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Using molecular tools to identify New Zealand endemic earthworms in a mine restoration project

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Using molecular tools to identify New Zealand endemic earthworms in a mine restoration project

(Oligochaeta: Acanthodrilidae, Lumbricidae, Megascolecidae)

Stephane Boyer, Stephen D. Wratten

Abstract. A restoration ecology project was commenced on the West Coast region of New Zealand to re-establish the local fauna of endemic *Powelliphanta* spp. carnivorous landsnails at an opencast coal mine site after mining activities. The aim of the current research is to provide recommendations for the use of earthworms to improve the restoration of ecological communities, especially the landsnails. To provide such recommendations, different aspects of the ecology and bio-systematics of the New Zealand endemic earthworm fauna have been studied using molecular techniques. About 1,500 earthworm individuals have been collected across 17 sampling sites in the Stockton mine area. In New Zealand, 173 endemic earthworm species are known. Only minor revisions to the earthworms' taxonomic status have been made since 1959. Species identification was performed by morphological analysis (following Lee's taxonomic key) and molecular analysis (using the mitochondrial 16S gene). The latter analyses conducted on a selection of 83 individuals revealed the existence of at least 17 different taxa, most of which are probably undescribed species. Some of these earthworm species are predated by an endangered carnivorous landsnail, *Powelliphanta augusta* Walker, Trewick & Barker. Because the conservation of *P. augusta* may rely greatly on the understanding of their diet, earthworm DNA was sought after in the snails' feces, using molecular analyses. Molecular analyses have been helpful in establishing an inventory of the species present in the study site, facilitating new species taxonomic descriptions and elucidating the predator-prey relationship.

Key words. Earthworms, landsnails, New Zealand, restoration, mining, DNA.

Introduction

Opencast mining causes several environmental impacts, which require land rehabilitation when mining operations are finished (DOWN & STOCKS 1977). For this reason, rehabilitation after extractive industries' work is common and has been well studied (e.g., LYLE 1987, HOSSNER 1988, SMYTH & DEARDEN 1998, BELL 2001, HÜTTL & WEBER 2001, KOCH 2007, XI-JUN et al. 2008). However, many ecological rehabilitation schemes do not examine to what extent complete and functioning ecosystems have been restored above and below ground (GRANT et al. 2007).

A research project in collaboration with Solid Energy New Zealand Limited (SENZ; www.coalnz.com) aims at using endemic earthworms to improve the restoration of ecological communities after opencast coal mining has finished. This mining in New Zealand presents particular environmental problems including threats to rare endemic flora and fauna. SENZ is engaged in an environmental policy that aims to minimize the ecological disruption caused by mining activities through rehabilitation of native flora and fauna to the mine site once the mine is retired.

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To reach these objectives, the rehabilitation of earthworm communities has been investigated. Indeed, earthworms have a major role in the comminution and mineralization of organic matter and greatly influence soil structure and chemistry (STOCKDILL 1982, EDWARDS 2004, SNYDER & HENDRIX 2008). The presence of a flourishing earthworm population is therefore likely to accelerate soil rehabilitation (EDWARDS & BOHLEN 1996) and consequently favour native flora restoration, especially in soils affected by mining activities (BOYER & WRATTEN 2010). At the same time, the substantial earthworm biomass in many terrestrial ecosystems (BROCKIE & MOEED 1986) represents an important food source capable of supporting large communities of vertebrate (MACDONALD 1983) and invertebrate (JUDAS 1989) predators.

The study site is the Stockton mine, which is located on the West Coast of New Zealand's South Island, in the Buller region. This area lies between 500 m and 1,100 m above sea level and is characterized by high rainfall (~6,000 mm p.a.), a low human presence and important endemic flora and fauna. Among the potential earthworm predators inhabiting the Stockton mine vicinity, kiwis (*Apteryx* spp.) and carnivorous landsnails (*Powelliphanta* spp.) seem to rely heavily on earthworms in their diet (REID et al. 1982, COLBOURNE & POWLESLAND 1988, SALES 2005, STRINGER et al. 2003, YEATES 2007). Although many other species probably feed on New Zealand endemic earthworms, kiwis and carnivorous landsnails are particularly important because of their endangered status (HITCHMOUGH et al. 2005) and the requirement of strict management plans for the populations that are likely to be affected by mining operations (BULLER DISTRICT 2009).

