Article DOI: http://dx.doi.org/10.3201/eid2305.161165

# Hidden Complexities of Source Attribution for *Legionella longbeachae* Infections Revealed by Population Genomics

## Technical Appendix

## Materials and Methods

### Genome Assemblies and Variant Calling

De novo assemblies of the *Legionella* isolates were produced using SPAdes 2.5.1. (*1*) (k values of 21, 33, 55, 77, 99 and 127), generating a median of 106 contigs per genome (range, 38–402 contigs), with an average of 4.16 Mb in length (3.98–4.52 Mb) and an average N50 of 130 Kb (29 Kb-291 Kb).

The error-corrected reads produced by SPAdes were mapped against the *Legionella longbeachae* reference genome of strain NSW 150 (GenBank accession number NC_013861) using BWA 0.5.9 (*2*) with default parameters. SNPs were called using Samtools 1.18 (*3*) and those absent in at least 30% of the reads, with quality below 30 and depth below 3 were filtered out. The output from this filtering was used to construct consensus genomes of all the isolates for further phylogenetic analyses.

### Analysis of Genome Content

The contigs were annotated using Prokka v1.10 (*4*) and orthologous genes were clustered using the algorithm OrthoMCL (*5*) integrated in the software Get_homologs (*6*). We selected the options –f 50 (filters by 50% length difference within clusters) and –t 0 (for reporting all the clusters), resulting in 1801 core genome clusters. This program was also run using the Sg1 isolates only as input, specifying the options minimum percentage coverage (-C 80) and percentage identity (-S 85), which generated a core genome of 2574 gene clusters.

We also used JSpecies (*7*) to compute the average nucleotide identity values (BLAST; ANIb) between several pairs of isolates. These ANIb results were represented on a plot where isolates were clustered according to the 16S rRNA phylogenetic tree. In addition, a pangenomic

tree of the OMCL binary matrix from get_homologs.pl using all the *Legionella* isolates was constructed using the compare_clusters.pl script.

### Evolutionary and Phylogenetic Analysis

To confirm the identity of the isolates, a Neighbor-Joining tree based on the 16S rRNA gene of the sequenced genomes and all the cultured type *Legionella* strains available in the Ribosomal Database Project (*8*) (as of 01/06/2015) was constructed. The RNAmmer 1.2 server (*9*) was used to identify the 16S rRNA genes in the de novo assemblies, which were then aligned using MUSCLE with default parameters (*10*). The Neighbor-Joining tree was estimated using the Hasegawa–Kishino–Yano model and 1000 bootstrap resampling replicates using the program Geneious 5.4.6 (*11*).

To construct a phylogeny based on the whole genome sequence data, the 1801 orthologous open reading frames identified using OrthoMCL were aligned using MUSCLE 3.8.31 (*10*). Individual protein alignments were translated back to DNA alignments using pal2nal v14 (*12*) and the resultant alignments were concatenated using catfasta2phyml.pl (*13*) into an 1110024 bp long super-alignment. A ML phylogenetic tree was estimated based on this alignment using RAxML. C-4E7 was excluded from the original clustering as the low quality of the assembly significantly reduced the size of the core genome.

The *L. longbeachae* phylogeny was reconstructed using a Neighbor-Joining approach in Splitstree4 (*14*). Phylogenies of *L. longbeachae* Sg1 isolates before and after removing recombination were reconstructed from the genome alignments using RAxML 7.2.6 (*15*).

### Detection of Recombination

Recombination was examined using the SplitsTree4 program (version 4.13.1) (*14*). A phylogenetic network was computed on the *L. longbeachae* Sg1 multiple genome alignment using the Neighbor-Net method implemented in this software. The statistical significance of the tree was confirmed using a Phi test (*16*). Recombination was detected on the core genome alignment of the Sg1 isolates using BratNextGen (*17*). After drawing a PSA tree, we selected a cutoff of 0.042, which split the tree into 8 clusters. We used 20 iterations for the recombination learning algorithm and after performing 100 replicate runs in a single processor we selected a threshold of 5% for estimating the significance of recombination. Finally, the *L. longbeachae* Sg1 ML tree and the whole genome alignment were used as input for ClonalFrameML (*18*) to generate a phylogeny with branch lengths corrected for recombination. 100 pseudo-bootstrap

replicates were used to estimate the uncertainty in the EM model and the option -ignore_user_sites with the list of non-core coordinates was parsed.

