Arachnology (2019) **18** (2), 94–96

First DNA barcode for the enigmatic *Leiobunum* sp. A (Opiliones)

Grant R. Brown

Dyers Brae,
University of St Andrews,
St Andrews, Fife, KY16 9TH
email: grb31@st-andrews.ac.uk

Abstract

The first DNA barcode for the as yet unnamed species of harvestmen known as *Leiobunum* sp. A is presented, in addition to a preliminary phylogeny of the genus based on mitochondrial sequence data. It is hoped that the availability of this sequence will further efforts to discover the source population of these harvestmen, and facilitate species delimitation and phylogenetic analyses.

Keywords: harvestman • invasive species • systematics • taxonomy

Introduction

A putatively new species of *Leiobunum* C.L.Koch 1839, known as *Leiobunum* sp. A, has been rapidly colonizing central and northern Europe over the last decade (Wijnhoven, Schönhofer & Martens 2007; Rozwałka, Żurawlew & Rutkowski 2017). By 2009, the species was established in the UK, and established populations are now known from Nottingham, Sheffield, and elsewhere in southern England and Wales (Spider Recording Scheme 2018). *Leiobunum* sp. A has a number of interesting morphological and ecological traits in comparison to other UK species including, in particular, the tendency to aggregate in large groups of up to several thousand individuals (Wijnhoven, Schönhofer & Martens 2007).

The impact of this species on UK native harvestmen remains unknown, although anecdotal observations reported in Wijnhoven, Schönhofer & Martens (2007) suggest that large aggregations of this species may exclude smaller species such as *Leiobunum rotundum* Latreille, 1798 and *L. blackwalli* Meade, 1861. Continued monitoring of this species, therefore, presents an ideal opportunity for study of species invasions in a typically neglected arthropod taxon.

Taxonomic descriptions and conservation are inherently linked, in that conservation interest is often dependant on species as the basic unit of measurement (Mace 2004). A putative species without a name is likely to receive less attention, and the use of changeable temporary names has knock-on effects for research reliant on bioinventories (Isaac, Mallet & Mace 2004). Taxonomy is often a slow process, however, and modern synthetic taxonomy demands synthesis of multiple sources of information; morphological, ecological and molecular (Pante, Schoelinck & Puillandre 2015; Sangster & Luksenburg 2015). Whilst is it unwise to rely solely on molecular data to define a species, the inclusion of DNA-based evidence has had considerable influence on the rate of species descriptions (Isaac, Mallet & Mace 2004; Hajibabaei *et al.* 2007). Arguably, the easiest method of incorporating DNA evidence into taxonomic decisions is via DNA barcodes.

DNA barcodes are short sequences of DNA that can be used to assign an organism to a particular species (Hajibabaei *et al.* 2007). In eukaryotes, DNA barcodes are typically generated from the cytochrome oxidase subunit I (COI) region of the mitochondria. Mitochondrial DNA is highly abundant in eukaryotic cells, is easily extracted via standard protocols, and evolves rapidly, ensuring commonality with other members of the same species. Whilst DNA barcodes were initially intended to facilitate species identification (Hebert & Gregory 2005), barcodes can also be used in a comparative manner as the basis of phylogenetic analyses, particularly for the study of recent divergence events (e.g. Labarque *et al.* 2015; Wood, Griswold and Spicer 2007; Kallal & Hormiga 2018).

The goal of this work was to produce a reference DNA barcode for *Leiobunum* sp. A to facilitate future species description and phylogenetic placement. In doing so, I also tested several commonly used PCR primer pairs and comment on their applicability for use with this species.

Material and methods

The analysis is based on a single adult male specimen collected in Sheffield (SK3751889013, 18th August 2018). Morphological consistency with the description in Wijnhoven, Schönhofer & Martens (2007) was ensured using a Leica MS-5 6.3×–40× zoom stereomicroscope.

