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ORIGINAL CONTRIBUTION

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Prevalence and Diversity of the *Streptobacillus* Rat-bite Fever Agent, in Three Invasive, Commensal *Rattus* Species from South Africa

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Rat-bite fever is an over-looked, global zoonotic disease that has a mortality rate of up to 13%, if untreated. Historically, this rat-borne disease has been attributed to one of two causative agents, *Streptobacillus moniliformis* or *Spirillum minus*. Given the confirmed presence of multiple invasive *Rattus* host species, high rat densities in urban, informal human settlements and increasing reports of rat bites in South Africa, we undertook a retrospective assessment of *Streptobacillus* in rats sampled from 16 urban sites, in Gauteng, the smallest but most populous Province in South Africa. Using a multi-gene PCR-sequencing approach, we confirmed *Streptobacillus* presence in 50.9% of oral swabs from three rat species and the presence of two *Streptobacillus* species, *viz. S. moniliformis* and *S. notomytis*. The two members of the cryptic *Rattus rattus* species complex (*R. rattus* and *R. tanezumi*), which are morphologically indistinguishable from each other, had markedly different colonization rates. Whereas 48.6% of rats from this species complex were *Streptobacillus*-positive, only 32.3% of *Rattus tanezumi* were positive compared to 61.5% *R. rattus. Rattus norvegicus* had an intermediate prevalence of 55.6%. Phylogenetic analysis of four gene regions (16S rRNA, *gyrB*, *groEL*, *recA*) identified two discrete lineages; *S. moniliformis* occurred exclusively in *R. norvegicus*, and *S. notomytis* was restricted to the two members of the *R. rattus* species complex; this represents the first report of *Streptobacillus* in *R. tanezumi*. These results highlight a largely overlooked zoonotic threat posed by invasive rats and confirm the presence of two discrete and potentially host-specific *Streptobacillus* lineages in South Africa.

INTRODUCTION

Streptobacillus moniliformis is one of the causative agents of the zoonotic disease, rat-bite fever (RBF), the other being *Spirillum minus*. This bacterial pathogen is considered to be a commensal in the oro-nasopharynx of rats [1,2]. However, carriers such as laboratory and pet-bred rats may display clinical signs of the disease [1,3,4]. Colonization rates for wild rats range from 50-100% [5] and from 10-100% for laboratory and pet-bred rats [6].

Human infection with *S. moniliformis* is possible through direct and indirect contact via rat bites and scratches or through contact with any excreta (eg, saliva, urine, feces) [6,7], with the potential to cause mass

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Abbreviations: RBF, rat-bite fever; +G, gamma distributed substitution rates; AIC, Akaike Information Criterion; GTR, General Time Reversible; ML, Maximum Likelihood; NJ, Neighbor-Joining; ANOVA, analysis of variance.

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outbreaks, as occurred at Haverhill in the US [7]. Due to non-specific presentation of RBF, the disease is most likely under-reported [7] and misdiagnosed [8]). In the industrialized world, RBF is believed to be restricted to individuals in frequent contact with rodents, such as laboratory personnel, but its pediatric relevance in low socio-economic settings has been recognized [2,8,9].

To date, three *Rattus* species have been recorded in South Africa [10] of which two, *R. rattus* and *R. tanezumi,* are morphologically indistinguishable and form part of the *R. rattus* species complex [11]. These cryptic *Rattus* species, which are not native to South Africa, have successfully established and expanded their distribution range beyond the point of initial introduction and as such are considered invasive species [10]. Rat population eruptions in the Johannesburg and Cape Town metropoles of South Africa have been linked to increasing reports of pediatric rat bites requiring medical attention and to a leptospirosis outbreak in a correctional facility, respectively [12,13]. A large proportion of the South African human population resides in informal settlements that are characterized by over-crowding, poor housing infrastructure, and inadequate waste removal services [14]. These conditions, which are conducive to rodent population eruptions, make it crucial to assess the infection status of invasive rats, particularly as it is precisely within these settings that the number of immune-compromised individuals is highest in South Africa [15] and for which effective RBF treatment may be complicated.

