Pyrosequencing of prey DNA in faeces of carnivorous landsnails to facilitate ecological restoration and relocation programmes

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*** Declaration of Authorship

This study was a part of a wider PhD project by BRW on the restoration of functioning ecosystems following opencast mining. BRW designed the experiment, performed the analysis, analysed the data, prepared figures, wrote the paper and collated author comments for the final manuscript. SB conceived and designed the study, designed the primers and performed molecular analysis. SDW conceived the study, advised throughout and was involved in funding. All authors have read, amended and approved the final manuscript.

Abstract

Identifying and understanding predator diets is of high importance in biological conservation. This is particularly true for the introduction, establishment and maintenance of predator populations in newly created or modified ecological communities, such as translocation sites or restored habitats. Conservation status of predators may not permit captive feeding trials or intrusive gut-content methods, so non-intrusive diet assessment is required, such as faecal analysis. However, prey such as earthworms leave no morphological clues suitable for accurately discriminating between species consumed through visual faecal analysis. This study uses non-intrusive molecular methods on earthworm DNA extracted from the faeces of the carnivorous landsnail *Powelliphanta patrickensis* to identify the earthworm diet and any seasonal trends. 454-pyrosequencing data revealed earthworm DNA in all samples ($n=60$). Sequences were compared to a DNA library created from published and unpublished studies of New Zealand's endemic earthworms and online databases. Unidentified earthworm sequences were clustered into molecular operational taxonomic units (MOTUs). 26 MOTUs were identified, 17 of which matched the library, and 9 did not. Similarity indices indicate that there were seasonal differences ($P<0.05$) in the earthworm communities represented in the summer and the winter diets. This study highlights the importance of utilising the vast data generated by pyrosequencing to investigate potential temporal diet shifts in protected species. The method described here is widely applicable to a wide range of predatory species of conservation interest and can further inform habitat restoration and relocation programmes to optimize the long-term survival of the target species.

Keywords: DNA, molecular diet analysis, molluscs, earthworms, next generation sequencing, metagenomics

Introduction

Earthworms are responsible for a number of key ecosystem functions, such as enhancing plant germination rate, seed dispersal, and seedling recruitment (Milcu et al. 2006), enhancing soil aggregation and stability (Scullion and Malik 2000), increasing plant growth rate and nutrient availability (Chaoui et al. 2003) and many others that make this group important in the resilience and recovery of ecosystems (Boyer & Wratten 2010). However, they also undertake an important function as prey for a range of organisms including mammals (Silcox and Teaford 2002), birds (Colbourne and Powlesland 1988), reptiles (Brown et al., 2012), and invertebrates (King et al., 2010).

The endemic *Powelliphanta patrickensis* Powell 1949 (Mollusca: Pulmonata: Rhytididae) occurs on the West Coast of New Zealand's South Island. It is a rare carnivorous land snail, the ecology of which is little known, aside from that it is believed to feed predominantly (perhaps exclusively) on earthworms, and that its distribution is restricted to a tussock grassland valley, and the plateau of the neighbouring mountain range. Although many Rhytididae are carnivorous and predate on earthworms (Efford 2000), other species within this molluscan family rely heavily on other invertebrates, such as *Wainuia urnula urnula* Pfeiffer, which specialises predominantly on leaf-litter amphipods (landhoppers *Parorchestia*

tenuis Dana 1852). Another species closely related to *P. patrickensis* is *Powelliphanta augusta* Walker Trewick & Barker 2008, which has a distribution adjacent but not overlapping with *P. patrickensis*. This species feeds almost exclusively on earthworms (Boyer et al. 2011c), but beyond this, little is known about diet in the *Powelliphanta* genus. Due to opencast mining operations, a large proportion of the current, limited distribution of *P. patrickensis* is subject to rapid, large-scale habitat loss and subsequently media and conservation attention. Subsequently, a relocation programme has been implemented to move as much of the *P. patrickensis* population as possible from its habitat prior to mining. Instead of holding individuals in captivity as with other species, *P. patrickensis* will be moved to temporary 'holding' sites of either 'untouched' native habitat, or areas of recently completed mining that have been restored. Once mining in the original tussock habitat is complete, and the area restored, the *P. patrickensis* population is to be returned. The relocation is targeted through systematic surveys across the valley to collect, record and relocate individuals.

