Host population structure and rare dispersal events drive leptospirosis transmission patterns among *Rattus norvegicus* in Boston, Massachusetts, US

Short Title: Brown rat population dynamics drive leptospirosis transmission in Boston, US

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Abstract

Leptospirosis (caused by pathogenic bacteria in the genus *Leptospira*) is prevalent worldwide but more common in tropical and subtropical regions. Transmission can occur following direct exposure to infected urine from reservoir hosts, such as rats, or a urine-contaminated environment, which then can serve as an infection source for additional rats and other mammals, including humans. The brown rat, *Rattus norvegicus*, is an important reservoir of leptospirosis in urban settings. We investigated leptospirosis among brown rats in Boston, Massachusetts and hypothesized that rat dispersal in this urban setting influences the movement, persistence, and diversity of *Leptospira*. We analyzed DNA from 328 rat kidney samples collected from 17 sites in Boston over a seven-year period (2016-2022); 59 rats representing 12 of 17 sites were positive for *Leptospira*. We used 21 neutral microsatellite loci to genotype 311 rats and utilized the resulting data to investigate genetic connectivity among sampling sites. We generated whole genome sequences for 28 *Leptospira* isolates obtained from frozen and fresh tissue from some of the 59 *Leptospira*-positive rat kidneys. When isolates were not obtained, we attempted *Leptospira* genomic DNA capture and enrichment, which yielded 14 additional *Leptospira* genomes from rats. We also generated an enriched *Leptospira* genome from a 2018 human case in Boston. We found evidence of high genetic structure and limited dispersal among rat populations that is likely influenced by major roads and/or other unknown dispersal barriers, resulting in distinct rat population groups within the city; at certain sites these groups persisted for multiple years. We identified multiple distinct phylogenetic clades of *L. interrogans* among rats, with specific clades tightly linked to distinct rat populations. This pattern suggests *L. interrogans* persists in local rat populations and movement of leptospirosis in this urban rat community is driven by rat dispersal. Finally, our genomic analyses of the 2018 human leptospirosis case in Boston suggests a link to rats as the source. These findings will be useful for guiding rat control and human leptospirosis mitigation efforts in this and other urban settings.

Introduction

Rats (*Rattus norvegicus and R. rattus*) are highly invasive species globally, persisting on all continents (except Antarctica) and in most habitats (1, 2). They cause devastating impacts to natural ecosystems (3), and are considered a serious public health threat (4, 5). *Rattus norvegicus* (the brown rat) is ubiquitous in urban environments (6, 7) and has been documented to carry/transmit (either directly or indirectly) an abundance of bacterial and viral pathogens in urban ecosystems, including *Leptospira* spp., *Bartonella* spp., *Borrelia* spp., *Yersinia pestis*, *Streptobacillus moiliformis*, antibiotic-resistant *Staphylococcus* spp., *Shigella* spp., *Campylobacter* spp., *Escherichia coli*, *Rickettsia* spp., *Salmonella* spp., influenza A virus, and Seoul hantavirus (8-11).

Multiple genetic studies utilizing various methods have assessed *R. norvegicus* population structure, movements, and dispersal patterns in urban settings in both temperate and tropical climates (2, 12-17), consolidated in a recent review (6). Despite this somewhat limited body of research, findings are generally uniform across all cities assessed (Vancouver, Canada; New York City, US; New Orleans, US; Baltimore, US; Salvador, Brazil; and Hauts-de-Seine, France): 1) individual *R. norvegicus* utilize small home ranges, display site fidelity, and display philopatry to their birth site; and 2) dispersal events are more common in adult rats (male or female), but rare overall, and often inhibited by major roads and rivers, which act as dispersal barriers.

Leptospirosis is the most globally widespread bacterial zoonoses resulting in over 1 million human infections annually and nearly 60,000 deaths (18,19). Brown rats are asymptomatic, chronic carriers of pathogenic *Leptospira* spp. (20-23) and the most important known source of human leptospirosis infections (24). Leptospires colonize and replicate in the proximal renal tubules of rats (20-22), with transmission occurring after leptospires are shed through urine, either via direct contact with the urine or indirectly via environmental or fomite contamination (24). Globally, >30% of *R. norvegicus* are estimated to be infected with *Leptospira* spp. (25).

126 Understanding how rat movements relate to *Leptospira* transmission in urban settings is of
127 vital importance to guide the rational design of disease mitigation strategies. A recent study of rat
128 population structure and pathogen density in an urban setting (Vancouver) suggested *L. interrogans*
129 prevalence was associated with rat population connectivity (16), wherein related rats sampled from
130 neighboring blocks shared the same pathogen status (*i.e.*, infected/not infected). Our study builds
131 upon this finding by pairing fine-scale population genetic analyses of rats in another urban city
132 (Boston, US) with fine-scale genomic analyses of the *Leptospira* they carry and transmit.
133 Historically, the acquisition of *Leptospira* genomes from reservoir (*e.g.*, rats) and incidental
134 (*e.g.*, humans) hosts has relied on the ability to obtain cultured isolates, which is inherently
135 challenging (26). Obtaining isolates from human leptospirosis cases is further inhibited by the
136 administration of antibiotics used to treat the disease (27), after which isolation of live leptospires is
137 almost impossible. As such, genomic analyses of infecting strains are lacking, and these data are
138 critical for improved understanding of leptospirosis ecology and epidemiology. We implemented two
139 new culture-independent techniques for the genetic/genomic characterization of infecting *Leptospira*
140 spp. in rats and humans in Boston: DNA capture and enrichment (28) and amplicon sequencing
141 (AmpSeq; described herein). Our goal was to gain a deeper understanding of the nuanced *Leptospira*
142 transmission network among urban *R. norvegicus* and potential spillover into humans. Our findings
143 are useful for guiding rat control and human leptospirosis mitigation efforts in urban settings.
144

145 # Results

146 ## *lipL32* qPCR in rodent samples

147 In total, 59 *R. norvegicus* kidney samples and one fresh urine sample (derived from an animal
148 that also had a positive kidney) were *lipL32* PCR positive, revealing an overall *Leptospira* infection
149 rate of 18.0% (59 of 328 animals) in this *R. norvegicus* dataset (**S1 Table**). *Leptospira*-positive

150 kidneys included 37 frozen samples (17.6%; 37/210) yielding an average Ct value of 28.77 ± 5.58,
151 and 23 fresh samples from 22 rats (18.6%; 22/118) yielding an average Ct value of 27.1 ± 6.06. Four
152 bycatch mice and one squirrel were *lipL32* PCR negative (**S2 Table**). Fifty-six of the infected *R.*
153 *norvegicus* were detected among 20/33 collections representing 12/17 sampling sites (**Fig 1 and S1**
154 **Table**) and positive rats were detected in all sampling years [2016=3/42 (7.1%), 2017=6/50 (12.0%),
155 2018=16/68 (23.5%), 2019=9/42 (21.4%), 2020=3/8 (37.5%), 2021=14/70 (20.0%), 2022=8/48
156 (16.7%)]. Precise locations were unknown for infected *R. norvegicus* collected in 2020 (samples B1,
157 B2, and B4; **S1 and S2 Tables**). Infected rats were more commonly adults (58/59) than juveniles
158 (1/59) (X^2 =60.68, p <0.001), and a higher proportion of infected rats were collected in fall (0.277)
159 and winter (0.283) compared to spring (0.196) and summer (0.123); a chi-square test indicated a non-
160 random association between *Leptospira* infection and season (X^2 =10.33, p <0.05). We also detected
161 a significant association between *Leptospira* infection and site (X^2 =26.58, p <0.05); however, this
162 association is likely driven by capture of a single rat from site 4 that was infected, and 3/5 infected
163 rats captured at site 15 (60%); all other sites ranged from 0-33.3% positivity. No other significant
164 associations were observed between other sampling variables (sex, sampling year, genetic group) and
165 the detection of *Leptospira*.
166
167 **Fig 1. *Rattus norvegicus* population assignment.** STRUCTURE population assignment of 311 *R.*
168 *norvegicus* with collections color coded according to genetic group (S1 Table); likely dispersal
169 barriers and major open spaces are indicated with black bars and green shading, respectively. A)
170 Inferred migrants are represented by one-sided arrows; shared genetic groups among collections from
171 sites 13, 14, and 16, and separately sites 9 and 10, are indicated by thin lines; and *lipL32* PCR
172 positivity at 12 of 17 sites is indicated with asterisks. Two migrants (sites 2 and 6) were both *lipL32*
173 PCR positive and are denoted by asterisks on one-sided arrows. B) Each individual *R. norvegicus*

174 (n=311) is assigned a probability of membership ($Q \geq 0.75$) to one of 12 genetic groups
175 (S3 Table). Collection ID is indicated on the X-axis and probability (Q) on
176 the Y-axis; each thin vertical line represents the Q value for an individual rat. The map in panel A
177 was created using ArcGIS software by Esri. ArcGIS and Arc-Map are the intellectual property of Esri
178 and are used herein under license. Copyright Esri. All rights reserved. For more information about
179 Esri software, please visit www.esri.com. Basemap: Light Gray Canvas Base v2
180 https://www.arcgis.com/home/item.html?id=291da5eab3a0412593b66d384379f89f.
181

182 # Culture

183 Twenty-eight isolates were obtained from *lipL32* PCR positive kidneys, including 15 from 37
184 frozen samples and 13 from 22 fresh samples. Nearly half of the collections (15/33) are represented
185 by these isolates as well as all sampling years (2016=1, 2017=2, 2018=6, 2019=3, 2020=3, 2021=7,
186 2022=6; **S2 Table**). The frozen samples were originally cultured in both T80/T40/LH and HAN
187 media, which were incubated at 29 and 37°C, respectively. However, it became evident that they
188 were heavily contaminated, and we observed that the contaminants grew better in HAN compared to
189 T80/T40/LH. Therefore, use of T80/T40/LH at 29°C was prioritized. Fresh kidneys were positive by
190 culture in both HAN and T80/40/LH media.
191

192 # Serotyping of *Leptospira* isolates

193 All isolates had high agglutination titers with reference antiserum specific for serogroup
194 Icterohaemorrhagiae (**S2 Table**). Isolates were completely seronegative (at 1:100 or above) (29) for
195 any other serogroup tested, except for three samples: MAR_57 had a titer of 1:102,400 against
196 Icterohaemorrhagiae and 1:100 against Pyrogenes; Cad103 had a titer of 1:51,200 against

197 Icterohaemorrhagiae, 1:200 against Djasiman, and 1:100 against Hardjo; and R304 had a titer of
198 1:52,400 against Icterohaemorrhagiae and 1:100 against Pyrogenes. We consider all three of these
199 isolates to be serogroup Icterohaemorrhagiae as it is not uncommon to see cross reactions with other
200 serogroups (30).
201

202 # Evaluation of virulence

203 Intraperitoneal inoculation of all hamsters with 10^8 leptospires derived from a frozen *R.*
204 *norvegicus* kidney (strain R47) resulted in acute disease requiring all hamsters to be euthanized
205 four days post-infection. Liver and kidney samples from each infected hamster were positive by both
206 culture and *lipL32* qPCR; Ct values for positive liver and kidney samples were on average
207 19.04 ± 0.63 and 23.64 ± 0.75, respectively.
208

209 # Rat MSAT results

210 ## MSAT marker validation

211 Twenty-one microsatellite (MSAT) markers were suitable for use in this population genetic
212 analyses of *R. norvegicus*. Only one marker (D12Rat76) deviated from Hardy-Weinberg equilibrium
213 in one collection (8f) at the Bonferroni corrected a =0.000595 (a =0.05/84 tests) and no signatures of
214 linkage disequilibrium were detected. Null alleles, large allelic dropout, or scoring errors due to
215 stuttering also were not detected, although we did detect excessive homozygosity in two markers
216 (D12Rat76 and D5Rat83) for collection 2c and one marker in four other collections (D5Rat33 in 5b,
217 D10Mit5 in 6g1, D7Rat13 in 12a, and D1Cebr3 in 12ab). No single marker displayed excessive
218 homozygosity in more than one collection. To check for allele scoring errors, we replicated PCRs for
219 all 21 loci across 94 *R. norvegicus* DNAs (30.2% of the final dataset) and detected an allele scoring
220 error rate of 0.23% (9 errors from 3,948 possible allele calls).

