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Molecular identification and distribution of native and exotic earthworms in New Zealand human-modified soils

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Abstract: Important knowledge gaps remain with regards to the ecology and the systematics of New Zealand's native earthworms. With many putative new species yet to be described, often specimens cannot be named, which makes species inventory, monitoring and community comparisons difficult. Our work aimed to identify new putative taxa of New Zealand native species of earthworms, and describe their distribution in selected human-modified ecosystems. A total of 24 earthworm taxa (13 native and 11 exotic) were identified using a DNA barcoding approach focusing on 16S rDNA and COI (cytochrome oxidase subunit 1). The combination of morphological and molecular analyses were complementary in elucidating species identity. However, of the 13 native taxa, eight could not be named and are likely to be undescribed species from the genera *Octochaetus*, *Maoridrilus* and *Deinodrilus*. Most native species appeared to have a restricted geographic distribution linked to soil conditions, in particular pH and organic matter.

Key words: 16S; COI; DNA barcoding; Lumbricidae; Megascolecidae; native earthworms; phylogeny

Introduction

Since the arrival of Polynesians c. 750 years ago, more than two-thirds of New Zealand's native forests and grasslands have been replaced by human-modified landscapes, resulting in depauperation of native fauna including earthworms. Following conversion of native habitat to agriculture, native earthworm communities have largely disappeared from newly established farmland (Lee 1961). Introduced European Lumbricidae (mainly *Aporrectodea caliginosa*, *A. longa*, *A. rosea*, *A. trapezoides*, *Lumbricus rubellus*, and *Octolasion cyaneum*) with more tolerance to environmental disturbances have become dominant (Lee 1985; Fraser et al. 1996; Springett et al. 1998). Native species in the Megascolecidae family are often restricted to protected habitats and remnants of native vegetation, but they are also found on the borders of agricultural land (Kim et al. 2015). Coexistence of native and exotic species has recently been reported where patches of native vegetation borders agricultural land (Kim et al. 2015; Bowie et al. 2016) or when native vegetation is restored on agricultural land (Boyer et al. 2016).

Of the 3700 species of terrestrial earthworm described worldwide, 173 were described in New Zealand prior to 2000 (Blakemore 2006; Glasby et al. 2009). The earthworm species list was mainly the result of Lee's monograph published in the late 1950s (Lee 1959a). Despite an extensive geographical coverage of New Zealand, Lee's work was restricted to areas that were relatively easily accessible at that time. As a result, recent studies have unearthed a number of putative undescribed native species particularly in remote locations where no previous searches had been conducted (Boyer et al. 2011; Buckley et al. 2011).

In many cases, research on earthworm taxonomy has been limited by a lack of standardised morphological characters, phenotypic variability, and difficulties in defining diagnostic characters at juvenile or cocoon stages (Decaëns et al. 2013). Some of these taxonomic difficulties may be alleviated by recent developments in imagery for the description of internal morphology using Micro-Computed Tomography (Fernández et al. 2014), but lack of taxonomic expertise remains limiting. In recent years, the introduction of DNA barcoding has effectively aided species discrimination, identification of new taxa, reconstruction of phylogeny, and biodiversity assessments in numerous invertebrate groups, including earthworms (King et al. 2008; Chang & James 2011; Decaëns et al. 2013). DNA barcoding can be particularly useful for resolving previous taxonomic confusion but also to accelerate new taxonomic acts. For example, a new species of *Hormogaster* (*H. abbatissae*) was reported by Novo et al. (2010) based on the combination of morphological information and phylogenetic position following DNA barcoding. Moreover, molecular tools can be used to support phylogeography analysis for single species or a group of closely related species (e.g. Chang & Chen 2005; Minamiya et al. 2009) as well as discriminating between native and exotic species (Cameron et al. 2008; Porco et al. 2013). In conjunction with phylogenetic analyses, DNA barcoding analyses not only contribute to the discovery of new species and the identification of specimens, but also enhance our understanding of earthworms' ecology, taxonomy and evolutionary history (Domínguez et al. 2015).