Current ecological restoration on the mine can be broadly categorized in to three main areas: stockpiled soil spreads, vegetation direct transfer (VDT), and artificial soils. Mine tailing stockpiles are the soil and rock removed to access the resources beneath that can be stored for several years in deep stockpiles (Abdul-Kareem and McRae 1984). Stockpile spreading is the redistribution of these tailings over completed mined areas and manually replanting with nursery-reared native vegetation. VDT is a process whereby approximately 1 m x 1 m areas of unmodified vegetation with ~30 cm soil is translocated to either a holding site or to an area of mine requiring restoration; the aim of which is piece together a replica

of the habitat from where it was taken (Boyer et al. 2011b). Artificial soils are a mixture of stockpiled mine tailings and biosolids – municipal sewage waste that has been dried and sterilised – that is then spread and manually replanted. The aim of this mix is to re-establish nutrients, organic matter and bulk volume lost in the mining process (Gardner et al. 2010). For the restoration of *P. patrickensis* habitat in particular, VDT will be the method used.

Understanding the ecology of a species of conservation concern is important in achieving conservation goals, and data on what a species eats are fundamental to the effective maintenance or enhancement of its population. Indeed many species exhibit seasonal (Hill 1997; García-Berthou and Moreno-Amich 2000) and spatial (Stehlik and Meise 2000; Lake et al. 2003) variation in diet, often linked to prey distribution and availability, but this can also be due to specialisation by predatory species on specific prey groups or species. Such differences in prey selection or availability should be reflected in the selection and management of receptor sites, and are primary considerations in selection of habitat restoration methods. For example, surveying restored areas or putative relocation sites for appropriate diet species that fulfill seasonal diet coverage would be important for species such as *P. patrickensis*; reconciling accurate diet data with existing surveys or literature could inform conservation measures; and detailed diet information could inform further research into the impacts of restoration methods on the persistence of key prey species.

Historically, accurately identifying prey items has been problematic (summarised in Symondson (2002); Deagle et al., (2009)). In particular, morphological analyses of prey gut or faeces contents relies on diagnostic hard parts, such as hair, bones, teeth or cuticular

fragments, that may be absent from a sample (King et al., 2010; Braley et al., 2010; Brown et al., 2012) due to liquid meals or soft-bodied prey, or the predator may be too small. Previous studies, for example, aiming to achieve comprehensive diet analysis for earthworm predators using morphological analysis have failed to discriminate between earthworm species using faeces, as only hair-like chaetae remain as evidence, which alone are not diagnostic features of many earthworm species (Colbourne and Powlesland 1988). Additionally, as with *P. patrickensis*, many organisms of interest may be afforded legal protection where handling is prohibited or only under license, and it may not be possible to acquire enough individuals for a reliable assessment of gut contents or feeding behaviour.

Such obstacles have, in part, been remedied with the advent of the DNA-based diet analysis utilising polymerase chain reaction (PCR), next-generation sequencing (NGS) and DNA sequences to simultaneously identify groups of consumed species accurately, which is the ultimate goal of diet analysis. These methods can generate large amounts of data relatively quickly and cost effectively, potentially allowing large numbers of individuals from a population to be sequenced simultaneously (Deagle and Tollit 2006). Many studies utilise faeces for DNA analysis, as they are relatively easy to acquire, often non-intrusive and often do not require direct contact with the organism. Furthermore, modern bioinformatics techniques combined with large NGS data sets has led to methodological developments to more accurately estimate diversity, and also account for specimens that have not been formally described by science; for example, molecular operational taxonomic units (MOTUs) in species diversity and molecular diet studies (Floyd et al. 2002; Bohmann et al. 2011).

Broadly, this study aims to use the conservation of *P. patrickensis* to inform wider ecosystem restoration and species conservation, but specifically aims to 1) identify the earthworm diet of *P. patrickensis* using next generation molecular methods on faecal samples; 2) investigate evidence for predation on other hard-bodied invertebrates through morphological examination of faeces; 3) investigate potential seasonal differences in the *P. patrickensis* diet; 4) highlight putative earthworm diversity by using predator faecal samples as indicators; and 5) investigate the use of habitats inhabited by related snails by comparing findings with the *Powelliphanta augusta* diet described by Boyer et al. (2013). An additional outcome of using a molecular approach to investigate diverse yet understudied sites, as that in this study and groups such as New Zealand earthworms, is to encounter earthworm species that have previously gone undetected. This study focuses on a previously unstudied species and improves on previous study of the *Powelliphanta* genus by investigating temporal differences in the diet and by reducing methodological labour for molecular and sequence analysis. Identifying the feeding preferences of *P. patrickensis* will contribute valuable information to the conservation of this species, highlight a key ecosystem function undertaken by endemic earthworms in this ecosystem, and contribute an increasingly cost-effective way to inform restoration and relocation programmes.