DNA was extracted from leg tissue in 100µl fly squishing buffer with 20mg/ml proteinase K (Gloor & Engels 1992). After overnight incubation at 55°C, samples were boiled for 2 minutes to inactivate the proteinase K. PCR was then performed on extractions using different primer pairs (Appendix Table 1). Each reaction tube contained 0.6µl 50µM/ml of each primer, 1.5µl of the DNA extract, 12.3µl mQ H_2O and 15µl Bioline MyTaq mix DNA polymerase. PCR was a single denaturation step at 94°C for 180 seconds, with 40 cycles of 15s at 94°C, 30s at 50°C and 40s at 72°C, finishing with a single 300s elongation step at 72°C (Miller *et al.* 2013). The size of each PCR product was assessed by gel electrophoresis. The PCR product was compared against a 100 bp ladder (Thermo Scientific GeneRuler 100 bp) after staining with orange G dye. Samples of the correct length (600–700 bp) for generating barcode sequences were then prepared for sequencing using Illustra ExoProStar 1-step. Each digest sample contained 3µl ExoProStar 1-step and 15µl of PCR product, and were placed in the thermocycler for one cycle of 15min at 37°C and 15min at 80°C.

The concentration of DNA in each sample following digestion was estimated by comparison against four Promega Lambda DNA standards (5, 10, 20 and 50 ng/µl), using 1.5% agarose gels. Samples for sequencing were a total volume of 6µl, consisting of 1µl (6.4µM/ml) primer (forward or reverse) and 5–20ng DNA. The total sample volume was made up to 6µl using mQ H_2O.

Twenty-eight pseudo-replicate samples were prepared in total, using different combinations of forward and reverse

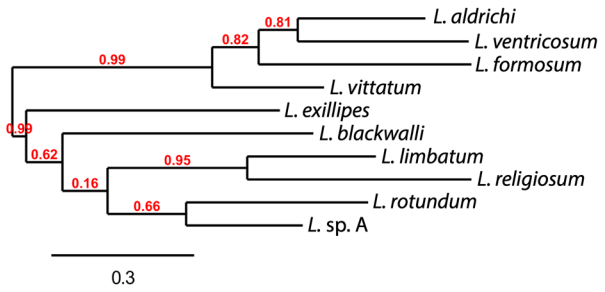

Fig. 1: Preliminary placement of *Leiobunum* sp. A within the genus *Leiobunum*, based on comparison of available DNA barcodes. Sequences were aligned and maximum likelihood tree constructed using the one-click tree builder tool on Phylogeny.fr (Dereeper *et al*. 2008). Bootstrap AIC values for node support are provided; higher values indicate increasing support, to a maximum of 1.0.

primers. Samples were sequenced by Edinburgh Genomics, Edinburgh (Edinburgh Genomics 2018). Sequence chromatograms were interpreted using FinchTV (Geospiza 2016) and CodonCode Aligner (CodonCode Corporation 2018). Sequences were trimmed to exclude primers, and base identities with Phred scores of less than 20 were removed. Phred scores of >20 are generally considered high quality and have been shown to be a reliable indicator of base identity (Ewing & Green 1998). Multiple sequence alignment was then performed using Clustal Omega multiple sequence alignment (European Molecular Biology Laboratory 2018) and a consensus read generated. In case of disagreement between different chromatogram reads, base identities with the highest Phred scores took precedence.

A BLAST search of the assembled barcode was then performed to confirm the novelty of the sequence against public and published data (NCBI, 2019). A preliminary phylogeny of the genus using the available *Leiobunum* spp. DNA barcodes (of length >630 bp) was generated via the one-click tree builder tool available at Phylogeny.fr (Dereeper *et al.* 2008; 2010). Only species with valid binomial names were included (sequence details are given in Appendix Table 2).