Due to its unique geography, New Zealand is potentially home to many yet to be described Megascolecidae inhabiting isolated remnants of undisturbed native vegetation (SB unpubl. data). Buckley et al. (2012) anticipated that about 100 cryptic taxonomic species may remain to be described and molecular tools are now instrumental to the taxonomic description of native earthworms in New Zealand. Boyer et al. (2011) used DNA barcoding and phylogenetic analyses (based on the 16S

rDNA and COI genes) to support their description of three new species of Megascolecidae (*Deinodrilus gorgon*, *Ma. felix*, *Octochaetus kenleei*).

The primary aim of this study was to identify New Zealand native earthworms, including undescribed species, through DNA barcoding and describe their occurrence, as well as that of introduced species, in human-disturbed soils in relation to soil physicochemical properties.

Materials and methods

Earthworm sampling

Earthworm collection was undertaken between 2012 and 2015 in remnants of native vegetation and at a number of restoration areas in the South Island of New Zealand (Table 1). The 13 sampling sites were located at Banks Peninsula, Bankside, Eyrewell and Lincoln in Canterbury; and Punakaiki on the West Coast. To avoid the dry season when soil is hard to excavate and earthworms are more difficult to find, sampling took place mostly between late autumn (May) and the beginning of summer (December).

Soil pits (20 × 20 × 20 cm) were dug using a spade and earthworms were hand-sorted in the field. Collected earthworms were brought back to the laboratory for morphological identification following Lee (1959a, b) as well as for DNA analysis and other experimental work. Specimens were first categorised into morphospecies based on their external morphology, size, colour and behaviour. A total of 32 specimens representing all morphospecies were then analysed through DNA barcoding using the COI and 16S genes in an attempt to confirm species status.

DNA extraction, PCR and sequencing

Molecular analyses were conducted following a modified method from Boyer et al. (2011). Earthworms were washed in distilled water, then tissue samples (muscular body wall) were taken from behind the clitellum (mostly the tip of the tail) and preseved in 98% ethanol. Genomic DNA was extracted using a GF-1 Tissue DNA extraction kit (Vivantis Technologies Sdn. Bhd., Malaysia) following the manufacturer's recommendation. DNA was eluted in 200 µl preheated elution buffer and stored at -20^{o}C until further analysis.

Universal invertebrate primers for 16S (LR-J-12887 and LR-N-13398; Simon et al. 1994) and COI (LC01490 and HC02198; Folmer et al. 1994) were used to amplify ~550 and ~650 base pair fragments of DNA respectively (see Table S1 in Supplementary Material). PCR reactions (10 µl) consisted of 5 µl GoTaq® Green Master Mix (Promega, Madison, WI, USA), 0.1 µl $MgCl_2$ [25 mM], 0.4 µl forward and reverse primers [10 µM], 1.5 µl DNA template and 2.6 µl DNA-free water. The thermocycling protocol comprised of an initial denaturation at 95°C (4 mins), 35 cycles of denaturation at 94°C (1 min), annealing at 52°C (1 min) and elongation at 72°C (1.5 mins), followed by a final elongation at 72°C (10 mins). Negative controls were included to detect potential contamination. PCR products were sequenced in both directions using BigDye® Terminator Cycle Sequencing Kit following the manufacturer's protocol (Thermo Fisher).

Delineation of molecular taxonomic units (MOTUs)

All DNA sequences generated as part of this study were submitted to the GenBank database (accession numbers: KP771668–KP771678, KP780261–KP780262, KP828823–KP828824). Sequences were manually edited using FinchTV 1.40 (Geospiza), and compared to existing sequences in the Genbank database as well as sequences from Buckley et al. (2011) and Boyer (2013). The sequences generated here and their best match in the existing databases were exported into MEGA6 (Tamura et al. 2013) and Geneious® 6.1.8 (Biomatters) for alignment using MUSCLE (Edgar 2004). This resulted in alignments of 49 sequences for COI and 48 sequences for 16S. Neighbour-Joining trees (Saitou & Nei 1987) were then prepared and p-distances were calculated to make taxonomic decisions. The R package SPIDER (Species Identity and Evolution in R) was used to determine species boundaries and estimate the number of species present (Brown et al. 2012). The threshold for interspecific distances was calculated using the function *localMinima* in SPIDER (Brown et al. 2012).