### Plasmid Analysis

We used PLACNET, a software that constructs a network of contigs interactions, for the identification and visualization of plasmids (*19*). Bowtie2 v2.0.6 (*20*) was first used to find all possible scaffold links of the contigs by mapping the reads to them. Length and insert sizes of the reads mapped were calculated using Picard-Tools v1.90 (*21*). These files and metrics were parsed as input for placnet.pl, which produced a plasmid network from which we extracted the scaffolds. We then performed a BLAST search of the contigs assembly files to a database containing all the bacteria and plasmids genomes available in the NCBI ftp site (*22*) (created in March 2015) and results were filtered as follows: contigs longer than 200 bp, with a bitscore below 1e-26 and that had at least a blast hit over 5% of the contig size. The results were further analyzed to classify the nodes into one of these categories: “hit completely to a single reference genome,” “split nodes that hit to a single reference genome” and “nodes that hit several genomes.” The hits and the scaffolds were combined into a network that was uploaded into Cytoscape (*23*). Recommendations given in the PLACNET manual were followed to visualize the chromosome and plasmids networks. BLAST was finally used to search for *Legionella* spp. plasmid related sequences in the contigs.

### References


1. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77.

2. Li H, Durbin R. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics. 2010;26:589–95.

3. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

4. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30:2068–9.

5. Li L, Stoeckert CJJ Jr, Roos DS. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 2003;13:2178–89.

6. Contreras-Moreira B, Vinuesa P. GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. Appl Environ Microbiol. 2013;79:7696–701.

7. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci U S A. 2009;106:19126–31.

8. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42(D1):D633–42.

9. Lagesen K, Hallin P, Rødland EA, Staerfeldt HH, Rognes T, Ussery DW. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 2007;35:3100–8.

10. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–7.

11. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9.

12. Suyama M, Torrents D, Bork P, Delbru M. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 2006;34:609–12.

13. Nylander JA. Nylander/catfasta2phyml. https://github.com/nylander/catfasta2phyml

14. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67.

15. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006;22:2688–90.

16. Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. Genetics. 2006;172:2665–81.

17. Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, et al. Detection of recombination events in bacterial genomes from large population samples. Nucleic Acids Res. 2012;40:e6.

18. Didelot X, Wilson DJ. ClonalFrameML: efficient inference of recombination in whole bacterial genomes. PLOS Comput Biol. 2015;11:e1004041.

19. Lanza VF, de Toro M, Garcillan-Barcia MP, Mora A, Blanco J, De Cruz F, et al. Plasmid flux in *Escherichia coli* ST131 sublineages, analyzed by Plasmid Constellation Network (PLACNET), a new method for plasmid reconstruction from whole genome sequences. PLoS Genet. 2014;10:e1004766.

20. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.

21. Picard-tools 1.9. https://sourceforge.net/projects/picard/files/picard-tools/

22. NCBI ftp site. ftp://ftp.ncbi.nlm.nih.gov/

23. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics. 2011;27:431–2.