Molecular detection of *S. moniliformis* by PCR is a sensitive and accurate means of determining prevalence in reservoir host species such as *Rattus* [7,16-19]. Clinical confirmation in humans is generally achieved by culture of the organism from blood, cerebrospinal fluid, and joint puncture [7,20,21], but this is hampered by the slow growth of the bacterium [7,21,22]. Molecular confirmation by PCR is increasingly reported [23,24] and is another mode for detection.

Until recently, *S. moniliformis* was the only species in the genus [25] and RBF epidemiology was largely limited to individual case reports. A recent study by Eisenberg et al. [19], which provided insights into the genotypic and phenotypic characteristics of the genus, highlighted the need for epidemiological studies on reservoir rodent hosts. Reports of RBF in Africa are limited to Kenya and thus far associated with *Spirillum minus* [26,27]. In this study we address the current dearth of information by extending preliminary 16S rRNA PCR confirmation of *Streptobacillus* presence in murid rodents from South Africa [28] through increased sampling of *Rattus* and expanded genetic characterization of *Streptobacillus* through sequencing of four bacterial genome targets. The results have implications for rodent pest management practices and emphasize the importance of assessing host and bacterial strain diversity in formulating regionally relevant control strategies.

MATERIALS AND METHODS

Sample Collection

Oral swab samples were collected from 106 rats (*Rattus* spp.) from 16 Gauteng Province sampling sites in South Africa (Table 1; Figure 1). Sampling occurred monthly for three consecutive days from August 2010 to September 2011. Animals were sacrificed by means of halothane inhalation as approved by the Animal Ethics Committee of the University of Pretoria (Ethics clearance number EC025-10). Standard measurements were recorded and kidney samples were secured for 62 of the 106 rats sampled. All animals were collected under permit number (CPF6 0032) issued by the Gauteng Department of Nature Conservation and with permission from landowners.

Rattus Species Identification and Age Analyses

Adult individuals of *R. norvegicus* were identified based on external morphology whereas the two cryptic species, *R. rattus* and *R. tanezumi*, were genetically identified on the basis of discrete monophyletic mitochondrial lineages [11]. This was achieved by mitochondrial cytochrome *b* (cyt *b*) gene amplification as previously described [10]. Rodents were aged based on the extent of the maxillary molar tooth-row wear on the upper molars [29,30] grouped in five age categories (I-V).

Molecular Detection and Characterization of Streptobacillus

Genomic DNA was extracted from 62 kidney and 106 oral swabs using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's protocol. Initial screening for *Streptobacillus* genome presence was performed with previously published diagnostic primers (S5 and AS2) that target a 269 bp region of the 16S rRNA gene [18] (Table 2).

In addition, *Streptobacillus* strain diversity was assessed in *Rattus* hosts by characterizing *gyrB*, *recA, groEL* and a larger (> 1.2 Kbp) fragment of the 16S rRNA gene. The latter was achieved by amplifying two over-lapping gene fragments using each of the diagnostic primers AS2 and S5 [18] in combination with a broad range universal primer set, 27F and 1522R [31], respectively. The 27F/AS2 primer combination yielded a 1206 bp fragment at an annealing temperature of 57ºC, whilst the S5/1522R primer combination yielded a fragment of 539 bp at an annealing temperature of 54ºC. Amplification of the *gyrB*, *recA*, and *groEL* gene regions was

Table 1. *Streptobacillus* in *Rattus rattus*, *R. tanezumi,* and *R. norvegicus* from 16 sampling localities in Gauteng Province, South Africa. The numbers indicated for each *Rattus* species correspond to positive animals and those in parenthesis represent sample size.