Soil analyses

Soil analyses were performed at eight of the 13 sampling sites (Table 1). To elucidate soil properties at those collection sites, 500 g of fresh soil was sampled from the pits at the time of earthworm sampling. All soils were analysed by Analytical

Table 1. Earthworms sampling sites and GPS coordinates. Punakaiki is located on the West Coast while the other four sampling sites are located in Canterbury.

Sampling sites		Location (latitude/longitude)	Sites where soil samples were collected
Punakaiki	Nikau Reserve	-42° 8'38.39"S / 171°19'50.36"E	✓
	Restored and unplanted land	-42° 8'26.74"S / 171°19'47.53"E	✓
Bankside	Bankside Scientific Reserve	-43°43'49.33"S / 172°09'34.60"E	
Banks Peninsula	Okuti Reserve	-43°47'07.98"S / 172°49'51.23"E	✓
	Bossu Road	-43°48'59.93"S / 172°51'49.46"E	✓
	Southern Summit Roadside	-43°44'15.41"S / 172°54'32.64"E	✓
	Kaituna Reserve	-43°44'37.23"S / 172°41'14.82"E	✓
	Ahuriri Reserve	-43°39'58.97"S / 172°37'26.37"E	✓
	Northern Summit Roadside	43°39'59.86"S / 172°37'28.63"E	✓
Eyrewell	DOC Scientific Reserve	-43°22'59.07"S / 172°11'39.78"E	
	Spencer Bower Reserve	-43°25'42.08"S / 172°25'48.10"E	
Lincoln	Liffey Spring	-43°38'18.64"S / 172°29'06.93"E	
	Lincoln University	-43°38'37.19"S / 172°27'43.77"E	

Services in the Department of Soil and Physical Sciences at Lincoln University using standard methodologies, with ASPAC Ring Test QA procedures. Available nitrogen was analysed on fresh soil following extraction with 2M KCl (Blakemore 1987) and was determined using a FIA star 5000 triple channel analyser (Foss Tecator AB, Sweden). The remaining soil was air-dried and sieved to <2 mm using a stainless steel sieve for further soil chemical analysis. Soil pH (1:5W) and electrical conductivity (EC) were measured using pH and EC meters (Mettler Toledo Seven Easy). For organic matter (OM) content, 10 g of oven dried (100°C) soil was processed through loss on ignition at 550°C in a muffle furnace (Blakemore 1987).

Statistical analysis

Soil properties, such as pH, EC, OM content and mobile nitrogen (NH_4^+ and NO_3^-), were analysed using one-way ANOVA followed by a Fisher's least-significant-difference post-hoc test. Data were analysed using Minitab 17 (Minitab Inc., State College, Pennsylvania, USA).

Results

Specimen identification

Species delineation thresholds calculated for the COI and 16S genes were 3% and 2.8%, respectively, meaning that specimens of a similar species have at least 97% similarity in their COI DNA sequence, and at least 97.2% similarity in their 16S DNA sequence. Based on these thresholds, 24 discrete taxa were identified from the 36 individuals analysed (Fig. 1).

Only two specimens could be confidently identified at species level using morphological characteristics. They belonged to the native Megascolescidae species: *Octochaetus multiporus* and *Ma. transalpinus* (Table 2). Eight more specimens could be identified at genus level based on their morphology: *Octochaetus*, *Maoridrilus*, and *Deinodrilus*. When using DNA analyses, 11 specimens could be identified at species level from their 16S sequence, and the same was true for COI although the specimens that could be identified by each marker were different. The combination of both molecular markers and morphology led to the identification of 17 specimens at species level, and three at genus level, leaving 16 specimens unidentified. Despite slight differences in the datasets and the trees, the analysis of COI and 16S sequences provided no contradictory diagnostic in terms of species identification (Fig. 1, Table 2).

Earthworm distribution and soil chemistry

A total of 13 native and 11 exotic taxa were sampled across all study sites with richness at the different sites ranging from two to six taxa (Fig. 2, Table S2 in Supplementary Material). The Punakaiki restoration area contained the greatest richness of native earthworms (six taxa) in the Nikau Reserve and the greatest richness of exotic earthworms (eight taxa) in the restored land. Five of the 13 sites had a mixture of native and exotic earthworms.