Materials and Methods

Site location and sample collection

The study site was a tussock grassland valley basin named 'Happy Valley' (41°42'12.91"S, 171°53'25.05"E), with dense scrub vegetation ascending the valley walls into podocarp forest. It is located within the footprint of a future opencast mine in the Buller coalfields on the West Coast of New Zealand. One faecal sample was collected from each of 60 sub-adult to adult *P. patrickensis* snails located in their natural habitat, typically on the fringes of the tussock grassland and scrub; 29 individuals were collected in summer (late November to December 2011); 29 in winter (July 2012); and 2 in autumn (March 2012) during pre-mining snail survey operations in the footprint of the proposed mine area. Collected snails were individually placed in clean plastic containers with damp tissue paper to prevent desiccation, and held for three days (the maximum holding time stipulated by the Department of Conservation, New Zealand) or until a faecal sample was produced, whichever was the sooner. GPS location, shell diameter and weight were recorded for each snail before they were translocated to their receptor site. Faecal samples produced within the three days were collected, individually packed in zip-lock bags, and frozen on site at approximately -19°C. Samples were then transferred to The Bio-Protection Research Centre, Lincoln University, New Zealand for analysis, where they were stored at approximately -80°C. Each sample was divided into two: one half for morphological analysis, and the other half for molecular analysis.

Morphological analysis

Sixty individual faecal samples were air dried and examined under a compound stereomicroscope at 80 x magnification for evidence of prey item remains. As previous

studies suggested that earthworms are a key component of the *Powelliphanta spp.* diet across all age classes (Boyer et al. 2011c), the presence or absence of earthworm chaetae was scored for each sample. Also, samples were examined for the remains of other potential prey items including hard-bodied arthropod remains and plant material, which were scored as present or absent.

Molecular analysis

As prey DNA present in faeces has passed through the gut of the predator, it is likely that longer fragments typically used for species identification have been degraded, so shorter gene regions should be targeted to maximize the DNA amplified (Deagle et al., 2010; Murray et al., 2011; Pompanon et al., 2012). Also, as mtDNA is present in multiple copies in a given cell it is therefore more likely to be present in partially-degraded environmental DNA samples (Symondson 2002; Pompanon et al. 2012). For these reasons a ~130 bp region within the 16S gene of mitochondrial DNA (mtDNA) was targeted. The targeted region is of variable size with total target amplicon range, including primers, of 165-169 bp. Primers were group-specific to New Zealand endemic earthworms, designed and used in previous studies (Boyer et al. 2011c; Boyer et al. 2013): forward primer (nz_worm_16S_int_F) 5'-AATTMGGTTGGGGCGACSHW-3', reverse primer (nz_worm_16S_int_R) 5'-AACATCGAGGTGCCAAWCCC-3'. A two-step molecular method was used to identify the earthworm species consumed by *P. patrickensis*. This differs from processes previously used by removing the nested-PCR step, thus reducing labour and the likelihood of introducing erroneous PCR artefacts. Steps consisted of PCR followed by high-throughput 454-

pyrosequencing. DNA was extracted from faecal samples using the Qiagen DNeasy Blood and Tissue kit. A positive control of native earthworm tissue, and a negative control of nuclease-free water were used to highlight false positives/negatives and for comparison of amplicon length. Extracted mtDNA was amplified using 10 µl PCR comprising 5 µl KAPA2G HotStart ReadyMix, 3 µl nuclease-free water, 0.5 µl forward primer [10 µM], 0.5 µl reverse primer [10µM], and 1µl DNA template. For pyrosequencing the same primer sequences were used, combined with the appropriate pyrosequencing adapter to create fusion primers, including a unique 10 bp Multiplex Identifier (MID) tag for each sample, thus allowing simultaneous processing of all samples. Pyrosequencing adapters and MID tags were as recommended by the manufacturer (*Roche Genome Sequencer FLX System*).