Sampling code	Locality (site number)	Land-use site	*R. norvegicus*	*R. rattus*	*R. tanezumi*
BSK	Boschkop, Pretoria	Peri-Urban	-	-	1 (6)
DS	Diepsloot, Alexandra	Urban	0 (4)	-	-
GF	Garsfontein, Pretoria	Urban	-	1 (1)	1 (1)
HF	Hatfield (1), Pretoria	Urban	-	-	0 (1)
HGP	Hammanskraal (1), Pretoria	Peri-Urban	-	-	3 (3)
HSY	Hammanskraal (2), Pretoria	Peri-Urban	-	-	0 (4)
HUP	Hammanskraal (3), Pretoria	Peri-Urban	-	-	4 (6)
ML	Menlyn, Pretoria	Urban	-	0 (2)	-
MV	Mountain View, Pretoria	Urban	-	-	0 (6)
RF	Rietfontein, Pretoria	Urban	-	-	1 (1)
TEM	Tembisa	Urban	20 (32)	-	-
TR	The Reeds, Pretoria	Urban	-	-	0 (1)
UPH	Hatfield (2), Pretoria	Urban	-	2 (5)	-
UPE	Hillcrest, Pretoria	Peri-Urban	-	21 (31)	-
VDG	Val De Grace, Pretoria	Urban	-	-	0 (1)
VL	Villiera, Pretoria	Urban	-	-	0 (1)
Total (%)			20 (55.6%)	24 (61.5%)	10 (32.3%)

achieved using published and newly designed primers that amplify a broad range of *Streptobacillus* species (Table 2). Positive and negative controls were included in all assays and the resulting products were run against a molecular weight marker (Fermentas, Waltham, MA, USA) on a 1.5% agarose gel. All PCR reactions were performed in a final volume of 50 µl containing 0.25 µM dNTP's (Fermentas, Waltham, Massachusetts, USA), 0.4 µM of each primer, 1U Taq Polymerase (Biotools B&M Labs, Madrid, Spain) and 3 µL template DNA.

Nucleotide Sequencing and Phylogenetic Analyses

Amplified PCR products were purified using the Roche PCR Product Purification Kit (Roche Diagnostics, GmbH, Mannheim, Germany) and cycle sequenced using BigDye v.3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, USA), with each of the external PCR primers in separate reactions. Samples were run on an ABI 3130 sequencer and sequence chromatograms were viewed and edited in MEGA 5 [31] prior to performing BLAST nucleotide searches (www.ncbi.nm.nih.gov/blast). Each of the sequence variants generated in this study have been submitted to the GenBank database (www.ncbi.nlm.nih.gov) and are available under the following accession numbers: (i) 16S rRNA: KF843829, KF843833, (ii) *gyrB*: KF843839, KU957988-90, (iii) *recA*: KU957991-93, (iv) *groEL*: KU957985-87.

For the individual gene phylogenies inferred using the 16S rRNA (24 taxa, 1225 nt), *gyrB* (19 taxa, 1712 nt), and *recA* (21 taxa, 601 nt) data sets, the Tamura-3-parameter (TN92) model (TPM3uf) with gamma distributed substitution rates (+G), was selected as the best-fit model of sequence evolution under the corrected Akaike Information Criterion (AIC) in jModeltest [32] whereas for the *groEL* dataset (18 taxa, 738 nt), the best-fit model was the General Time Reversible (GTR) +G (Supplementary Figure S1: Appendix A). For the concatenated dataset, the GTR+G model of sequence evolution was used, with sequence statistics for each of the individual 15-taxon datasets that make up the concatenated dataset being summarized in Supplementary Table S1: Appendix A. Phylogenies were inferred using Maximum Likelihood (ML) and Neighbor-Joining (NJ) in MEGA 5 [33] and nodal support was assessed by 10,000 non-parametric bootstrap replications. Bayesian inference (BI) was performed with MrBayes v.2.1.3 [34] with priors guided by the best-fit model of sequence evolution and run for 10,000,000 generations. Tracer plots were viewed in Tracer v.1.5 [35], after which 25% of trees were discarded as burn-in.

