Taxonomic Description template

Escherichia ruysiae sp. nov., isolated from an international traveller

Boas C.L. van der Putten^{1,2#}, S. Matamoros¹, D.R. Mende³, COMBAT consortium[†], C. Schultsz^{1,2}

¹Department of Medical Microbiology, Amsterdam UMC, University of Amsterdam, the Netherlands

²Department of Global Health, Amsterdam Institute for Global Health and Development, Amsterdam UMC, University of Amsterdam, the Netherlands

³Department of Applied Evolutionary Biology, Amsterdam UMC, University of Amsterdam, the Netherlands

[#]Corresponding author, email address: boas.vanderputten@amsterdamumc.nl

[†]Members listed in supplementary file 1

Keywords: *Escherichia*, bacterial taxonomy, Enterobacteriaceae

ABSTRACT

The *Escherichia* genus comprises four species and at least five lineages currently not assigned to any species, termed '*Escherichia* cryptic clades'. We isolated an *Escherichia* strain from an international traveller and resolved the complete DNA sequence of the chromosome and an IncI multi-drug resistance plasmid using Illumina and Nanopore whole-genome sequencing (WGS). Strain OPT1704^T can be differentiated from existing *Escherichia* spp. using biochemical (VITEK2) and genomic tests (average nucleotide identity [ANI] and digital DNA:DNA hybridisation [dDDH]). Phylogenetic analysis based on alignment of 16S rRNA sequences and 682 concatenated core genes showed similar results. Our analysis further revealed that strain OPT1704^T falls within *Escherichia* cryptic clade IV, and is closely related to cryptic clade III. Combining our analyses with publicly available WGS data of

cryptic clades III and IV from Enterobase confirmed the close relationship between clades III and IV (>96% interclade ANI), warranting assignment of both clades to the same novel species. We propose *E. ruysiae* sp. nov. as a novel species, encompassing *Escherichia* cryptic clades III and IV (type strain OPT1704^T = NCCB 100732^T = NCTC 14359^T).

Author notes

The Genbank accession number for the 16S rRNA gene sequence of strain OPT1704^T is LR745848. The Genbank accession number for the complete genome sequence of strain OPT1704^T is CABVLQ000000000.

Introduction

Within the *Escherichia* genus, four species are recognized; *E. coli* (1), *E. fergusonii* (2), *E. albertii* (3) and most recently, *E. marmotae* (4). Several species were assigned to the *Escherichia* genus previously, but have now been moved to other genera, such as *E. vulneris* (now *Pseudoescherichia vulneris* (5)), *E. blattae* (now *Shimwellia blattae* (6)), *E. adecarboxylata* (now *Leclercia adecarboxylata* (7)) and *E. hermannii* (now *Atlantibacter hermannii* (8)). All four *Escherichia* species have been associated with the potential to cause animal and/or human disease (9–12). Several *Escherichia* strains cannot be assigned to any of the four existing species. Based on analysis of genetic data, these strains cluster into several groups, which were termed ‘*Escherichia* cryptic clades’ (13,14). Recently, cryptic clade V was formally recognized as a separate species (*E. marmotae*), leaving five cryptic clades that have not been delineated at the species level. Here we report the novel species *Escherichia ruysiae* sp. nov., isolated from faecal material of an international traveller. *Escherichia ruysiae* sp. nov. encompasses the closely related *Escherichia* cryptic clades III and IV.

49

50 Isolation and Ecology

51 We discovered a cryptic clade IV strain in our collection, previously identified as extended spectrum
52 beta-lactamase (ESBL) producing *E. coli* as part of the COMBAT study, which investigated the
53 acquisition of ESBL-producing Enterobacteriaceae (ESBL-E) during international travel (15). This
54 isolate, OPT1704^T, was further characterized in detail.

55 The strain was isolated from a human faecal sample provided immediately after an individual's
56 return from a one-month journey to several Asian countries. No ESBL-E were detected in a faecal
57 sample collected immediately before departure, suggesting the ESBL gene, and possibly strain
58 OPT1704^T, were acquired during travel. The traveller reported diarrhoea during travel but no
59 antibiotic usage. No ESBL-E were isolated in follow-up faecal samples, suggesting loss of the
60 OPT1704^T strain or the ESBL gene within one month after return from travel.

