DNA Barcoding Reveals Cryptic Diversity in *Lumbricus terrestris* L., 1758 (Clitellata): Resurrection of *L. herculeus* (Savigny, 1826)

Samuel W. James1,3, David Porco2,3, Thibaud Decaëns3, Benoit Richard3, Rodolphe Rougerie2,3, Christer Erseús4

1 Biodiversity Institute, Kansas University, Lawrence, Kansas, United States of America, 2 Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, Guelph, Canada, 3 Laboratoire d’Ecologie, UPRES-EA 1293 ECO DIV, FED SCALE, Bâtiment IRESE A, UFR Sciences et Techniques, Université de Rouen, Mont Saint Aignan, France, 4 Department of Zoology, University of Gothenburg, Göteborg, Sweden

Abstract

The widely studied and invasive earthworm, *Lumbricus terrestris* L., 1758 has been the subject of nomenclatural debate for many years. However these disputes were not based on suspicions of heterogeneity, but rather on the descriptions and nomenclatural acts associated with the species name. Large numbers of DNA barcode sequences of the cytochrome oxidase I obtained for nominal *L. terrestris* and six congeneric species reveal that there are two distinct lineages within nominal *L. terrestris*. One of those lineages contains the Swedish population from which the name-bearing specimen of *L. terrestris* was obtained. The other contains the population from which the syntype series of *Enterion herculeum* Savigny, 1826 was collected. In both cases modern and old representatives yielded barcode sequences allowing us to clearly establish that these are two distinct species, as different from one another as any other pair of congeners in our data set. The two are morphologically indistinguishable, except by overlapping size-related characters. We have designated a new neotype for *L. herculeus*. The newly designated neotype and a syntype of *L. herculeus* yielded DNA adequate for sequencing part of the cytochrome oxidase I gene (COI). The sequence data make possible the objective determination of the identities of earthworms morphologically identical to *L. terrestris* and *L. herculeus*, regardless of body size and segment number. Past work on nominal *L. terrestris* could have been on either or both species, although *L. herculeus* has yet to be found outside of Europe.

Citation: James SW, Porco D, Decaëns T, Richard B, Rougerie R, et al. (2010) DNA Barcoding Reveals Cryptic Diversity in *Lumbricus terrestris* L., 1758 (Clitellata): Resurrection of *L. herculeus* (Savigny, 1826). PLoS ONE 5(12): e15629. doi:10.1371/journal.pone.0015629

Editor: Dirk Steinke, Biodiversity Institute of Ontario, University of Guelph, Canada

Received: August 26, 2010; Accepted: November 15, 2010; Published: December 29, 2010

Copyright: © 2010 James et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Most of this research was supported through grants from Natural Science and Engineering Research Council of Canada (NSERC) and Genome Canada to Paul Hebert (Biodiversity Institute of Ontario). S. W. James was supported by United States National Science Foundation Grant DEB 0516439 and by a Marie Curie France Regions grant from the Région Haute Normandie. R. Rougerie and D. Porco were supported by post-doctoral fellowship grant from the Conseil Régional de Haute Normandie through the Grand Réseau de Recherche Sciences de l’Environnement et Gestion des Risques. T. Decaëns, B. Richard, R. Rougerie, D. Porco, and S. James were supported by the Sciences Appliquées a L’Environnement research federation through the Functions and Determinants of BIODiversity program. C. Erseús was supported by ArtDatabanken (Swedish Taxonomy Initiative) and The Royal Society of Arts and Sciences in Göteborg. None of these funding agencies had any role in the conducting of the study or in the preparation of the manuscript. It is fair to say than until this work is published, most of them remain unaware of its existence. Paul Hebert, as director of the Canadian Centre for DNA Barcoding at the Biodiversity Institute of Ontario, made it a priority to direct the necessary resources toward the study, but the resources were ultimately derived from grants by NSERC and Genome Canada. Dr. Hebert cannot be cast in the role of a funding agency, but rather in the role of a laboratory director making decisions about how to use existing support.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sjames@ku.edu

Introduction

*Lumbricus terrestris* L., 1758 occupies an important place in the nomenclature of earthworms, having been the first earthworm named [1], and has an important place in biological science and science education. It has been used in many studies of earthworm anatomy, behaviour, physiology and ecology, achieving the status of model organism long before this term came into common use in the last few decades. Virtually every student of biology in secondary or higher education systems of the Western world, as well as many places influenced by textbooks produced therein, has been presented the example of “*Lumbricus terrestris*” as an object of study. In some cases it may be doubted that *L. terrestris* was in fact on the dissection tray, but no matter; the name was used and biologists everywhere recall this as “the earthworm.” Darwin [2] referred loosely to earthworms in this way, though it is likely that one of the species whose activities he observed was *L. terrestris*. The species came into further prominence as an economic resource through the fish bait trade, notably in North America, where astonishing numbers are gathered from Canadian golf courses for domestic bait use and export to the United States of America [3]. Finally, *L. terrestris* is now considered an invasive species and has a prominent role in transforming soils and organic matter accumulations where it has invaded ecosystems previously devoid of earthworms, or replaced species with a comparable ecology [4,5]. It routinely reaches population densities capable of consuming the entire annual leaf fall of north temperate deciduous forests, which is far more than above-ground herbivores normally do except in massive pest outbreaks [4,6,7].