Population genetic analyses: determination of genetic groups and population assignment using STRUCTURE

The delta-*K* method estimated 2 and 12 genetic groups as the most likely number of populations sampled in the 311 individual rats from 33 collections/17 sites (**S1A Fig**). The *K* of 12 is more consistent with what is known about the philopatric nature of *R. norvegicus* (6, 12) and physical distances among our collection sites. Variance among the L(K) values is very low at *K* =12 but rapidly increases beyond this number (**S1B Fig**); this phenomenon is discussed in the STRUCTURE manual and can be expected to occur after the real *K* is reached (31). Furthermore, the higher delta-*K* value at *K* =2 could simply be that *K* =1 is consistently a very poor solution and the model improved dramatically between *K* =1 and 2. The 12 genetic groups were each assigned an arbitrary color in STRUCTURE (**Fig 1**) and that color was retained across all analyses, figures, and tables. Thus, we refer to the genetic groups by the following color designations: Blue, Brown, Evergreen, Green, Light Blue, Orange, Pink, Purple, Red, Steel, Tan, Yellow, and admixed (indicated by the color White) (**Fig 1 and S1 Table**). To aid in visualizing patterns across space and time, collections were color coded according to the most dominant genetic group (≥50%) present in that collection (**defined in S1 Table and displayed in Figs 1 and 2**). In total, 247 rats were assigned to one of these genetic groups at *Q* ≥0.75 (**see Materials and Methods**), whereas 64 rats were assigned to the admixed group (**S3 Table**). Due to repeated sampling over multiple years at sites 1, 5, 6, 8, 9, 10, 12, and 17, we detected stable rat populations at 7/8 sites for up to five years: site 1 (Blue group: 2016-2018), site 5 (Light Blue group: 2016-2017), site 6 (Tan group: 2018-2022 and Yellow group: 2021-2022), site 8 (Red group: 2017-2018 and Steel group: 2021-2022), site 9 (Brown group: 2021-2022), site 12 (Green group: 2016-2017), and site 17 (Pink group: 2018-2019); as well as population turnover/invasion

245 followed by persistence at two sites: site 6 (Tan group in 2018 to Yellow and Tan groups from 2020-
246 2021) and site 8 (Red group from 2017-2018 to Steel group from 2021-2022) (**Fig 1**).
247
248 **Fig 2. *Leptospira interrogans* serogroup Icterohaemorrhagiae whole genome phylogeny**. A
249 maximum likelihood phylogeny of 28 *Leptospira interrogans* serogroup Icterohaemorrhagiae isolate
250 genomes from rats, two publicly available *L. interrogans* serogroup Icterohaemorrhagiae serovar
251 Copenhageni complete genomes (GenBank accession# GCA_002073495.2 and GCA_010978155.1),
252 and 13 enriched genomes (12 rats and 1 human) based upon a concatenated SNP alignment of 127
253 positions out of a core genome size of 2,123,028 nucleotides (nts). The phylogeny was rooted with
254 reference genome *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz
255 L1-130 (GenBank accession# GCA_000007685.1). Four major clades were observed (1-4 in white
256 text) and bootstrap values are indicated at specific nodes. The color of the genome ID corresponds to
257 the rat genetic groups in **Fig 1**; black text indicates that no genetic group was assigned. One inferred
258 migrant (indicated with a black arrow) from site 6c was trapped at site 2c and carried *Leptospira*
259 *interrogans* identical to its source collection (see R335-Migrant in clade 1).
260
261 Using Q values ≥0.75, we detected potential *R. norvegicus* migrations as
262 indicated by the trapping of a rat assigning to a distinct genetic group among a collection of rats
263 belonging to a different genetic group; any highly admixed individuals were not treated as migrants
264 due to the potential for mis-assignment. In total, we detected eleven such migration events, seven of
265 which were adult rats (5 females, 2 males) and four were juveniles (1 female, 3 males). Rat
266 movement distances ranged from ~0.8-5.3 km. Migrations were suspected from collection 17c or 17d
267 to collection 14d (1 juvenile female, Q =0.93); collection 6c to collection 2c (2 juveniles males, Q
268 =0.91 and 0.95, and 1 adult male, Q =0.76); collection 13d, 14d, or 16d (all belonging to the

269 Evergreen genetic group) to collection 15d (1 adult female, *Q* =0.81); collection 5a or 5b to
270 collection 12a (1 adult female, *Q* =0.92); collection 9g1, 9g2, or 10c (Brown genetic group) to
271 collection 6g1 (1 adult male, *Q* =0.76); collection 15d to collection 10d (1 adult female, *Q* =0.97);
272 collection 12a or 12b to collection 5a (1 juvenile male, *Q* =0.82); collection 8b or 8c to collection 1b
273 (1 adult female, *Q* =0.83); and collection 13d, 14d, or 16d (Evergreen genetic group) to collection 7d
274 (1 adult female, *Q* =0.87) (**Fig 1 and S3 Table**). Two migrants (R335 from collection 2c and Cad89
275 from 6g1) were *lipL32* positive (**Fig 1 and S3 Table**). Some suspected migrations could also result
276 from home range overlap among neighboring collections.
277

278 ## Population genetic analyses: genetic structure

279 Principal Coordinates Analysis (PCoA) generally supported the 12 *R. norvegicus* genetic
280 groups identified by STRUCTURE and indicated two genetic groups (Blue and Steel) may be more
281 genetically isolated from the other ten groups (**S2 Fig**). The collections comprising these two genetic
282 groups are geographically separated from each other and the other genetic groups by major roadways
283 (**Figs 1 and 3).** This PCoA analysis of standardized genetic distances mirrors the geographic
284 distribution of these genetic groups (**Fig 1)**, suggesting a relationship between *R. norvegicus* genetic
285 and geographic distance in this study system.
286

287 **Fig 3. *Rattus norvegicus* trap locations.** Map indicating 64 trap locations (white circles) at 17 sites
288 (numbers), likely dispersal barriers (black bars), and major open spaces (green shading). Circle size
289 corresponds to the number of total rat samples collected from each trap location. This map was
290 created using ArcGIS software by Esri. ArcGIS and Arc-Map are the intellectual property of Esri and
291 are used herein under license. Copyright Esri. All rights reserved. For more information about Esri

software, please visit www.esri.com. Basemap: Light Gray Canvas Base v2 https://www.arcgis.com/home/item.html?id=291da5eab3a0412593b66d384379f89f.

Fairly high estimates of global F_{ST} were observed in the FSTAT analysis, with θ =0.184 (95% CI =0.155, 0.214). Of 66 pairwise F_{ST} estimates among 12 *R. norvegicus* genetic groups, all were significant at a =0.05 and only 16/66 were not significant at the Bonferroni corrected a =0.000758. That said, pairwise F_{ST} values were high among the 66 pairwise comparisons, ranging from 0.0826-0.2922 (**S4 Table**). All significant p-values (16/16) were associated with the Purple, Red, Orange, and Brown genetic groups, a result in agreement with the pairwise analysis of similarities (ANOSIM) (see below). Likewise, an Analysis of molecular variance (AMOVA) generated a moderate yet significant PhiPT value of 0.312 (p <0.001), revealing that ~31% of the molecular variance in this dataset was present among *R. norvegicus* genetic groups (*i.e.,* intersite variation), whereas 69% was present within them (*i.e.,* intrasite variation).

Population genetic analyses: isolation by distance and fine-scale genetic structure

To explore signatures of isolation by distance, we used the RELATE function in PRIMER v5.2.9 and the standardized genetic distances and geographic locations (latitude and longitude) of the 247 individual *R. norvegicus* forming the 12 genetic groups (**see Materials and Methods**). The Spearman's rank correlation coefficient (Rho) was calculated as 0.232 (p <0.001), identifying a statistically significant relationship between *R. norvegicus* genetic distance and geographic distance. The global ANOSIM R statistic was 0.921 (p <0.0001), documenting that some of the 12 genetic groups formed distinct geographic groups. Most of the pairwise ANOSIM comparisons among the 12 genetic groups (61 of 66) were significant even with a very conservative Bonferroni corrected a =0.000758, indicating that most of the 12 genetic groups formed distinct geographic groups. Only

316 5/66 pairwise comparisons were not significant at *a* =0.000758 (Brown-Orange, Orange-Purple,
317 Purple-Red, Pink-Purple, and Purple-Evergreen), suggesting the Purple group has a genetic
318 connection with three other groups (Red, Pink, and Evergreen).

319 Consistent with the signal of *R. norvegicus* genetic isolation by distance across all sites,
320 microsatellite data revealed clear evidence of fine-scale spatial genetic structure within collection
321 sites. First, the spatial autocorrelation analysis yielded correlation values significantly greater than
322 zero in the first six distance classes (0-100 m out to 0-600 m), suggesting that, on average, relatives
323 are generally living in close proximity (**S3 Fig**). This is supported by the significantly high value of
324 average relatedness of individuals within collections, at 0.313 (95% CI = 0.268, 0.357), which
325 indicates relatedness ranging from half (0.25) to full (0.5) siblings (**S4 Table**). Therefore, dispersing
326 *R. norvegicus* would need to travel >600 m, on average, to move beyond sites occupied by relatives.
327 Finally, F_{IS} values were only positive in three genetic groups [Blue (0.11), Yellow (0.078), and Red
328 (0.006)], suggesting inbreeding within collections at sites 1 and 2 (Blue), and perhaps site 6 (Yellow)
329 (**Fig 1 and S4 Table**).