An optimised three-stage PCR thermo-cycle was used to amplify DNA extracts consisting of initial denaturation of 3 min. at 95°C, followed by 40 cycles of 15 sec. denaturation at 95°C, 15 sec. annealing at 63°C, and 15 sec. elongation at 72°C. The final elongation step was 72°C for 3 minutes, after which PCR products were stored at ~4°C. Following visualization of DNA by gel electrophoresis, PCR products were purified using Zymoclean™ Gel DNA Recovery Kit. Products were then used as a template (1 µl) for a short PCR using the same reagents as above to incorporate the pyrosequencing tags. The thermo-cycle consisted of 3 min. 95°C denaturation, then 10 cycles 15 sec. denaturation at 95°C, 15 sec. annealing at 61°C, and 15 sec. elongation at 72°C, final elongation step was 72°C for 3 minutes, followed by purification with the aforementioned purification kit. DNA concentration was quantified using a Qubit Fluorometer (Invitrogen). Samples were then diluted with ultrapure nuclease-free water to the same concentrations, and 2 µl of each sample was pooled to create an

equimolar pool. The equimolar pool was sent to New Zealand Genomics Limited and sequences were generated on a GS Junior (454 Life Sciences, Branford, CT, USA).

Library preparation

A local library of 148 New Zealand native and endemic earthworm species was created from sequences of earthworms available in GenBank and those previously collected from the study site by Boyer & Wratten (2010) and various New Zealand locations by Buckley et al., (2011). This library included species not likely present on the study site. Sampling of over 1,500 individual earthworms on the study site yielded 28 putative new species (three of which were subsequently described (Boyer et al. 2011a)). An integrated taxonomic approach of morphological and molecular methods was used to group these putative species into distinct operational taxonomic units (OTUs), and representative sequences were gathered by Sanger sequencing. Where appropriate these library OTUs will be ascribed pseudonyms of OTU 1, OTU 2 and so on.

Sequence analysis

Following quality checking, pyrosequencing data were compared to this library using Basic Local Alignment Search Tool (BLAST) with a $\geq 95\%$ similarity threshold (Altschul et al., 1990; Brown et al., 2012). Sequences that did not match the local library were clustered into molecular operational taxonomic units (MOTUs) (Floyd et al. 2002) to an 85% threshold based on consistent divergence across a number of measures: mean interspecific nearest-

neighbour relatedness of 82.7%, average p-distance of 15% (*i.e.* 85% similarity), and mean percent identification of 85%, calculated based on the local library using SPIDER package for R (Brown et al., 2012) and Geneious® Pro v. 6.1.4 (Biomatters). Representative sequences from these clusters were vetted by aligning them to the existing local library, comparing them to GenBank using BLAST, constructing a neighbour-joining tree, and discarding non-earthworm sequences. The remaining sequences were considered MOTUs when one or more representative sequence grouped in the tree independently of existing library sequences. Cluster thresholds were chosen, and the multiple comparisons undertaken, to ensure confidence in the MOTU threshold and to deliver a conservative estimate of the number of MOTUs either consumed or present on the study site. Finally, rare earthworm haplotypes were screened by removing those with <5 sequences in a sample from that sample, and by removing all species or MOTUs that appeared in only one sample. These measures were taken to avoid inclusion of artifacts or chimeras generated by stochastic PCR or sequencing errors. Gene alignment and tree-building were undertaken in Geneious®.

Post-bioinformatics sample data analysis was undertaken in R v 3.0.1 (The R Development Core Team 2011) and PRIMER (Clarke and Gorley 2006), which included a species accumulation curve for sample number based on cumulative earthworm species data using bootstrap methods to predict diet coverage. Total contribution to *P. patrickensis* diet of each earthworm species was calculated based on presence/absence of each species in a given sample. For seasonal analysis data were grouped into summer and winter categories, excluding autumn samples from the analysis due to lack of replication, and compared using a test of Jaccard similarity using permutational analysis of variance (PERMANOVA) in PRIMER.

This method was also used for comparison of diet between *P. patrickensis* and *P. augusta*.

Evenness was compared using the Gini coefficient (Damgaard and Weiner 2000) that ranges from zero to one, where higher values represent a larger degree of unevenness. The Gini coefficient was calculated in R using the method of Buckley & Damgaard (2012).

The application of NGS to diet identification is a growing area of research, but there are numerous recent studies disputing the reliability of quantification estimates (relating sequence proportions to actual biomass consumed) due to a broad range of influencing factors (summarised in Pompanon et al. 2012). Unfortunately, validating quantification with feeding trials is not possible for many species with high conservation profiles, or no captive populations such as *P. patrickensis*, and therefore this type of analysis was not undertaken.

