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An integrative taxonomic approach to the identification of three new New Zealand endemic earthworm species (Acanthodrilidae, Octochaetidae: Oligochaeta)

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Abstract

This work adds three new species to the ca. 200 currently known from New Zealand. In Acanthodrilidae is *Maoridrilus felix* and in Octochaetidae are *Deinodrilus gorgon* and *Octochaetus kenleei*. All three are endemics that often have restricted ranges; however, little is yet known of their distribution, ecology nor conservation status. DNA barcoding was conducted, which is the first time that New Zealand endemic holotypes have been so characterized. The barcoding region COI (cytochrome c oxidase subunit 1) as well as the 16S rDNA region were sequenced using tissue from the holotype specimen to provide indisputable uniqueness of the species. These DNA sequences are publically available on GenBank to allow accurate cross checking to verify the identification of other specimens or even to identify specimens on the basis of their DNA sequences alone. Based on their 16S rDNA sequences, the position of the three newly described species in the phylogeny of New Zealand earthworms was discussed.

The description of new species using this approach is encouraged, to provide a user-friendly identification tool for ecologists studying diverse endemic faunas of poorly known earthworm species.

Key words: Morphological description, DNA barcoding, phylogeny, COI, 16S rDNA

Introduction

The definitive study of New Zealand earthworms by Ken Lee (1959) was updated and checklisted by Blakemore (2004, 2006) as modified by Blakemore in Lee *et al.*, (2000). While the enduring work by Lee (1959) listed approximately 193 species, the current list has about 200 taxa with natives now given separate family status in either Acanthodrilidae, Octochaetidae or Megascolecidae *sensu* Blakemore (2000).

Because of this high diversity, ecological studies focusing on New Zealand earthworms require the expertise of a taxonomist for accurate identification of the species. Indeed, earthworm taxonomy is based on complexe and variable morphological diagnostic characters, which require a high level of expertise (Pop *et al.*, 2007).

Modern molecular-based species identification is a promising way for researchers with basic molecular knowledge to identify earthworm species using barcoding regions of the genome (Pop *et al.*, 2007). However, this requires a comprehensive database of earthworm DNA sequences. More than 6,000 species of earthworm have been named to date and this figure is continuously growing as illustrated in the current study. At the same time, the Genbank database (http://www.ncbi.nlm.nih.gov/genbank/) contains barcoding sequences (COI) for only earthworm 600 taxa (i.e., 10% of the species), and for most of these (~400) the DNA description is not associated with any morphological taxonomic description and no valid species name is provided.

Although thousands of described species are still requiring DNA barcoding, molecular ecologists are releasing many barcoding sequences for new undescribed species, with little or no taxonomic support. Because of this imbalance between the taxonomically described species and the 'DNA barcoded' species, earthworm DNA barcoding can rarely be used for its original purpose: identifying species (Hebert *et al.*, 2003). In ecological studies it is often

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restricted to the determination of species richness in particular areas (e.g., Boyer & Wratten, 2010b), or the so-called discovery of cryptic species (e.g., King *et al.*, 2008 cf. Blakemore *et al.*, 2010). However, this does not provide species names, so interpretation and comparison to other ecological studies is difficult.

This paper aims at combining morphological description and DNA barcoding techniques to achieve a better taxonomic description of earthworm species. Such approach, using multiple and complementary perspectives to delimit the units of life's diversity is known as integrative taxonomy (Dayrat 2004). The adoption of integrative taxonomy will allow ecologists to identify earthworm species with standard molecular techniques. Because DNA analyses are directly conducted on the type specimen, this integrative approach also circumvents the release of species DNA codes originating from mis-identified non-type specimens (Blakemore *et al.*, 2010). In addition, by inferring evolutionary history from DNA sequences, one can confirm that the taxonomical diagnostic on a new species matches with its position in the phylogeny.

Classification follows Blakemore (2000); nomenclature follows ICZN (1999). In addition to the barcoding region of COI, molecular analyses were also conducted on the 16S rDNA region, which has complementary taxonomic value at genus and species level for earthworms (Pop *et al.*, 2003). These two markers have been often used in earthworm molecular ecology, protocols are easily accessible in the literature (e.g., Pop *et al.*, 2003; Chang and Chen, 2005; Huang *et al.*, 2007; Pop *et al.*, 2007; Chang *et al.*, 2008; Blakemore *et al.*, 2010; Boyer and Wratten 2010b). The 16S rDNA region was also chosen by Buckley *et al.* (2011) in their recent phylogenetic analysis of New Zealand endemic earthworms. Because this study provides the only comprehensive dataset of DNA sequences for New Zealand earthworms, it was necessary to use the same molecular marker to permit phylogenetic analysis of the specimens described here.

Material and methods

Earthworms were collected by excavation and hand sorting of soil samples (20×20 ×20 cm) from the tussock grassland of 'Happy Valley' in the Upper Waimangaroa Valley, Buller Region, West Coast, New Zealand), on the 'footprint' of the proposed Cypress coal mine. Specimens were sketched, dissected and described under low power microscope using the techniques and conventions noted in Blakemore (2002, 2008). Small tissue samples (muscular body wall) were taken from behind the clitellar region and placed in 98% ethanol for molecular analyses.