330

331 *Leptospira* genomics and genotyping

332 In total, 52 of 59 *lipL32* PCR positive *R. norvegicus* and one of two positive human samples
333 were assigned to *L. interrogans* (86.9%) using the genotyping methods described herein (**S2 Table**).
334 Whole genome analyses revealed all isolates derived from frozen or fresh kidneys (n=28) and all
335 successful enrichments (n=15) were *L. interrogans* (**S2 Table**) and most like other serogroup
336 Icterohaemorrhagiae serovar Copenhageni/Icterohaemorrhagiae genomes (**S4 Fig**). Enriched
337 genomes for samples R47 and R316, which were included as genomic analysis controls, were
338 identical to their isolate genome counterparts (**Fig 2**). Phylogenomic analysis of all 28 isolates and 12
339 enrichments [the latter selected due to average breadth and depth of coverage values >63% and >18x,

340 respectively (**S5 Table**)], revealed four distinct clades that largely associate with the genetic groups
341 of the *R. norvegicus* from which they were derived (**Fig 2**). For example, 14 of 15 *L. interrogans*
342 genomes assigning to clade 1 were obtained from rats belonging to the Yellow, Tan, and Green
343 genetic groups, all occurring in or near Boston Common Garden, and the Tan and Yellow groups co-
344 occur at site 6. However, the Tan group, the most prominent in Boston Common Garden in 2018,
345 was replaced or modified as indicated by the emergence of the Yellow group in 2021 and 2022.
346 Likewise, clade 3 contains mostly *L. interrogans* genomes from the Red and Steel genetic groups (6
347 of 7). Both genetic groups contain rats from the same site (8) but different collections and years (8b
348 and 8c =Red, 8f and 8fg =Steel), perhaps indicating the removal of one rat genetic group (Red) and
349 replacement with another (Steel). Similarly, clade 2 contains mostly *L. interrogans* genomes from
350 rats belonging to the Evergreen genetic group (3 of 5); this clade also contains the *L. interrogans*
351 genome derived from a human leptospirosis case in 2018 (MA-2). Finally, clade 4 contains only *L.*
352 *interrogans* genomes from rats belonging to the Pink genetic group, comprised of the most
353 geographically isolated collections (17c and 17d) (**Fig 1**). Our phylogenetic placement of two lower
354 coverage enriched genomes (R289 and R292; **see S5 Table**) placed them into clade 1 among other *L.*
355 *interrogans* genomes derived from rats from collection 6c and belonging to the Tan genetic group
356 (**S5 Fig**).

357 Our higher resolution [3,280,516 nucleotides (nts)] phylogenomic comparison of *L.*
358 *interrogans* clade 2 revealed the human derived genome (MA-2) from a 2018 leptospirosis case
359 differed by only two single nucleotide polymorphisms (SNPs) from three *L. interrogans* genomes
360 obtained from rats collected in 2019, 2021, and 2022, respectively, when comparing a core genome
361 of >3.2 mb (**Fig 4**). The four *L. interrogans* genomes comprising this clade were from rats belonging
362 to the Evergreen genetic group or admixed group and were collected at sites 9 (collections 9f and
363 9g1) and 16 (collection 16d) (**Fig 1 and S1 Table**).

364

365 **Fig 4. *Leptospira interrogans* serogroup Icterohaemorrhagiae clade 2 whole genome phylogeny.**
366 Maximum likelihood phylogeny of four *R. norvegicus* and one human derived *L. interrogans*
367 genomes from clade 2 (**see Fig 2**) based upon the concatenated SNP alignment of 33 positions out of
368 a core genome of 3,280,516 nucleotides. The color of the genome ID corresponds to the rat genetic
369 group in **Fig 1**; black text indicates that no genetic group was assigned.

370

371 Our phylogenomic analysis of 24 whole genome assemblies representing all 18 P1 clade
372 pathogenic *Leptospira* spp. placed the 24 assemblies into 18 unique species clades, as expected (**S6A**
373 **Fig**). All nine isolate genomic DNAs subjected to AmpSeq analysis were accurately placed into their
374 respective species clades (**S6B Fig**), which were conserved in the AmpSeq phylogeny despite the
375 large reduction in size of the SNP alignment used to infer it (WGS =53,132 vs. AmpSeq =375 nts)
376 and the core genome included in the analyses (WGS =167,849 vs. AmpSeq =1,195 nts) (**S6 Fig**). We
377 note the proportion of variable sites (variable sites/core genome) represented in both the whole
378 genome and AmpSeq phylogenies are essentially equal (WGS =0.317 vs. AmpSeq =0.314), which
379 likely accounts for the similar branch lengths separating the species clades. The average number of
380 reads that mapped for each isolate was 44,994 ± 12,348 and the number of AmpSeq loci that
381 amplified for each isolate ranged from 22 for *L. kmetyi* to 39 each for *L. interrogans* and *L.*
382 *santarosai* (**S6 Table**).

383 Of the 20 *R. norvegicus* and one human sample subjected to AmpSeq
384 analysis, 13 rats yielded adequate PCR amplification and sequencing
385 coverage (≥7 loci with >10x reads) of *Leptospira* loci to confidently assign a species
386 classification of *L. interrogans* (**S6 Table**). Nine displayed enough coverage (≥25 loci
387 with >10x reads) to assign within species phylogenetic placement to the

388 *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni/Icterohaemorrhagiae clade (**S7**
389 **Fig**). The remaining seven rats and one human sample were not assigned a
390 species classification due to inadequate PCR amplification and/or
391 sequencing coverage (≤2 loci with >10x reads); two rats (R66 and R108)
392 had no mapped loci with >10x reads, two (R324 and R379) had one mapped
393 locus, and four samples (Cad1, MAR_36, MAR_5 and MA-1) had two mapped
394 loci (**S6 Table**). Regardless, the few loci that did amplify for samples R324, R379, Cad1,
395 MAR_36, MAR_5, and MA-1 all mapped to *L. interrogans* references except for MAR_5 and Cad1,
396 which had one and two loci, respectively that mapped to *L. borgpetersenii* (**data not shown**). The *L.*
397 *interrogans* serovar Copenhageni strain Fiocruz L1-130 gDNA control amplified robustly at all 42
398 loci (1,444,384 assigned reads in total) and was accurately phylogenetically placed among other *L.*
399 *interrogans* serovar Copenhageni strains (**S7 Fig**).
400

401 # Discussion

402 Our population genetic analyses of *R. norvegicus* in Boston revealed the existence of distinct
403 rat populations for periods of up to five years, most likely due to low dispersal rates among rat
404 populations and inbreeding. Our analyses revealed significant relatedness among individuals within a
405 genetic group and, furthermore, that related individuals are generally living within close proximity to
406 each other (**S3 Fig**), an observation consistent with other studies of *R. norvegicus* home ranges,
407 dispersal, and site fidelity in urban settings (6, 7, 14, 17, 32-35). In an analysis of gene flow among
408 *R. norvegicus* in two US, one Canadian, and one Brazilian city, Combs et al., found that rats were
409 highly related to each other within 500 m (12). Our findings in Boston were similar, as rats would
410 need to travel, on average, >600 m to encounter an unrelated individual. Although dispersal events

411 were observed in this study (**Fig 1 and S1 and S3 Tables**), they were rare [only 11/311 rats (3.5%)
412 were presumptive migrants], and this low rate is consistent with the observed patterns of strong
413 genetic structure and stability of genetic groups over time (**Fig 1**).

414 The 11 instances of inferred migrations included seven trapped adults (5 females, 2 males)
415 and four juveniles (1 female, 3 males) (**S1 and S3 Tables**). Rat migration distances ranged from
416 ~0.8-5.3 km in Boston, but because of the methodology we used to assign rats to specific collections
417 for population genetic analyses (**see Materials and Methods**), short-distance migrations (< 0.5 km)
418 would only be detected among adjacent sites separated by major roads (*e.g.,* sites 8 and 9 in **Fig 3**).
419 Although short-distance migrations are thought to be more common (6, 7, 36), long-distance
420 migrations of up to 11.5 km have been documented in brown rats within urban settings (14) and
421 could be anthropogenically facilitated (6, 37). We detected a juvenile female that migrated over 5.3
422 km from site 17 (Pink group) to site 14 (Evergreen group) (**Fig 1**). Since both sites are connected by
423 a large road and subway line, and adjacent to the same large river, it is possible human mediated
424 transport on a car, truck, train, or water vessel facilitated migration. We also detected migration of
425 three males (2 juveniles, 1 adult) from site 6 (Boston Common Garden) to site 2 in 2017 (**Fig 1 and**
426 **S1 and S3 Tables**). These migration events were <1 km and all three rats (R335, R336, and R337)
427 were collected on the same day (30-Nov-2018) at the same trap location (Government Center-1) (**S1**
428 **and S2 Tables**). We are unsure if this finding represents a group migration or if it is the result of a
429 common dispersal throughway between sites 6 and 2, or some other unknown dispersal mechanism.
430 To our knowledge, group migrations have not been described previously in the literature. In general,
431 our observed rate of inferred migrations (3.5%) was similar to other studies of *R. norvegicus*
432 population genetics in urban ecosystems; Gardner-Santana et al., reported a migration rate of 4.7% in
433 Baltimore, US (14), and Kajdacsi et al., reported 6.8% in Salvador, Brazil (15).