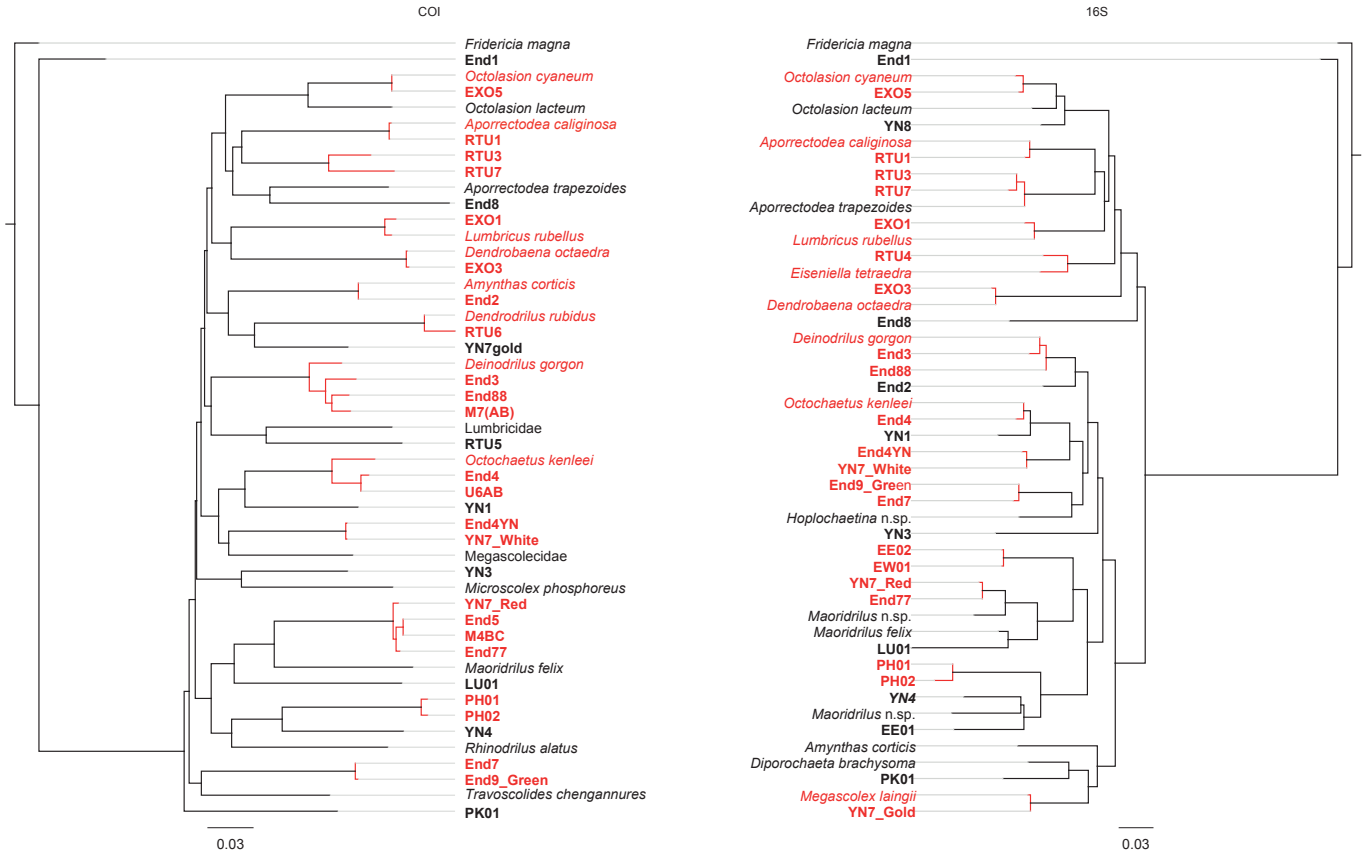

Figure 1. Neighbour joining tree for COI (left) and 16S (right) based on 36 earthworm specimens collected as part of this study (names in bold), along with their closest matches according to a MegaBLAST search against the Genbank library and sequences from Boyer (2013) and Buckley et al. (2011). The enchytraeid species *Friderica magna* was used as an outgroup. Trees are drawn to scale, with horizontal branch lengths corresponding to percentage difference (see scale). The evolutionary distances were computed using the Kimura 2-parameter substitution model. Specimens linked by red lines are considered to be of the same species based on species identify thresholds of 3% and 2.8% for COI and 16S, respectively.