With all the scientific, educational and popular attention devoted to *L. terrestris*, it is rather surprising to find that we do not really know what it is. Some of the problems stemmed from
the brief original description and lack of a type specimen, but this was rectified in a detailed consideration of the nomenclatural history and identities of various earthworms cited as *L. terrestris*, with the designation of a neotype from the probable type locality in Uppsala, Sweden [8]. That neotype is now missing (The Natural History Museum, in litt.). Savigny [9], in describing *Enterion herculeum* Savigny, 1826 deposited a series of specimens which exists to this day. However, *E. herculeum* was later placed in the synonymy of *L. terrestris* [8,10]. Richard et al. [11] detected two genetic clusters within nominal *L. terrestris*, reopening the debate. In this paper we revisit the question of the identities of the earthworms known as *L. terrestris* and *L. herculeus*, with the application of molecular and morphological data. We designate a new neotype for *L. terrestris* and provide a DNA barcode record for a syntype of *L. herculeus*. The latter is from Savigny’s specimens, which were automatically syntypes because no holotype was fixed by the author. This barcoded specimen is here designated as the lectotype of *L. herculeus*.

**Materials and Methods**

We examined 200 specimens of “*L. terrestris*” recently collected in Europe and North America, four specimens topotypic to the former neotype [8] of *L. terrestris* collected in Uppsala, Sweden in 1972; a syntype of *L. herculeus* and several specimens of five congeneres: *L. castaneus* (Savigny, 1826); *L. centralis* Bouché, 1972; *L. festivus* Savigny, 1826; *L. friendi* Cognetti, 1904; and *L. rubellus* Hoffmeister, 1843 (Table 1). Morphological examinations were confined to nominal *L. terrestris* (including *L. herculeus*), including four specimens topotypic to the 1973 neotype of *L. terrestris*, six specimens from Parc du Chateau Brunoy, and five specimens collected in 2008 from Parc du Gally on the grounds of the Versailles Palace, the location of Savigny’s material from the environs of Paris (M.B. Bouche, pers. comm., based on notes of Savigny). Tissue samples were obtained from three of the topotypic Uppsala *L. terrestris* collected in 1972, a syntype of *L. herculeus*, the five congeneres, and the 198 recent specimens of “*L. terrestris*” one of which is the new neotype (GenBank HM3838349; BOLD EW-ECO-0533) and topotypic to the former neotype of *L. terrestris*.

In total, 230 specimens from 6 species of *Lumbricus* were used for genetic examination of the divergence within the genus *Lumbricus* (Table 1). All these worms were processed for the campaign ‘Barcoding Earthworms’ (BCEW) at two different laboratories.

**Samples processed at Canadian Centre for DNA Barcoding**

Lysis of the tissues was carried out in 50μl volume of lysis buffer and proteinase K incubated at 56°C overnight. DNA extraction followed a standard automated protocol on 96-well glass fiber plates (Ivanova et al. 2006). The 5′ region of COI used as a standard DNA barcode was amplified using M13 tailed primers LCO1490 and HCO2198 [12]. Failed samples from this first pass were amplified with a pair of internal primers combined with full length ones LepF1-MLepR1 and MLepF1-LepR1 [13]. A standard PCR reaction protocol was used for PCR amplifications and products were checked on a 2% E-gel 96Agarose (Invitrogen). Unpurified PCR amplicons were sequenced in both directions using M13 tails as primers. The sequencing reactions followed standard protocols of the Canadian Centre for DNA Barcoding (CCDB) [14], with products subsequently purified using Agencourt CleanSEQ protocol (Agencourt) and processed using BigDye version 3.1 on an ABI 3730 DNA Analyzer (Applied Biosystems).

The specimen from the type series of *Lumbricus herculeus* from the Savigny 1821 collection and the 1972 topotypic *L. terrestris* specimens were sampled for DNA (hereafter referred to as the museum specimens). The age and preservation of the specimens from which these tissues were sampled, however, demanded a different approach for extraction and amplification. Extraction was done manually with the Nucleospin tissue extraction Kits and PCR amplification was done with 6 pairs of primers in order to amplify overlapping fragments of about 160bp (Table 2). The same primers and the standard protocol of the CCDB [14] were used for the sequencing of those fragments.