61

62 Genome Features

63 The whole-genome sequence of strain OPT1704^T was determined using a combination of the
64 Illumina HiSeq and Oxford Nanopore Technologies (ONT) sequencing platforms. The Illumina
65 sequencing run yielded a total of 6.3×10^6 paired-end reads, with a mean read length of 151 bp.
66 Illumina reads were downsampled using seqtk (version 1.3-r106, <https://github.com/lh3/seqtk>) to
67 provide a theoretical coverage depth of 100X with the assumption that the OPT1704^T has a genome
68 size of approximately 5×10^6 bp. The ONT sequencing run yielded a total of 2.5×10^4 reads, with a
69 mean read length of 9078 bp before filtering. ONT reads were filtered on length and on read identity
70 using Filtlong (version 0.2.0, <https://github.com/rrwick/Filtlong>) with Illumina reads as a reference,
71 leaving 1.5×10^4 reads with a mean length of 12580 bp. This provided a theoretical coverage depth of

~38X of ONT reads. The combined assembly using Unicycler (version 0.4.6 (16)) of Illumina and Nanopore reads resulted in a completely assembled genome, consisting of one circular chromosome and one circular plasmid. The GC content of the complete OPT1704^T genome was 50.6%.

Putative resistance and virulence genes were predicted from the draft genome using ABRicate with the CARD (17) and VFDB (18) databases. OPT1704^T harbours 6 resistance genes on its IncI plasmid, associated with reduced susceptibility to fluoroquinolones (*qnrS1*), aminoglycosides (*aph(6)-Id* & *aph(3'')-Ib*), cephalosporins (*blaCTX-M-14*), trimethoprim (*dfrA14*) and sulphonamides (*sul2*), corresponding with its reduced susceptibility to fluoroquinolones (norfloxacin, MIC: 2 mg/L and ciprofloxacin, MIC: 0.5 mg/L), cephalosporins (cefuroxime, MIC: >32 mg/L and cefotaxime, MIC: 4 mg/L) and trimethoprim-sulfamethoxazole (MIC: >8 mg/L), assessed using VITEK2 (BioMérieux). However, strain OPT1704^T was susceptible to tobramycin (MIC: ≤1 mg/L) and gentamicin (MIC: ≤1 mg/L) despite presence of aminoglycoside resistance genes. Furthermore, several putative virulence genes were predicted from the genome sequence associated with siderophore function (*chuX*, *entS*, *fepABD*), fimbriae (*fimBCDGI*), a type II secretion system (*gspGHI*) and capsular polysaccharide biogenesis (*kpsD*). These predicted virulence genes, when present in *Escherichia coli*, are not typically associated with a specific clinical syndrome such as diarrhoeal disease.

Physiology and Chemotaxonomy

Strain OPT1704^T formed circular, grey-white colonies on a Columbia sheep (COS) blood agar plate. Individual cells were observed under a light microscope and were rod-shaped and approximately 1 by 2 µm in size. The strain was shown to be Gram-negative, non-motile, oxidase-negative and catalase-positive. The strain was capable to grow in the absence of oxygen. On COS blood plates, it showed growth in the temperature range of 20-42 °C. The strain was also able to grow in NaCl concentrations ranging from 0% to 6% in lysogeny broth. MALDI-TOF (Bruker) and VITEK2 (BioMérieux) systems both identified OPT1704^T as *E. coli* with high confidence scores (score>2 for

MALDI-TOF and “Excellent identification” for VITEK2). Comparison of the output of the VITEK2 biochemical test with published biochemical reactions of other *Escherichia* species revealed that *E. ruysiae* sp. nov. str. OPT1704^T is distinct from other *Escherichia* species based on a biochemical profile (table 1) (2–4,19). One biochemical reaction, lysine decarboxylation, cannot be performed by strain OPT1704^T, but can be performed by all other *Escherichia* species. This reaction is typically mediated by the *cadA* gene (20), which is missing in OPT1704^T but present in other *Escherichia*.

102

103 16S rRNA and whole-genome phylogeny

Next, we calculated 16S rRNA sequence similarities, ANI values and digital DNA:DNA hybridisation (dDDH) values between OPT1704^T and type strains of the four other *Escherichia* species, representative genomes of the other three *Escherichia* cryptic clades, and *S. enterica* serovar Typhimurium (table 2). Representative genomes for the *Escherichia* cryptic clades I, II, III and VI were selected from Enterobase (21), using the genomes with the highest contiguity. Clades VII and VIII in Enterobase only consisted of a single strain and were not used in further analyses. We used three separate tools to calculate average nucleotide identity (ANI) (fastANI (22), OrthoANIu (23) and ANI calculator from Enveomics (24)) and the DSMZ Genome-to-Genome Distance Calculator to calculate digital DNA:DNA hybridisation (dDDH) (25). 16S rRNA genes were extracted from whole genomes using barrnap (version 0.9, <https://github.com/tseemann/barrnap>) and similarity was assessed using snp-dists (version 0.6, <https://github.com/tseemann/snp-dists>).