Little research has been carried out on the ecology of the New Zealand earthworm fauna since the seminal work of LEE (1959a,b). At present, there is insufficient knowledge to facilitate the re-establishment of earthworms in post-mining land. Among the 193 earthworm species considered present in New Zealand, 23 are exotic and 170 are endemic (BLAKEMORE et al. 2009). Endemic species belong to the Megascolecidae and Acanthodrilidae families, while 15 of the introduced species belong to the Lumbricidae. Most of the exotic species have been introduced from Europe, first accidentally and then for agricultural purposes (STOCKDILL 1982). Introduced species are now established where human influence is obvious and agriculture is extensive. Because this study site is isolated from any agricultural land, only endemic earthworm species are expected to occur. LEE (1959b) recorded only 16 endemic species from the West Coast region of New Zealand. Although Lee's geographical coverage of the country was comprehensive, it is likely that some species remain to be collected and identified. This is because all but a few of New Zealand's endemic earthworm species are confined to only small areas of undisturbed soils under predominantly native vegetation.

Molecular analyses have been widely used for species identification, based on the genetic code of particular genes that are considerably conserved within species but quite variable between them. The most famous one is the barcode project based on the COI gene (HEBERT & RATNASINGHAM 2007); however, depending on the studied organism and the tested hypotheses, other genes have been successfully used such as ITS, 16S (DE ROJAS et al. 2007), 18S, 28S (CRUICKSHANK 2002), etc. These methods provide valuable help in taxonomical and ecological studies.

The aim of this paper is to outline the benefits of DNA-based identification in earthworms and its application in the context of an opencast mine rehabilitation research project in New Zealand. Molecular techniques have been used to identify earthworms in various parts of the world (e.g., JAMIESON et al. 2002, ADMASSU et al. 2006, HUANG et al. 2007); but no pub-

lished data exist on the genetics of New Zealand endemic species. The molecular analyses reported here were conducted to achieve three main objectives:

- Determine the family and genus of putative undescribed species by comparing their DNA to that of museum type specimens.
- Identify collected juveniles to species.
- Identify which species of earthworms are part of the diet of the newly described carnivorous landsnail species, *Powelliphanta augusta* (WALKER et al. 2008).

Methods

A total of 250 soil blocks of 20 x 20 cm and 20 cm deep were collected across 17 sites in the Stockton mine area. Earthworms were hand-sorted in the laboratory, and DNA samples were collected from individuals that were big enough to endure amputation of a minimum of 2 mg of tissue from the abdominal tip. This quantity is required for DNA extraction (AXYGEN BIOSCIENCES 2009). Landsnails' fecal samples were collected from free living animals during several translocation operations of the *Powelliphanta augusta* population in 2006 and 2007, i.e., before the species was formally identified (WALKER et al. 2008).

Molecular analyses were conducted using the mitochondrial marker 16S. This gene comprises about 500 base pairs and is frequently used to distinguish species one from one another and to establish species-specific sequences (DE ROJAS et al. 2007). Previous research has highlighted the value of 16S in earthworm taxonomy reconstruction at genius and species levels (POP et al. 2003, 2007). Sequences were aligned using MUSCLE (EDGAR 2004) and phylogenetic trees were built with MEGA (TAMURA et al. 2007) using the Neighbour-Joining (NJ) method (SAITOU & NEI 1987). A selection of 83 earthworms and 10 feces from landsnails were used in the molecular analyses.

Results

Molecular analysis of museum type specimens

About 1,500 individuals were collected from 250 soil samples in the Stockton mine. Only 53 of these earthworms were adults. Morphological identification of these species revealed that they all belong to the Megascolecidae or Acanthodrilidae families. Four species were identified: two of them had been recorded by Lee from the West Coast area: *Sylvodrilus gravus* Lee, 1959 and *Diplotrema rossi* (Lee, 1959); the other two species are new to this area: *Deinodrilus gracilis* Ude, 1905 and *Ochtochaetus sylvestris* Lee, 1952. However, half of the collected adult specimens did not match any morphological description and are likely to belong to new undescribed species (see Fig. 1).

Taxonomic description of these putative new species requires the proposition of morphological characteristics that distinguish them from closely related species. Comparing the DNA of the collected specimens to museum type specimens would facilitate this by determining the genus to which they belong. Furthermore, it would allow detection of field variants and avoid the description of synonymous species.

Since the inventory of LEE (1959a), there have been few studies on New Zealand earthworms in native vegetation. Lee's type collection is maintained by the *Te Papa Tongarewa* Museum in Wellington. Thomas Buckley from Landcare Research, Auckland is analyzing these type specimens using DNA techniques appropriate for ancient DNA.