Genomic DNA was extracted using the DNeasy® Tissue Isolation Kit from Qiagen. Universal invertebrate 16S mitochondrial DNA primers (LR-J-12887 and LR-N-13398, Simon *et al.* 1994) were used to amplify a ~500 base pair fragment of DNA. Universal invertebrate COI mitochondrial DNA primers (LC01490 and HC02198, Folmer *et al.* 1994) were used to amplify a ~650 base pair fragment of DNA.

The 25µl polymerase chain reaction (PCR) contained 5µl Qiagen Q solution, 2.5µl 10X buffer (Invitrogen), 2.5µl dNTPs [2.5mM], 1µl $MgCl_2$ [25mM], 1µl Bovine Serum Albumin [10mg/ml], 0.5µl forward and 0.5µl reverse primers [10µM], 0.3µl Invitrogen *Taq* DNA polymerase [5unit/µl], 1µl DNA template and 10.7µl nanopure water. The thermocycler protocol consisted of an initial denaturation at 95°C (4 min), 35 cycles of denaturation at 94°C (1 m) annealation at 51°C (1 m) and elongation at 72°C (1.5 m), followed by a final elongation at 72°C (10 min). PCR products were purified (Qiagen Qiaquick© PCR purification kit), and sequenced using Big Dye Terminator Cycle Sequencing Kit according to the manufacturers' protocol (Applied Biosystems, CA, USA). DNA sequences were submitted to the GenBank database (see accession numbers in results).

The 16S rDNA sequences obtained for the three newly described species were compared to similar sequences obtained by Buckley *et al.* (2011). One representative for each major clade of New Zealand endemic earthworms was included in the analysis (see Buckley *et al.* 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Mei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.* 2007).

Results

Acanthodrilidae Claus, 1880

Maoridrilus felix Blakemore sp. nov.

Material examined. Museum of New Zealand Te Papa Tongarewa W.002908 (Holotype). From the tussock grassland of 'Happy Valley' (Upper Waimangaroa Valley, Buller Region, West Coast, New Zealand). Collected by S. Boyer, 2010. Mature, complete, fixed in ethanol 98% and placed in propylene glycol.

Etymology. Adjectival Latin for "Happy", after the location name.

External characters. Body circular in anterior, squaring off in mid-body and dorsally canaliculate in the posterior 50 or so segments. Pigment dark, especially dorsum chocolate brown with darker mid-dorsal stripe. Length 170 mm with 199 segments. Prostomium tanylobous. Setae lumbricine. Clitellum faintly marked 15-19,½20. Dorsal pores wanting. Nephropores, after the first few segments, alternate regularly between c and b lines with anterior segmental distributions: 3–7c, 8 c or b, 9–10c, 11b, 12c, 13b, etc. Spermathecal pores in mid-ab lines in 7/8 and 8/9. Female pores faint, just anterior to b setae on 14. Prostatic pores approximately in a lines on 17 and 19 with protuberant penial setae. Male pores not located within concave seminal grooves, although likely central between retained ab setae. Genital markings absent, but setae ab on 16 with slight pale tumescence as on 20lhs. Genital setae absent; penial setae longish, curving with spoon-shaped tips [one of their functions, if not primary function, is to scrape out or disrupt any prior semen from spermathecal diverticula that often correspond in depth to the setal length (see Blakemore 2000)].

Internal morphology. Pharyngeal mass anterior to 4/5. Septa mostly thin and translucent. Proventriculus wide and S-shaped in 5. Gizzard muscular in 6. Dorsal blood vessel single thoughout. Heart paired in 10–13. Nephridia holoic with long, sausage-shaped vesicles. Spermathecae in 8 and 9 each with a multiloculate diverticulum (inseminated) transcending anterior septum. Testes free, posterio-ventrally in 10 and 11. Seminal vesicles saccular, anterio-dorsally in 11 and 12. Ovaries compact sheets in 13 with large oviducts; ovisacs not found. Prostates tubular in 17 and 19 exiting through muscular ducts with ectal penial setal sheathes and tendons. Vasa deferentia seen to 18. Oesophagus dilated in 11–15 with blood vessels attaching dorsally but not saccular and not construed as calciferous glands. Intestinal origin in 18. Typhlosole not detected to about 26. Gut contains colloidal organic matter.

Ecology: Lack of dorsal pores is more usually associated with a semi-aquatic habitat. Unidentified nematodes were found near the prostates (cf. Yeates *et al.*, 1985). Specimen was found under 10 to 20 cm of soil. Dark colouration on the dorsum suggests at least partial surface exposure on topsoil, gut content suggests topsoil geophagy. This species is likely to be anecic.

Remarks. Quintessentially *Maoridrilus* due to its alternating nephridiopores, this species appears unique in its lack of dorsal pores (although more information is needed on several other congeners), gizzard in 6, lack of oesophageal glands, and genital marking absence. Multiloculate spermathecae appear characteristic of the genus and in the current species their form is almost identical to *Maoridrilus thomsoni* Benham, 1919: fig. 4 from D'Urville Island in Cook Strait. Lee (1959) held this species, along with similar *M. intermedius* Michaelsen, 1923 and *M. mauiensis* Benham, 1904, as *incertae sedis* because original descriptions were inadequate. Permanence of the name *M. felix* depends on redescription of *M. thomsoni*, however, the manifestly larger penial setae and lack of oesophageal glands in 14–16 seem to separate the current species. *Maoridrilus nelsoni* Lee, 1959 differs in its prostatic pores in b lines, and its prominent tuberculae pubertatis ventrally on segments 10 and 16. *Maoridrilus uliginosus* (Hutton, 1877) differs, not least, in its paired dorsal blood vessel.