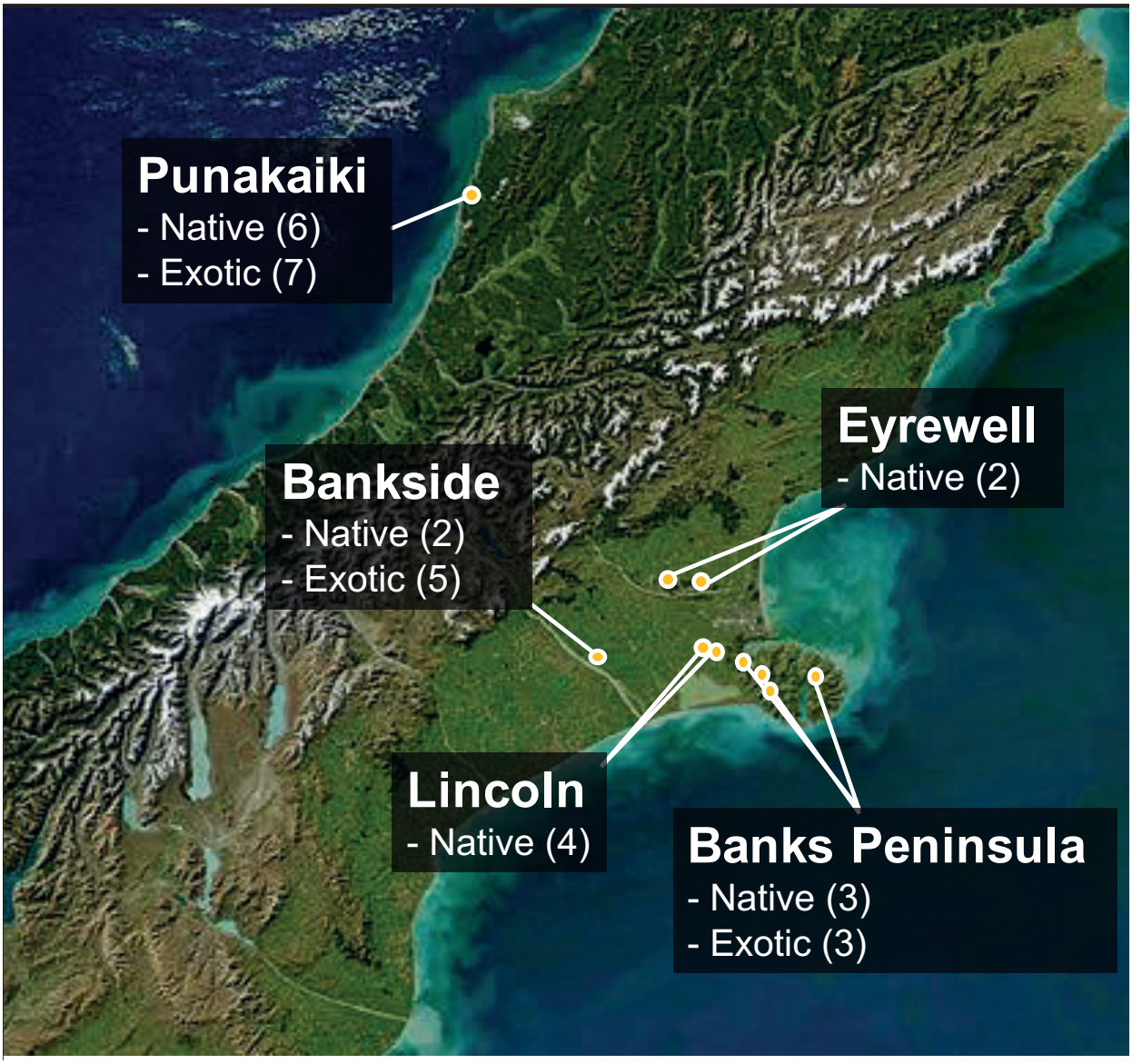

Figure 2. Distribution of native and exotic earthworms sampled in five regions of the South Island (Punakaiki, Bankside, Banks Peninsula, Eyrewell, and Lincoln). Number of species is in brackets. Underlying picture modified from Norman Kuring. commons.wikimedia.org/wiki/File:Turbid_Waters_Surround_New_Zealand_-_crop.jpg