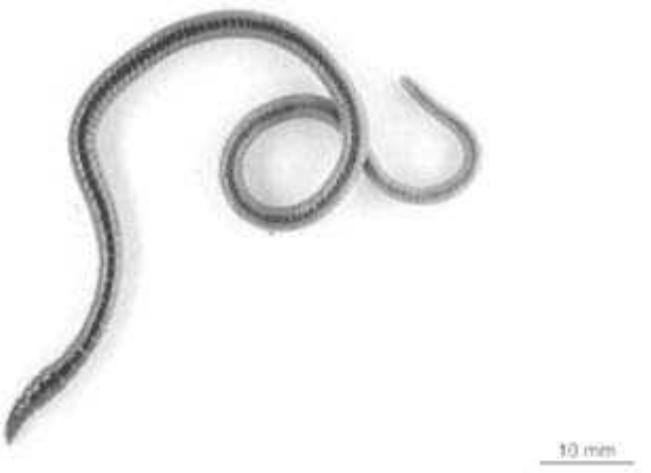

Fig. 1. Example of an undescribed earthworm species collected in the Stockton mine (West Coast, New Zealand).

Species identification of juveniles

Because the New Zealand earthworm identification key is based on sexual characteristics (LEE 1959b), only fully mature adults can be identified morphologically. Therefore, the majority of the collected individuals cannot be used for further analyses unless DNA identification is conducted.

Molecular analysis of the 16S gene was conducted on 83 earthworm individuals (adults and juveniles) collected in the Stockton mine area. The bootstrap consensus NJ tree (inferred from 2000 tree replicates) is taken to represent the evolutionary history of the taxa analyzed (FELSENSTEIN 1985). It displays 15 different groups, or clades, and two isolated individuals of New Zealand endemic earthworms with genetic differences between groups greater than 10% (Fig. 3). Such a genetic difference in the 16S gene is very likely to correspond to different species (POP et al. 2003). The observed diversity is relatively high compared with Lee's samples that report a range of one to 15 species, rarely more than nine, and most commonly two to five in one community (LEE 1985).

Two adult specimens used in the molecular analysis were also identified morphologically. The specimen coded *MA3_1a* was *Sylvodrilus gravus*, and the one coded *MA6_1a* was *Diplotrema rossi* (Fig. 2). For these two species, the DNA code for 16S is now described and can be used to identify juveniles. Therefore, the specimens belonging to the clade 13 (*CAM10_3*, *MAN5_3*, *MAN4_1*, *ST5* and *STH5*), which share the same DNA code as *MA3_1a,* belong to the same species (i.e., *Sylvodrilus gravus*). Similarly, specimens belonging to clade 8 are all *Diplotrema rossi*.

Molecular analysis of adults allows the construction of a DNA library where every species name is associated with a specific DNA code. Juveniles can then be identified to species by comparing their DNA code to the ones recorded in the library (Fig. 2).