**Specimens processed at University of Gothenburg**

Twenty-four specimens morphologically identified as *L. terrestris* were collected in Scandinavia (21 in Sweden, two in Denmark, and two in Norway), in 2008–2009. DNA was extracted from a tissue sample of each worm with the QIAamp DNeasy® Blood & Tissue Kit, after which PCR reactions were performed using the COI primers LCO1490 (forward) and HCO2198 (reverse) [12],

| Species          | Country     | Region             | N  |
|------------------|-------------|--------------------|----|
| Lumbricus castaneus | France      | Seine Maritime     | 9  |
|                   | Andorra     | Santa Julia        | 1  |
| Lumbricus centralis | France      | Provence-Alpes-Cote d’Azur | 10 |
| Lumbricus festivus | France      | Seine Maritime     | 8  |
|                   | France      | Ile de France      | 1  |
| Lumbricus friendi | France      | Midi-Pyrenees      | 1  |
| Lumbricus rubellus | France      | Haute Normandie    | 9  |
| Lumbricus terrestris | Canada      | Ontario            | 63 |
|                   | Denmark     | Jutland, Arhus     | 1  |
|                   | France      | Languedoc-Roussillon | 1  |
|                   | France      | Ile de France      | 7  |
|                   | France      | Haute Normandie    | 41 |
|                   | France      | Bretagne           | 2  |
|                   | Norway      | Nordland           | 1  |
|                   | Norway      | Hordaland          | 1  |
|                   | Sweden      | Jämtland           | 1  |
|                   | Sweden      | Scania             | 2  |
|                   | Sweden      | Småland            | 2  |
|                   | Sweden      | Uppland            | 2  |
|                   | Sweden      | Västervången       | 1  |
|                   | Sweden      | Västergötland      | 2  |
|                   | United States | Ohio              | 7  |
|                   | United States | Iowa              | 8  |
|                   |             |                    | 144|
| Lumbricus herculeus | Denmark     | Jutland, Arhus     | 1  |
|                   | France      | Seine Maritime     | 24 |
|                   | France      | Ile de France      | 22 |
|                   | Sweden      | Scania             | 9  |
|                   |             |                    | 56 |

The text references to lineages 1 and 2 correspond to *L. terrestris* and *L. herculeus*, respectively. doi:10.1371/journal.pone.0015629.t001
the reverse primer sometimes replaced by COI [15]; all following standard protocols. PCR products were purified using an Omega E.Z.N.A. cycle-pure kit, and sent to Macrogen, South Korea, for sequencing.

Sequence analysis

Sequences were assembled with Sequencer 4.5 (GeneCode Corporation, Ann Arbor, MI, USA) and aligned by eye using BIOEDIT version 7.0.5.3 [16]; we observed no indels in this coding region of the mitochondrial genome and therefore all base positions were aligned with confidence in positional homology. Distance analyses were conducted with MEGA4 [17] using a Neighbor-Joining [18] algorithm and distances corrected with the Kimura-2 parameter model [19]. The robustness of nodes was evaluated through bootstrap re-analysis of 1000 pseudoreplicates.

Results

Barcode data

230 full length barcodes were obtained ranging between 508 and 658 bp. From the syntype of L. herculeus we obtained 5 of the 6 fragments with 4 consecutive overlapping ones producing a continuous sequence of 480 bp. Only one of the 1972 Uppsala museum specimens of L. terrestris yielded a sequence, a 144 bp fragment. A complete COI barcode was obtained from the specimen collected in Uppsala in 2009 (the replacement neotype, GenBank HM388349; BOLD EW-ECO-0533). Sequences are publicly available on BOLD [20]; http://www.barcodinglife.org) within the project LTERH and in GenBank (Table S1).

The mean intraspecific and interspecific variations for COI in the genus Lumbricus are 1.24% and 19.81%, respectively, except for nominal L. terrestris which exhibits the highest intraspecific value in the dataset at 8.93% and a range of 0% to 19%. These extreme values are due to the presence of two highly divergent groups of individuals within the nominal species. Separating the two lineages, divergence within L. terrestris s.s is 3.37%, and within L. herculeus it is 1.54% (Table 3). Comparing the distribution of the L. terrestris intraspecific divergences to what is exhibited among the other species in the genus (Figure 1) we see clearly that the divergence between the two groups found in nominal L. terrestris is comparable to the distances among other species of the genus. The mean interspecific divergence between the two L. terrestris lineages is 17.5%.

An unrooted Neighbor-Joining (NJ) tree of Lumbricus barcode sequences placed all nominal L. terrestris in two well-supported and

Table 2. Primers used to obtain short overlapping barcode sequence fragments.

|   | 5' - 3'                                      |
|---|------------------------------------------------|
| 1st pair | LCO1490_t1 TGTAAAAACGAGCCCAATGGTGAACAACTTCCATTTGATATTGG |
|       | EWLT1R GCACCATTAGACTGGTATYAC                  |
| 2nd pair | EWLT2F TTATACAATACATCGTTACTGC                 |
|       | EWLT2R GAACTAAGAAATAAGGAGGG                   |
| 3rd pair | EWLT3F CATAGATTTGTACCTTCTRCC                  |
|       | EWLT3R AGRATAGAGAYGACCTGC                    |
| 4th pair | EWLT4F CTGGCCAGRAATCTCGGCA                    |
|       | EWLT4R ACAAYAGAGGATCTGCTAG                   |
| 5th pair | EWLT5F TCCCTCATTGGACAGGGGC                   |
|       | EWLT5R GTTARGATATTGTGATTGGCYCCK              |
| 6th pair | EWLT6F AATTACAGTAGTYCCTCCTC                  |
|       | HCO2198_t1 CAGGAAAAGACGTAGATCAACTTCAGGGTACCAAAAATACA |

doi:10.1371/journal.pone.0015629.t002

Table 3. Kimura 2-parameter mean genetic distances (%) between and within Lumbricus spp.