Statistical Analyses

The prevalence of *S. moniliformis* among host species was tested by a one-way analysis of variance (ANO-

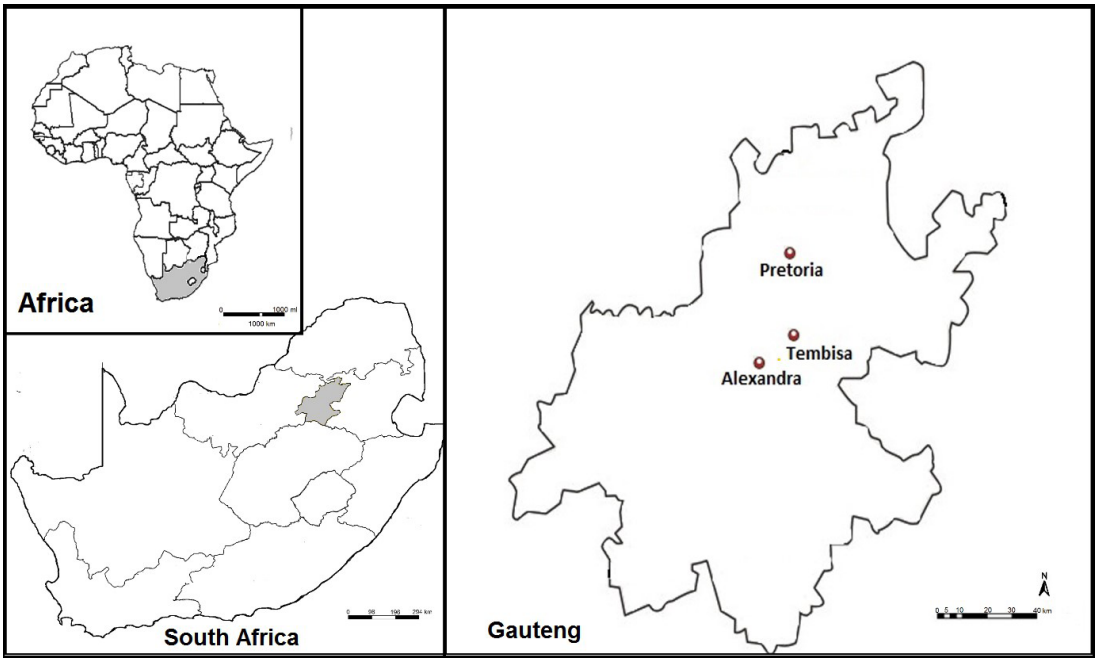

Figure 1. A map depicting the Gauteng Province sampling sites of *Rattus novergicus, R. rattus,* and *R. tanezumi* in South Africa. Of the 16 samples sites, 14 are in the Pretoria area (Table 1) and the remaining two in the Tembisa and Alexandra areas.

Table 2. Oligonucleotide primers used in this study.

Gene region	Primers	Sequence 5'-3'	Target amplicon size (bp)	Tm (ºC)	Reference
	S5	ATACTCGGAATAAGATGG	269	48	[18]
	AS2	GCTTAGCTCCTCTTTGTAC		50.5	[18]
16S rRNA					
	27F	AGAGTTTGATCCTGGCTCAG	1500	57	[31]
	1522R	AAGGAGGTGATCCAGCCGCA		61	[31]
	MZK-F	AAGATAGGGTAATGCTTACAGAAGGAG	616	62	[17]
	MZK-R	AATCTACCTTGTTTTGCAGATCCAC		60	[17]
gyrB					
	XiGyrB-F1	TCTTCAAGAGGATTACATCA	1710	47	This study
	XiGyrB-R2	TTATCTGCATATTGTGCATC		47	This study
recA	XiRecA-F	TATGGTGAAGGCTCTATAATGA	835	51	This study
	XiRecA-R	CAGTACCATAACTAAACCATGA		51	This study
groEL	XiGroEL-F1	TTAGGACCTCGTGGTAGAAATGT	800	58	This study
	XiGroEL-R1	CTCCTGTTAATATTGCAATATCTTC		59	This study

Tm: Melting temperature; bp: base pairs

VA) followed by Tukey's *post hoc* analyses to identify non-significant differences ($P > 0.05$) among species. The influence of land-use, sex, and age on *S. moniliformis* prevalence among host species was investigated by a three-way ANOVA. Land-use sites were categorized as either urban or peri-urban areas, dependent on building density and natural vegetation (Supplementary Table S2: Appendix A). As *R. norvegicus* was only sampled at urban sites, there was no variation in land-use for this species and only *R. rattus* and *R. tanezumi* were considered when evaluating this aspect. Age category I was considered as juvenile, categories II-III as sub-adult and IV-V as adult. Seasonal aspects could not be evaluated due to the opportunistic nature of sample collection.