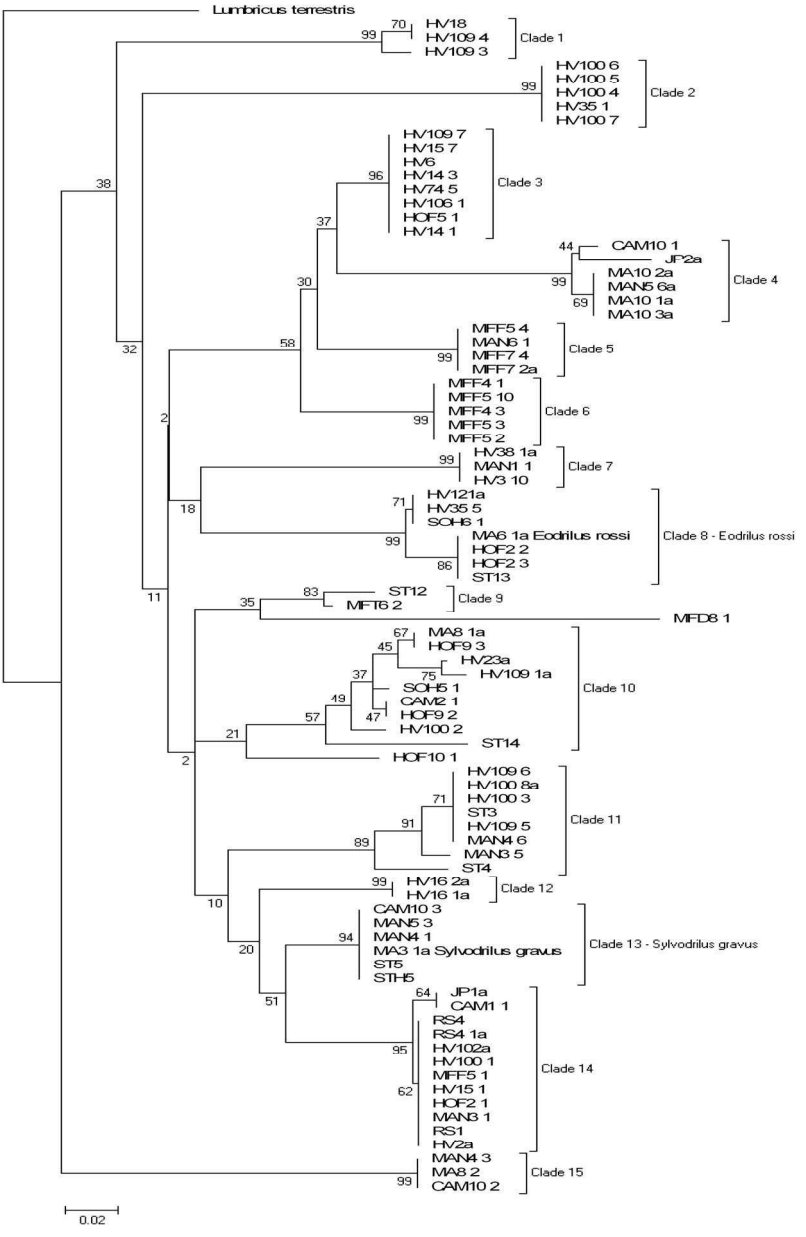

Fig. 2. Neighbour-joining bootstrap tree based on 16S mitochondrial rDNA for 83 New Zealand endemic earthworms and one out-group (*Lumbricus terrestris*). Each line corresponds to one individual designated by a unique code. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. The tree is drawn to scale, with horizontal branch lengths corresponding to percentage of difference (see scale for 2% of difference). The evolutionary distances were computed using the Kimura 2-parametermethod.

Molecular analysis of predator diet

Earthworm chaetae were recently found by Yeates in *Powelliphanta augusta* feces (YEATES 2007). The large quantities of chaetae (up to 400 in a single fecal string) indicate that this landsnail species does feed on earthworms. The identification of the predated earthworm species was not possible using the morphology of the chaetae. Therefore, molecular techniques were investigated to try to identify the predated species.

There are two major issues facing molecular analysis of faecal materials: (1) the highly degraded state of the DNA, and (2) the presence of non-prey DNA. (1) Because DNA is degraded during digestion, little prey DNA can be retrieved in the feces and only small fragments of it remain (JARMAN et al. 2004). Therefore, for optimal efficiency, the PCR primers must target a rather small DNA fragment. Good PCR results from digested DNA have been observed using fragments of less than 300 base pairs (AGUSTÍ et al. 1999, SYMONDSON et al. 1999, CHEN et al. 2000, HOOGENDOORN & HEIMPEL 2001). Furthermore, mitochondrial DNA, which is present in numerous copies per cell, is more likely to be retrieved after digestion than is nuclear DNA (SYMONDSON 2002). (2) Feces may contain a mix of DNA from prey, gut bacteria, the predator itself and accidentally-swallowed elements. Therefore group-specific primers are required to select only DNA from the prey (e.g., earthworms). A target fragment of DNA was selected using mitochondrial 16S sequences from 42 endemic earthworms collected in the Stockton mine that belong to 12 genetic different clades. This target fragment is internal to the mitochondrial 16S gene; it is 150 base pairs long and is variable enough to distinguish earthworm species one from one another. Specific primers were designed to bind exclusively to this target fragment of New Zealand endemic earthworm DNA and not with the European species *Lumbricus terrestris*. Because they do not amplify DNA from closely related species of earthworms, these group-specific primers will not amplify any DNA from distantly-related species such as other invertebrates or bacteria.

Samples from the feces of 10 *Powelliphanta augusta* individuals were analyzed. DNA from these feces was extracted, amplified by PCR using New Zealand endemic earthworm group-specific primers and sequenced. Two of the samples displayed accurate sequences and could be added to the phylogenetic tree of New Zealand endemic earthworms (Fig. 3). The other eight samples displayed non-interpretable sequences with large zones of low accuracy due to double peaks in the sequence, which are characteristic of the presence of a mixture of DNA from different species. Although only earthworm DNA is selected by the group-specific primers, DNA from different earthworm species may be present. It is likely that one landsnail will predate different earthworm species prior to fecal collection. The amplification of this mix of DNA will cause standard sequencing to fail. Indeed, the latter requires there to be thousands of copies of the same DNA fragment and produces a consensus sequence of the DNA fragments. If the sample displays high heterogeneity, the consensus sequence has low accuracy and is meaningless.