OPT1704^T showed 98.7–98.9% 16S rRNA sequence similarity to *E. coli* ATCC 11775^T, *E. fergusonii* ATCC 35469^T and *E. marmotae* HT073016^T, which would not warrant assignment to a novel species based on the current threshold for species delineation (less than 98.7% sequence similarity). However, the threshold for species delineation on the basis of 16S rRNA sequence has changed often and thresholds of up to 99% sequence similarity have been proposed previously (Kim 2014). In

contrast, ANI analysis and dDDH did support assignment of OPT1704^T to a novel species, together with the representative strain of *Escherichia* cryptic clade III (table 2). The analyses also confirmed that OPT1704^T falls within the *Escherichia* genus. This novel species, encompassing both *Escherichia* cryptic clades III and IV, was assigned *E. ruysiae* sp. nov. with OPT1704^T as the proposed type strain.

To gain a better understanding of the *Escherichia* genus, we produced two phylogenies, based on 16S rRNA sequence (Fig. 1) and on an alignment of 682 core genes (Fig. 2). In short, rRNA genes were predicted from whole genomes using barrnap (version 0.9, <https://github.com/tseemann/barrnap>) and a tree was generated using FastTree (version 2.1.10 (26)). For the core gene alignment, genomes were first annotated with Prokka (version 1.14.0 (27)) and a core gene alignment was produced using Roary (version 3.12.0 (28)) and MAFFT (version 7.307 (29)). The phylogeny was inferred using a generalised time reversible model using base frequencies from the SNP alignment and free rate heterogeneity (GTR+F+R4 model) in IQ-tree (version 1.6.6 (30)), as advised by ModelFinder (31). Phylogenies were rooted on the *Salmonella enterica* serovar Typhimurium str. LT2^T genome. Both phylogenies showed that strain OPT1704^T clusters closely with the strain MOD1-EC7259 from *Escherichia* cryptic clade III, and away from the current *Escherichia* species.

Chun et al. (32) proposed that strains with >95-96% genome-wide ANI between each other should be assigned to the same species. If cryptic clades III and clade IV would share >95-96% ANI, this would mean both clades should be assigned to the same novel species, *E. ruysiae*. To assess this for a larger number of strains than the type strains presented in table 2, we downloaded all available WGS from clade III and clade IV strains from Enterobase and compared ANI between all genomes using fastANI (version 1.1 (22)). This analysis revealed that within 32 clade III genomes, the median ANI is 98.6% (range: 97.7%-99.9%), while within 31 clade IV genomes, the median ANI is 98.9% (range: 98.6%-99.9%). Between clade III and clade IV genomes, the median ANI is 96.6% (range 96.2%-96.8%). This suggests clades III and IV should be assigned to the same novel species, *E. ruysiae* sp. nov.

Currently, no IJSEM guidelines exist for the delineation of subspecies based on genomic data. However, *E. ruysiae* could potentially be delineated further into two subspecies (representing the current clades III and IV, respectively) in the future, after a type strain for cryptic clade III has been identified.

Description of *E. ruysiae* sp. nov.