DNA sequences

COI, GenBank accession number HQ529282 (submitted 01 November 2010)
AAGATATTGGAACTCTATATTTCATTTTAGGTGTTTGGGCCGGTATAATTGGAGCTGGGATAAGATTATT
AATTCGAATTGAATTAAGACAGCCTGGTGCTTTTCTAGGGAGTGATCAACTATATAATACTATTGTCACT
GCCCACGCTTTTTTAATAATCTTTTTCTTAGTGATACCAGTATTTATCGGAGGATTCGGAAATTGGTTAC
TACCCCTAATACTTGGAGCACCTGACATGGCTTTTCCACGATTAAACAATATAAGATTTTGATTACTGCC
CCCATCTCTTATTCTCCTAGTTTCTTCCGCAGCCGTAGAAAAGGGGGCTGGAACAGGGTGAACTGTAT

ACCCCCCCTTAGCAAGCAATCTAGCTCATGCTGGACCCTCTGTAGATCTTGCAATCTTCTCCCTCCACC
TAGCTGGTGCCTCATCAATTTTAGGTGCTATTAATTTTATTACAACCGTTATTAATATGCGGTCAGTTGG
ATTACGGTTAGAGCGAGTACCACTATTCGTTTGAGCGGTATTAATTACAGTAGTATTACTACTCTTGTCC
CTACCAGTATTAGCCGGTGCTATCACCATATTACTAACAGACCGAAATTTAAATACATCATTTTTTGACC
CGTCAGGAGGGGGTGACCCAATTCTATATCAACACTTATTCTGATTTTTTG

16S, GenBank accession number HQ529285 (submitted 01 November 2010)
CAAAAACATTGCTTTTTGAATAACTATAAAAAGTAATTTCCTGCCCAGTGACAAAGTTAAACGGCCGC
GGAACCCTAACCGTGCAAAGGTAGCATAATCACTTGCCTATTAATTGTAGGCTAGAATGAACGGATGA
ACGAAATAGAGACTGTCTCAGTCAGCTCACTAAAAATTAACACATGCATGAAGAGTTGCAAATAAAGT
CGAAGGACAAGAAGACCCTATAGAGCTTTATTTTAACTATAGATACAACTATACAAAAATTCGGTTGGG
GCGACCAAGGACATAGAGAATATCAACCTAAACAAAAATGATAAATTAATCTATAAACTGACCCTTATA
AAGAACATAAAAATAAGCTACCTTAGGGATAACAGGCTAATCCTATTTGAGAGTCCATATCTACAATAG
GGTTTGGCACCTCGATGTTGGCTTAGGGTATCAATATGGCGCAAAAGTTGTATGAAGATGGTTTGTTCA
ACCAGTAACTCCCTACATGAGCTGA

Octochaetidae Michaelsen, 1900 *sensu* Blakemore, 2000

***Deinodrilus gorgon* Blakemore sp. nov.**

Material examined. Museum of New Zealand Te Papa Tongarewa W.002909 (Holotype). From the tussock grassland of "Happy Valley" (Upper Waimangaroa Valley, Buller Region, West Coast, New Zealand). Collected by S. Boyer, 2010. Mature, posterior amputee, fixed in ethanol 98% and placed in propylene glycol.

Etymology. Noun alluding to Greek mythical monsters with sharp fangs, staring eyes and, similar perhaps to the ring of diverticula on each spermatheca – a belt of serpents.

External characters. Body circular in anterior. Pigment dark, especially dorsum with paler setal auriolae; clitellum and male field white. Length 55+ mm with 73+ segments (amputee). Prostomium tanylobous. Setae perichaetine, 12 per segment, evenly spaced. Clitellum pale, tumid ⅔13–16. Dorsal pores from 10/11. Nephropores not found. Spermathecal pores in b lines in 7/8 and 8/9, small but gaping. Female pores anterio-ventral to a setae on 14 in common field. Prostatic pores at b on 17 and 19. Male pores within concave seminal grooves lateral to b. Genital markings as large eye-shaped papillae paired on 10; with smaller markings on 13rhs, 16 rhs and two additional pairs on 18 as figured. Genital and penial setae not found.

Internal morphology. Pharyngeal mass anterior to 4/5. Septa 8/9–10/11 with some thickening. Gizzard muscular in 6 (weak septum 6/7 can be carefully teased off to base). Dorsal blood vessel doubled. Heart paired in 10–13. Nephridia meroic; equatorial forests especially obvious around clitellar segments. Spermathecae in 8 and 9 each with a thin duct to multiple, finger-like diverticula, five per spermatheca (inseminated) surrounding duct from where it thickens before reaching yellowish, knob-like ampulla. Testes free, posterio-ventrally in 10 and 11. Seminal vesicles small saccular in 9 (vestigial?) and larger racemose anterio-dorsally in 11 and 12. Ovaries fan-shaped in 13 with several strings of largish eggs; ovisacs vestigial in 14. Prostates compacted tubular in 17 and 19 exiting through muscular ducts. Vasa deferentia seen to exit unceremoniously in 18. Oesophagus dilated in 15–17 but lacking internal lamellae and thus not construed as calciferous glands. Intestinal origin in 18. Typhlosole thin, lamellar becoming deeper from 19. Gut contains colloidal soil and organic matter.