The proportional contribution of each variable to the observed differences in prevalence of each respective host

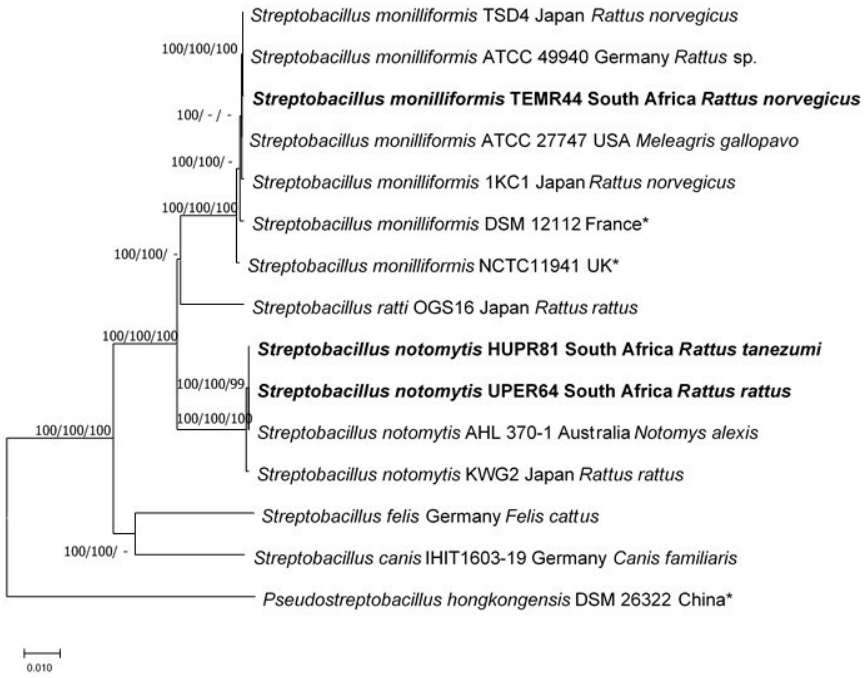

Figure 2. *Streptobacillus* phylogeny inferred using a concatenated dataset, 3125 nucleotides (nt) in length, comprising of four partial gene regions corresponding to the 16S rRNA (1225 nt), rec*A* (601 nt), gyr*B* (1046 nt) and gro*EL* (253 nt) genes. Taxon names include the *Streptobacillus* species, followed by the strain name, country and host species. Sequences generated in this study are indicated in bold. Bootstrap support values (≥70%) from NJ and Maximum Likelihood (ML) analyses, and posterior probabilities (≥95%) from Bayesian Inference (BI) are indicated NJ/ML/BI above the relevant node. Nodal support values below these cut-offs are denoted by a - and * indicates strains reported in humans.

was undertaken by partitioning the sum of squares (SSQ) of each source of variation obtained from a three-way ANOVA table. Percent SSQ of the four potential sources of variation in the data, namely, sex, land-use site, age, and error (= residual) where available were obtained by dividing the SSQ associated with each source of variation by the total SSQ. All statistical analyses were performed in STATISTICA v.10 (StatSoft Inc. Tulsa, OK, USA).

RESULTS

The 106 rats examined in this study comprised 36 *R. norvegicus* (33.96%), 39 *R. rattus* (36.79%) and 31 *R. tanezumi* (29.25%). Of the 106 oral swabs tested, 54 (50.94%) were confirmed positive for *Streptobacillus* through amplification and nucleotide sequencing of all diagnostic 16S rRNA [18] gene products. For the subset of rats for which both oral and kidney samples were also available (n = 62), oral swab prevalence was 69.35%, whereas only one (1.61%) of the kidney samples of a *R. rattus* individual was positive. Although differences in oral swab prevalence (Table S2: Appendix), were statistically significant among *Rattus* hosts ($F_{(2,103)} = 3.30$; n = 106; $P = 0.04$), *post hoc* analyses revealed this was due to statistically significant differences between *R. rattus* (61.54%) and *R. tanezumi* (32.26%).