**Technical Appendix Table.** Isolates examined in the current study

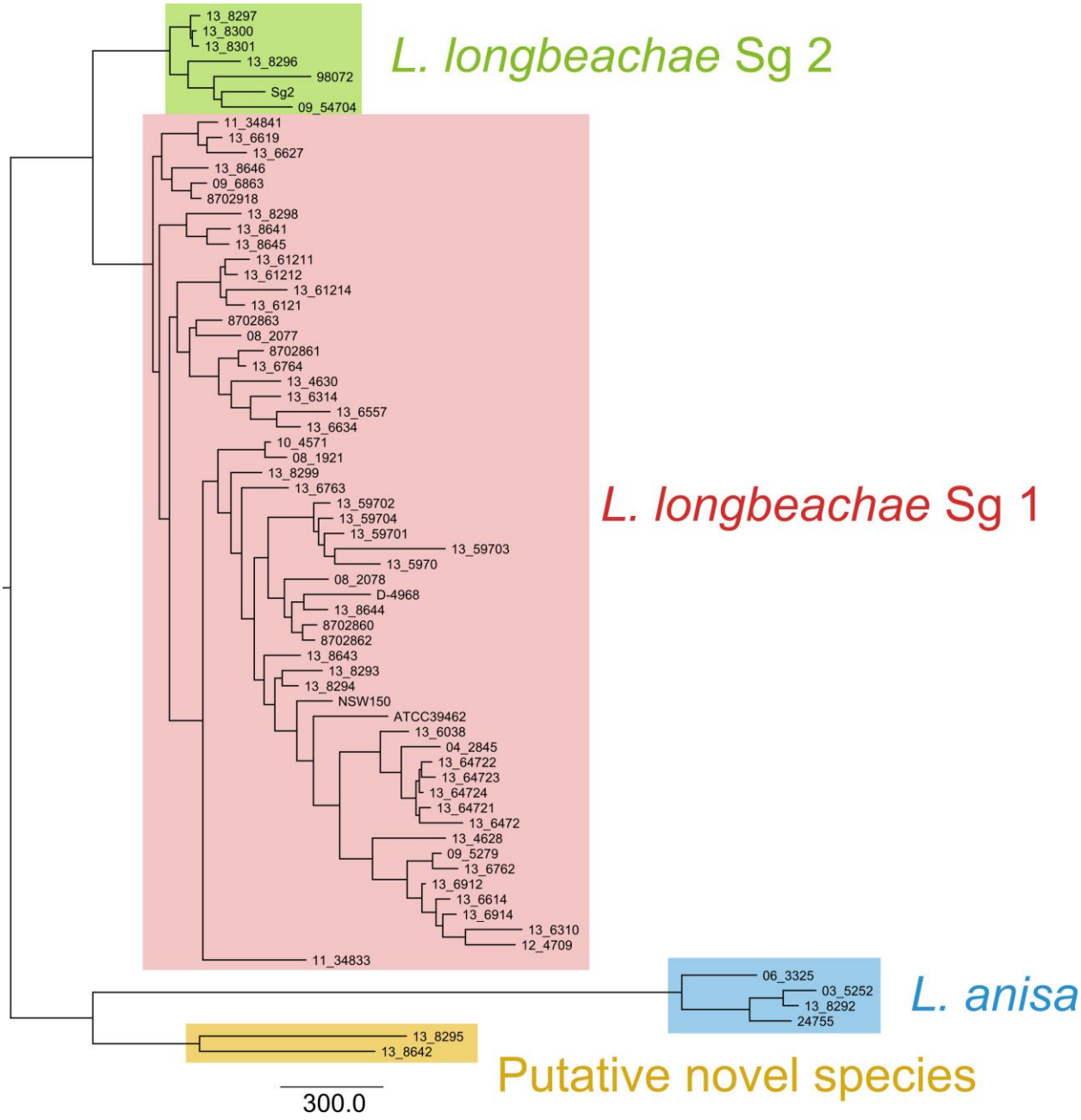
| Identifier | Species/serogroup | Date* | Source | Country | Linked to† |
|---|---|---|---|---|---|
| 02.4755 | *L. anisa* | Sep 24, 2002 | Hot water supply | Scotland | - |
| 03.5252 | *L. anisa* | Nov 12, 2003 | Patient | Scotland | - |
| 04.2845 | *L. longbeachae* Sg1 | Jun 7, 2004 | Patient | Scotland | - |
| 06.3325 | *L. anisa* | Jul 18, 2006 | Patient | Scotland | - |
| 08.1921 | *L. longbeachae* Sg1 | Apr 1, 2008 | Patient | Scotland | 08.2077,08.2078 |
| 08.2077 | *L. longbeachae* Sg1 | Apr 17, 2008 | Compost-IMS | Scotland | 08.1921 |
| 08.2078 | *L. longbeachae* Sg1 | Jul 31, 2008 | Compost Direct | Scotland | 08.1921 |
| 09.5279 | *L. longbeachae* Sg1 | May 20, 2009 | Patient | Scotland | 09.5470–4 |
| 09.5470–4 | *L. longbeachae* Sg2 | Jun 2, 2009 | Compost-Direct | Scotland | 09.5279 |
| 09.6863 | *L. longbeachae* Sg1 | Nov 19, 2009 | Compost | Scotland | Patient negative |
| 10.4571 | *L. longbeachae* Sg1 | Mar 19, 2010 | Patient | Scotland | Compost negative |
| 11.3483(3) | *L. longbeachae* Sg1 | May 13, 2011 | Compost | Scotland | 11.3484(1) |
| 11.3484(1) | *L. longbeachae* Sg1 | May 13, 2011 | Compost | Scotland | 11.3483(3) |
| 12.4709 | *L. longbeachae* Sg1 | Jun 18, 2012 | Patient | Scotland | No compost |
| 13.8641 | *L. longbeachae* Sg1 | Jun 18, 2012 | Compost | Scotland | 13.8644/45 |
| 13.8642 | New species | Jun 18, 2012 | Compost | Scotland | 13.8643 |
| 13.8643 | *L. longbeachae* Sg1 | Jun 18, 2012 | Compost | Scotland | 13.8642 |
| 13.8644 | *L. longbeachae* Sg1 | Jun 18, 2012 | Compost | Scotland | 13.8641/45 |
| 13.8645 | *L. longbeachae* Sg1 | Jun 18, 2012 | Compost | Scotland | 13.8641/44 |
| 13.8646 | *L. longbeachae* Sg1 | Jun 26, 2013 | Compost | Scotland | - |
| 13.4628 | *L. longbeachae* Sg1 | Jun 26, 2013 | Compost | Scotland | - |
| 13.4630 | *L. longbeachae* Sg1 | Aug 23, 2013 | Compost | Scotland | - |
| 13.5970‡ | *L. longbeachae* Sg1 | Aug 23, 2013 | Patient | Scotland | 13.6038 |
| 13.59701‡ | *L. longbeachae* Sg1 | Aug 23, 2013 | Patient | Scotland | 13.6038 |
| 13.59702‡ | *L. longbeachae* Sg1 | Aug 23, 2013 | Patient | Scotland | 13.6038 |
| 13.59703‡ | *L. longbeachae* Sg1 | Aug 23, 2013 | Patient | Scotland | 13.6038 |
| 13.59704‡ | *L. longbeachae* Sg1 | Aug 27, 2013 | Patient | Scotland | 13.6038 |
| 13.6038‡ | *L. longbeachae* Sg1 | Aug 30, 2013 | Top soil | Scotland | 13.5970 |
| 13.6121‡ | *L. longbeachae* Sg1 | Aug 30, 2013 | Patient | Scotland | 13.6619/27 |
| 13.61211‡ | *L. longbeachae* Sg1 | Aug 30, 2013 | Patient | Scotland | 13.6619/27 |
| 13.61212‡ | *L. longbeachae* Sg1 | Aug 30, 2013 | Patient | Scotland | 13.6619/27 |
| 13.61214‡ | *L. longbeachae* Sg1 | Sep 6, 2013 | Patient | Scotland | 13.6619/27 |
| 13.6310‡ | *L. longbeachae* Sg1 | Sep 6, 2013 | Patient | Scotland | 13.6762 |
| 13.6314‡ | *L. longbeachae* Sg1 | Sep 13, 2013 | Patient | Scotland | 13.6619 |
| 13.6472‡ | *L. longbeachae* Sg1 | Sep 13, 2013 | Patient | Scotland | No compost |
| 13.64721‡ | *L. longbeachae* Sg1 | Sep 13, 2013 | Patient | Scotland | No compost |
| 13.64722‡ | *L. longbeachae* Sg1 | Sep 13, 2013 | Patient | Scotland | No compost |
| 13.64723‡ | *L. longbeachae* Sg1 | Sep 17, 2013 | Patient | Scotland | No compost |
| 13.64724‡ | *L. longbeachae* Sg1 | Sep 19, 2013 | Patient | Scotland | No compost |
| 13.6557‡ | *L. longbeachae* Sg1 | Sep 19, 2013 | Patient | Scotland | No compost |
| 13.6614‡ | *L. longbeachae* Sg1 | Sep 19, 2013 | Compost | Scotland | - |
| 13.6619‡ | *L. longbeachae* Sg1 | Sep 20, 2013 | Compost | Scotland | 13.6121 |
| 13.6627‡ | *L. longbeachae* Sg1 | Sep 25, 2013 | Compost | Scotland | 13.6121 |
| 13.6634‡ | *L. longbeachae* Sg1 | Sep 25, 2013 | Patient | Scotland | No compost |
| 13.6762‡ | *L. longbeachae* Sg1 | Sep 25, 2013 | Compost | Scotland | 13.6310 |
| 13.6763‡ | *L. longbeachae* Sg1 | Oct 1, 2013 | Compost | Scotland | - |
| 13.6764‡ | *L. longbeachae* Sg1 | Oct 1, 2013 | Compost | Scotland | - |
| 13.6912 | *L. longbeachae* Sg1 | May 29, 2014 | Compost | Scotland | - |
| 13.6914 | *L. longbeachae* Sg1 | May 7, 2014 | Compost | Scotland | - |
| 8702918 | *L. longbeachae* Sg1 | May 8, 2014 | Patient | Scotland | 870286x |
| 8702860 | *L. longbeachae* Sg1 | May 9, 2014 | Soil | Scotland | 8702918 |
| 8702861 | *L. longbeachae* Sg1 | May 10, 2014 | Soil | Scotland | 8702918 |
| 8702862 | *L. longbeachae* Sg1 | Apr 1, 2008 | Soil | Scotland | 8702918 |
| 8702863 | *L. longbeachae* Sg1 | Apr 17, 2008 | Soil | Scotland | 8702918 |
| 13.8292 | *L. anisa* | 2010 | Compost | New Zealand | 13.8293 |

