WP
Fabaceae	1.74	1.05	6.14	0.67	6.78	LB	WP
Podocarpaceae	0	2.38	5.34	0.83	5.9	LB	WP
Podocarpaceae	0.71	2.38	4.82	0.99	5.88	OB	WP
Fabaceae	1.74	1.17	4.87	0.81	5.37	LB	OB
Fabaceae	1.17	1.05	4.31	0.84	5.26	OB	WP

Datafile S1. Table of read BLAST hits and assigned MEGAN taxa with reads reclassified at the family or order level by filtering on read length to alignment length ratio and percent identity.

Datafile S2. Table of read BLAST hits and assigned MEGAN taxa with no filters applied.

Supplemental Figures

Fig S1. Correlation of read accuracy with alignment characteristics. Only rat reads exhibit a clear positive relationship between accuracy and percent identity. (A) indicates the relationship for reads assigned to *Rattus* and (B) for *Mus*. The orange line indicates a running median; the red dotted line is the y=x line, which is expected if accuracy corresponds exactly to percent identity.

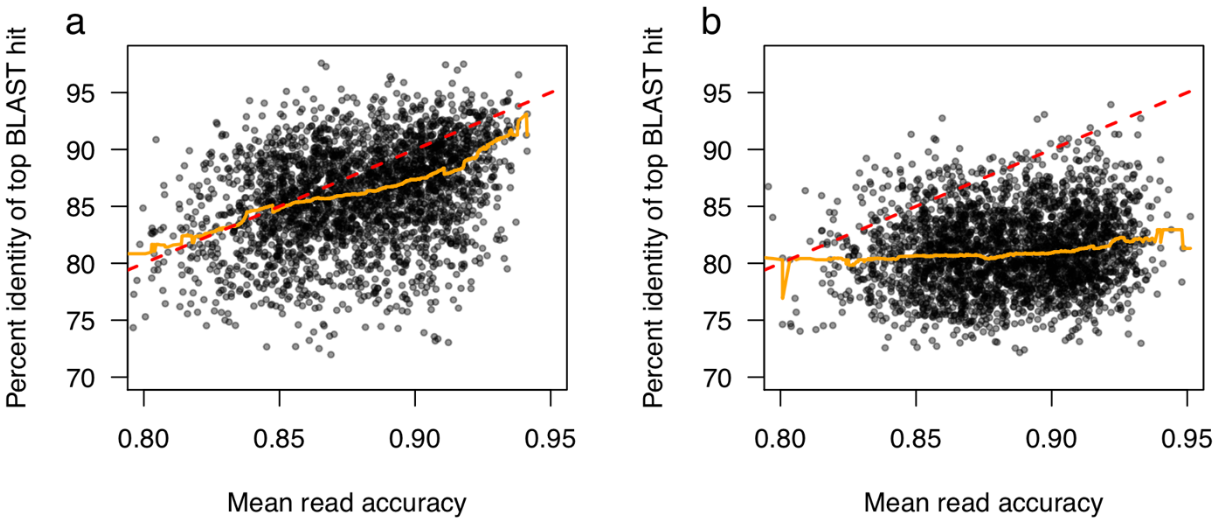
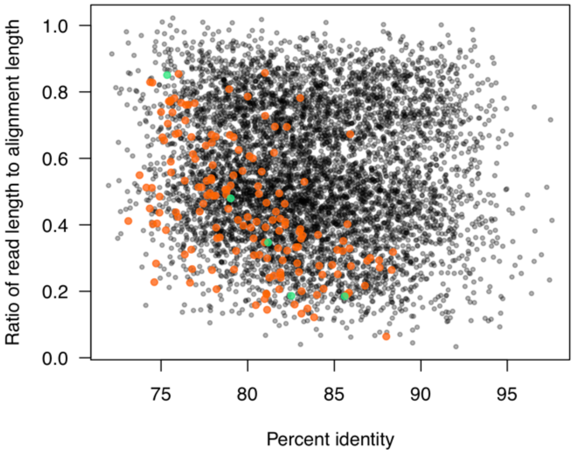


Fig S2. Alignment characteristics of true positive and false positive taxon assignments at the family level. False positive taxon assignments (*Cricetideaa*, orange, and *Spalacidae*, green) have lower percent identity and shorter alignment lengths than true positive taxon assignments (*Muridae*, black). Only a single false positive taxon assignment had a read length to alignment length ratio greater than 0.5 and a percent identity greater than 85%. This suggests that with further filters or methodologies (e.g. decision tree analysis using different read and alignment characteristics) could, if necessary, decrease false positive rates even further.


Ratio of read length to alignment length
Percent identity