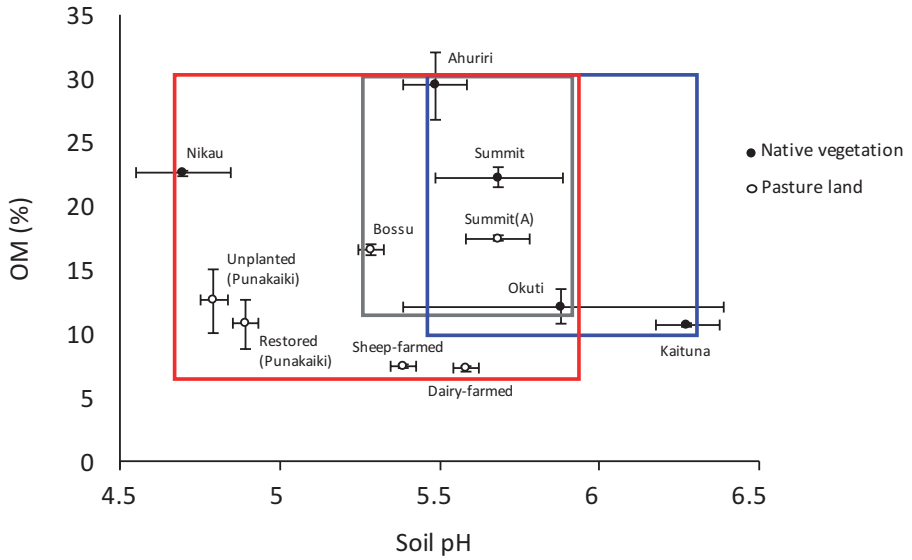

Figure 3. Soil pH and OM content at sampling sites in which native earthworms, including *Maoridrilus transalpinus* (blue box) and *Octochaetus multiporus* (grey box), and exotic earthworms, including *Octolasion cyaneum*, *Octolasion lacteum*, *L. rubellus*, and *Aporrectodae caliginosa* (red box), were found to co-occur. Each dot corresponds to values for one sampling site (mean ± SE). In addition to the sites analysed as part of the current study, soil data for sheep- and dairy-farmlands collected as part of previous studies (Fraser et al. 1996; Kim et al. 2015) were added to the graph.

Table 2. Species affiliation after morphological identification and DNA-based identification. When species could not be identified, specimens were marked with No Match (i.e. the closest hit on Genbank was greater than the species delineation threshold) or X (no DNA sequence available). For clarity, specimens are presented in the same order they appear in the neighbour-joining tree (Fig. 3).

Specimen name	Morphological ID (if any)	DNA-based identification (COI)	DNA-based identification (16S)	Native / Exotics
End1		No Match	No Match	Native
EXO5		*Octolasion cyaneum*	*Octolasion cyaneum*	Exotics
RTU1		*Aporrectodea caliginosa*	*Aporrectodea caliginosa*	Exotics
RTU3		No Match	*Aporrectodea trapezoides*	Exotics
RTU7		No Match	*Aporrectodea trapezoides*	Exotics
End8		No Match	No Match	Native
EXO1		*Lumbricus rubellus*	*Lumbricus rubellus*	Exotics
RTU4		X	*Eiseniella tetraedra*	Exotics
EXO3		*Dendrobaena octaedra*	*Dendrobaena octaedra*	Exotics
End2		*Amynthas corticis*	No Match	Exotics
RTU6		*Dendrodrilus rubidus*	X	Native
YN7gold		No Match	*Megascolex laingii*	Exotics
End3	*Deinodrilus*	*Deinodrilus gorgon*	*Deinodrilus gorgon*	Native
End88	*Deinodrilus*	*Deinodrilus gorgon*	*Deinodrilus gorgon*	Native
M7(AB)	*Deinodrilus*	*Deinodrilus gorgon*	X	Native
RTU5		No Match	X	Native
End4	*Octochaetus*	*Octochaetus kenleei*	*Octochaetus kenleei*	Native
U6AB	*Octochaetus*	*Octochaetus kenleei*	X	Native
YN1	*Octochaetus multiporus*	No Match	No Match	Native
End4YN		No Match	No Match	Native
YN7_White		No Match	No Match	Native
YN3		No Match	No Match	Native
EE02		X	No Match	Native
EW01		X	No Match	Native
YN7_Red	*Deinodrilus* (sp.1)	No Match	No Match	Native
End5		No Match	X	Native
M4BC		No Match	X	Native
End77		No Match	No Match	Native
LU01	*Maoridrilus* (sp.2)	X	No Match	Native
PH01	*Maoridrilus* (sp.1)	X	No Match	Native
PH02		X	No Match	Native
YN4	*Maoridrilus transalpinus*	No Match	No Match	Native
EE01		X	No Match	Native
End7		No Match	No Match	Native
End9_Green		No Match	No Match	Native
PK01		No Match	No Match	Native