| Identifier | Species/serogroup | Date* | Source | Country | Linked to† |
|---|---|---|---|---|---|
| 13.8293 | *L. longbeachae* Sg1 | 2010 | Patient | New Zealand | 13.8292 |
| 13.8294 | *L. longbeachae* Sg1 | 2004 | Patient | New Zealand | - |
| 13.8295 | New species | 2013 | Patient | New Zealand | - |
| 13.8296 | *L. longbeachae* Sg2 | 2012 | Sump drain | New Zealand | - |
| 13.8297 | *L. longbeachae* Sg2 | 2007 | Compost | New Zealand | 13.8301 |
| 13.8298 | *L. longbeachae* Sg1 | 1996 | Compost | New Zealand | - |
| 13.8299 | *L. longbeachae* Sg1 | 2003 | Compost | New Zealand | - |
| 13.8300 | *L. longbeachae* Sg2 | 2011 | Compost | New Zealand | - |
| 13.8301 | *L. longbeachae* Sg2 | 2007 | Patient | New Zealand | 13.8297 |
| Sg2 | *L. longbeachae* Sg2 | - | - | - | - |
| NSW 150 | *L. longbeachae* Sg1 | - | Patient | Australia | - |
| C-4E7 | *L. longbeachae* Sg2 | - | Patient | Australia | - |
| D-4968 | *L. longbeachae* Sg1 | - | Patient | USA | - |
| ATCC39642 | *L. longbeachae* Sg1 | - | Patient | USA | - |
| 98072 | *L. longbeachae* Sg2 | - | Patient | USA | - |