434 Major roads were likely the largest contributor to low rates of migration of *R. norvegicus*
435 among neighboring sites in Boston (**Figs 1 and 3**). We observed several examples of major roads
436 acting (and smaller roads failing to act) as dispersal barriers among three intensely sampled sites (6,
437 8, and 9). We compared the dispersal dynamics within Boston Common Garden (site 6: sampled
438 2016-2022) that spans ~0.83 km and is bisected by a 3-lane road with those between Melnea Cass
439 Blvd SE (site 8: sampled 2017-2022) and Melnea Cass Blvd N (site 9: sampled 2021-2022), which
440 are directly adjacent to each other but separated by an 8-lane road (**Figs 1 and 3**). The 3-lane road
441 bisecting the northeastern and southwestern portions of Boston Common Garden did not act as a
442 dispersal barrier for *R. norvegicus* as both the Tan and Yellow groups were detected ubiquitously
443 throughout the park. In contrast, the 8-lane road separating sites 8 and 9 prevented admixing among
444 them and migration between them (**Fig 1**), despite the sites being separated by only ~0.25 km.
445 Roadways have frequently been described as significant barriers to rat dispersal (6, 7, 17, 35, 38, 39)
446 and larger roadways deter dispersal more than smaller ones (40). We also observed a pattern of
447 genetic connectivity among sites separated by >1 km (see Evergreen group; sites 13, 14, and 16 in
448 **Fig 1**) but connected by a large park complex containing green spaces, open water, and a network of
449 river underpasses (**Figs 1 and 3**). Taken together, these results suggest different dispersal dynamics
450 in commercial/residential areas (shorter movements) vs. park settings (longer movements) in this
451 urban ecosystem, likely associated with road size and access to dispersal throughways. These
452 observations are in concert with previous findings of *R. norvegicus* movement patterns in urban
453 ecosystems (6, 12, 13, 41).
454 *Rattus norvegicus* population turnover occurred at two sites (6 and 8), followed by robust
455 expansion of the new/invading population (**Fig 1**). In Boston Common Garden we observed a large
456 population in 2018 (collection 6c: Tan group) that was mostly displaced by the emergence of the
457 Yellow group in 2021 (collection 6f). Both genetic groups remained (6g1 and 6g2) at the conclusion

458 of sampling in 2022 although the Yellow group was most often detected in 2021 and 2022 (**Fig 1B**).
459 We also observed genetic signatures of admixture among the Tan and Yellow genetic groups
460 (individuals assigning to both groups or neither) in the later collections (6f and 6g2 in **Fig 1B**), which
461 could suggest these genetic groups are in the process of merging. Complete population turnover
462 occurred at site 8 between October 2018 (Red group) and June 2021 (Steel group) (**Fig 1**). A rat
463 abatement campaign took place at site 8 but did not begin until 5-Nov-2021, suggesting population
464 turnover from the Red to Steel group occurred before that campaign began. Furthermore, the Steel
465 group persisted throughout the campaign (collections 8fg and 8g in **Fig 1**). It is unknown what
466 occurred at site 8 between October 2018 and June 2021 that led to the population change, but our
467 results suggest that rat abatement is unlikely to lead to population turnover unless complete
468 eradication can be achieved, as populations can rapidly rebound to pre-intervention sizes once
469 interventions cease (42-45). However, if an intervention resulted in removal of dominant individuals
470 followed by immigration of other individuals into this open niche, population turnover could occur
471 (7, 42). We suspect an open niche once recolonized would result in the rapid expansion of a new
472 genetic group due to rapid urban rat reproduction rates (up to five litters per year) (7) paired with the
473 tendency of *R. norvegicus* to breed with individuals from their same genetic group, a result of
474 dispersal barriers, site fidelity, and small home ranges (6, 12).

475 *Leptospira interrogans* persists locally in *R. norvegicus* populations in Boston. We detected
476 pathogenic *Leptospira* DNA in 18.0% of rats representing 20/33 collections and 12/17 sampling sites
477 (**Fig 1 and S1 Table**) with 98.3% of infected rats classified as adults. To put these number into
478 context, several other studies that examined *Leptospira* carriage in *R. norvegicus* in urban ecosystems
479 reported 7-82% in the highly endemic tropical climate city of Salvador, Brazil (46-48), 11.1% in the
480 temperate city of Vancouver, Canada (49), and 35.7% in New Orleans, US (50). Furthermore, a 2019
481 review of *Leptospira* carriage in rats globally reported an overall carriage rate in *R. norvegicus* of

482 30.3% (25), with higher carriage rates associated with tropical climates. Of the studies that assessed
483 the relationship between *Leptospira* positivity and age or sexual maturity, all reported significantly
484 higher proportions of positive adults than juveniles (47-49). Using a suite of molecular methods, we
485 determined the *Leptospira* species circulating in *R. norvegicus* in Boston was *L. interrogans* and
486 most similar to other serogroup Icterohaemorrhagiae genomes (**S4 and S7 Figs**). The 28 cultured
487 isolates were confirmed to belong to serogroup Icterohaemorrhagiae via the microscopic
488 agglutination test (MAT) (**S2 Table**), whereas the samples subjected to enrichment (n=14) and
489 AmpSeq (n=9) could only be inferred based upon phylogenetic placement (**Fig 2 and S4, S5, and S7**
490 **Figs**). Detection of only *L. interrogans* serogroup Icterohaemorrhagiae in this rat dataset was not
491 surprising. Indeed, the exclusive detection of *L. interrogans* serogroup Icterohaemorrhagiae in *R.*
492 *norvegicus* from the US was documented in a recent review (25). In that same article, it was also
493 reported that the most frequently identified *Leptospira* in rats globally (in 36 of 43 countries) belong
494 to serogroup Icterohaemorrhagiae. To that point, in the broad phylogenetic framework of *L.*
495 *interrogans,* the genomes from Boston *R. norvegicus* formed a monophyletic clade with four other *L.*
496 *interrogans* serogroup Icterohaemorrhagiae genomes from Saint Kitts (SK1), Brazil (203 and L1-
497 130), and Malaysia (898) (**S4 Fig**); this analysis included diverse *L. interrogans* representing >9
498 serovars. Importantly, monophyletic groupings like this can be teased apart with clade-focused whole
499 genome analysis, which was demonstrated by Santos et al., in a comprehensive study of 67 *L.*
500 *interrogans* serogroup Icterohaemorrhagiae isolates originating from >15 countries across the globe
501 (51). At that scale, they describe the detection of 1,072 SNPs and 258 indels among the 67 isolates,
502 and phylogenetic clusters that broadly associated with geographic locations. Likewise, by using
503 clade-focused whole genome analysis we identified >100 SNPs and four distinct clades among *L.*
504 *interrogans* serogroup Icterohaemorrhagiae genomes from *R. norvegicus* in Boston (**Fig 2 and S5**
505 **Fig**).

506 Distinct clades of *L. interrogans* correspond to specific geographic locations and rat
507 populations in Boston and persist locally through time despite population turnover (**Fig 2**). Rats from
508 sites 6 and 12 exclusively carried *L. interrogans* from clade 1 (**Fig 2 and S5 Fig**), the virulence of
509 which was validated in the hamster model of leptospirosis, despite being separated by >1.3 km and
510 belonging to three different rat genetic groups (Tan, Yellow, and Green); at site 6 the rat population
511 shifted from the Tan to Yellow group between 2018 and 2021 (**Fig 1**). Furthermore, collections from
512 sites 6 and 12 spanned seven years (2016-2022) (**S1 Table**). This finding suggests connectivity
513 among these sites and/or that stable rat populations at these sites maintain endemic clades of *L.*
514 *interrogans* over time. A greenbelt containing a public walkway connects these two sites almost
515 directly, and although eight roads bisect this greenbelt, none exceed 4-lanes and six are only 2-lanes,
516 suggesting movement between these sites is possible (40). It is also important to consider that
517 although these two sites contain three distinct genetic groups, the Green group from site 12 was
518 sampled in 2016 and 2017, whereas the Tan and Yellow groups from site 6 were sampled in 2018-
519 2022. It is therefore possible that the Green group no longer existed beyond 2017 and that if rats were
520 trapped at site 12 in 2018-2022, they would resemble the Tan and Yellow genetic groups, or –
521 alternatively – that the Green group was present at site 6 prior to 2018. A single rat was trapped at
522 site 6 in 2016 (Cad18) and although it was not assigned to a genetic group due to Q <0.75 (*i.e.,*
523 admixed), the most probable assignment was to the Green group with Q =0.358 (**S3 Table**). Under
524 either scenario, the exclusive detection of clade 1 *Leptospira* at sites 6 and 12 over a period of seven
525 years suggests a connection between them. Likewise, *L. interrogans* from clade 2 was exclusively
526 found in rats from sites 9 and 16 (**Fig 2 and S5 Fig**). These sites were separated by nearly 2.5 km and
527 the samples were collected over different years; the site 16 rats were trapped in 2019 and the site 9
528 rats in 2021-2022. As with clade 1, we see two distant locations sharing a single *L. interrogans*
529 genotype, suggesting a connection between them, but in this case, there is no obvious dispersal

530 throughway linking them. That said, we see evidence of long-distance migrations throughout this
531 study system (**Fig 1**), so it is plausible that a *Leptospira*-positive migrating rat from site 16 to site 9
532 (during or post 2019) led to the establishment of the clade 2 genotype at site 9 that we observed in
533 2021-2022. Population turnover of the rats with persistence of the same *Leptospira* genotype was
534 also observed at site 8 (**Fig 2 and S5 Fig**) where the rats displayed a genetic change from Red to
535 Steel between 2018 and 2021, yet the *L. interrogans* clade 3 genotype remained the same. Finally, *L.*
536 *interrogans* clade 4 contained only rats from site 17 collected in 2018-2019. Site 17 is the most
537 geographically distant site in this study being >4.5 km away from its nearest geographic neighbor,
538 site 16 (**Figs 1 and 3**), so it is not surprising to see an exclusive clade of *L. interrogans* among rats
539 from site 17. These results provide a clearer picture of *Leptospira* transmission among rat
540 populations and provide compelling evidence for local persistence of distinct *L. interrogans*
541 serogroup Icterohaemorrhagiae genotypes among *R. norvegicus* in Boston.

542 *Leptospira* genotypes can be dispersed between populations by migrating rats**.** We detected
543 the migration of a *Leptospira*-positive adult rat (R335) from site 6 to site 2 in 2018 (**Fig 1**) and the
544 genetic signature of the *Leptospira* it carried (clade 1) further supported this assignment (**Fig 2**).
545 Importantly, this event clearly illustrates a mechanism by which *Leptospira* genotypes could be
546 transferred between rat populations. A *Leptospira*-positive migrant (Cad89) that moved from site 9 to
547 site 6 (**S3 Table and Fig 1**) was also detected. Unfortunately, a genome was not obtained from this
548 sample so fine-scale genomic assignment was not possible. However, AmpSeq assigned it to the *L.*
549 *interrogans* serogroup Icterohaemorrhagiae clade (**S7 Fig**). Regardless, it illustrates movement of
550 *Leptospira* via rat migrations between sites 9 and 6 and it is therefore reasonable to assume that
551 migration of a *Leptospira*-positive rat could have occurred in the opposite direction (site 6 to 9),
552 ultimately resulting in a rat trapped at site 9 carrying a *L. interrogans* clade 1 genotype. We also
553 observed single rat populations that carried diverse *L. interrogans* that assigned to multiple locations

554 in the phylogeny (*e.g.*, 15d, 9g1, 8f) (**Fig 2**), suggestive of movement of *L. interrogans* between rat
555 populations. For example, a single rat trapped at site 9 (Cad91) contained *L. interrogans* from clade 1
556 (**Fig 2**). Although this rat was not assigned to a genetic group the most probable assignment was to
557 the Brown group (Q =0.589) (**S3 Table**). We see evidence of rat mediated *L. interrogans* movement
558 at site 8 where three *L. interrogans* genotypes were recovered (**Fig 2**). Because the clade 3 genotype
559 was the most common at site 8 and maintained over three years despite the turnover of the rat
560 population from the Red to Steel group, we suggest the clade 3 *L. interrogans* is established at that
561 site, whereas the other two genotypes (n=1 each) were the result of *Leptospira*-positive rat migrations
562 into site 8. Finally, we found clade 3 *L. interrogans* in a rat from site 13 that clearly assigned to the
563 Evergreen genetic group (**Fig 1**); clade 3 genotypes were otherwise only found in rats from site 8
564 (**Fig 2**). Taken together, these data provide strong support for *R. norvegicus* migrations being a
565 mechanism by which *L. interrogans* serogroup Icterohaemorrhagiae genotypes are transferred among
566 rat populations in Boston, an important consideration when designing rodent control interventions or
567 construction, as both can drive long-distance migrations and intraspecific antagonism (42) and
568 human interventions may increase rat population pathogen prevalence (52).