Results

A complete 660 base pair DNA barcode was generated for *Leiobunum* sp. (Appendix Fig. 1). In general, the highest quality reads were obtained when using the forward primer LCO1490, whilst HCO2198 generated reads of generally lower quality overall. The primers C1J1517F and CIN2568R failed to produce usable sequences (Appendix Table 1). A BLAST search of this sequence confirmed this barcode was novel (NCBI, 2019); the nearest matches in terms of base similarity were *Lophopilio palpinalis* Herbst, 1799, *Opilio parietinus* Degeer, 1778, and *Mitopus morio* Fabricius, 1799 (83%). Within the genus *Leiobunum*, the closest match was to *L. exillipes* Wood, 1878 (81%; Appendix Table 3).

Phylogenetic analysis revealed a close alignment between *Leiobunum* sp. A and the widespread species *L. rotundum* Latreille, 1798, although support for this clade was relatively weak (bootstrapped AIC: 0.66; Fig. 1).

Discussion

This paper presents the first DNA barcode for the enigmatic *Leiobunum* sp. A, based on a specimen collected in the UK (Appendix Fig. 1). The most consistent and reliable results were obtained using the universal barcode pairs proposed by Folmer *et al.* (1994), whilst limited success was achieved using more degenerate primers, such as CIN2568R (Hedin & Maddison 2001; Appendix Table 1). Preliminary phylogenetic analysis of the available *Leiobunum* spp. mitochondrial DNA sequences suggests a close affinity between *Leiobunum* sp. A and *L. rotundum* (Fig. 1). Although support for this node was relatively weak (AIC = 0.66), the degree of morphological similarity between these species (Wijnhoven, Schönhofer, & Martens 2007) in conjunction with sequence overlap suggests close affinity.

It is hoped that provision of a reliable sequence barcode for *Leiobunum* sp. A (Wijnhoven, Schönhofer, & Martens 2007), using a cheap and rapid DNA extraction methodology, will stimulate interest in this species in the UK and Europe, and facilitate formal description of this species.

Acknowledgements

I would like to thank Paul Richards (University of Sheffield) for sending me specimens, and Hay Wijnhoven for helpful discussion concerning the current taxonomic status of this species. Two anonymous reviewers also provided helpful comments on the manuscript.

References

ASTRIN, J. J., HÖFER, H., SPELDA, J., HOLSTEIN, J., BAYER, S., HENDRICH, L., HUBER, B. A., KIELHORN, K.-H., KRAMMER, H.-J., LEMKE, M., MONJE, J. C., MORINIÈRE, J., RULIK, B., PETERSEN, M., JANSSEN, H. & MUSTER, C. 2016: Towards a DNA Barcode reference database for spiders and harvestmen of Germany. *PloS one*, **11**: e0162624-e0162624.

CODONCODE CORPORATION 2018: *CodonCode Aligner version 8.02*, online at https://www.codoncode.com

DEREEPER, A., GUIGNON, V., BLANC, G., AUDIC, S., BUFFET, S., CHEVENET, F., DUFAYARD, J. F., GUINDON, S., LEFORT, V., LESCOT, M. & CLAVERIE, J. M. 2008: Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* **36**: W465–W469.

DEREEPER, A., AUDIC, S., CLAVERIE, J. M. & BLANC, G. 2010: BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evolutionary Biology* **10**: 8.

EUROPEAN MOLECULAR BIOLOGY LABORATORY 2018: *EMBL-EBI: Clustal Omega Multiple Sequence Alignment*, online at https://www.ebi.ac.uk/Tools/msa/clustalo

EWING, B. & GREEN, P. 1998: Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* 8: 186–194.

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. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

GEOSPIZA 2016: *FinchTV, version 1.4.0*. Geospiza Inc.

GLOOR, G. B. & ENGELS, W. R. 1992: Single-fly DNA preps for PCR. *Drosophila Information Service* **71**: 148–149.

HAJIBABAEI, M., SINGER, G. A. C., HEBERT, P. D. N. & HICKEY, D. A. 2007: DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics* **23**: 167–172.

HEBERT, P. D. N., PENTON, E. H., BURNS, J. M., JANZEN, D. H. & HALLWACHS, W. 2004: Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the USA* **101**: 14812–14817.