Escherichia ruysiae (ruy'si.æ N.L. fem. n. after Anna Charlotte Ruys, professor of microbiology at the University of Amsterdam from 1940 to 1969). Cells are Gram-negative, facultatively anaerobic, non-sporulating, non-motile rods with a size of approximately 1 by 2 µm. Colonies are circular, convex, grey-white and semi-transparent when grown overnight at 37 °C on COS sheep blood agar plates. The species is catalase-positive and oxidase-negative and grows at temperatures between 20 and 42 °C and NaCl concentrations between 0% and 6% w/v. In the VITEK2 GN biochemical test set it yields a positive result for Beta-Galactosidase, D-Glucose, D-Maltose, D-Mannitol, D-Mannose, D-Sorbitol, D-Trehalose, Saccharose/Sucrose, D-Tagatose, Gamma-Glutamyl-Transferase, Fermentation Glucose, Tyrosine Arylamidase, Succinate Alkalinisation, Alpha-Galactosidase, Ornithine Decarboxylase, Courmarate, Beta-Glucuronidase, O/129 Resistance (Comp.Vibrio.) and Ellman and negative for Ala-Phe-Pro-Arylamidase, Adonitol, L-Pyrrolydonyl-Arylamidase, L-Arabitol, D-Cellobiose, H₂S Production, Beta-N-Acetyl Glucosaminidase, Glutamyl Arylamidase Pna, Beta-Glucosidase, Beta-Xylosidase, Beta-Alanine Arylamidase Pna, L-Proline Arylamidase, Lipase, Palatinose, Urease, Citrate (Sodium), Malonate, 5-Keto-D-Gluconate, L-Lactate Alkalinisation, Alpha-Glucosidase, Beta-N-Acetyl-Galactosaminidase, Phosphatase, Glycine Arylamidase, Lysine Decarboxylase, L-Histidine Assimilation, Glu-Gly-Arg-Arylamidase, L-Malate Assimilation and L-Lactate Assimilation.

The 16S rRNA sequence is deposited in ENA under accession LR745848. Raw Illumina and Nanopore whole-genome sequencing data, as well as the complete genome assembly are deposited under project PRJEB34275.

AUTHOR STATEMENTS

Funding information

The COMBAT study was funded by Netherlands Organization for Health, Research and Development (ZonMw; 50-51700-98-120) and EU-H2020 programme (COMPARE, 643476).

Acknowledgements

The authors would like to thank Rob Weijs and Patricia Brinke for their help in phenotypic characterization of type strain OPT1704^T of *Escherichia ruysiae* sp. nov. and Arie van der Ende for the helpful discussions. We thank SURFsara (www.surfsara.nl) for the support in using the Lisa Compute Cluster.

Ethical statement

Not required.

Conflicts of interest

None.

ABBREVIATIONS

ONT: Oxford Nanopore Technologies

188 MIC: Minimum Inhibitory Concentration

189 COS: Columbia agar + Sheep blood

190 dDDH: digital DNA:DNA hybridisation

191 ANI: Average Nucleotide Identity

192 SNP: Single Nucleotide Polymorphism

193

194 REFERENCES

- 195 1. Castellani A, Chambers AJ. Manual of tropical medicine. William Wood; 1919.
- 196 2. Farmer JJ, Fanning GR, Davis BR, O'Hara CM, Riddle C, Hickman-Brenner FW, et al. *Escherichia*
197 *fergusonii* and *Enterobacter taylorae*, two new species of Enterobacteriaceae isolated from
198 clinical specimens. J Clin Microbiol. 1985 Jan 1;21(1):77.
- 199 3. Huys G, Cnockaert M, Janda JM, Swings J. *Escherichia albertii* sp. nov., a diarrhoeagenic species
200 isolated from stool specimens of Bangladeshi children. Int J Syst Evol Microbiol.
201 2003;53(3):807–10.
- 202 4. Liu S, Jin D, Lan R, Wang Y, Meng Q, Dai H, et al. *Escherichia marmotae* sp. nov., isolated from
203 faeces of *Marmota himalayana*. Int J Syst Evol Microbiol. 2015;65(7):2130–4.
- 204 5. Alnajar S, Gupta RS. Phylogenomics and comparative genomic studies delineate six main clades
205 within the family Enterobacteriaceae and support the reclassification of several polyphyletic
206 members of the family. Infect Genet Evol. 2017;54:108–127.
- 207 6. Priest FG, Barker M. Gram-negative bacteria associated with brewery yeasts: reclassification of
208 *Obesumbacterium proteus* biogroup 2 as *Shimwellia pseudoproteus* gen. nov., sp. nov., and
209 transfer of *Escherichia blattae* to *Shimwellia blattae* comb. nov. Int J Syst Evol Microbiol.
210 2010;60(4):828–833.