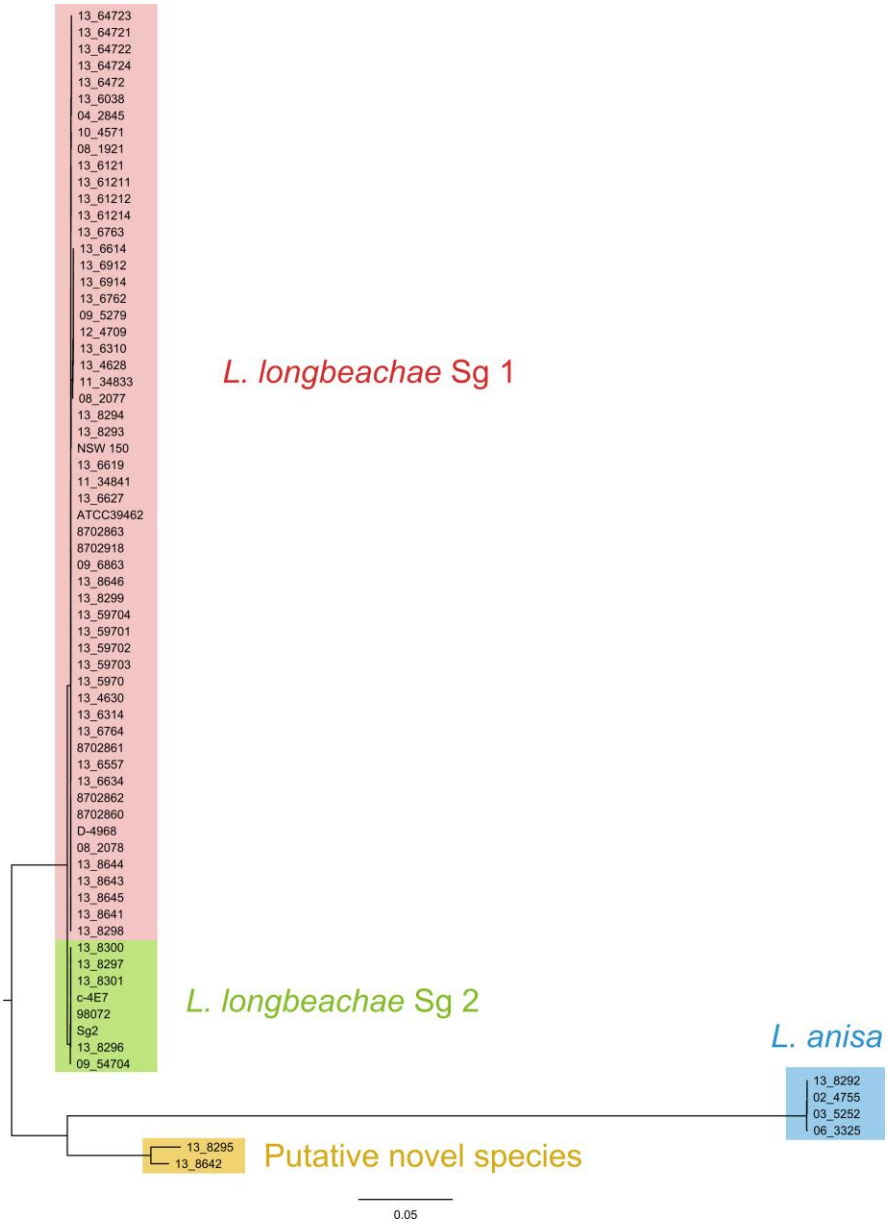
*Date received in the reference laboratory.