Results

Morphological analysis

Large numbers of earthworm chaetae were present in all samples. Four samples contained arthropod exoskeletal remains. These consistently comprised very small isolated body parts. For example a single Pseudoscorpionida pedipalp <1 mm in length in one sample, and a single (<1 mm) possible leg section of an unidentified arthropod in the other three samples; in all four cases there was no evidence of any other body parts or exoskeleton. In addition to earthworm remains, several samples contained sphagnum moss and other unidentified plant parts.

Molecular analysis

86,838 sequences were retrieved from pyrosequencing, 62,842 of which were earthworm sequences following quality control, with a mean of 1047.36 ($\pm$ SE 54.25) sequences per sample. 26 unique earthworm MOTUs were identified. Initial BLAST yielded 17 MOTUs (totaling 51,110 sequences) matching the local library with the remaining 11,853 clustered into 9 unknown MOTUs. Discarded sequences had been compared to the GenBank database using BLAST and identified as bacterial. Cumulative earthworm species data (Fig. 1) estimated that 98.5% of the total *P. patrickensis* earthworm diet was identified based on the bootstrap estimated projection of 26.4 ($\pm$ SE 0.602) species present in the diet in total. Considering only the 20 most common species found (98% of the diet), 15 of these were identified using the library. Each faecal sample contained a mean of 4.7 ± 0.305 unique earthworm MOTUs, ranging from 1 to 10 per sample. A marginal difference in diet composition between summer and winter was observed (PERMANOVA, $P = 0.065$, $F = 1.646$; Fig. 2b&3). Presence/absence data were collated to assess the proportion of samples in which earthworm species occurred (Fig. 2a). This showed a relatively steady decrease in earthworm species occurrence, with no particular earthworm species dominating the diet as no species exceeded 45% occurrence overall. A similar pattern was observed in both summer and winter diets, however some of the most frequently consumed species differed from one season to another (Fig. 2b). Gini coefficients of evenness for summer (0.407), winter (0.395) and overall (0.372) did not differ greatly. Comparison between *P. patrickensis* and the previously assessed *P. augusta* (Boyer et al. 2013) indicated that *P. patrickensis*

consumes a larger number of species (Fig. 4), and that the diet composition significantly differs between snail species (PERMANOVA, $P < 0.001$, $F = 19.86$; Fig. 3b).

Discussion

Diet composition for P. patrickensis

Powelliphanta patrickensis feeds by rapidly snatching prey when in close enough proximity and swallowing it whole (personal observation; Efford 2000), suggesting it is unlikely that it feeds on liquid prey or by removing liquid from prey items. Arthropod hard parts were observed in four out of 60 samples, suggesting that these remains can survive the digestive process. Little other evidence of predation on other organisms but the presence of multiple earthworm species and highly abundant earthworm chaetae in all samples strongly suggests that earthworms probably form the major component of the diet throughout the year. However, predation on other soft-bodied organisms such as slugs, which were not investigated in this study, cannot be ruled out. Given the scarcity of invertebrate hard parts, consumption may have been either opportunistic or incidental as a byproduct of earthworm consumption, where earthworms or the snails themselves may have ingested a single arthropod appendage in the soil (Pearce 1978). With respect to moss fragments, *P. patrickensis* is often found in deep sphagnum moss beds, which are abundant throughout their habitat. The snails may encounter earthworm prey whilst in this habitat, and the way in which this species strikes prey suggests that moss and plant fragments may have been collaterally consumed.

Twenty-six earthworm MOTUs were identified and their occurrence in the diet, both overall and seasonally, declines relatively consistently reflecting what might be considered a typical rank abundance curve; *i.e.* a negative binomial (Fig. 2 a&b). Such patterns may suggest that *P. patrickensis* does not selectively predate particular earthworms; however this would require validation with additional information on the earthworm community and relative abundance on the study site. Despite this, there are some clear differences between the summer and winter diets (Fig. 2b). For example, OTU 1, *Ochtochaetus kenleei* Blakemore, OTU 4 and *Deinodrilus gorgon* Blakemore feature heavily in the summer diet, whilst are almost non-existent in the winter diet (Fig. 2b). Conversely, the opposite is the case for OTU 2. It seems therefore that the marginal difference in seasonal diet composition is driven by only a handful of species, which is also supported by a lack of clustering in the MDS plot (Fig. 3a). These differences may be attributable to seasonal availability of the individual earthworm species, given that there does not appear to be a strong preference for any particular species. Alternatively, as *P. patrickensis* requires damp or wet conditions in which to hunt (M. Hamilton, personal communication), foraging behaviour may change with seasonal weather patterns, such as day length and moisture; indeed the differences could be explained by a combination of these factors. Such differences had little impact on the diet as a whole. This may be explained by the relative constancy of the West Coast climate in New Zealand, characterised by mild winter temperatures and relatively consistent levels of rainfall throughout the year (National Institute of Water and Atmospheric Research 2013).