Ecology. Specimen was found under 10 to 20 cm of soil. Dark colouration suggests at least partial surface exposure on topsoil, gut content suggests topsoil geophagy. This species is likely to be anecic.

Remarks. Of the eight currently known *Deinodrilus* species, only two have tanylobous prostomia: *D. gracilis* Ude, 1905 from Stephen Island and *D. parvus* Lee, 1959 from Mangamuku Range. Both also have 5 or 6 spermathecal diverticula however, *D. gracilis* has copulatory setae, oesophageal glands and intestine from 19; while *D. parvus* has a saddle-shaped clitellum in 12–16, and all its reproductive pores are in a or ab. Further, their gizzards are in 6–7 and 5, respectively, rather than single in 6 as in the current species. *D. montanus* Lee, 1959 from Rimutaka Range is similar to *D. parvus* and differs for similar reasons. The current species appears unique in the distribution of its eye-like genital markings that are especially noticeable on segment 10.

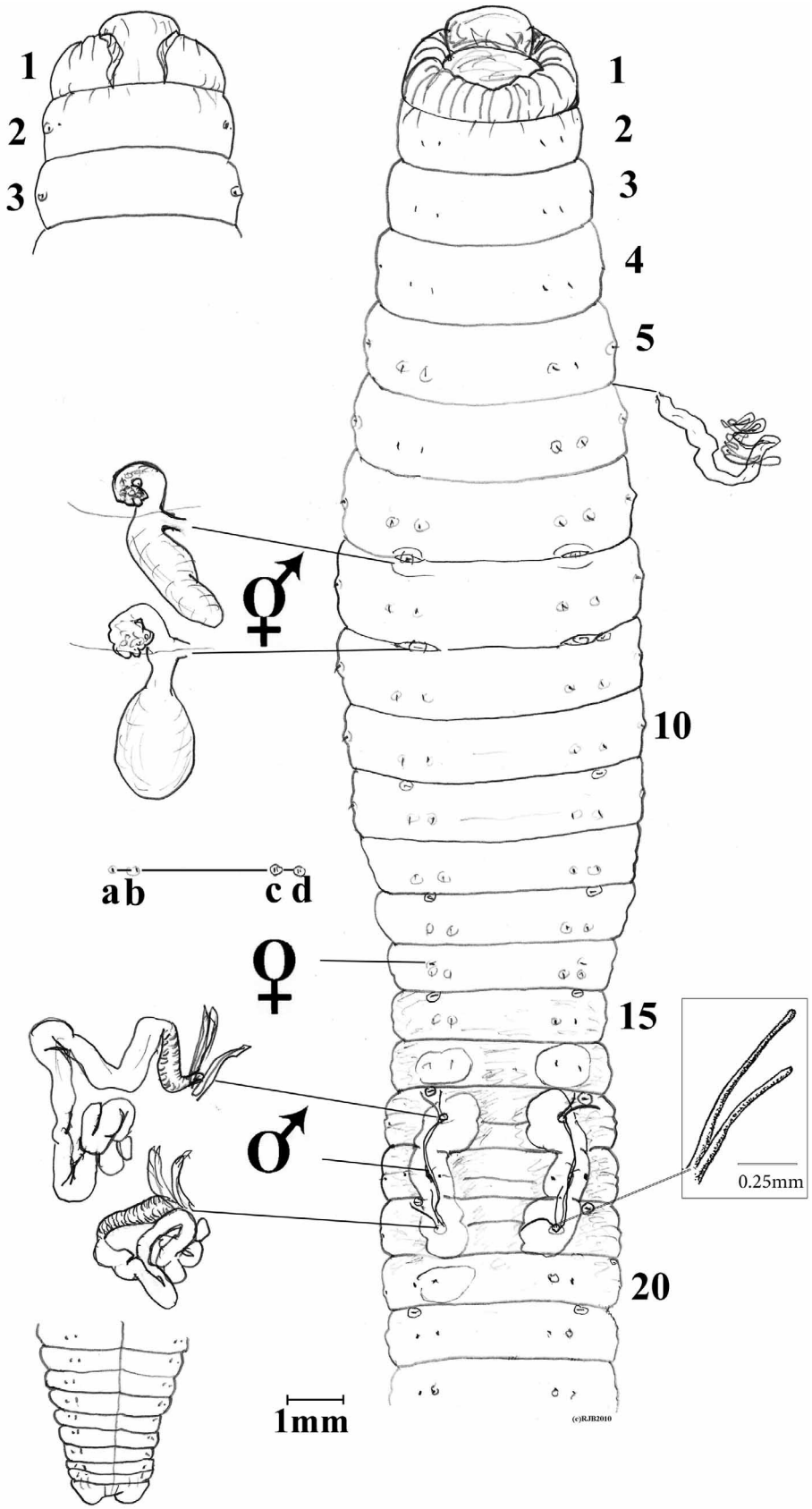

FIGURE 1. *Maoridrilus felix* Holotype showing dorsal view of prostomium and pygidium, and ventral view of body with spermathecae and prostates *in situ*; nephridium of 6lhs is as seen from dorsal dissection; enlarged penial seta 19rhs.