†Isolates that are linked to each other, as patient and cognate compost samples. Multiple isolates from each of the three patients or environmental samples share common identifiers (13.5970, 13.6121, 13.6472, 870286).

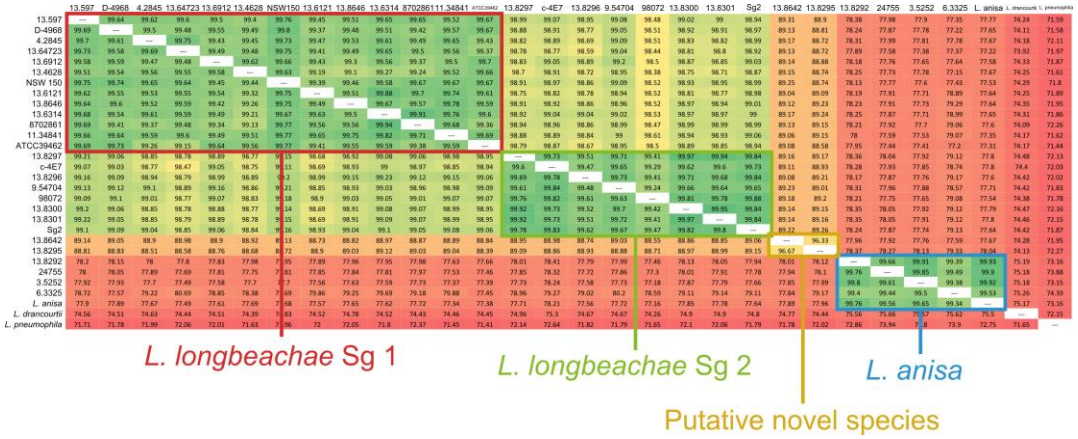
‡Isolates from the 2013 summer cluster of diseases in Scotland.