Comparison of prevalence between land-use sites for each member of the *R. rattus* species complex revealed that *Streptobacillus* prevalence was higher in peri-urban areas than in urban areas, but this was only statistically significant for *R. rattus* ($F_{(1,37)} = 4.74$; $n = 39$; $P = 0.036$). Similarly, age effects were only statistically significant at $P \leq 0.05$ for *R. rattus* with older rats having higher colonization rates. However, subsequent assessment of the sources of the variation (% SSQs) (Table S2: Appendix A) revealed that differences among hosts were not related to the variables investigated (sex, site, and age), but were due to unknown residual factors.

Similar tree topologies were recovered across all gene regions and methods of analysis (Figure 2, Figure S1: Appendix A). A concatenated dataset, 3125 nt in length, comprising of the 16S rRNA (1225 nt), *gyrB* (1047 nt), *recA* (601 nt), and *groEL* (252 nt) gene regions was subsequently inferred, for which the best-fit model under the AICc was GTR+G (Figure 2; Table S1: Appendix A). *Streptobacillus* strains recovered from *R. nor-*

vegicus from South Africa grouped with *S. moniliformis* whereas those from the *R. rattus* species complex (*R. rattus* and *R. tanezumi*) grouped with *S. notomytis*. There were no discernible genetic differences in *Streptobacillus* strains in the two members of the *R. rattus* species complex across the gene regions characterized in the study (Figure 2, Figure S1: Appendix A).

DISCUSSION

The low *Streptobacillus* prevalence in *Rattus* kidney tissue suggests that excretion of the bacteria in urine likely only occurs when rodents are bacteremic and confirms the oro-nasopharyngeal cavity as the maintenance site of the bacterium. However, secondary contamination of the urethra/urine cannot be discounted. Since the formal recognition of *S. notomytis* in 2015 [19], both *Streptobacillus* species identified in this study have previously been associated with human RBF cases outside of Africa and present with similar clinical outcomes [36,37].

In South Africa, rat bites are reported to occur frequently in rural areas [38], however, a recent report confirms an increasing trend in urban areas [12]. RBF is a systemic disease [16] and immune-compromised individuals (children, the elderly, and those living with HIV and AIDS), in particular, may develop complications including polyarthritis, endocarditis, and pneumonia [22,24,39]. RBF is likely underreported as it is frequently misdiagnosed due to non-specific presentation [6,7] and because rat bite victims rarely seek medical attention [37]. Fortunately, the disease is relatively easy to treat if accurately diagnosed [2,7,19], but may be fatal (7-13% mortality) if left untreated [6].

Streptobacillus strains present in *R. tanezumi* and *R. rattus* from South Africa were genetically indistinguishable across the four gene regions characterized, and fell within the *S. notomytis* lineage, whereas *S. moniliformis* was only detected in *R. norvegicus*. These two *Streptobacillus* species have previously been detected in *Rattus* hosts by Kimura et al. [18] based on the 16S rRNA gene region. The additional gene regions (*gyrB*, *recA*, and *groEL*) characterized in this study are functional genes that have been used for species-level identification [19] and suggest the possibility of host specificity for each of the two *Streptobacillus* species identified. The strain associated with *R. norvegicus* in both this and a prior Japanese study [18] was also detected in turkeys in the US, whereas the *R. rattus* species complex strain from our study is identical to a *R. rattus* strain from Japan and Germany [18,40]. The latter was recognized as a novel species, *S. notomytis* (= AHL370-1^T) being named after the Australian spinifex hopping mouse (*Notomys alexis*) in which it was initially detected in 1981 [25,41]. This and another elevated strain, *S. ratti* (= OGS16^T) [42] together with two newly described species namely, *S. felis* [43] and *S. canis* [44], underscores the largely overlooked genetic diversity within what was formerly a monotypic genus. Our results suggest that the *S. notomytis* and *S. moniliformis* strains may be associated with discrete *Rattus* reservoir hosts, those of the *R. rattus* species complex and *R. norvegicus*, respectively. Although a geographical component cannot be ruled out as the three species did not occur sympatrically, the recovery of *S. moniliformis* from *R. norvegicus* sampled from both South Africa and Japan (Figure 2) and *S. notomytis* carriage in *R. rattus* sampled from diverse localities in South Africa and from Japan [18], suggest the likelihood of a strong host-association. This study represents the first report of *S. notomytis* in *R. tanezumi*, an invasive *Rattus* species that likely has a greater global footprint than currently acknowledged because it cannot be distinguished morphologically from the better-known *R. rattus* invader.