| Species            | B          | W          | L. terrestris s.s | L. herculeus |
|--------------------|------------|------------|-------------------|-------------|
| Lumbricus castaneus| 0.45       |            |                   |             |
| Lumbricus centralis| 22.99      | 0.36       |                   |             |
| Lumbricus festivus | 23.46      | 17.72      | 18.55             |             |
| Lumbricus friendi  | 0.36       | 17.54      |                   |             |
| Lumbricus rubellus | 21.04      | 20.29      | 17.50             | 17.54       |
| Lumbricus terrestris| 23.74     | 21.62      | 18.23             | 17.54       |

The principal diagonal has intraspecific distances; all others are interspecific. doi:10.1371/journal.pone.0015629.t003

Figure 1. Boxplots of intraspecific (W; gray bars) and interspecific (B; black bars) genetic distances (K2P) for Lumbricus species with corrected taxonomy separating L. terrestris from L. herculeus. Each boxplot represents: the discarded outliers (external dots), the smallest and largest observations (external bars), the lower and upper quartiles (limits of the box) and the median (within-box black line). The boxplots are notched and indicate that medians differ if the notches do not overlap. doi:10.1371/journal.pone.0015629.g001
divergent clusters (Figure 2A). All other species represented by more than one individual also fell into well-supported clusters.

In a second step the short barcode sequences from the 1972 museum specimen of *L. terrestris* and from the syntype of *L. herculeus* were introduced in the dataset. Although both the sequences were short, they allowed an accurate assignment of each specimen to one of the *L. terrestris* lineages (Figure 2B). The discriminating power of mini-barcodes is established [13] and here we used these short sequences in favorable conditions as the divergence between the two lineages of *L. terrestris* is very high (17.5%). Thus each of the type-related specimens was successfully assigned to a lineage in the NJ analysis, one to the *L. terrestris* cluster and one to the *L. herculeus* cluster (Figure 2B). From this point forward in the results and discussion, we use the two species names in the restricted sense supported by these data, unless enclosed in quotation marks.

In a separate NJ analysis (tree not shown) the cytochrome oxidase I gene barcode region of the complete “*L. terrestris*” mitochondrial genome sequence [21; GenBank NC001673.1] fell within the *L. terrestris* cluster.

**Morphology**

Morphological examination of the fresh specimens, each registered in such manner that a COI barcode sequence can be matched to an individual worm, indicates that there are differences in segment number (125 vs 143, *L. herculeus* and *L. terrestris* respectively), body mass (1.7g vs 3.2g), and body length (107mm vs. 148 mm) between the two groups (Figures 3A–C). These differences are not as clear-cut as the genetic differences, there being overlap in the distributions of the three measurements. Put simply, small *L. terrestris* can be smaller than large *L. herculeus*, but they have strongly divergent COI sequences. The two species are illustrated in Figure 4.

The specimen of *L. terrestris* in the vial labeled as neotype (Natural History Museum, London; Register No. 1973.1.1) is shorter by 12 mm and has 6 fewer segments than the neotype described by Sims [8]. The other specimens of the same series from Uppsala are all either longer or shorter, or have more or fewer segments than the specimen described in Sims (1973) (E. Sherlock, in litt.), so the Sims [8] neotype is presumed lost.

**Figure 2.** Neighbor joining trees (K2P) for 6 species of the genus *Lumbricus*, based on the COI 5’ ‘barcoding fragment’; bootstrap support values for each cluster shown on its subtending branch. The upper and lower sides of each triangle represent respectively the maximum and minimum genetic distances within a species. A. Without type or museum material of the two *L. terrestris* lineages. B. Reconstruction with type specimens and museum material. Higher genetic variation of *L. terrestris* L1 in B. is due to the short sequence (144bp) of the 1972 museum specimen.

doi:10.1371/journal.pone.0015629.g002
Swedish sites, the Danish site and three French sites had both species, while others had only one.

**Neotypes**

We turn now to the means of defining these two very similar species. As we have indicated, size is the only morphological difference, and it is not reliable in the overlapping sections of the size distributions. Above we noted that the neotype of *L. terrestris* collected at Uppsala in 1972 is no longer in the Natural History Museum (London). Neotypes can be replaced “…when no name-bearing type specimen (i.e. holotype, lectotype, syntype or prior neotype) is believed to be extant and an author considers that a name-bearing type is necessary to define the nominal taxon objectively.” [22, Art. 75.1].

If the designation of a new neotype would only replace one morphologically undifferentiated specimen with another, there is little or no justification for the designation. However, we have successfully isolated and sequenced some DNA from a contemporary member of the same population (GenBank HQ024541) as the missing neotype. We also obtained sequence data from specimen CE6377M collected at the same location (GenBank HM388349; BOLD EW-ECO-0533). Then we unequivocally clustered these resulting sequences with those of numerous other individuals which by size are generally identifiable as *L. terrestris*. We also demonstrated a substantial genetic difference between the *L. terrestris* cluster and the related and cryptic congener *L. herculeus*.