- 211 7. Tamura K, Sakazaki R, Kosako Y, Yoshizaki E. *Leclercia adecarboxylata* Gen. Nov., Comb. Nov.,
212 formerly known as *Escherichia adecarboxylata*. *Curr Microbiol.* 1986;13(4):179–184.
- 213 8. Hata H, Natori T, Mizuno T, Kanazawa I, Eldesouky I, Hayashi M, et al. Phylogenetics of family
214 Enterobacteriaceae and proposal to reclassify *Escherichia hermannii* and *Salmonella*
215 *subterranea* as *Atlantibacter hermannii* and *Atlantibacter subterranea* gen. nov., comb. nov.
216 *Microbiol Immunol.* 2016;60(5):303–311.
- 217 9. Russo TA, Johnson JR. Medical and economic impact of extraintestinal infections due to
218 *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes Infect.* 2003
219 Apr 1;5(5):449–56.
- 220 10. Savini V, Catavittello C, Talia M, Manna A, Pompetti F, Favaro M, et al. Multidrug-Resistant
221 *Escherichia fergusonii*: a Case of Acute Cystitis. *J Clin Microbiol.* 2008 Apr 1;46(4):1551.
- 222 11. Ooka T, Seto K, Kawano K, Kobayashi H, Etoh Y, Ichihara S, et al. Clinical significance of
223 *Escherichia albertii*. *Emerg Infect Dis.* 2012 Mar;18(3):488–92.
- 224 12. Liu S, Feng J, Pu J, Xu X, Lu S, Yang J, et al. Genomic and molecular characterisation of
225 *Escherichia marmotae* from wild rodents in Qinghai-Tibet plateau as a potential pathogen. *Sci*
226 *Rep.* 2019;9(1):1–9.
- 227 13. Gangiredla J, Mammel MK, Barnaba TJ, Tartera C, Gebru ST, Patel IR, et al. Draft Genome
228 Sequences of *Escherichia albertii*, *Escherichia fergusonii*, and Strains Belonging to Six Cryptic
229 Lineages of *Escherichia* spp. *Genome Announc.* 2018 May 3;6(18):e00271-18.
- 230 14. Walk S. The “Cryptic” *Escherichia*. *EcoSal Plus* [Internet]. 2015; Available from:
231 <https://www.asmscience.org/content/journal/ecosalplus/10.1128/ecosalplus.ESP-0002-2015>
- 232 15. Arcilla MS, van Hattem JM, Haverkate MR, Bootsma MCJ, van Genderen PJJ, Goorhuis A, et al.
233 Import and spread of extended-spectrum β -lactamase-producing Enterobacteriaceae by
234 international travellers (COMBAT study): a prospective, multicentre cohort study. *Lancet Infect*
235 *Dis.* 2017 Jan 1;17(1):78–85.
- 236 16. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from
237 short and long sequencing reads. *PLOS Comput Biol.* 2017 Jun 8;13(6):e1005595.
- 238 17. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The Comprehensive
239 Antibiotic Resistance Database. *Antimicrob Agents Chemother.* 2013 Jul;57(7):3348–57.

- 240 18. Chen L. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res.* 2004 Dec
241 17;33(Database issue):D325–8.
- 242 19. Abbott SL, O'Connor J, Robin T, Zimmer BL, Janda JM. Biochemical properties of a newly
243 described *Escherichia* species, *Escherichia albertii*. *J Clin Microbiol.* 2003;41(10):4852–4854.
- 244 20. Snider J, Houry WA. MoxR AAA+ ATPases: a novel family of molecular chaperones? *J Struct*
245 *Biol.* 2006;156(1):200–209.
- 246 21. Zhou Z, Alikhan N-F, Mohamed K, Fan Y, Achtman M, Brown D, et al. The EnteroBase user's
247 guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and
248 *Escherichia* core genomic diversity. *Genome Res.* 2020;30(1):138–152.
- 249 22. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis
250 of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun.* 2018 Nov
251 30;9(1):5114.
- 252 23. Yoon S-H, Ha S-M, Lim J, Kwon S, Chun J. A large-scale evaluation of algorithms to calculate
253 average nucleotide identity. *Antonie Van Leeuwenhoek.* 2017 Oct;110(10):1281–6.
- 254 24. Rodriguez-R LM, Konstantinidis KT. The enveomics collection: a toolbox for specialized analyses
255 of microbial genomes and metagenomes. *PeerJ Prepr.* 2016 Mar;4:e1900v1.
- 256 25. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation
257 with confidence intervals and improved distance functions. *BMC Bioinformatics.* 2013
258 Feb;14(1):60.
- 259 26. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large
260 alignments. *PloS One.* 2010;5(3).
- 261 27. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics.* 2014 Jul
262 15;30(14):2068–9.
- 263 28. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale
264 prokaryote pan genome analysis. *Bioinformatics.* 2015 Nov 15;31(22):3691–3.
- 265 29. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements
266 in performance and usability. *Mol Biol Evol.* 2013;30(4):772–780.