569 The mechanism(s) by which identical *Leptospira* genomes becomes established in rat
570 populations at these sites is unclear. *Rattus norvegicus* are well known chronic and asymptomatic
571 carriers of *L. interrogans* serogroup Icterohaemorrhagiae (20-22), and the transmission cycle via
572 carriage of *Leptospira* in renal tubules, excretion through urine, environmental contamination,
573 ultimately leading to direct or indirect exposure to other rats as well as other susceptible mammals,
574 including humans, is well established (24). During chronic carriage in the rat, one might expect to
575 observe the accumulation of mutations over time corresponding to an inherent *L. interrogans*
576 mutation rate (to our knowledge, this is unknown). Indeed, we do observe this pattern across certain
577 rats (R269, R289, R292 in clade 1; R365, R366 in clade 2; R336-19 in clade 4) (**Fig 2 and S5 Fig**),

presumably the result of long-term chronic infection where *L. interrogans* was in a replicative state. More often, however, we observed identical genomes from rats spanning up to a seven-year period, suggesting a dormant or very low replication state of *L. interrogans* in this urban ecosystem. A possible mechanism for this could be biofilm formation (53) in the renal tubules of the rats (54). However, laboratory analysis of *R. norvegicus* infected with *L. interrogans* serovar Copenhageni revealed they shed leptospires continuously (10^5 -10^7 leptospires per mL) in their urine for the duration of the experiment (159 days) (22), suggesting that in this laboratory setting there was no such dormant state. Alternatively, *Leptospira* biofilm formation could take place in the environment (55, 56). It is important to note that because we are only analyzing the shared genomic space among all the rat derived *L. interrogans* genomes (core genome >2.1 million nts) there could be mutations or acquired/lost pan genomic content outside of the core genome that we did not detect. That said, if replication were occurring at a constant rate, we would still expect to observe mutations accumulating in the core-genome. Additionally, when we increased our resolution for clade 2 to >3.2 million nts (**Fig 4**), we observed the same identical genomes shared among three rats (MAR-47, Cad86, R367). Furthermore, our genomic analysis of clade 2 also included a human derived *L. interrogans* genome (MA-2 in **Figs 2 and 4**). This genome was enriched from urine DNA obtained from a human leptospirosis case in 2018 and only differs by two SNPS (core genome >3.2 million nts) from *L. interrogans* genomes obtained from three rats at sites 9 and 16 in 2019 and 2022 (**Fig 4**). Due to the clonality of these *L. interrogans* genomes derived from two host species [human (incidental) and rat (reservoir)] and collected over a five-year period (2018-2022) paired with the knowledge that rats are recognized globally as carriers of serogroup Icterohaemorrhagiae [while other hosts (*e.g.*, dogs) are incidental carriers], we suggest the most likely explanation for the human infection is exposure to a fomite or the environment that was contaminated by rats from sites 9 or 16 or another *R. norvegicus* population carrying the same *L. interrogans* clade 2 genotype in the city.

602 That said, any attempts to link this human case to a specific site should be interpreted with caution
603 given that *L. interrogans* dispersal between sites does occur (**Figs 1 and 2**).
604 This is the first study to pair fine-scale population genetic analyses of *R. norvegicus* in an
605 urban ecosystem with genomic analysis of the *L. interrogans* they harbor. This analysis was possible
606 due to improvements in culturing techniques (described herein) enabling high rates of *Leptospira*
607 recovery (47.5%) paired with advances in culture independent molecular capabilities (28) that
608 together enabled the acquisition of 40 *L. interrogans* genomes from rats (out of 59 *lipL32* PCR
609 positives) and one from a human. Importantly, these genomes were derived not just from fresh
610 samples, but also frozen and archived samples (tissues and DNA), thereby greatly expanding the
611 types of samples that could be included in these genomic analyses. A particularly salient outcome
612 was the acquisition of a human derived *L. interrogans* genome from trace amounts of archived DNA
613 (22.4 ng total). Although not much is known about the origin of this urine sample beyond it
614 originating from a human leptospirosis case in Boston in 2018 due to patient privacy rights, our
615 analysis placed it among rat derived genomes collected exclusively at sites 9 and 16 (**Fig 4**),
616 suggesting the source of infection was localized to a relatively small geographic area within Boston
617 (**Fig 1**) that was accessible to rats from both sites.
618 We have previously applied AmpSeq to assess the genetic diversity of *lipL32* sequences from
619 pathogenic leptospires [original design described by Stone et al., (56)]. AmpSeq is based upon
620 traditional PCR methods, Illumina short-read sequencing (both technologies that are accessible to
621 researchers and clinicians across the world) and requires <10 µL of a *Leptospira*-positive clinical
622 DNA sample. Furthermore, it is relatively inexpensive on a per-sample basis due to the ability to
623 simultaneously sequence hundreds of samples on a single Illumina run. For this study, we
624 implemented and assessed an expanded *Leptospira* AmpSeq panel that is capable of providing
625 *Leptospira* species identification among all 69 currently described and validated *Leptospira* spp.

626 from the P1, P2, S1, and S2 clades (57) (**S1 File**), as well as sub-species phylogenetic placement
627 within *L. interrogans* (*i.e.*, serogroup associated clades) (**S7 Fig**). Although we did not extensively
628 assess sub-species placement among other P1 *Leptospira* spp., we expect a similar outcome for *L.*
629 *kirschneri*, *L. noguchii*, *L. alexanderi*, *L. santarosai*, *L. weilii*, *L. borgpetersenii*, and *L. mayottensis*
630 due to the high number of discriminating loci (<35) expected to amplify in each species (**S1 File**).
631 Furthermore, our phylogenetic analysis of P1 clade pathogenic *Leptospira* spp. via sequences
632 generated with AmpSeq suggests sub-species placement (**S6B Fig**), even with as few as 22
633 amplicons (**S6 Table**). Importantly, the AmpSeq method can identify mixtures of *Leptospira* spp.
634 within a single sample (56), which is an under-characterized aspect of *Leptospira* ecology. Molecular
635 advances such as these will greatly facilitate an increased understanding of mixed infections and their
636 potential role in leptospirosis infection and disease ecology. Thus, we suggest it could serve as an
637 applicable tool for species and sub-species identification of circulating and clinically relevant
638 leptospires in regions throughout the world.
639

640 # Conclusion

641 In this study, we paired population genetic analyses of *R. norvegicus* in an urban setting with
642 genomic analysis of the infecting leptospires they transmit to gain a deeper understanding of the
643 nuanced transmission patterns of *Leptospira* among rats and spillover into humans. We found that
644 rats in Boston displayed strong signals of population structure, likely associated with behavioral
645 norms and dispersal barriers. We also found the *Leptospira* carried by rats in Boston was *L.*
646 *interrogans* and most similar to other serogroup Icterohaemorrhagiae isolates, and that there was
647 fine-scale genetic structure within that phylogenetic group that associated with distinct rat
648 populations. We found evidence that long-distance dispersal events lead to the movement of unique
649 *L. interrogans* genotypes throughout the city, but these events were rare. Our analysis identified

650 highly clonal genomes of *L. interrogans* that persisted within certain populations of rats for up to
651 seven years. Finally, our genomic analysis of *L. interrogans* obtained from a human leptospirosis
652 case using DNA capture and enrichment strongly suggests a link to rats as the source and provides a
653 likely geographic origin within Boston where this transmission event might have occurred, although
654 we cannot rule out the possibility that the same genotype of *L. interrogans* was also carried by rats in
655 an unsampled location in Boston.
656

657 Materials and Methods

658 Study location and rodent trapping

659 The City of Boston, Massachusetts (125.4 sq. km), in the eastern US has an estimated
660 population of ~650,000 people (US Census Bureau 2020 Decennial Census) with a temperate climate
661 (cold winters and hot, humid summers). *Rattus norvegicus* collections between July 2016 and
662 November 2020 were conducted in collaboration with the City of Boston's Inspectional Services
663 Department (COB-ISD), as described previously (10, 11).
664 For rats trapped between 2021-2022, 6-10 traps were placed at each site (see site definitions
665 below) at least 15 meters apart in locations with evidence of recent rat activity (*i.e.*, near burrows or
666 along runs with evidence of fresh rat droppings/urine). Rats were trapped using a commercial lethal
667 snap trap (T-Rex, Bell Labs, Windsor, WI, US) fixed to a remote sensing device (RATMO®, Arctic
668 Systems, Copenhagen, DK) with zip-ties. Traps were baited with meat jerky (Slim-Jim original
669 flavor, Conagra Brand, Chicago, IL, US) and placed inside a weighted, locked commercial bait
670 station (Protecta EVO Express Bait Station, Bell Labs, Windsor, WI, US). When rats were trapped,
671 the remote sensing device alerted the study team in real time for prompt retrieval and reset of the
672 trap. Traps were placed in the evening, attended from dusk until dawn, and deactivated and shut

673 down the following morning. Trapping was conducted in accordance with protocols reviewed and
674 approved by the Cummings School of Veterinary Medicine IACUC (G2020-126).
675 Rat carcasses were stored in a cooler with icepacks until necropsies were performed within
676 six hours of trapping. Rats were weighed, sexed, dipped in 70% ethanol, and placed in dorsal
677 recumbency. Sterile tissue scissors and forceps were used to reflect the abdominal skin and a new set
678 of sterile instruments were used to open the abdomen and aseptically harvest the kidneys. For
679 samples collected in 2021-2022, one fresh kidney was placed into 50 mL conical tubes containing 10
680 mL liquid HAN medium (26) and shipped overnight at ambient temperature to the National Centers
681 for Animal Health (NCAH) in Ames, IA. Archived frozen kidneys (2016-2020) were stored at -80°C
682 until they were shipped overnight from Tufts University to NCAH on dry ice.
683