Designation of a neotype must demonstrate exceptional need. We believe this to be the case. All of the points raised by Sims [8] regarding stability of nomenclature are still valid today. As Sims [8] indicated in his arguments for the designation of a neotype, *L. terrestris* occupies not only an important historical position in the nomenclature of earthworms as the first earthworm described and type species of *Lumbricus* and the Lumbricidae, it has also been a model organism for research and education in Biology. It is of considerable importance for the stability of nomenclature that it be possible to determine the identity of an earthworm matching the physical characters of *L. terrestris* and *L. herculeus*. Our results indicate that morphological examinations are not sufficient for the identification of these species, but that DNA sequences are. We have established the utility of the barcode fragment of the COI gene [23, 24] for this purpose but do not confine the method to this gene. The proposed neotype now has a COI sequence tag which can unequivocally be used to characterize and recognize the taxon *L. terrestris* in a way hitherto impossible. To date there are no known *Lumbricus* species with a sufficiently similar sequence to cause any confusion in DNA-based identification of species within this genus, let alone in the discrimination of *L. terrestris* and the morphologically virtually identical *L. herculeus*.

To satisfy the provisions of ICZN [22] Art. 75.3.1-7, the qualifying conditions for validly designating a neotype, we offer the following points:

1. Our designation of a neotype is necessary to clarify the taxonomic status of *L. terrestris*, in order that it can be distinguished from *L. herculeus*.
2. The characters differentiating *L. terrestris* from *L. herculeus* are differences in aligned, positionally homologous COI gene DNA sequence bases.
3. The partial COI sequence derived from the neotype (GenBank HM388349; BOLD EW-ECO-0533), and the physical description in Sims [8], which is identical in all but measurements to the designated neotype, and the measurements given here below are sufficient to ensure correct recognition of the specimen designated.
4. The prior neotype is missing from the collection of the Natural History Museum (London) and the staff made a thorough search of the premises, but failed to locate the specimen.

5. Other than the particular body size and segment number measurements, the designated specimen is anatomically consistent with the prior neotype.

6. One of the collectors of the prior neotype (Tryggve Persson) was consulted on the collection event location of 13 October 1972, and the new neotype was taken from the same locality in the Botanical Gardens at Uppsala, Sweden. The location was chosen because that was the location satisfying this condition (proximity to original collection site of Linnaeus) for the prior neotype. We follow this established precedent, which is in any case the valid type locality following the designation of the prior neotype.

7. The new neotype has been deposited in the Swedish Museum of Zoology, with catalogue number given below.

Our choice of neotype specimen is not one of the specimens collected at the same time as the prior neotype, even though we were able to obtain a short (144 bp) sequence of the COI gene from one of three attempts. Therefore regarding “Recommendation 75A. Choice of neotypes...” [22] we designate a new specimen preserved in a manner that allows extraction of high-quality DNA. Thus the definition and delimitation of the taxon need not be based only on the short “mini-barcode” obtained from the 1972 specimen, because future researchers will be able to use small samples of the newly designated neotype for further genetic data. In short, we maintain that for purposes of molecular definition and delimitation of the taxon, the 1972 material is in poor condition.

The following synonymy is modified from: http://earthworms.elt.hu/Hungary/lumbricus.htm by removal of the references to poor condition. The definition and delimitation of the taxon, the 1972 material is in genetic data. In short, we maintain that for purposes of molecular.

36. The prior neotype is missing from the collection of the Natural History Museum (Stockholm) catalogue number SMNH Type-0035.

Other material: 4 clitellates, Sweden, Uppland, Uppsala, Uppsala Botanical Garden., 13 October 1972. B. Axelsson, U. Lohm, T. Persson collectors; 1 clitellate, France, Parc du Château de Versailles, Ile de France, 48° 43′ 41.61′′ N, 2° 06′ 56.74′′ 1 November 2008, M. Hedde collector; 2 clitellates, France, Essonne, Ile de France, Parc du Château de Brunoy, 48° 48′ 41.61′′ N, 2° 29′38.25′′ 11 November 2008, M. Hedde and T. Decaens collectors.

Description of neotype and other material: The neotype is in two fragments, the anterior consisting of 59 segments and the posterior of 95 segments, for a total of 154 segments, with total length (strongly contracted) 89 mm. There are herniations on the right side at 15/16 and 28/29, and slight abrasions to the left side of segments 46–52. This damage was present on the specimen at the time of collection from a walkway. The clitellum is at 32–37, the tubercula pubertas at 33–36, and there are genital markings surrounding enlarged AB genital setae on segments 31–37 and right side of segment 38. The first muscular septum is always 19/20, which is displaced about a half-segment length posteriorly to lie close to septum 20/21.

Examining the 1972 and the French material, the typhlosolar convolutions are very distinctive. From the beginning of the typhlosole in XXII it has lateral flaps oriented vertically. The ventral edges of each flap bifurcate and fuse with the split sections of the flaps anterior and posterior to the flap in question. The fused parts form a short bar extending across the center ventral face of the typhlosole to meet the lateral flaps of the other side, which are also split and fused as just described. The short bars take the appearance of the rungs of a ladder whose lengthwise components are made of the fusion points of the lateral flaps. This pattern originates in segments 23–24 and gradually fades out over two or three segments between 47 and 52, after which the typhlosole has a smooth surface and a circular to oval cross-section. The typhlosole ends abruptly over one or two segments anywhere from 99 to 117, though most commonly in 100–108.