- 267 30. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: A Fast and Effective Stochastic
268 Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol Biol Evol.* 2014 Nov
269 3;32(1):268–74.
- 270 31. Kalyaanamoorthy S, Minh BQ, Wong TK, von Haeseler A, Jermiin LS. ModelFinder: fast model
271 selection for accurate phylogenetic estimates. *Nat Methods.* 2017;14(6):587.
- 272 32. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, et al. Proposed minimal
273 standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol*
274 *Microbiol.* 2018 Jan 1;68(1):461–6.

275

276

FIGURES AND TABLES

Table 1. Comparison of biochemical markers which differentiate *E. ruysiae* sp. nov. from other

Escherichia species. + and – indicate that ≥85% of tested strains is positive or negative for that

biochemical marker, respectively. Data for *E. albertii*, *E. coli*, *E. fergusonii* and *E. marmotae*

summarised from literature (Abbott 2003, Huys 2003, Farmer 1985, Liu 2015).

	<i>E. ruysiae</i>	<i>E. albertii</i>	<i>E. coli</i>	<i>E. fergusonii</i>	<i>E. marmotae</i>
ONPG	+	+	+	+	?
Lysine decarboxylase	-	+	+	+	+
Ornithine decarboxylase	+	+	+	+	?
Fermentation of:					
Adonitol	-	?	?	+	?
d-Xylose	-	?	+	+	+
Cellobiose	-	?	?	+	?
d-Sorbitol	+	?	+	?	+

*50-85% of *E. coli* possess this biochemical property.

284 **Table 2.** Comparison of OPT1704^T 16S rRNA and whole-genome sequence with type strains of *E.*
 285 *albertii*, *E. coli*, *E. fergusonii*, *E. marmotae*, representative genomes of *Escherichia* cryptic clades I, II,
 286 III and VI and *S. enterica* serovar Typhimurium. In bold are the values that warrant assignment of
 287 OPT1704^T to a novel species (<98.7% 16S rRNA sequence similarity, <95-96% ANI, <70% dDDH). ANI:
 288 average nucleotide identity, dDDH: digital DNA:DNA hybridisation.

	<i>E. ruysiae</i> sp. nov.				
	OPT1704 ^T				
	16S rRNA sequence similarity (%)	ANI (% fastANI)	ANI (% OrthoANIu)	ANI (% ANI calculator Enveomics)	dDDH (%)
<i>E. albertii</i> NBRC 107761 ^T	98.6	90.0	90.0	89.2	39.8
<i>E. coli</i> ATCC 11775 ^T	98.7	92.8	92.4	92.0	48.3
<i>E. fergusonii</i> ATCC 35469 ^T	98.9	89.4	88.2	89.7	36.7
<i>E. marmotae</i> HT073016 ^T	98.9	92.2	92.2	91.4	47.1
<i>S. enterica</i> Typhimurium LT2 ^T	97.5	82.1	80.7	81.8	24.0
<i>Escherichia</i> cryptic clade I 89-3506	99.0	92.5	92.1	91.8	47.8
<i>Escherichia</i> cryptic clade II MOD1-EC7253	99.2	92.0	91.7	91.0	45.5
<i>Escherichia</i> cryptic clade III E4694	99.7	96.6	96.5	96.3	70.8
<i>Escherichia</i> cryptic clade VI UHCL_3L	98.4	91.6	91.7	91.3	45.9

289

290 **Figure 1.** 16S phylogeny of *E. ruysiae* str. OPT1704^T with type strains of other *Escherichia* spp., other
 291 *Escherichia* cryptic clades and *Salmonella enterica* serovar Typhimurium as outgroup. Numbers
 292 indicate bootstraps on a scale of 0 to 1. Phylogeny available at
 293 <https://itol.embl.de/tree/14511722611197561579189697>

294

295

296 **Figure 2.** Phylogeny based on 682 concatenated core genes including *E. ruysiae* OPT1704T with type
 297 strains of other *Escherichia* spp., other *Escherichia* cryptic clades and *Salmonella enterica* serovar
 298 Typhimurium as outgroup. Numbers indicate bootstraps on a scale of 0 to 100. Phylogeny available
 299 at <https://itol.embl.de/tree/14511722711358151579253758>

Tree scale: 0.1