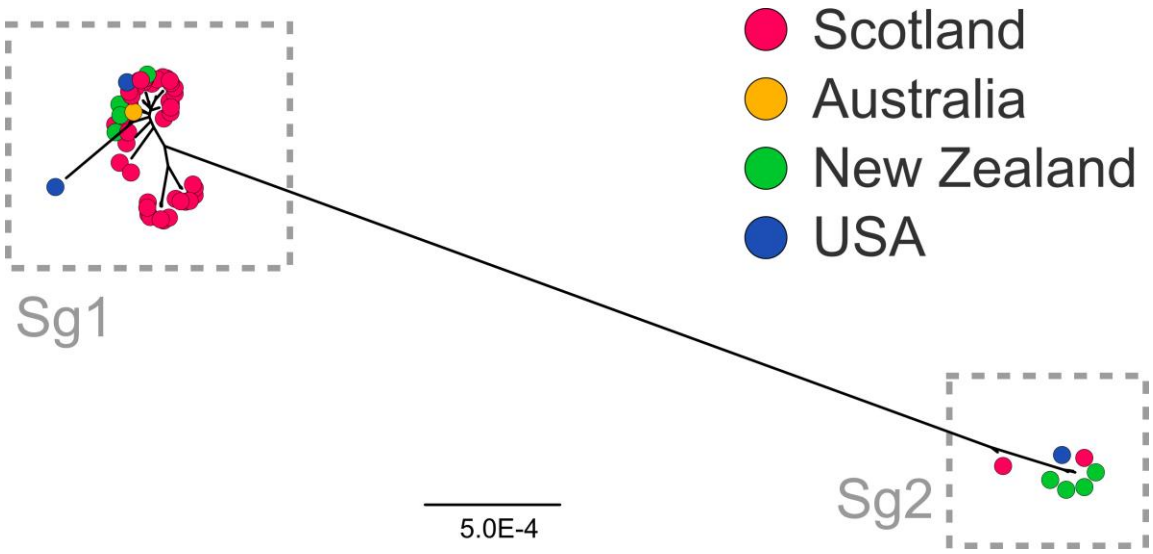

**Technical Appendix Figure 1.** Parsimony based tree of the OMCL pangenomic matrix obtained for all the sequenced genomes. Scale bar indicates the gene content differences.