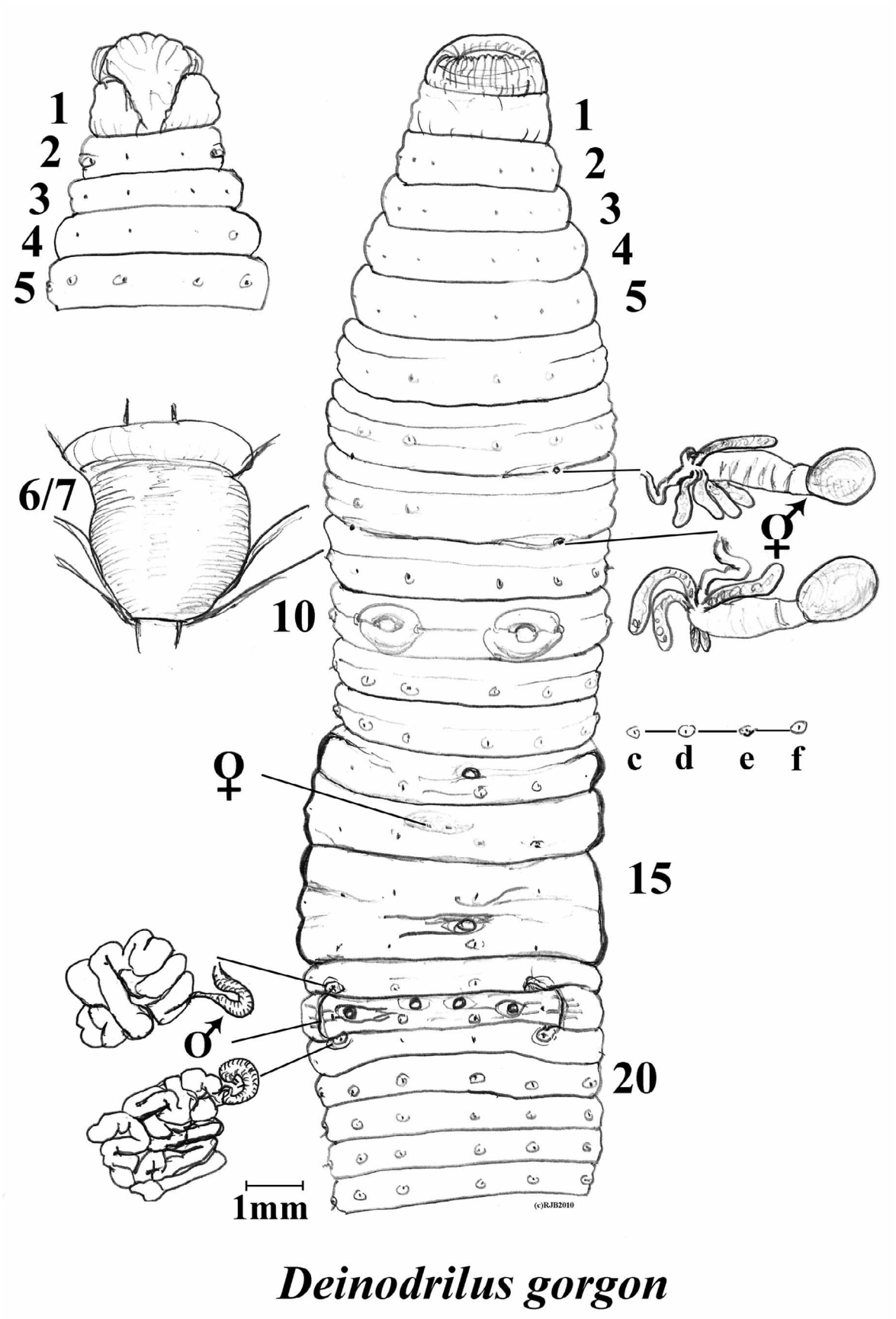

FIGURE 2. *Deinodrilus gorgon* Holotype showing dorsal view of prostomium and ventral view of body with spermathecae and prostates *in situ*; gizzard is in 6.

DNA sequences:

COI, GenBank accession number HQ529284 (submitted 01 November 2010)
AAAGATATTGGAACACTATATTTTATCTTAGGAGTTTGAGCAGGTATGATCGGAGCCGGAATAAGACTA
TTAATCCGAATCGAATTGAGCCAACCTGGAGCCTTCCTAGGAAGCGATCAATTATATAATACAATTGTT
ACAGCACACGCATTTCTAATAATCTTCTTTTTAGTAATACCAGTATTTATTGGGGGGTTTGGAAACTGAC
TACTTCCATTAATACTAGGAGCACCAGACATAGCATTTCCACGACTTAATAATATAAGATTCTGATTGTT
GCCCCCATCTCTAATTCTTCTAGTATCCTCTGCAGCCGTCGAAAAAGGAGCTGGAACAGGATGAACAG
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TCTAGCAGGTGCATCATCTATTTTAGGAGCAATCAACTTTATTACAACTGTAATTAATATGCGATGAACA
GGTCTACGACTAGAGCGAGTTCCTTTATTTGTATGAGCTGTATTAATTACAGTAGTACTTCTTCTTCTAT
CCTTACCAGTACTAGCTGGTGCCATTACCATACTCCTTACAGACCGAAATCTAAATACCTCATTCTTTGA
TCCCTCAGGAGGGGGAGATCCCATTCTATATCAACACTTATTTTGATTTTTTG