Diet comparison between P. patrickensis and P. augusta

The current data suggest that *P. patrickensis* requires seasonal availability of different earthworm species in its translocation and restored habitats regardless of whether indiscriminately consuming available earthworm species it encounters, or selectively shifting foraging behaviour across seasons. Reconciling the data in this study with that of *P. augusta* in Boyer et al. (2013) reveals a significant difference in prey communities consumed. However, it also reveals that there is an overlap in many of the species consumed, albeit in differing proportions (Fig. 3b&4). Boyer et al. (2013) suggest that *P. augusta* indiscriminately predate on earthworms that are available, however to conclude this for both species would require further information on the earthworm community in the respective habitats, and the behaviour of earthworms themselves (anecic, epigeic, or endogeic in the case of earthworms (Bouché 1972)). Until this information is available, it may be remiss to assume that habitat sufficient to support one species of *Powelliphanta* would be suitable for another. In management and restoration terms this means ensuring that the relevant earthworm community is present in relocation sites, achieved through site surveys, and ensuring restored sites contain or are stocked with the correct species as compared to the current study.

Consequences for habitat restoration and snail conservation

Specifically on the mine site, VDT is the preferred method for restoring the snail habitat and aims to translocate entire sections of ecosystems, including soil fauna to restoration areas. Assessing earthworm communities in VDT sites through surveys and reconciling this with the

current study can inform whether this restoration method is truly sufficient for earthworm and snail conservation. VDT is not yet implemented globally, but work by Boyer et al. (2011b) suggests that it is comparable to unmodified habitat for maintenance of earthworm biomass and species richness, which is promising for restoration, conservation of *P. patrickensis*, but also conservation of a diverse endemic earthworm community. As the aforementioned was a short-term study, further study would be required to confirm that this is a long-term success.

The use of charismatic individual species as focal- or 'flagship' species to achieve wider conservation has been previously debated and is unlikely to consistently deliver positive broad results (Simberloff 1998; Andelman and Fagan 2000; Williams et al. 2000). However, ecological restoration as defined by the Society for Ecological Restoration (2013) '... is an intentional activity that initiates or accelerates an ecological pathway—or trajectory through time—towards a reference state', where in the case of the current study the reference state is the impacted tussock grassland as it stands prior to mining. Thus, focusing on *P. patrickensis* and/or *P. augusta* that have garnered substantial media attention, does not aim to neglect wider ecosystem recovery or other important species nor utilise focal-species as indicators or conservation surrogates. Indeed, comparison of the diets of *P. patrickensis* and *P. augusta* reveals that focusing on single species conservation may not be sufficient to fulfill the dietary needs of even closely related species. Instead, in combination with the definition of ecological restoration, these species serve to draw attention to the issue of restoration on the study site, and ensure that it is appropriately addressed and built upon empirical evidence to achieve ecologically far-reaching restoration (Lindenmayer et al. 2002; Walpole

and Leader-Williams 2002). Study of *P. patrickensis*, earthworms and their interactions is therefore a part of wider research into restoring the impacted ecosystem as a whole.

Limitations of the molecular approach

The advantages of using a metagenomic approach is that it is possible to rapidly assess poorly studied or, as in the current study, imminently threatened sites and species. However collecting data in this way may lead to use of MOTUs (Floyd et al. 2002), a consideration of which is how one might go about defining the MOTU threshold. Much of the DNA library for Happy Valley was based on 1,500 earthworms collected for one study (Boyer et al., 2011), and so may be that full intraspecific variation and some taxa were overlooked. Based on this information, we used a conservative threshold so as not to overestimate the number of species consumed or on site, calculated using the distance measures available for the reference library. As mining has begun, sampling and site surveys are subject to strict time constraints and therefore sampling of entire communities may not be possible prior to complete ecosystem removal. In such cases, data mining combined with site surveys, although potentially limited, is often the only information on which to base such thresholds. Nevertheless, our data indicates that despite extensive sampling there may be a highly diverse endemic earthworm community at the mine site that has not yet been fully discovered. This further extends the need for adequate conservation and restoration that is not focused on very few focal-species, but the ecosystem holistically.