Remarks: The neotype and the 1972 specimens agreed in all other particulars with the description in Sims [8], except for the musculature of septa behind the gizzard. It is possible that Sims [8] mistook the two closely-spaced septa for one and misjudged the count because of the displacement. To be certain, we counted anteriorly from segment 25, where septum 24/25 is in line with its external segment boundary. No morphological differences from the nominal L. terrestris as traditionally defined were detected. The sequences given (Genbank HQ024541, HM388349) are the only present means of objective determination of the species, in relation to the slightly smaller L. herculeus.

Lumbricus herculeus

Savigny, 1826 (synonymy modified from http://earthworms.elt.hu/Hungary/lumbricus.htm)

Eisenia herculeana: Savigny, 1826 Mem. Acad. Sci. Inst. Fr., 5: 180.

Lumbricus terrestris

(part.); Orleay 1885 Entek. term. tud. körőböl, 15: 30.

Lumbricus studeri

Ribaucourt, 1896 Rev. suisse Zool., 4: 5.

Lumbricus terrestris

(part.); Michaelsen 1900 Das Tierreich, 10: 511.

Lumbricus terrestris

(part.); Szuts 1909 Allattani Közlemények, 8: 142.

Lumbricus terrestris

(part.); Zicsi 1959 Acta zool. hung., 5: 433.

Lumbricus terrestris

(part.); Zicsi 1968 Opusc. Zool. Budapest, 8: 130.

Lumbricus terrestris

(part.); Sims 1973. Bull. Zool. Nom. 30:32.

Lumbricus terrestris

(part.); Zicsi 1982 Acta zool. hung., 28: 443.

Lumbricus terrestris

(part.); Easton 1983 Earthworm Ecology, p. 482.

Lumbricus terrestris

(part.); Zicsi 1991 Opusc. Zool. Budapest, 24: 173.

Lumbricus terrestris

(part.); Mršić 1991 Acad. Sci. Art. Slov. (Hist. Nat.), 31: 481.

Lumbricus terrestris

(part.); Qiu & Bouché 2000 Doc. pedozool. integrál, 4: 192.

Lumbricus terrestris

(part.); Michaelis 1900 Das Tierreich, 10: 412.

Lumbricus terrestris

(part.); Zicsi 1959 Acta zool. hung., 5: 433.
Lumbricus terrestris (part.); Bouček 1972 Inst. Nat. Rech. Agron. p. 352.

Lumbricus terrestris (part.); Sims 1973. Bull. Zool. Nom. 30:32.

Lumbricus terrestris (part.); Zícs 1968 Opusc. Zool. Budapest, 8: 130.

Lumbricus terrestris (part.); Zícs 1982 Acta zool. hung., 28: 443.

Lumbricus terrestris (part.); Easton 1983 Earthworm Ecology, p. 482.

Lumbricus terrestris (part.); Zícs 1991 Opusc. Zool. Budapest, 24: 173.

Lumbricus terrestris (part.); Mršíč 1991 Acad. Sci. Art. Slov. (Hist. Nat.), 31: 481.

Lumbricus terrestris terrestris (part.); Qiu & Bouček 2000 Doc. pedo zoool. integral, 4: 192.

Lectotype: Clitellate specimen. In the general area of Paris France. 1821. Collector J.C. Savigny. Paris, Musee Nationale d’histoire Naturelle. Label data: Enterion herculeum Savigny, Paris 1821.

Other material: 4 clitellates, France, Parc du Château de Versailles, Ile de France 48° 43’43.32” N, 2° 06’56.74” 1 November 2008, M. Hedde collector; 4 clitelates, France, Essonne, Ile de France, Parc du Château de Brunoy, 48° 40’41.61” N, 2° 29’30.25” 11 November 2008, M. Hedde and T. Decaëns collectors.

Lectotype: Three fragments in one vial, consisting of the first 22 segments, segments 23–50, and segments 51 to 145. There is a partial cut in the 5th segment and a small knotted thread is inserted in the 9th segment, in the manner of those used for tagging larger animal specimens to have fewer atypical segments than indistinguishable other than a slight tendency of pigmentation is visible. No dissection was performed on this specimen is GenBank HQ024540.

The other material examined had no differences from the L. terrestris specimens other than measurements (Figs 3A–C, which include additional specimens to those examined in detail). Septal musculature, and typhlosole morphology and termination were all indistinguishable other than a slight tendency of L. herculeus specimens to have fewer atyphlosole segments than L. terrestris. However these numbers overlapped, like the other quantitative measures.

Savigny did not designate any type specimen(s), so here we defined this species in the most simple and direct manner possible at this time. The somewhat softened clitellate specimen was not subjected to further examination, and is now designated as the lectotype of L. herculeus. The description of L. terrestris by Sims [8] serves as a source of morphological details. Our Figures 3A–C, the observations on the specimens examined for the above descriptions, and the descriptive data of Bouček and Beugnot [25] give the extent of morphological differences between this species and L. terrestris.