Among the three *Rattus* species in South Africa, *R. rattus* and *R. norvegicus* had the highest colonization rates; 61.5% and 55.6%, respectively. This contrasts with the study by Kimura et al. [18] in Japan which found higher levels of colonization in *R. norvegicus* (92%) than in *R. rattus* (58%) when assessing *Streptobacillus* PCR-positivity with the same PCR assay as was used in this study. This geographic variation in host colonization rates stresses the importance of regionally specific studies for determining relative risk of transmission following a rat bite. The almost two-fold higher *Streptobacillus* prevalence in the *R. rattus* versus *R. tanezumi* was surprising, not only because they are members of the same species complex, but also because they were sampled from sites in close proximity to one another, yet are colonized with phylogenetically indistinguishable *Streptobacillus* variants (Figure 2). Because these two *Rattus* species are morphologically indistinguishable, this disparate prevalence may be linked to genetic and/or behavioral differences [2] and warrants further investigation. Confirmation of RBF-associated *Streptobacillus* presence in > 50% of rats sampled in peri-urban formal and informal human settlements is of public health relevance and highlights the need for renewed pest control efforts and increased awareness amongst health practitioners treating rat bite cases.

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Appendix A: Supplementary Tables

Table S1. Summary statistics of the individual 16S rRNA, *gyrB*, *recA* and *groEL* gene datasets

Gene region	Sequence length (nt)	No. of taxa	Variable/Pi sites	Average base pair composition	Transition: transversion ratio (R)
16S rRNA	1225	24	10/10	AT rich	1.24
gyrB	1712	19	101/101	AT rich	1.89
recA	601	21	59/59	AT rich	0.64
groEL	738	18	52/51	AT rich	0.91

Pi: parsimony informative; nt: nucleotide

Table S2. *F*-value and percentage sum of squares (% SSQ) of potential sources of variation in *Streptobacillus moniliformis* oral infection rates for three invasive *Rattus* host species (*R. norvegicus*, *R. rattus* and *R. tanezumi*) derived from a three-way analysis of variance (ANOVA)

	F-value			% SSQ			
Host species	Sex	Site	Age	Sex	Site	Age	Error
R. norvegicus	2.00	-	2.02	5.4	-	5.5	89.1
R. rattus	0.95	4.74*	14.00***	1.7	8.7	25.6	64.
R. tanezumi	2.69	2.67	1.00	8.1	8.0	3.0	80.9
Mean ($\bar{x}$)				5.1	8.3	11.4	78.0