Table 3. Physicochemical soil properties (mean ± SE, n=3) in Punakaiki and Banks Peninsula, where widespread exotic and native species were co-occuring. Native species include *M. transalpinus* and *O. multiporus* and exotic species include *O. cyaneum*, *L. rubellus*, and *A. caliginosa* (c.f. Table 3). EC: Ecectrical conductivity; OM: Organic Matter content.

Sample site		Vegetation type	Soil pH (1:5W)	EC ($dS \cdot cm^{-1}$)	OM (%)	NH_4-N ($mg\ kg^{-1}$)	NO_3-N ($mg\ kg^{-1}$)
Punakaiki	Nikau Reserve	Forest	4.7 ± 0.2	0.19 ± 0.03	23 ± 0.2	20 ± 1.4	18 ± 0.5
	Restored land	Pasture	5.44 ± 0.01	0.04 ± 0.01	11 ± 1.9	2.0 ± 1.2	0.7 ± 0.2
Banks Peninsula	Okuti Reserve	Forest	5.9 ± 0.5	0.08 ± 0.02	12 ± 1.3	4.0 ± 1.3	9.7 ± 2.3
	Southern Summit Road	Forest	5.7 ± 0.2	0.10 ± 0.01	22 ± 0.8	0.9 ± 0.3	9.7 ± 1.3
	Kaituna Reserve	Forest	6.3 ± 0.1	0.06 ± 0.01	11 ± 0.1	0.4 ± 0.2	2.6 ± 0.1
	Ahuriri Reserve	Forest	5.5 ± 0.1	0.08 ± 0.01	30 ± 2.6	0.9 ± 0.1	3.9 ± 0.5
	Bossu Road	Pasture	5.3 ± <0.1	0.04 ± 0.01	17 ± 0.7	1.1 ± 0.4	0.4 ± 0.1
	Northern Summit Road	Pasture	5.7 ± 0.1	0.04 ± 0.01	18 ± 0.6	0.5 ± 0.2	2.1 ± 0.2

The Nikau Reserve soil was more acidic and contained higher concentrations of mobile N (ammonium and nitrate) than soils from Canterbury sites (Table 3) and contained the greatest diversity of native earthworms (six taxa). In contrast, less acidic Canterbury soils (Banks Peninsula), which displayed moderate concentrations of mobile N, often harboured only three indigenous species (*Maoridrilus* spp. and *Octochaetus multiporus*).

Discussion

Specimen identification

A total of 15 Megascolecidae species were identified based on morphology and DNA analyses. Of these, *Am. corticis* and *Me. laingii* have been described from Australia but are considered to be exotic in New Zealand (Lee 1959b; Blakemore 2006).