Discussion

These results indicate that “L. terrestris” as traditionally identified is composed of two species that have not been discriminated in the literature. Morphological examination can only make reliable distinctions between average or larger L. terrestris and average or smaller L. herculeus. Bouček and Beugnot [25] reached the same conclusion regarding what they considered as two sympatric populations of “L. herculeus” with nomenclature following Bouče’s 1970 advocacy of that name over “L. terrestris.” Our collection includes 18 individuals from the Parc du Château de Brunoy location sampled by Bouček and Beugnot (1972), of which 16 fall in L. herculeus and the other 2 in L. terrestris. The segment number and size variations Bouček and Beugnot [25] reported are the same as we observed between L. herculeus and L. terrestris. The two species are best distinguished by molecular data, which will work on all sizes and life stages of the individuals, from egg capsules to adults. The sequences from 1972 and 2009 specimens topotypic to the missing Sims [8] L. terrestris neotype were in the L. terrestris cluster.

In a similar situation, that of earthworms unquestionably separable by size, body coloration, and some genital papillae, Chang et al. [26] found that molecular data strongly supported separation of two species from nominal Ancythias wulinensis Tsai, Shen and Tsai, 2001. In the A. wulinensis case, the size, color, and papillae characters giving the initial indications of lineage diversity are traditionally not considered reliable in Asian earthworm taxon-omy. Eisenia fetida (Savigny, 1826) and E. andrei Bouček, 1972 are only sometimes separable by color, but are two genetically distinct and isolated species [27].

Savigny [9] did not attribute an author to Enterion herculeum and did not indicate that it was a new species, which was not required in his time. Nor did he expressly indicate that Enterion herculeum is a replacement name for some other nominal species group [25, Art. 72.7]. Therefore the two names are not objective synonyms, and L. herculeus is a junior subjective synonym of L. terrestris. In any case the brief description by Savigny is a valid indication [25, Art. 12.2.] of the identity of the worm and intent of the author. The effect of our work is to restore a junior subjective synonym (L. herculeus) to species status. In consideration of the molecular data and nomenclatural procedure, we remove L. herculeus from the synonymy of L. terrestris, and thereby restrict L. terrestris to the cluster whose members are larger.

Apparently, Savigny was describing a new species, named Enterion herculeum, among 21 other names in his document. Had his specimen been L. terrestris s.s., the name herculeus would definitely pass into synonymy. By happenstance, he collected and applied a name to what is now defined as a separate species. Where Linnaeus worked in Sweden he saw earthworms on the surface at night (1: p. 648: “ascendit noctu”). Sweden has both species, with L. herculeus only found in Scania so far, but regardless of which species Linnaeus saw, L. terrestris was defined by the Sims [8] neotype and is now defined by its replacement. On the other side of the Atlantic, the brisk trade in fish bait and classroom specimens is so far known to consist only of L. terrestris. North American investigators may rely on the lack of records of L. herculeus, but do so at their peril. We would not be surprised to find L. herculeus in North America or other continents where “L. terrestris” is known to occur.

This revision introduces doubt about the true identity of the species involved in any publication on “L. terrestris,” even if vouchers were deposited. Larger L. terrestris can be fairly certainly identified, as can smaller L. herculeus. Otherwise, old vouchers fixed in un-buffered formaldehyde solutions may not yield usable DNA, and therefore may not be identifiable. However, the worm used in Booze and Brown [21] for a complete mitochondrial genome sequence (GenBank NC001673.1) was apparently L. terrestris.

At this point it is an open question whether or not research on these two highly similar species has been tainted by the taxonomic confusion of the last 200 years. Are there conflicting results from similar studies, which could be resolved by establishing the true identities of the earthworms involved? Here we do not speak of the instances where careless study of “the earthworm, Lumbricus terrestris” actually referred to some other species, but to those in which a perfectly honest error was made, because taxonomists had no access to the types of data necessary to make the distinctions we are making here, and are now easily obtained. The two species
seldom co-occur in northern France, which could be due to competitive exclusion in various habitats that favor one or the other species. Alternatively they could be different enough to have distinct habitat preferences, and seldom come into competition. All northern European populations have been established by human-aided and natural dispersal since the retreat of the last European ice sheet. Thus we are not speaking of natural allopatric distributions but of a combination of accidents of arrival and competitive exclusion [28].

The obvious consequence of the revision is that any future identification of _Lumbricus_ species closely resembling _L. terrestris_ and _L. herculeus_ should be accomplished in part by comparing DNA sequences including the COI barcode region. The genetic “gap” between the two is large and there are no known intermediate populations, so the results should be very clear.

**Supporting Information**

Table S1

(DOC)

**References**

1. Linnaeus C (1758) Systema Naturae (10th edition). 1:1–824. Salvi: Holmiae. 824 p.

2. Darwin C (1863) The Formation of Vegetable Mould through the Action of Worms, with Observations on their Habits. London: Murray. 326 p.