Conclusion

Molecular analyses have proven to be helpful in establishing an inventory of the species present in the study site, facilitating new species taxonomic descriptions and elucidating the predator-prey relationship.

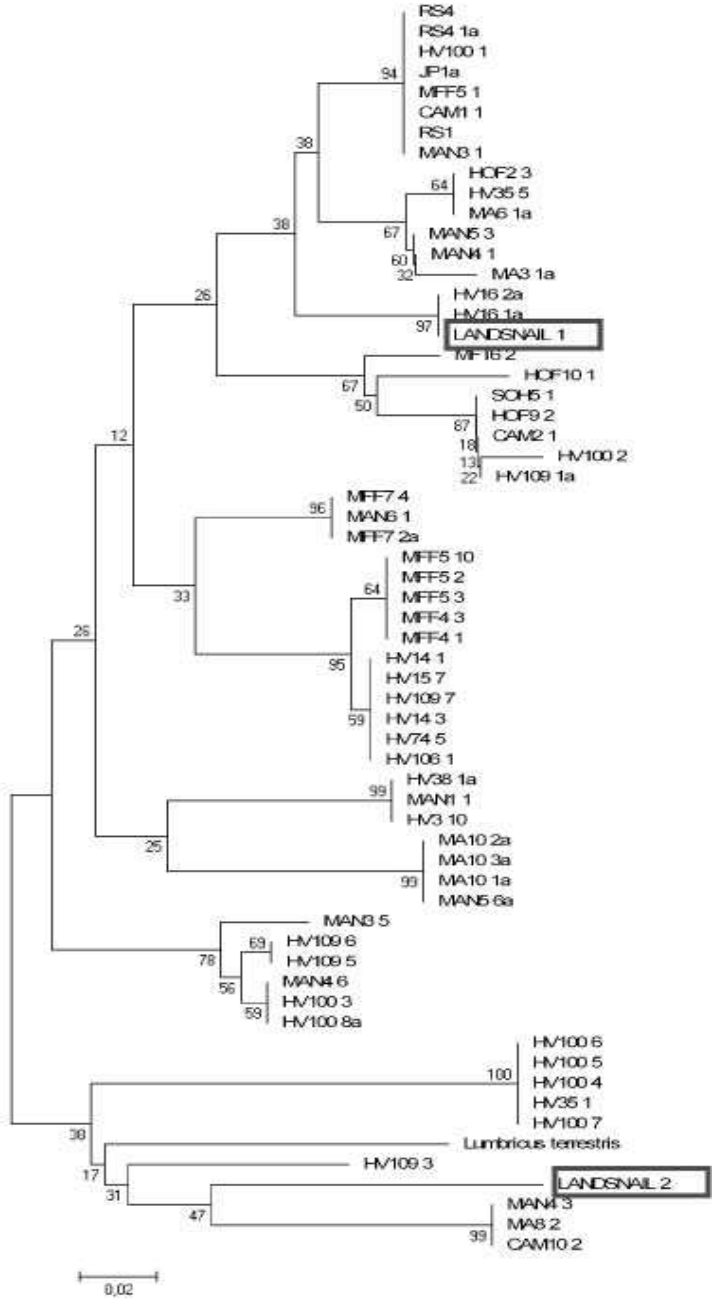

Fig. 3. Neighbour-joining bootstrap tree based on 16S mitochondrial rDNA for 61 New Zealand endemic earthworms and one outgroup (*Lumbricus terrestris*). Earthworm DNA retrieved from *P. augusta* landsnail feces is in red boxes. See Fig. 2 for legend.

Juveniles make up a high proportion of the population for many invertebrate species. However, identification keys are traditionally based on sexual characteristics and therefore require adult specimens (e.g., LEE 1959b, JAMIESON 1995). The use of molecular techniques for invertebrate species identification is becoming indispensable to maximize information obtained from each sample, which consequently minimizes population disturbance. In the current study, the identification of juvenile earthworms provides a significant increase in the amount of information obtained from sampling.

The description of putative new endemic species collected from the Stockton mine will be greatly facilitated by the molecular comparison between the collected individuals and the museum type specimens collected by LEE (1959a).

The detection of New Zealand endemic earthworm DNA in *P. augusta* faeces confirms that the snails feed on these earthworms. The identification of the predated species is of great importance for the conservation of this endangered landsnail species, particularly in relation to the choice of suitable translocation areas.

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