The 17 unidentified specimens (forming eight species according to the DNA analysis) are thought to be undescribed indigenous Megascolecidae as they did not match the morphology of any described species and had no corresponding reference in existing DNA libraries. Therefore, our work confirms previous suggestions that many New Zealand native earthworm species are yet to be described (Boyer et al. 2011; Buckley et al. 2011). Many New Zealand native earthworms have restricted distributions. In this study, none of the native species found at Punakaiki were present in Canterbury, which suggests that the alpine chain between the two regions forms a barrier to earthworm dispersion as illustrated by the distribution of many species (Lee 1959a; SB unpubl. data). One exception is *Ma. transalpinus*, a widespread species whose distribution spans from the South Island West Coast to Banks Peninsula via Arthur's Pass (SB unpubl. data; Kim et al. 2015) and therefore is now classified as a non-threatened species (Buckley et al. 2015). Another dominant species on the eastern side of the Southern Alps is the deep burrowing endogeic *Octochaetus multiporus* that is found in Canterbury reserves (Kim et al. 2015) as well as in agricultural pastures on ridges of Banks Peninsula. This species has long been reported to occur in agricultural pastures (Springett et al. 1998). Other native species had more restricted distributions and were only found at one location, or two nearby locations.

With regards to exotic earthworms, nine species were identified, four of which were Lumbricidae: *Am. corticis*, *Ap. caliginosa*, *D. octaedra*, *F. magna*, *L. rubellus*, *Lumbricidae* spp., *Me. laingii*, *Octolasion cyaneum*, and *Octolasion lacteum*. These species are known to be widespread in West Coast soils as well as in Canterbury (Table S2; Hahner et al. 2013; Kim et al. 2015; Smith et al. 2016). Sites in the Punakaiki region contained the greatest richness of both native and exotic earthworms, six and eight taxa, respectively. The high OM content of the litter from the local luxuriant broadleaf vegetation at these sites (Hahner et al. 2013; Rhodes et al. 2013) may have promoted a greater richness of native earthworms filling a variety of ecological niches. In the restored agricultural land, the smaller scale, less intensive nature of agriculture when compared to the Canterbury sites may have contributed to a greater diversity of exotic earthworms.

Soil pH and OM are both vital factors for earthworm feeding activity and survival (Curry 2004). Two native species (*Ma. transalpinus* and *Octochaetus multiporus*) that are known to have a wide geographic distribution (SB; unpubl. data; Lee 1959a; Buckley et al. 2015) were collected at several of the sampling sites. Both of these species occurred in soils that contained similar OM content but had quite different soil pH (Fig. 3). Temperate climate species are generally found in soil where pH is between 4.5 and 7.4 (Bouché 1972). *Maoridrilus transalpinus* was collected from soils of pH 5.5 to 6.3 and *Octochaetus multiporus* was found in soils of pH 5.3 to 5.9. Springett et al. (1998) also estimated that *Octochaetus multiporus* was distributed in soils of pH 4.9 in native forests to 5.7 in hill pastures. It seems that the endogeic *Octochaetus multiporus* was more likely to have stronger resistance to acidification than the anecic species. Exotic species such as *L. rubellus*, *L. terrestris*, *Ap. caliginosa*, *A. rosea* and *Octolasion cyaneum* occurred over a much broader range of environmental conditions, including more acidic (pH 4.7) and less organic soils (7.3% of OM) than those where native species were found. Fraser et al. (1996) reported exotic earthworms in agricultural soils in Canterbury containing 4.3% to 5.5% OM.

Conclusion

A total of 179 indigenous earthworm species belonging to 26 genera of the Megascolecidae family have been described previously from New Zealand (see Table S3 in Supplementary Material). However, many more undescribed species may be present in remote, difficult to access locations or places that simply have never been sampled before. A large number of undescribed species of earthworm have been reported in recent studies (e.g. SB unpubl. data; Waterhouse et al. 2014) and a significant taxonomic effort is required to complete the list of New Zealand native earthworms. The present study illustrates the rudimentary nature of much of our existing knowledge. Even when species have not been described, molecular analyses can provide a first insight into not only their evolutionary history, but also their distribution and environmental requirements. However, widespread sampling, the consolidation of existing DNA databases, and the sequencing of a variety of genetic makers (e.g. 16S, 28S, and COI) will be necessary to make informed decisions for the conservation of native earthworms, of which 59% of the formally described species are currently classified as 'data deficient' by the Department of Conservation (Buckley et al. 2015).

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Supplementary Material

Additional supporting information may be found in the online version of this article:
Table S1. Primers used for PCR and DNA sequencing in this study.
Table S2. Distribution of 24 earthworm taxa, 13 endemic and 11 exotic species, collected from soils in New Zealand's South Island.
Table S3. Synthetic classification of New Zealand earthworms based on their family and genus groups.

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