684 *lipL32* qPCR detection of *Leptospira* in rodent samples

685 We screened for the presence of *Leptospira* DNA in DNA extracted from 210 frozen kidneys,
686 118 fresh kidneys, and one fresh urine sample (kidney was also evaluated from this individual) from
687 328 *R. norvegicus* rats collected in Boston from 2016-2022; four kidneys from bycatch mice and one
688 kidney from a gray squirrel were also included. Kidneys were transferred to a 150 mm petri dish, the
689 capsule (fibrous outer layer of the kidney) removed, and then placed in a 710 mL Whirl-Pak bag
690 (World Bioproducts, LLC, Madison, Wisconsin, US) containing 10 mL of liquid HAN, and
691 immediately macerated manually. DNA was extracted using 500 µL of kidney macerate or 200 µL of
692 urine, and the Maxwell RSC Purefood Purification Pathogen kit (Promega Corporation, Madison,
693 Wisconsin, US). We screened all samples for the presence of pathogenic *Leptospira* spp. using a
694 TaqMan® (Thermo Fischer Scientific, Waltham, MA, US) qPCR assay specific for the *lipL32* gene
695 as previously described, using primer set LipL32-47Fd and LipL32-301Rd with LipL32-189P probe
696 (58-60). To control for qPCR inhibitors, we added TaqMan® exogenous Internal Positive Control to

697 the master mix to confirm DNA amplification, detect false negatives, and qualitatively detect
698 presence of amplification inhibitory substances. All samples were assayed in triplicate and
699 considered positive when duplicate or triplicates were positive with Ct values <40 (58, 60). Using
700 chi-square tests of independence, we examined associations between *Leptospira* positive rats and six
701 sampling variables: sex, maturity (juvenile vs. adult), sampling season [spring (19-Mar to 20-Jun),
702 summer (20-Jun to 22-Sept), fall (22-Sept to 21-Dec), and winter (21-Dec to 19-Mar)], year, site, and
703 rat genetic group (assigned herein).
704

705 # Culture

706 Remaining tissue from frozen and fresh kidneys positive by *lipL32* qPCR was used for
707 culture. Macerate from frozen kidneys (200 µL) was inoculated into 5 mL of semisolid T80/40/LH
708 media (61) and incubated at 29°C. Two 10-fold serial dilutions were made from the fresh kidney
709 macerate into liquid HAN media in 3% CO_2 (26) and T80/40/LH media (61) and incubated at 37°C
710 and 29°C, respectively. Cultures were maintained for up to six months and checked periodically by
711 dark-field microscopy for the presence of leptospires.
712

713 # Serotyping of isolates

714 *Leptospira* isolates were serotyped by the MAT with a panel of polyclonal rabbit reference
715 antisera representing 13 serogroups: Australis, Autumnalis, Ballum, Bataviae, Canicola,
716 Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Mini, Pomona, Pyrogenes, Sejroe, and Tarassovi.
717 The serogroup of each isolate was assigned according to the reference antiserum yielding the highest
718 agglutination titer.
719

720 # Evaluation of virulence

721 One rodent isolate (designated strain R47) was propagated in liquid HAN medium at 37°C in
722 3% CO_2 and evaluated for virulence by intraperitoneal injection of 10^8 leptospires in a final volume
723 of 1 mL into one group of four female golden Syrian hamsters (*Mesocricetus auratus*). Negative
724 control hamsters were inoculated with media alone. Hamsters were monitored daily for clinical signs
725 of disease, including weight loss, lethargy, bloody discharge from the nose or urogenital tract, and
726 sudden death; and were euthanized when moribund. At time of euthanasia, liver and kidney tissue
727 were harvested for culture and *lipL32* qPCR. These experiments were conducted in accordance with
728 protocols as reviewed and approved by the Animal Care & Use Committee at the NADC and as
729 approved by USDA institutional guidelines.
730

731 *Rattus norvegicus* collection definition

732 *Rattus norvegicus* were trapped over seven years (2016-2022) at 64 trap locations (**S7 Table**)
733 within 17 geographic sites in Boston (**Fig 3**). Site numbers were assigned from east
734 to west and defined as trapping grids separated by ≤0.5 km. Exceptions
735 to this were trapping locations ≤0.5 km apart but separated by major
736 roadways, which are known dispersal barriers for *R. norvegicus* and *R. rattus* (6);
737 these were considered separate sites. A large city park complex (~0.83 km northeast to southwest)
738 comprised of two smaller parks [Boston Common (northeast) and Boston Public Garden (southwest)]
739 was considered a single site and is referred to hereafter as Boston Common Garden. We grouped
740 individual rats into 33 collections based upon site ID and sampling date, whereby samples collected
741 >4 months apart were considered unique collections. These criteria were selected based upon the ~3
742 months needed to reach sexual maturity for *R. norvegicus* (62) and previous work describing genetic
743 relatedness among *R. norvegicus* in urban settings with distances of up to 0.5 km (12). Thus,
744 nomenclature for collections is as follows: site is indicated by numerals ranging from 1-17 ordered

745 from east to west followed by letters corresponding to year of collection where a=2016, b=2017,
746 c=2018, d=2019, e=2020, f=2021, and g=2022. If multiple collections were obtained within a year, a
747 second numeral follows the letter designation (*e.g.,* 9g2 indicates the second collection at site 9 in
748 2022). These definitions resulted in 33 collections from 17 sites (**S1 Table**).

749 Three sites were sampled intensely between August 2021 and August 2022: Boston Common
750 Garden (site 6), Melnea Cass Blvd SE (site 8), and Melnea Cass Blvd N (site 9) (**Fig 3**). Boston
751 Common Garden is a heavily landscaped urban park system with primarily mowed grass ground
752 cover and concrete foot paths. The southwest and northeast portions are bisected by a 3-lane road,
753 and the southwest portion is the site of a historical municipal burial ground. The two Melnea Cass
754 Blvd sites are heavily developed urban areas of mixed commercial and residential use and separated
755 by an 8-lane road. These three sites were identified in consultation with the COB-ISD as having
756 active rat populations and burrow systems. They were selected for intensive sampling to facilitate a
757 deeper understanding of the relationship between rat population genetics and leptospirosis
758 transmission in parks versus mixed commercial/residential environments, and to assess how a major
759 road may impact dispersal among neighboring rat populations, which we hypothesize will impact the
760 movement of pathogenic *Leptospira*. Furthermore, we used these sites to assess how genetic
761 signatures change over time across stable rat populations (sites 6 and 9) and the effects of a rat
762 control intervention (baiting and trapping) at site 8 that began 5 November 2021.

763

764 *Rattus norvegicus* microsatellite development and genotyping

765 Marker selection, optimization, and multiplexing

766 Twenty-one MSAT markers were utilized for population genetic analyses of *R. norvegicus*
767 samples (**Table 1**). All markers have been previously published (14, 15, 17, 32, 63) and were
768 selected for use in this study based upon: 1) geographic regions where previously utilized (*i.e.*,

769 eastern US cities as a proxy for presumed genetic compatibility with our Boston study site), 2)
770 number of described alleles in other study systems (prioritizing markers with multiple alleles), and 3)
771 successful use among diverse populations of *R. norvegicus* as inferred by inclusion in multiple
772 studies from diverse western hemisphere countries (*i.e.,* US and Brazil). The 21 markers were
773 optimized using a panel of six *R. norvegicus* DNA samples from different sites in Boston, one
774 Sprague Dawley rat gDNA control (Zyagen, San Diego, CA, US), and a non-template control
775 (molecular grade water).

776 We generated multi-locus genotypes for 328 rat kidney DNAs using these 21 MSAT markers
777 amplified in nine PCR reactions (six multiplexes and three singles, **Table 1**). All PCRs were carried
778 out in 10 µL volumes containing the following reagents (given in final concentrations): 10-20 ng of
779 DNA template, 1x PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.8 U Platinum Taq polymerase
780 (Invitrogen, Carlsbad, CA, US), and 0.1-0.5 µM of each primer pair (see **Table 1**). Thermocycling
781 used the following conditions: 10 minutes at 95°C to release the Platinum Taq® antibody, followed
782 by 38 cycles of 60 s at 94°C, 30 s at the annealing temperature (Ta) and 30 s at 72°C. The Ta,
783 dilution, multiplex, and pooling schemes for each marker are provided in **Table 1**. Diluted PCR
784 products were electrophoresed on an ABI 3130xl sequencer with LIZ®-1200 size standard (Applied
785 Biosystems, Foster City, CA, US) and analyzed using GeneMarker v2.6.7 (SoftGenetics, State
786 College, PA, US).

787 **Table 1. *Rattus norvegicus* MSAT marker primers and conditions.**

Marker	T_a	Mix	Primer pair conc. (µM)	Post-PCR dilution	Dye	*A*	Allele sizes (bp)	Citation and primer sequence (5'-3')
D12Rat76	60	1	0.5	1/50	NED	10	86, 88, 90, 92, 94, 96, 98, 100, 102, 104	[1] F-TGCCTTTTAAAATGATGTGCA R-ATTGGCAATGCACTCATGTG
D1Wox23	60	1	0.3	1/50	VIC	10	186, 190, 194, 198, 202, 206, 210, 214, 218, 222	[2] F-TCTGACCCATACTTGTACTTTGC R-AATTTCTGCCTCTTTTTCTCAG
D7Rat97	60	1	0.3	1/50	6FAM	11	172, 174, 176, 178, 180, 184, 186, 188, 192, 196, 198	[3] F-CAAGTTTTCCTCTGCCCAAG R-GCTGTCATTCCACTGGGTTT
D11Mgh5	60	2	0.4	1/75	6FAM	9	216, 226, 228, 230, 232, 234, 236, 238, 248	[1] F-CAGCTCTAATTCCAGAAAGGTTT R-GAATCGATTGACAGATGTCTGTG
D3Cebr3	60	2	0.3	1/75	PET	5	163, 165, 167, 177, 181	[4] F-CAGGGAATGCAGAAGATACAG R-GTGGCTTTAGGACTCTGGAG