3. Tomlin AD (1983) The earthworm bait market in North America. In: Satchell JE, ed. Earthworm Ecology. London: Chapman and Hall. pp 331–338.

4. Hale CM, Frelich LE, Reich PB, Pastor J (2005) Effects of European earthworm invasion on soil characteristics in the Great Lakes region. Gen. Tech. Rep. NE-277. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northeastern Research Station. pp 117–123.

5. Hendrix PF, Callaham MA, Jr., Drake J, Huang C-Y, James SW, et al. (2008) A new species of _Pandora_ in northeastern North America: the global implications of introduced earthworms. Ann Rev Ecol Evol Syst 39: 503–613.

6. Landsber J, Olmatt C (1989) Levels of insect defoliation in forests: Patterns and concepts. Trends Ecol Evol 4: 96–100.

7. Muzika RM, Liebhold AM (2001) Effects of gypsy moth defoliation on forest dynamics. Gen. Tech. Rep. NE-227. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northeastern Research Station. pp 117–123.

8. Sims RW (1973) _Lumbricus terrestris_. Linnaeus 1758 (Annelida, Oligochaeta): designation of a neotype in accordance with accustomed usage. Problems rising from the misidentification of the species by Savigny (1822 & 1826). Bull Zool Nomenclature 6: 959–964.

9. Savigny JC In: Cuvier J, ed (1826) Analyses de travaux de l’Académie Royale des Sciences pendant l’année 1821, partie physique. Zoologie. Mém Acad Sci Inst France [Hist] 5: 176–184.

10. Michalsen W (1900) Oligochaeta. In Spengler JW, ed. Das Tierreich. Vol. 10. Berlin: Friedländler.

11. Richard B, Decaën T, Rougerie R, James SW, Porco D, et al. (2007) Re-integrating earthworm juveniles into soil biodiversity studies: species identification through DNA barcoding. Mol Ecol Res 10: 606–614.

12. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299.

13. Hajibabaei M, Smith MA, Janzen DH, Rodriguez JF, Whittfeld JB, et al. (2006) A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Notes 6: 959–964.

14. Hajibabaei M, deWaard JR, Ivanova NV, Ramasingham S, Deho RT, et al. (2005) Critical Factors for assembling a high volume of DNA barcodes. Phil Trans R Soc B 360: 1959–1967.

15. Bely AE, Wray GA (2004) Molecular phylogeny of naidid worms (Annelida; Clitellata) based on cytochrome c oxidase I. Mol Phylogenet Evol 30: 50–63.

16. Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98.

17. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.

18. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425.

19. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120.

20. Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). Mol Ecol Notes 7: 355–364.

21. Boyle JL, Brown WM (1995) Complete sequence of the mitochondrial DNA of the annelid worm _Lumbricus terrestris_. Genetics 141: 305–319.

22. International Commission on Zoological Nomenclature (1999) International Code of Zoological Nomenclature. London: The International Trust for Zoological Nomenclature. 306 p.

23. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc B 270: 313–321.

24. Hebert PDN, Ramasingham S, deWaard JR (2005) Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. Proc R Soc B 270(Suppl.): 896–899.

25. Bouché MB, Beugnot M (1972) La complexité taxonomique de _Lumbricus herculeus_ illustrée par les caractéristiques de populations de stations de la R.C.P. 40. Rev Ecol Biol Sol 9: 697–704.

26. Chang C-H, Lin Y-H, Chen I-H, Chuang S-C, Chen J-H (2007) Taxonomic reevaluation of the Taiwanese montane earthworm _Amynthas wulinensis_ Tsai, Shen and Tsai, 2001. (Oligochaeta: Megacoleidae): polytypic species or complex? Org Divers Evol 7: 231–240.

27. Pérez-Losada M, Eiroa J, Mato S, Domínguez J (2005) Phylogenetic species delimitation of the earthworms _Eisenia fetida_ (Savigny, 1826) and _Eisenia andrei_ Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences. Pedobiologia 49: 317–324.

28. Decaën T, Marjerje P, Aubert M, Hedde M, Bureau F (2008) Assembly rules within earthworm communities in North-Western France - a regional analysis. Appl Soil Ecol 39: 321–335.

**Acknowledgments**

Paul Hebert of the Biodiversity Institute of Ontario and the Canadian Centre for DNA Barcoding allocated the resources necessary to complete the majority of the research. Isabelle Mesnier helped design the mini-barcode primers. The following people assisted in field or laboratory work related to this paper: Anna Ansebo, Thomas Cedhagen, Thomas Dahlgren, Pierre De Wit, Arne Larsson, Emeline Lindsjö, Maria Lindström, and Anna-Stina O gren. Jimmy Ca Jason and Emma Sherlock provided loans from the Paris and London museums, respectively. We dedicate this paper to our friend and colleague, Marcel Bouché, un homme avec un bon nez pour les vers (a man with a good nose for worms).

**Author Contributions**

Conceived and designed the experiments: SWJ DP RR TD BR CE. Performed the experiments: SWJ DP RR TD BR CE. Analyzed the data: SWJ DP TD BR CE. Contributed reagents/materials/analysis tools: SWJ DP BR CE. Wrote the paper: SWJ TD DP.