16S, GenBank accession number HQ529287 (submitted 01 November 2010)
AAAAACATTGCTTTATGAAAAACCATATAAAGTAATTCCTGCCCAGTGACAACTGTTCAACGGCCGCG
GTATCCTAACCGTGCAAAGGTAGCATAATAACTTGCCTATTAATTGTAGGCTAGAATGAACGGATAAAC
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GAAGGACAAGAAGACCCTATAGAGCTTTATTTAAAATCTAGATACTCTAGACAAAAATTCGGTTGGGG
CGACCAAGGGCACTATAAAACACCCTGAAAAAAAAAGATATATTAATCTACATGAATGACCCTAATAA
GATCACAAGATCAAGCTACCTTAGGGATAACAGGCTAATCCTATTTAAGAGTCCATATCAATAATAGGG
TTTGGCACCTCGATGTTGGCTTAGGGTATCGATATAGCGCAAAAGTTATACATGGATGGTTTGTTCAAC
CAATAATACCCTACATGAGCTG

***Octochaetus kenleei* Blakemore sp. nov.**

Material examined. Museum of New Zealand Te Papa Tongarewa W.002910 (Holotype). From the tussock grassland of "Happy Valley" (Upper Waimangaroa Valley, Buller Region, West Coast, New Zealand). Collected by S. Boyer, 2010. Mature, complete, fixed in ethanol 98% and placed in propylene glycol.

Etymology. In patronymic tribute to the foremost earthworm eco-taxonomist of New Zealand, Dr Kenneth Earnest Lee (1927–2007).

External characters. Body circular but posterior slightly square. Pigment lacking and generally fair. Length 220 mm with 270 segments. Prostomium prolobous. Setae lumbricine, 8 per segment, evenly spaced. Clitellum not well marked, perhaps in some or all of 14–20. Dorsal pores from 14/15, small. Nephropores not clear, some possibly in c and d lines or rather irregular. Spermathecal pores segmental, lateral to b lines on 8 and 9 on small mounds. Female pores just anterior to setae a on 14. Prostatic pores at b on 17 and 19. Male pores within concave seminal grooves lateral to b. Genital markings as small lens-shaped hollows paired in 8/9 and 9/10 near b lines and in 15/16 in a lines with a unilateral marking in 18/19lhs; area bb in 19/20–22/23 tumid. Genital and penial setae not found.

Internal morphology. Pharyngeal mass anterior to 4/5. Septa 8/9–10/11 with some thickening. Gizzards muscular in 5 and 6. Dorsal blood vessel appears single on gizzards but is doubled from 7 posteriorly. Heart paired in 10–13. Nephridia meroic as a few (ca. 4 per side) small tufted clusters in each segment. Spermathecae in 8 and 9 saccular each with small discrete and interlocular diverticula (inseminated) ringing exit. Testes free, posterio-ventrally in 10 and 11. Seminal vesicles large finely racemose anterio-dorsally in 11 and 12. Ovaries composed of several strings of largish eggs in 13; ovisacs absent. Prostates tubular in 17 and 19 exiting through narrow ducts. Vasa deferentia exits in 18. Oesophagus dilated as annular calciferous gland in 17 with several internal lamellae but not especially vascularized. Intestinal origin in 20 (valval in 19). Typhlosole large inverted T-shape developing from 21. Gut contains colloidal soil with a few quartz grits and woody fragments.

Ecology. Specimen was found under 10 to 20 cm of soil. Large size, pale colouration and gut contents suggest subsoil geophagy. This species is likely to be endogeic.

Remarks. The current species is compared to *Octochaetus thomasi* Beddard, 1892, widespread in the Canterbury Plains, that is the only other congener known to have gizzards in 5–6. As with all other members, it has spermathecal pores in 7/8/9 and on this character alone the current species is differentiated. *Neodrilus campestris*(Hutton, 1877) from Dunedin has segmental spermathecal pores (on 8) but differs, not least, by qualifying for inclusion in Acanthodrilidae due to its holoic nephridia.