D6Cebr1	60	2	0.4	1/75	NED	7	220, 224, 226, 230, 232, 234, 236	[4] F-TGGTTTGGTTGGGGAGAA R-GTGCTGTCAGGGAAAGATGTA
D4Wox7	55	3	0.1	1/50	VIC	5	130, 134, 138, 142, 146	[2] F-GATAGCATAAAATCCCTAGAGGTT R-TCGATTTATCTGAAACCATCAC
D6Wox2	55	3	0.3	1/50	6FAM	10	96, 100, 104, 108, 116, 120, 124, 128, 132, 136	[2] F-CCAGTCCATACTTATCCATCTG R-CATTTAGATAGGTGATAGATTCAG
D1Cebr9	55	3	0.3	1/50	6FAM	6	261, 263, 265, 267, 269, 271	[4] F-GGATTTGGCTCCCTTTAAG R-CAGTAACTCTGGTTCATGTACTCC
D7Rat13	64	4	0.4	1/25	NED	12	128, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164	[5] F-GACTTCTGCTACACGCCACA R-CAGCCCTAGAAGGAAATGCA
D5Rat83	64	4	0.4	1/25	VIC	11	164, 168, 170, 178, 184, 186, 190, 192, 194, 196, 200	[5] F-ACTTGGAAACAGGGAGATGG R-GGGTCTTCAGGATGGCAATGT
D12Wox1	64	4*	0.4	1/50	6FAM	8	392, 396, 400, 404, 408, 412, 416, 420	[2] F-GACATTAAGGGGTCTTCCTAAG R-TATCTTTGCAACGCTGAGG
D10Rat20	64	5	0.4	1/25	PET	6	109, 113, 115, 117, 119, 123	[1] F-AGTGATTGCCATACCTGCCT R-GAAATGGCCAGGATAAACCA
D20Rat46	64	5	0.4	1/25	6FAM	13	140, 142, 144, 148, 154, 156, 160, 162, 164, 168, 172, 174, 180	[1] F-AAGTACTGAGTGGGCTGCGT R-GGCAAAACACCAATGCCTAT
D10Mit5	64	5	0.2	1/25	NED	9	135, 137, 139, 141, 143, 145, 147, 149, 151	[1] F-TGCTGGGTGAACCAGAGAG R-CTGCCCTCCAAACCACC
D9Rat13	64	5	0.4	1/25	VIC	5	101, 109, 119, 121, 123	[5] F-CCCATCTTTACACCTCCCAA R-GGAAAGGAAACTGGAGGGTC
D19Wox11	66	6	0.3	1/15	NED	5	212, 216, 220, 224, 228	[2] F-CTACCCACCCATCTATTCATCC R-GTTTCCAGCACCCATGTCC
D1Cebr3	66	6	0.3	1/15	VIC	6	79, 81, 83, 105, 107, 109	[4] F-CTTGGGAGCTGGGAGTGT R-GAAGGCTGAGGTATGAAGACTG
D16Rat81	66	6	0.4	1/15	PET	7	148, 150, 152, 154, 156, 158, 160	[1] F-GAGCCTTAGCACAGTGGCTT R-GGCCCACATGTGCATGTATA
D5Rat33	66	6*	0.4	1/15	6FAM	15	102, 106, 110, 112, 116, 118, 120, 122, 124, 126, 130, 132, 134, 138, 140	[3] F-TGGAGAAAAGAAGAACCTCCA R-GTGCCCTCAGACTGAACTC
D1Cebr4	66	6*	0.4	1/30	VIC	8	278, 284, 286, 288, 290, 292, 294, 296	[4] F-GACCTCCTGCCCTTCACTG R-TGAAAAATGAATTGCTTGTG

[a][1] (64), [2] (65), [3] (66), [4] (67), [5] (63).

[b]Additional single PCRs are pooled into a corresponding mix (*i.e.*, multiplex PCR) prior to capillary electrophoresis. $\boldsymbol{T_a}$ = PCR annealing temperature. $\boldsymbol{A}$ = number of observed alleles in Boston, MA *Rattus norvegicus* (2016-2019 and 2021-2022).

MSAT sample data filters

794 Our final MSAT dataset included only rats with complete genotypes
795 at ≥19 markers; one individual sample failed to meet this criterion and
796 was removed. We also removed three individuals with evidence of DNA
797 cross contamination as indicated by the presence of >2 alleles at
798 multiple markers. Finally, location provenance was incomplete for 13
799 individuals (including all eight rats collected during 2020), which were
800 also removed. This resulted in a final MSAT dataset of 311 *R. norvegicus*
801 representing 33 collections from 17 sites collected between 2016-2019 and 2021-2022 (**S1 Table**).
802

803 **MSAT marker validation analyses**

804 We ran a series of tests on the 21 MSAT markers to validate neutrality and utility in
805 population genetic analyses of *R. norvegicus* from Boston using all 311 individuals
806 organized into 33 collections. Validations included a query for identical
807 multi-locus genotypes in GenAlEx (68) and a test for linkage
808 disequilibrium in FSTAT v2.9.4 based on the log-likelihood ratio G
809 statistic (69). We used MICRO-CHECKER (70) to test for null alleles, large
810 allelic dropouts, and scoring errors due to PCR stuttering using the
811 following settings: maximum expected allele size of 420 bp, a 95%
812 confidence interval (CI), and 1000 repetitions. We performed a test of
813 Hardy-Weinberg equilibrium for each marker, also in GenAlEx, but using
814 only collections with ≥20 rats (*i.e.*, 5b, 6c, 6g2, and 8f, **see S1 Table**). Finally, we ran

815 PCR replicates on a subset of 94 *R. norvegicus* DNAs (30.2%) from the final dataset to check for
816 allele scoring errors.

817

818 Population genetic analyses: determination of genetic groups and population 819 assignment using STRUCTURE

820 We determined the most likely number of genetic groups and conducted population
821 assignment analyses using STRUCTURE v2.3.4 analysis (71) on the MSAT genotypes obtained from
822 the 311 *R. norvegicus* DNAs. We set Bayesian parameters to assume genetic admixture and
823 correlated allele frequencies in the study system, and to ignore collection sites. These parameters
824 were run at *K* values from 1–15, with 25,000 burn-in iterations followed by 100,000 run iterations.
825 Bayesian analysis was repeated ten times for each *K*. Resulting log(Prob K) values were analyzed
826 with the delta-*K* method (72) to estimate the most likely number of genetic populations. We assigned
827 rats to a specific genetic group and identified potential migrants if their probability of membership
828 (*Q*) to a specific genetic group was ≥0.75. Individual rats not meeting
829 these criteria were grouped broadly into an "admixed" group, regardless
830 of collection site (**S3 Table**). Using these results, we sorted all 311 rats according to their
831 predicted genetic groups [12 distinct genetic groups (n=247) and one admixed group (n=64)] for
832 additional population genetic analyses [~24% of individuals (64 admixed and 11 potential migrants,
833 **see Results**) were no longer grouped according to their initial collection site].

834

835 Population genetic analyses: estimates of genetic structure at two scales

836 We estimated overall genetic structure among Boston *R. norvegicus* using PCoA and three
837 different estimators of F_{ST}. For PCoA, we used standardized genetic distances among all pairs of

838 individuals in GenAlEx (68). We then conducted *a posteriori* F_{ST} analyses using only individuals
839 from the 12 genetic groups identified in STRUCTURE (n=247) but also removed juveniles (weight
840 <140g) (73) and migrants, which could artificially increase or decrease, respectively, F_{ST} estimates
841 among the 12 predicted genetic groups. Collection 11b was the single exception because only
842 juveniles were trapped and they appeared to form a distinct genetic group (Orange, **see Results**).
843 These filters resulted in a final dataset for this analysis of 119
844 individual rats among the 12 genetic groups; all genetic groups were
845 represented by ≥4 individuals (up to 19; **S3 Table**). We generated F_{ST} estimates of
846 theta (θ) in FSTAT version 2.9.4 using Weir and Cockerham's method (74) to estimate population
847 structure. Pairwise population F_{ST} values were also estimated in FSTAT and *p*-values for all
848 comparisons were generated using a =0.05. We performed AMOVA on this same reduced set
849 (n=119) using standardized genetic distances in GenAlEx with 999 permutations to estimate PhiPT
850 (an F_{ST} analog). The third F_{ST} estimator was a one-way global ANOSIM in PRIMER v5.2.9
851 (PRIMER-e, Albany, AK, NZ), conducted on a reduced set of 247 individual rats from the 12 distinct
852 genetic groups (the 64 individuals forming the admixed group were removed but migrants and
853 juveniles were included; **S3 Table**); the R statistic was generated using 10,000 permutations.
854 Afterwards, a post-hoc test was conducted to determine the genetic groups contributing to R, wherein
855 pairwise comparisons among all genetic groups were conducted. The global alpha was divided by 66
856 (number of pairwise comparisons) to calculate a conservative Bonferroni corrected a =0.000758.
857

858 **Population genetic analyses: isolation by distance**

859 Because *R. norvegicus* relatives often live in close proximity, we performed a fine-scale
860 analysis of spatial genetic structure (SGS) on all 311 rats using spatial autocorrelation in GenAlEx
861 with options set to SinglePOP, even distance classes (n=20), and 999 permutations. Distance class

862 intervals were increased in 100 m increments (0-100 m, 0-200 m, etc.) up to a maximum of 0-2,000
863 m. We assessed isolation by distance using the RELATE function in PRIMER v5.2.9 and
864 standardized genetic distances and geographic locations (latitude and longitude) of the 247 rats
865 forming the 12 distinct genetic groups (**S3 Table**). We generated Spearman's rank correlation
866 coefficient (Rho) using 10,000 permutations. Inbreeding coefficient F_{IS} was estimated for each
867 genetic group and global relatedness was estimated among all groups using the reduced set of 119
868 rats (no juveniles or migrants) in FSTAT (**S3 Table**).
869

870 # *Leptospira* genomics and genotyping

871 ## Whole genome sequencing of isolates

872 Whole genome sequencing of DNA extracted from 5 mL cultures of each isolate was
873 performed as previously described (75, 76). Briefly, each isolate was propagated in HAN liquid
874 media at 29°C, which was harvested by centrifugation at 10,000 x *g* for 15 min and DNA extracted
875 using the Maxwell RSC Purefood Purification Pathogen kit. For Illumina library preparation, the
876 concentration of reconstituted genomic DNA was determined with a Qubit dsDNA HS assay using a
877 Qubit® 3.0 fluorometer (Invitrogen, Carlsbad, CA, US). Whole-genome sequences were obtained
878 (MiSeq Desktop Sequencer, 2x250 v2 paired-end chemistry and the Nextera XT DNA Library
879 Preparation Kit, Illumina, San Diego, California, US) per manufacturer's instructions.
880

881 ## Capture and enrichment of *Leptospira* DNA from rat and human samples

882 We attempted *Leptospira* genome capture and enrichment from 22 PCR positive rat samples
883 collected between 2017-2019, 20 culture negative and two culture positive (R316 and R47); the latter
884 served as genome analysis controls. We also attempted enrichment on two separate human samples
885 from Boston: one serum from 2017 (MA-1) and one concentrated urine from 2018 (MA-2). These