HEDIN, M. C. & MADDISON, W. P. 2001: A combined molecular approach to phylogeny of the jumping spider subfamily Dendryphantinae (Araneae: Salticidae). *Molecular Phylogenetics and Evolution* **18**: 386–403.

ISAAC, N. J. B., MALLET, J. & MACE, G. M. 2004: Taxonomic inflation: its influence on macroecology and conservation. *Trends in Ecology & Evolution* **19**: 464–469.

KALLAL, R. J. & HORMIGA, G. 2018: An expanded molecular phylogeny of metaine spiders (Araneae, Tetragnathidae) with description of new taxa from Taiwan and the Philippines. *Invertebrate Systematics* **32**: 400–422.

LABARQUE, F. M., SOTO, E. M., RAMÍREZ, M. J. & ARNEDO, M. A. 2015: Chasing ghosts: the phylogeny of Amaurobioidinae ghost spiders (Araneae, Anyphaenidae). *Zoologica Scripta* **44**: 550–561.

MACE, G. M. 2004: The role of taxonomy in species conservation. *Philosophical Transactions of the Royal Society of London, Series B* **359**: 711–719.

MILLER, J. A., BEENTJES, K. K., VAN HELSDINGEN, P. & IJLAND, S. 2013: Which specimens from a museum collection will yield DNA barcodes? A time series study of spiders in alcohol. *Zookeys* **365**: 245–261.

NCBI (National Center for Biotechnology information) BLAST. 2018: Bethesda: US National Library of Medicine, online at https://blast.ncbi.nlm.nih.gov/Blast.cgi

PANTE, E., SCHOELINCK, C. & PUILLANDRE, N. 2015: From integrative taxonomy to species description: one step beyond. *Systematic Biology* **64**: 152–160.

ROZWAŁKA, R., ŻURAWLEW, P. & RUTKOWSKI, T. 2017: First record of the expansive harvestmen *Leiobunum* sp. A (Arachnida: Opiliones) in Poland. *Fragmenta Faunistica* **60**: 113–118.

SANGSTER, G. & LUKSENBURG, J. A. 2015: Declining rates of species described per taxonomist: slowdown of progress or a side-effect of improved quality in taxonomy? *Systematic Biology* **64**: 144–151.

SPIDER RECORDING SCHEME 2018: British Arachnological Society, online at http://srs.britishspiders.org.uk

WIJNHOVEN, H., SCHÖNHOFER, A. L. & MARTENS, J. 2007: An unidentified harvestman *Leiobunum* sp. alarmingly invading Europe (Arachnida: Opiliones). *Arachnologische Mitteilungen* **34**: 27–38.

WOOD, H. M., GRISWOLD, C. E. & SPICER, G. S. 2007: Phylogenetic relationships within an endemic group of Malagasy 'assassin spiders' (Araneae, Archaeidae): ancestral character reconstruction, convergent evolution and biogeography. *Molecular Phylogenetics and Evolution* **45**: 612–619.

Appendix

> *Leiobunum* sp. A [Wijnhoven, Schönhofer & Martens 2007] COI voucher cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

GACAATATATATAATTTTTGGGATTTGAGCAGCTATAGTTGGCT
CAGCACTAAGTATTCTAATTCGAACAGAATTAGGTCAACCAG
GTTCTCTAATAAATGATGACCAAATCTATAATGTTATTGTAA
CAGCCCATGCATTTGTTATAATTTTTTTCATAGTTATACCAAT
TATAATGGGTAGATTTGGTAATTGGTTAGTTCCTTTAATATTAGG
GGCACCTGACATAGCTTTCCCACGATTAAATAACATAAGATTTT
GATTATTACCCCCTTCATTTTTATTATTATTAAGATCAACTATAG
TAGAAAGAGGGGCAGGAACAGGATGAACAGTATATCCTCCT
TTATCTGGCAACTCAGCACATAGAGGACCCTCAGTAGATTTAA
CAATCTTTTCGTTACATCTAGCCGGTATCTCCTCAATTTTAG
GAGCAATTAATTTTATTACTACTATTATTAATATACGAACTACAG
GTATAATTTACGAACGAGTACCTTTATTCGTTTGGTCAATTAA
GATCACAGCTATTCTTCTCTTACTATCACTACCTGTCTTAGCAG
GAGCTATTACTATGCTCCTTACTGACCGAAACTTCAATACATCT
TTCTTTGACCCAGCAGGGGGGGGGGATCCTATCCTCTACCAA
CATCTATTTTG