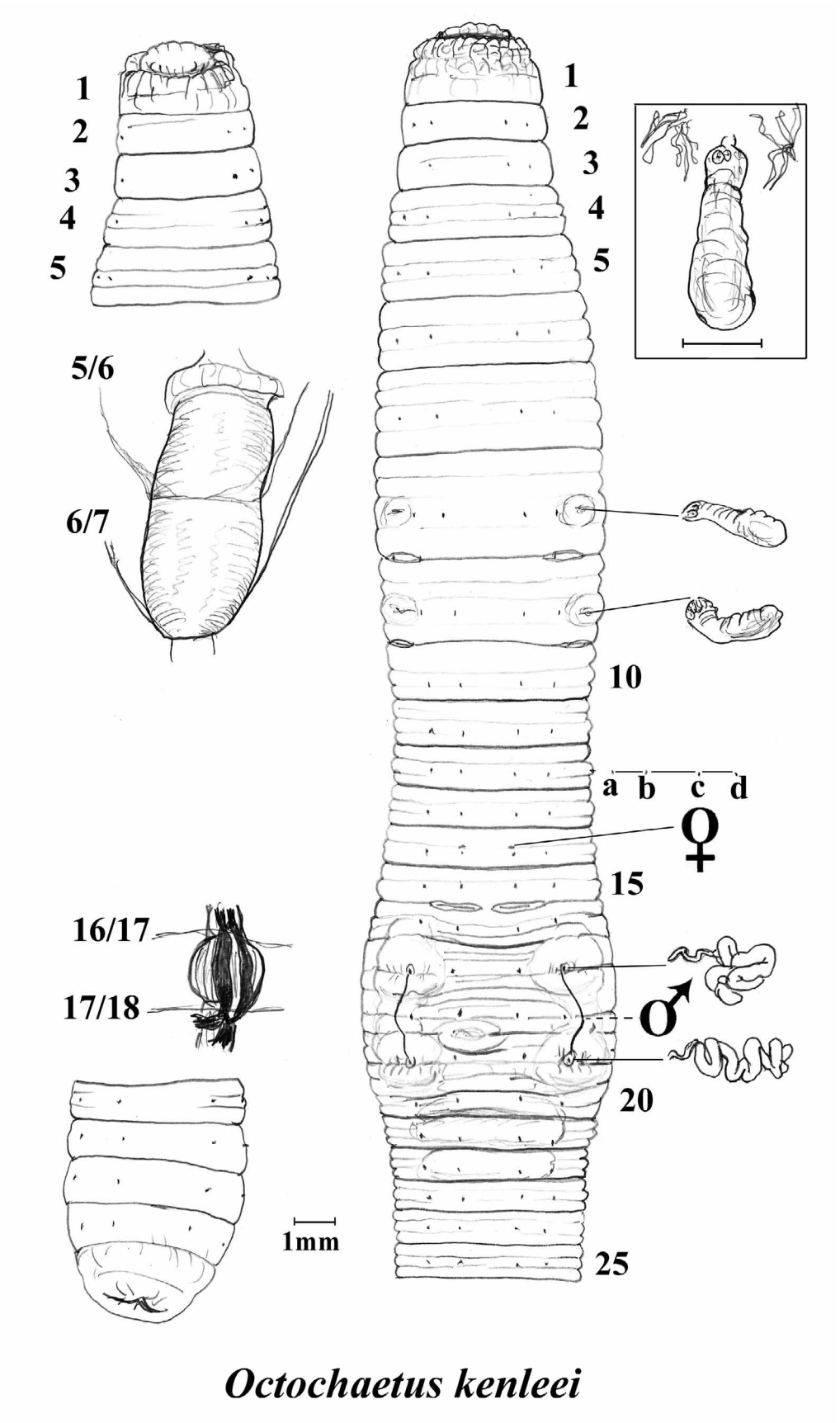

FIGURE 3. *Octochaetus kenleei* Holotype showing dorsal view of prostomium and pygidium, and ventral view of body with spermathecae and prostates *in situ*; gizzards are in 5 and 6 and calciferous gland is in 17; enlarged 9rhs spermatheca shows nephridia on either side.

DNA sequences

COI, GenBank accession number HQ529283 (submitted 01 November 2010)
AAAGATATTGGAACCCTTTACTTCATTCTAGGAGTCTGAGCAGGAATAATCGGTGCAGGAATAAGACT
TCTTATTCGTATCGAGCTCAGCCAACCTGGAGCCTTTTTAGGAAGAGACCAACTATATAATACAATCGT
AACCGCTCATGCATTTTTAATAATTTTCTTTCTAGTAATACCAGTATTTATTGGAGGATTTGGAAACTGA
TTACTACCTTTAATACTAGGAGCCCCCGACATAGCTTTTCCACGACTAAATAATATAAGATTCTGACTAT
TACCGCCATCATTAATCTTACTAGTCTCTTCCGCGGCCGTTGAAAAAGGTGCTGGAACAGGATGAACA
GTATATCCACCTCTTGCTAGAAATATGGCACATGCAGGACCATCAGTAGATCTCGCAATTTTTTCATTAC
ATTTAGCAGGTGCCTCATCAATTTTAGGGGCCATCAACTTTATTACAACTGTAATTAATATACGATGAGC
AGGATTACGACTAGAACGAGTACCACTATTTGTATGAGCTGTTGTAATTACGGTAGTTCTTCTATTATTA
TCCTTACCAGTTCTAGCAGGAGCTATTACTATACTTTTAACAGATCGAAACCTCAATACTTCATTCTTTG
ACCCATCTGGAGGAGGAGATCCAATTTTATATCAACATCTATTTTGATTTTT

16S, GenBank accession number HQ529286 (submitted 01 November 2010)
AAAACATTGCTTTCTGAAATTTTATAGAAAGTAATTCCTGCCCAGTGACAACTGTTCAACGGCCGCGG
TATCCTAACCGTGCAAAGGTAGCATAATAACTTGCCTATTAATTGTAGGCTAGAATGAACGGGTAAACG
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ACCCAGGAATTTATAAAACATCCTAAAAAACAAAGATCTATAAATCTATTAAATGACCCTTATTAAGATC
AAAAGACAAAGCTACCTTAGGGATAACAGGCTAATCCTATTTAAGAGTCCATATCAATAATAGGGTTTG
GCACCTCGATGTTGGCTTAGGGTATCAATATAGCGCAAAAGTTATATAAAGATGGTTTGTTCAACCAAT
TATTCCCTACATGAGCTGA

Phylogeny

Taxonomical diagnostic for the three newly described species matches with the main clades in the phylogeny of New Zealand earthworms. The phylogenetic tree in Fig. 4 was built using the DNA sequence of one specimen for each of the main New Zealand clades (Buckley *et al.* 2011). Within the clades of interest, specimens that were most closely related to the species described here were selected.