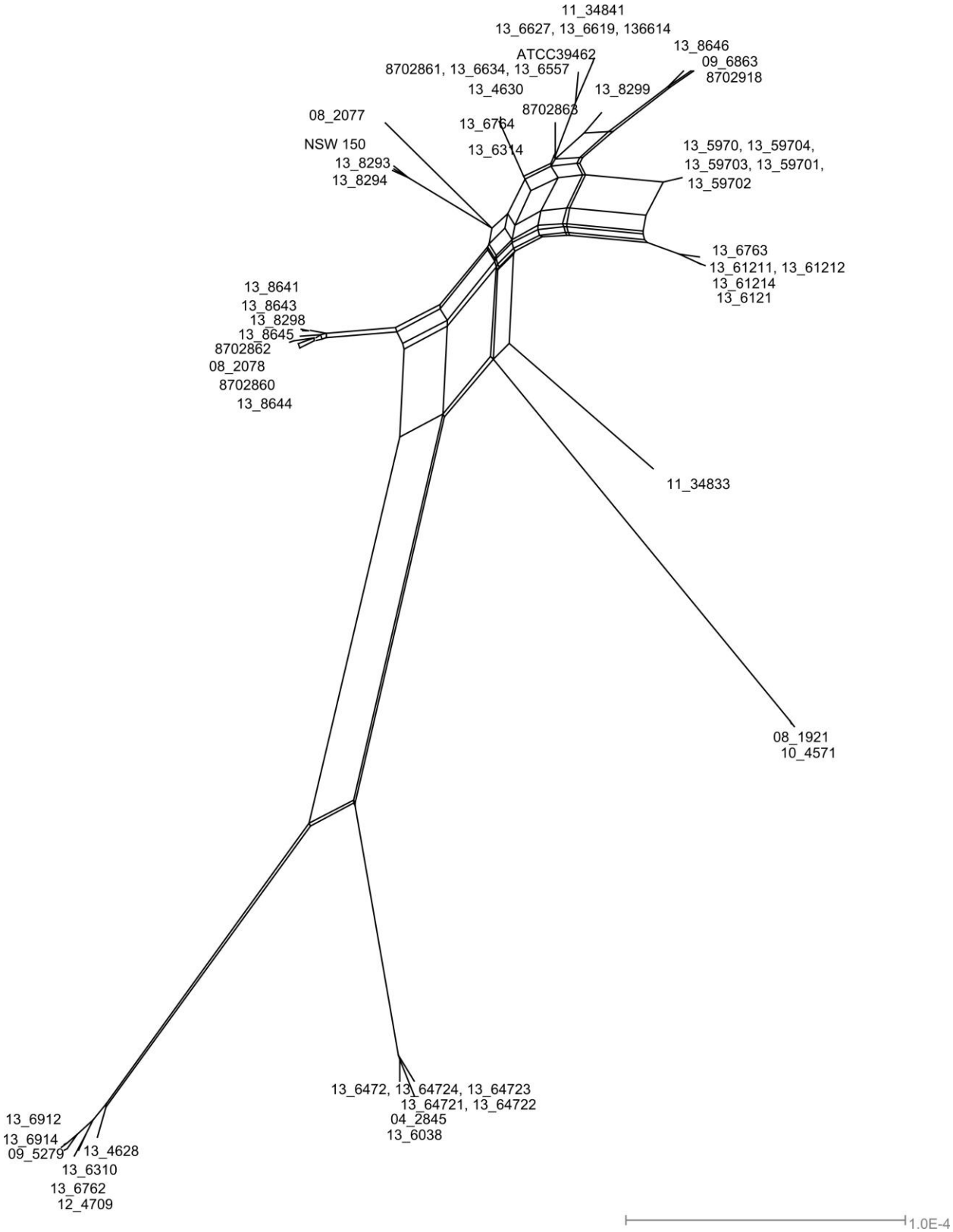

**Technical Appendix Figure 2.** Maximum likelihood tree of a core gene alignment of all the isolates included in the study. The tree shows the same clusters as the 16S rRNA gene based tree and the parsimony pangenome tree. Scale bar indicates the mean number of nucleotide substitutions per site.



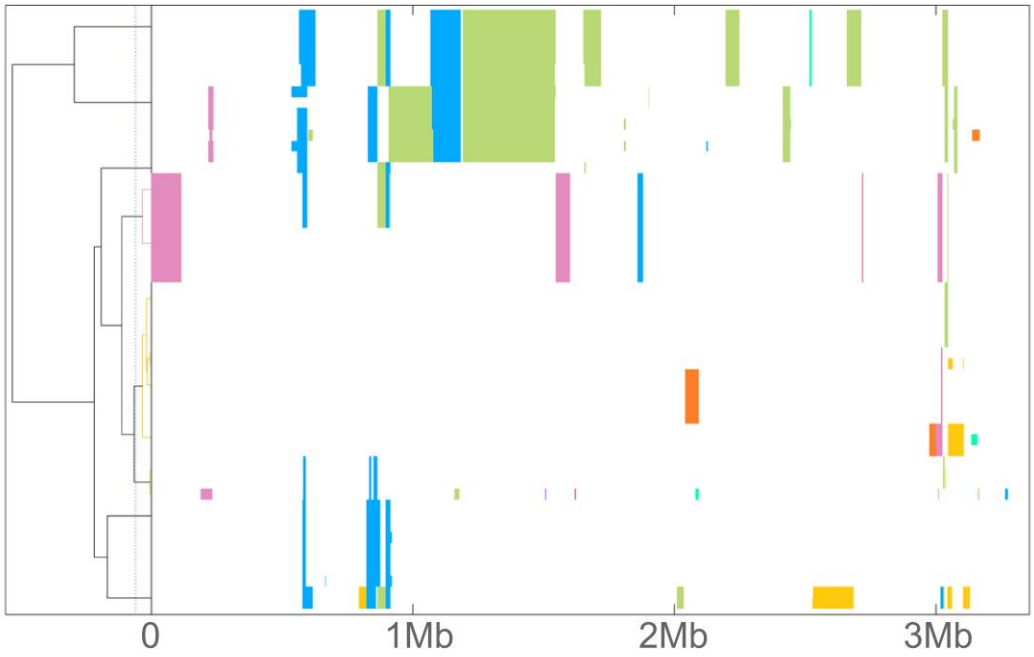

**Technical Appendix Figure 3.** Average Nucleotide Identity (ANI) comparison matrix of several genomes of the study sorted by similarity. Green, high ANI values; Red, low ANI values. The genomes of *Legionella drancourtii* and *Legionella pneumophila* were included in the matrix for comparative purposes, but none of the isolates showed an ANI higher than 76% with these two species.




**Technical Appendix Figure 4.** Neighbor-Joining phylogeny based on the core genome of *Legionella longbeachae* isolates. Isolates are colored by geographic source, and dashed boxes indicate the defined or predicted serogroups to which the isolates belong.




**Technical Appendix Figure 5.** Neighbor-Joining split network for the *Legionella longbeachae* Serogroup 1 isolates based on the consensus alignment obtained from mapping every isolate to the reference chromosome NSW 150. Scale bar indicates the mean number of nucleotide substitutions per site.




**Technical Appendix Figure 6.** Recombinant regions of the core genome alignment of 55 *L. longbeachae* Sg1 isolates as identified using BratNextGen. On the left, a clustering tree of the isolates with colored branches indicating cluster relationships. On the right, significant recombinant segments predicted, with similar color in a column representing recombinant regions for those isolates have the same origin.