* indicates $P < 0.05$, ***$P < 0.001$

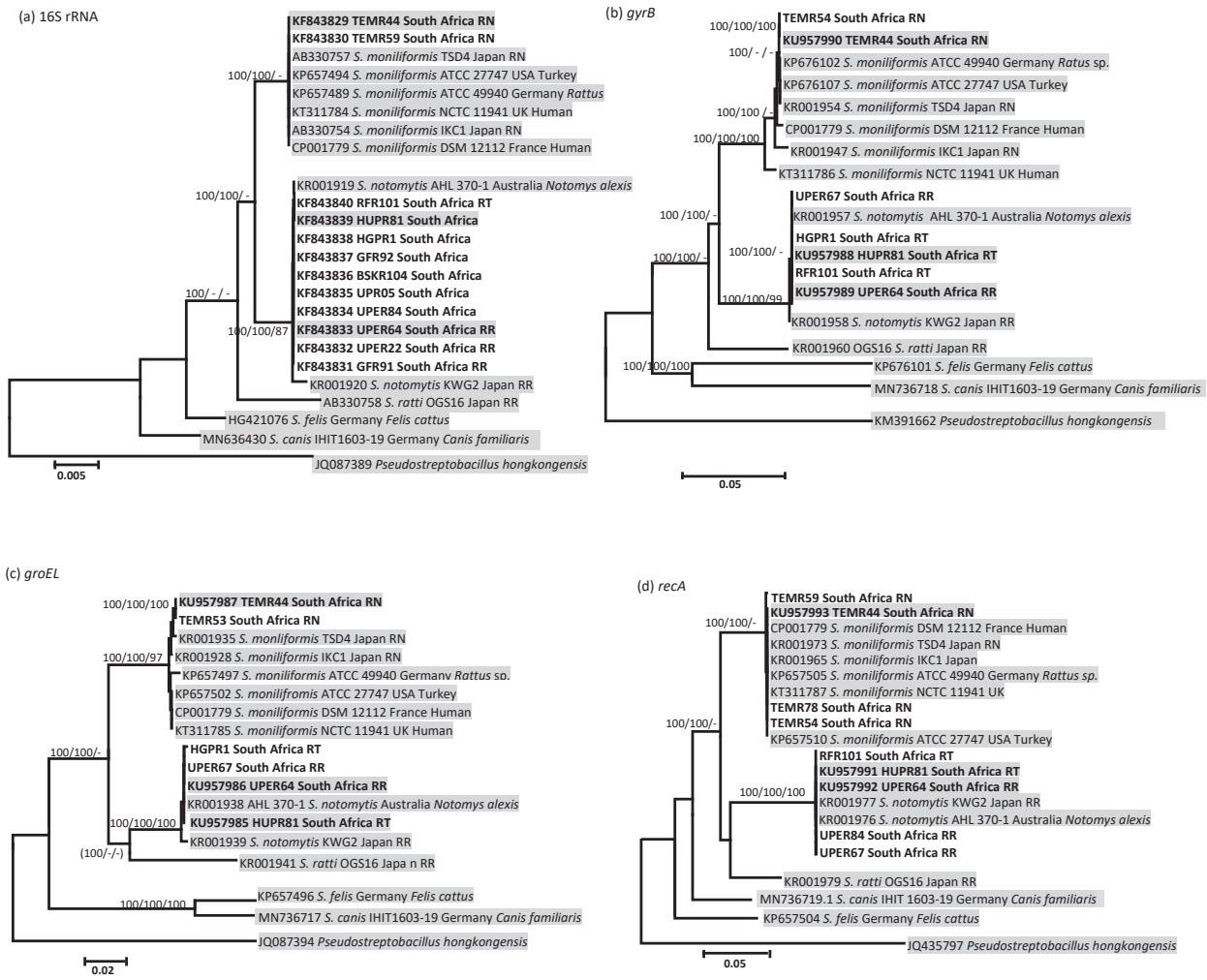

Figure S1. Neighbor-Joining trees depicting *Streptobacillus* taxon relationships based on aligned partial nucleotide (nt) sequences for each of the gene regions characterized in this study, viz. (a) the 16S rRNA (1225 nt), (b) *gyrB* (1047 nt), (c) *groEL* (252 nt) and (d) *recA* (601 nt) gene regions. The three *Rattus* species hosts (if known) are abbreviated: *R. rattus* (RR), *R. tanezumi* (RT) and *R. norvegicus* (RN). Sequences generated in this study (denoted by TEMR/HGPR/UPER/HUPR sample codes) are indicated in bold text to differentiate them from reference sequences. All sequences highlighted in grey correspond to taxa included in the concatenated gene phylogeny. Bootstrap support values and posterior probabilities, expressed as percentages, from Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) analyses are indicated NJ/ML/BI next to the relevant the node. The scale bar below each tree depicts genetic distance.