D. gorgon was very close to a specimen of *D. montanus* (~2% of difference on the 16S NJ tree). This specimen of *D. montanus* was collected from the Denniston area, about 10km South-West from Happy Valley (Buckley *et al.* 2011). Important geographical and DNA proximity between *D. gorgon* and *D. montanus* could indicate a potential synonymy. However, the fact that *D. gorgon* is tanylobous and the presence of eye-like genital markings on segment 10 are sufficient to consider it as a new species. Direct morphological comparison between these two specimens and the holotype of *D. montanus* (Lee 1959) could help confirming these results.

Among the specimens sequenced by Buckley at al. (2011) the one closest to *O. kenleei* was *Octhochaetus* n. sp., collected from the Okarito region (about 220 km South-West of Happy Valley). Genetic distance between those two specimens was less than 2%. They are therefore likely to be the same species.

M. felix was genetically quite different to any of the species sequenced by Buckley *et al.* (2011). The specimen that was closest to *M. felix* was *M. parkeri*, collected from the Chinamans Bluff (about 430 km South-West from Happy Valley).

Discussion

In this study, morphology-based taxonomy and molecular techniques were used to describe in a taxonomically integrated way three new species of earthworms from the West Coast of New Zealand's South Island. The barcoding region COI and the 16S rDNA region were sequenced using tissue from the holotype specimens to provide indisputable uniqueness of the species. Morphological identification of new specimens can now be verified using the DNA sequences publically available on GeneBank. In addition, when morphological identification cannot be used (i.e., for juveniles, pieces of earthworms or environmental samples), species are still identifiable on the sole basis of their DNA sequences. Further DNA analyses targeting different markers are also possible since type specimens were all fixed in ethanol 98% and preserved in propylene glycol. This conforms to the 'genetype' concept suggested by other authors (e.g., Tautz *et al.*, 2003; Chackrabarty 2010).

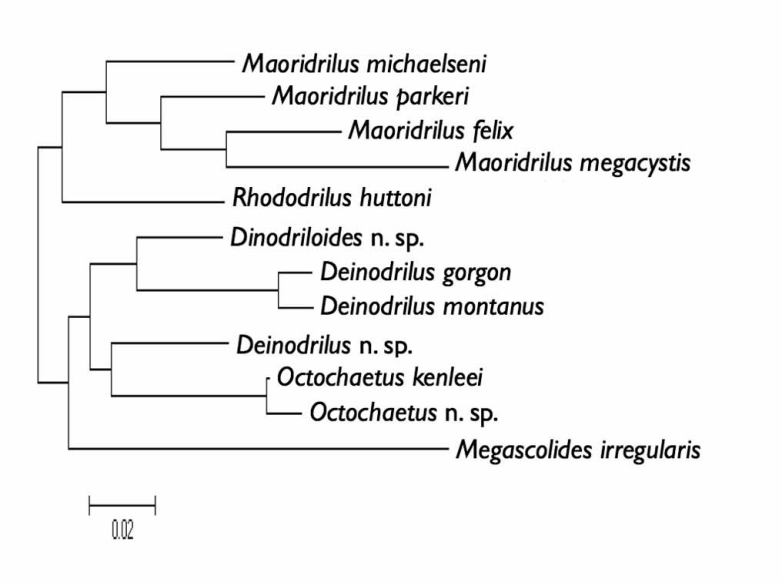

FIGURE 4. Simplified phylogeny of New Zealand earthworms including the three newly described species *M. felix, D. gorgon* and *O. kenleei* (NJ tree based on 16S rDNA). The 16S rDNA sequences obtained for the three newly described species were compared to similar sequences obtained by Buckley *et al.* (2011). One representative for each major clade of New Zealand endemic earthworms was included in the analysis (see Buckley *et al.* 2011). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. There were a total of 441 positions in the final dataset.

The DNA sequencing of holotypes helps the description of a new species by confirming that the taxonomical diagnostic matches with its position in the phylogeny. In addition, potential incongruence and synonymies are easily detectable on a phylogenetic tree.

Earthworm taxonomists and ecologists are encouraged to use this procedure for future species description and to apply DNA barcoding methods to type specimens of previously described species.

The generalization of this approach for earthworm species descriptions would provide a valuable tool for ecologists wanting rapidly to compare what they suspect to be undescribed species, new populations, or species in synonymy. Also, such database could serve to resolve questions about the taxonomic identity of juvenile specimens (Richard *et al.*, 2010) or for identification of earthworm DNA in environmental samples (e.g., Boyer *et al.*, unpublished).

An integrative taxonomic description, combining morphological description, DNA barcoding and phylogeny is particularly useful when working on highly diverse endemic faunas. These faunas are usually poorly known and only a few internationally recognized taxonomists can identify them. Yet many endemic earthworm species, which often represent high diversity (e.g., Blakemore, 2000; Chang *et al.*, 2008), substantial biomass (e.g., Brockie and Moeed, 1986), and have crucial roles in the functioning of native ecosystem (Boyer & Wratten 2010a), are likely to be threatened with extinction.

In New Zealand, most endemic earthworm species have been rarely recorded or studied and only few specimens have been found. Therefore, only three species are listed as New Zealand's threatened invertebrates (McGuinness, 2001) while 167 species are qualified as 'data deficient' (Hitchmough *et al.*, 2005).

Some of the new species described here could be under threat as opencast coal mining is about to begin in 'Happy Valley'. Their conservation may rely on the environmentally-driven habitat management and specific ecological rehabilitation measures conducted by the mining company Solid Energy New Zealand Limited (Boyer *et al.*, unpublished). For other species in New Zealand, further studies such as those of Springett & Grey (1998) are needed.

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