Of the 26 earthworm MOTUs detected in the diet of *P.patrickensis*, four species had already been formally described (*O. kenleei*, *D. gorgon*, *Maoridrilus felix*, *Diprochaeta cathamensis*) 13 were known library OTUs but undescribed species, and 9 were new MOTUs. This highlights several key aspects of diet studies of this type. They may accurately identify species consumed and aid in the understanding of the predator ecology, such as resource or temporal partitioning with other predators (Bohmann et al. 2011; Razgour et al. 2011) or feeding behaviour (Suzuki et al. 2008). Also, elucidating aspects of predator ecology, such as feeding preferences, prey selectivity, or identifying prey diversity can provide valuable insights for species conservation. But in the event of non-described prey species, as found here, prey morphology, ecology or habitat is unknown. If the aim is to use such studies to inform relocation or conservation programmes, then it may be sufficient to match the prey inhabitants of the proposed relocation area to those found in the diet analysis. Although diet analysis is indicative of the earthworm diversity likely on site, further study of the earthworm community would be required to answer the remaining ecological questions surrounding the predator-prey interactions, for example selective feeding, nocturnal foraging on anecic earthworm species present on the surface at night (Brown et al., 2012), or epigeic species at other times.

Conclusion

The use of pyrosequencing to identify key components of predator diets has been proven valuable in this study as in many others, and is particularly effective in identifying prey species that leave no distinguishable remains. A large amount of data can be generated

relatively quickly and inexpensively, and can therefore lead to many opportunities for community and population-level studies. We have shown that this method can be used at the population level to identify seasonal variation in a predator diet, which, in principle, can be applied to almost any predator diet. Indeed, understanding how a predator diet shifts and provisioning for such changes is essential for the long-term persistence of translocated predator populations in restored or relocation sites. The current results are valuable as they can be used to inform conservation and relocation programmes for effective conservation of target *P. patrickensis* populations, and begin to tease apart aspects of *P. patrickensis* ecology. Furthermore, it shows that although earthworms may not be the sole source of nutrition, they are a key component of the diet of *P. patrickensis*, and therefore highlights the need for conservation of earthworms as important prey items as well as the predator itself. In the case of New Zealand there is a highly diverse endemic earthworm community, which has conservation value in its own right, not just for its functional roles in native ecosystems as prey and ecosystem engineers.

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Data Accessibility

Earthworms from the local DNA library that are available on GenBank have accession numbers HQ232489 to HQ232687 (from Buckley et al., 2011), and HQ529285 to HQ529287 (Boyer et al. 2011a).

Figure Legends

Figure 1: Species accumulation curve representing the cumulative number of earthworm MOTUs discovered against the number of faecal samples ($n=60$). The horizontal solid line represents the total number of MOTUs (26.4 ± 0.602 ; SE represented by dashed horizontal lines) expected with limitless sampling, based on bootstrapped estimates. The vertical

dashed line indicates sampling effort for the current experiment, intercepting at 26 MOTUs, 98.5% of the projected total.

Figure 2: Rank abundance for the proportion of samples in which each earthworm species was found for (a) all samples and (b) summer (light bars) and winter (dark bars). OTUs are the library sequences taken from sampling of over 1,500 earthworms on the study site grouped into operational taxonomic units using an integrated molecular and morphological approach. MOTUs are molecular operational taxonomic units for which no library match.

Figure 3: Multidimensional scaling plots (MDS) of Jaccard similarity coefficients showing similarity between snail diet composition between (a) summer (▲) and winter (▼) ($P = 0.065$), and (b) between *Powelliphanta patrickensis* (▼) and *Powelliphanta augusta* (▲) ($P > 0.001$). Stress for both plots = 0.12.

Figure 4: Comparison of proportion of samples in which an earthworm species occurs, between diets of *Powelliphanta patrickensis* (dark bars) and *Powelliphanta augusta* (light bars). Diets between species significantly differed ($P < 0.001$).

Tables and Figures

Figure 1:

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