Fig. 1: DNA barcode (COI region, 660bps) in FASTA format for *Leiobunum* sp. A (Wijnhoven, Schönhofer & Martens 2007) based on consensus between multiple chromatogram reads (forward and reverse).

Sample	Primer pair	Sequence length	Sequence quality (no. bases Phred >20)
1	HCO2198 + LCO1490	659	614
2	HCO2198 + LCO1490	662	619
3	HCO2198 + LCO1490	*	*
4	HCO2198 + LCO1490	659	641
5	HCO2198 + LCO1490	660	613
6	HCO2198 + LCO1490JJ2	*	*
7	HCO2198 + LCO1490JJ2	*	*
8	HCO2198 + LCO1490JJ2	*	*
9	HCO2198 + LCO1490JJ2	*	*
10	HCO2198JJ2 + LCO1490	*	*
11	HCO2198JJ2 + LCO1490	659	636
12	HCO2198JJ2 + LCO1490	660	641
13	HCO2198JJ2 + LCO1490	660	635
14	HCO2198JJ2 + LCO1490	663	581
15	HCO2198JJ2 + LCO1490JJ2	*	*
16	HCO2198JJ2 + LCO1490JJ2	662	604
17	HCO2198JJ2 + LCO1490JJ2	660	620
18	HCO2198JJ2 + LCO1490JJ2	659	613
19	C1J1517F + CIN2568R	**	**
20	C1J1517F + CIN2568R	**	**
21	C1J1517F + CIN2568R	**	**
22	C1J1517F + CIN2568R	**	**
23	LCO1490 + CIN2568R	**	**
24	LCO1490 + CIN2568R	**	**
25	LCO1490 + CIN2568R	**	**
26	LCO1490 + CIN2568R	**	**
27	LCO1490 + CIN2568R	**	**
28	LCO1490 + CIN2568R	**	**

Table 1: Sequence qualities obtained for different primer pairs. * denotes primer pairs for which either a single primer read, or both, were of sufficiently low quality that a contiguous set (forward and reverse read) could not be generated. ** denotes sequences which failed PCR amplification.

Leiobunum species	BOLDv4 / NCBI record number	Sequence length
L. aldrichi	ARSO038-08	658bp
L. ventricosum	ASAAR498-15	658bp
L. vittatum	BBLZA251-16	658bp
L. limbatum	GBBSP877-15	658bp
L. rotundum	GBBSP256-15	658bp
L. religiosum	GBBSP1974-15	658bp
L. blackwalli	GBBSP1905-15	658bp
L. exillipes	GenBank: KM835163.1	633bp
L. formosum	GenBank: MH587764.1	638bp

Table 2: Voucher sequence details for those used to produce the phylogeny in Fig. 1.

Species	Reference sequence record title	Similarity (% base matches)
Lophopilio palpinalis Herbst, 1799	BIOUG13170-D12 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. Sequence ID: MF811216.1	83
Lophopilio palpinalis Herbst, 1799	BIOUG:OPILION-74 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. Sequence ID: HQ577662.1	83
Opilio parietinus Degeer, 1778	Cytochrome oxidase subunit I gene, partial cds; mitochondrial gene for mitochondrial product. Sequence ID: AF370832.1	83
Mitopus morio Fabricius, 1799	ZFMK-TIS-19118 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. Sequence ID: KY269018.1	83
Mitopus morio Fabricius, 1799	BC ZSM ARA 00038 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. Sequence ID: KX537278.1	83

Table 3: The top five closest matches following a BLAST search of the *Leiobunum* sp. A barcode with their relevant voucher details (NCBI 2019).