886 sample DNAs were provided by the CDC and had *lipL32* PCR Ct values of 38.03 and 31.6,
887 respectively. DNA from these 24 samples were subjected to pan pathogenic *Leptospira* DNA capture
888 and enrichment as previously described (28). Briefly, sample DNAs were diluted separately to ~4
889 ng/µL in a volume of 55 µL, sonicated to an average size of 285 bp using a Q800R2 sonicator
890 (QSonica, Newtown, CT, US), and then single indexed libraries were prepared using Agilent Sure-
891 Select methodology (XT-HS kit, Agilent, Santa Clara, CA, US). The libraries (some pooled) were
892 subjected to one or two rounds of DNA capture and enrichment (**S5 Table**) and sequenced on an
893 Illumina MiSeq instrument using either a MiSeq v3 600 cycle (2x300 reads) or v2 300 cycle kit
894 (2x150 reads). Three samples (R309, R334-19, and R379) failed to generate successful sequencing
895 libraries and were excluded from DNA capture and enrichment and Illumina sequencing and six
896 others failed to enrich *Leptospira* DNA and were excluded from sequencing (R66, R67, R108, R318,
897 R324 and MA-1) (**S2 and S5 Tables**). These unsuccessful attempts were associated with low
898 abundance samples [PCR Ct values >38 (n=2)], enzymatic inhibition [failed library preparation
899 (n=3)], or perhaps contained divergent or non-pathogenic species of *Leptospira* or some other
900 genetically similar species not compatible with our pan pathogenic *Leptospira* DNA capture and
901 enrichment system (n=4 including all three *Leptospira*-positive rats from collection 5b). Pooling
902 schemes, rounds of DNA capture and enrichment, and Illumina sequencing kits used for each sample
903 are provided in **S5 Table**.
904

905 ## Read classifications, read mapping, SNP calling, and phylogenomics

906 To estimate the percentage of *Leptospira* reads in enriched sequences and for taxonomic
907 classification of enrichments and isolates, reads were mapped against the standard Kraken database
908 with Kraken v2.1.2 (77). To determine breadth of coverage for enriched genomes, reads were aligned
909 against *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GenBank accession#

910 GCA_000007685.1) with minimap2 v2.22 (78) and the per base depth of coverage was calculated
911 with Samtools v1.6 (79). Two *Leptospira* phylogenies were built. The first included a diverse set of
912 *L. interrogans* (representing >9 serovars) for the purpose of contextualizing Boston rat and human
913 derived genomic diversity within *L. interrogans*. Single nucleotide polymorphisms were identified
914 among 28 genomes from cultured isolates (GenBank accession numbers provided in phylogeny), 21
915 publicly available *L. interrogans* genomes, and 13 enriched genomes (12 rats and 1 human) by
916 aligning reads against reference genome *L. interrogans* serovar Copenhageni strain
917 Fiocruz L1-130 using minimap2 v2.22 and calling SNPs from the BAM file
918 with GATK v4.2.2 (80) using a depth of coverage ≥3x and a read proportion
919 of 0.9. SNPs that fell within duplicated regions, based on a reference
920 self-alignment with NUCmer v3.1 (81), were filtered from downstream
921 analyses. All methods were wrapped by NASP v1.2.1 (82). A maximum
922 likelihood phylogeny was inferred on the concatenated SNP alignments
923 using IQ-TREE v2.2.0.3 with default parameters (83), the "-fast" option,
924 and the integrated ModelFinder method (84); the phylogeny was midpoint
925 rooted. Second, to provide increased genomic resolution within *L.*
926 *interrogans* and assess fine scale relatedness among the Boston genomes we replaced the 21 diverse
927 *L. interrogans* genomes with two complete, publicly available *L. interrogans* serovar Copenhageni
928 genomes (GenBank accession# GCA_002073495.2 and GCA_010978155.1). A maximum likelihood
929 phylogeny was inferred on the concatenated SNP alignments using IQ-TREE v2.2.0.3 as described
930 above, except with 1000 bootstrap replicates; the phylogeny was rooted with *L. interrogans* serovar
931 Copenhageni strain Fiocruz L1-130.
932

933 ## *Leptospira interrogans* reference phylogeny and WG-FAST placement of enriched
934 samples

935 Two enriched samples (R289 and R292) had lower breadth and depth of coverage than the
936 other enrichments (**S5 Table**) and were inserted into the phylogeny with *WG-FAST* v1.2 (85) using
937 default settings. Reads were simulated from genome assemblies with ART vMountRainier (86) using
938 the command "-p -na -ss MSv3 -l 250 -f 75 -m 300 -s 30". Based on the position of the enriched
939 reads in the complete *L. interrogans* phylogeny (**see Results**), simulated reads were aligned against
940 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 with minimap2 v2.24 (78) and SNPs were
941 called with GATK v4.2.6.1 (80). A maximum likelihood phylogeny was inferred on the concatenated
942 SNPs with RAxML-NG v. 1.1.0 (87) and rooted with *L. interrogans* serovar Copenhageni strain
943 Fiocruz L1-130.

944

945 ## Human and rat phylogenomic comparison

946 To provide additional resolution among *Leptospira* genomes derived from four *R. norvegicus*
947 and one human that grouped together within a single *L. interrogans* serovar Copenhageni clade
948 (designated as clade 2, **see Results**), we conducted a phylogenomic analysis using NASP v1.2.1 and
949 IQ-TREE v2.2.0.3 as described above, except *L. interrogans* serovar Copenhageni strain SK1
950 (GenBank accession# GCA_010978155.1) was used as the reference and root.

951

952 # AmpSeq methods

953 We performed highly multiplexed amplicon sequencing (AmpSeq) on two subsets of rat
954 samples that were PCR positive for *Leptospira* but not included (or were unsuccessful) in the above
955 steps for culturing or genome enrichment. The first subset consisted of rats from 2016 and 2020-2022
956 that were PCR positive but culture negative (n=11); we included one culture positive rat (Cad99) to

957 serve as an analysis control. The final subset for AmpSeq contained any 2017-2019 positive samples
958 that failed DNA capture and enrichment (n=8 rats and n=1 human) (**S2 Table**).
959 In total, 42 primer sets were designed and validated for the *Leptospira* AmpSeq system.
960 Thirteen primer sets were either modified from existing primer sets (60, 88-98) targeting genes
961 commonly used for molecular typing or detection of *Leptospira* (*lipl32*, 16S, *secY*, *Loa22*, *OmpL1*,
962 *flaB*, LIC_11108, LFB1, *ligB*), or redesigned from MLST loci (*glmU*, *lipL41*, *icdA*, *sucA*) (99-101) to
963 be compatible with AmpSeq (153-415 bp in length) (**S8 Table**). We also designed 29 new primer
964 sets targeting loci determined, via comparative genomic analyses, to be SNP-rich and highly
965 discriminatory among 778 genome assemblies from 64 pathogenic and non-pathogenic *Leptospira*
966 spp. (**S9 Table**). Assemblies were downloaded from the NCBI RefSeq database (102, 103) using
967 ncbi-genome-download v0.3.1 https://github.com/kblin/ncbi-genome-download/tree/0.3.1 and
968 aligned to reference genome *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 using
969 NUCmer (104), and SNPs were identified using NASP v1.2.0 (82). The SNPs were analyzed using
970 VaST (105) to identify a set of targets maximizing discriminatory power among the assembled
971 genomes. To avoid paralogous sequence variants and loci with highly variable alleles, we only
972 analyzed SNPs located within a genus-wide core genome (106). To maximize amplification of these
973 targets across *Leptospira* species, primers were designed with ambiguous bases
974 for variants that occurred in ≥5% of genome assemblies.
975 To generate a conservative estimate of species coverage and discrimination for this AmpSeq
976 system, we used *in silico* PCR to predict amplification for each of the 42 loci across 69 *Leptospira*
977 spp. genome assemblies listed on the International Leptospirosis Society's resource webpage
978 (accessed 15-Jan-2024; https://leptosociety.org/resources/); these genomes represent all validated
979 pathogenic (P1 and P2) and saprophytic (S1 and S2) *Leptospira* spp. (57, 107). Genomes were
980 downloaded with the ncbi-genome-download tool (https://github.com/kblin/ncbi-genome-download)

981 from the RefSeq database (102) and renamed based on annotation information in the RefSeq record.
982 PCR primers were screened against the 69 *Leptospira* genomes with the -search_pcr2 method in
983 USEARCH v11.0.667 (108) with maximum number of SNP mismatches allowed in each primer set
984 to two, using parameters: "-strand both -maxdiffs 2 -maxamp 500". Predicted genome hits were
985 compiled for each primer set. This *in silico* analysis predicted amplification in all 69 *Leptospira*
986 species. The number of expected loci for each species varied, ranging from a single locus to all 42
987 loci. The pathogenic P1 clade was well represented with predicted amplification of 18-42 loci in all
988 20 species (**S1 File**).

989 AmpSeq was performed on two sample sets. The first set was used to validate the method and
990 consisted of nine genomic DNA extracts obtained from isolates from seven P1 clade *Leptospira* spp.
991 [*L. interrogans* (n=2), *L. kirschneri*, *L. santarosai*, *L. noguchii* (n=2), *L. borgpetersenii*, *L.*
992 *alexanderi*, and *L. kmetyi*]. The second set contained unknown *Leptospira* spp. and consisted of DNA
993 from 20 *Leptospira* PCR positive *R. norvegicus* kidney samples and one human serum sample (MA-
994 1) (**S2 Table**) along with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (ATCC,
995 Manassas, Virginia US, catalog# BAA-1198D-5), which served as a PCR and analysis control, and
996 also molecular grade water as a non-template negative control. The 42 loci were arranged into four
997 multiplexes and PCRs were carried out in 10 µL volumes containing the following reagents (given in
998 final concentrations): 10-20 ng of DNA template, 1x PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs,
999 0.8 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA, US), and 0.1-0.4 µM of each primer pair
1000 (**see S8 Table**). Thermocycling utilized the following conditions: 10 minutes at 95°C to release the
1001 Platinum Taq® antibody, followed by 38 cycles of 60 s at 94°C, 30 s at 55°C and 30 s at 72°C. PCRs
1002 were pooled together in equal amounts in a final volume of 25 µL (6.25 µL of each multiplex) and
1003 sequence libraries were prepared for all positive samples as previously described (56, 109). Uniquely

1004 indexed sample libraries were pooled together in equimolar amounts and sequenced in parallel on an
1005 Illumina NextSeq instrument using a 600 cycle (2x300) P1 kit (Illumina, San Diego, CA, US).
1006

1007 AmpSeq analysis

1008 Read mapping and classification

1009 Illumina sequencing reads for each individual sample were analyzed using the Amplicon
1010 Sequencing Analysis Pipeline (ASAP; https://github.com/TGenNorth/ASAP), wherein reads from
1011 each locus are mapped against a reference database using Bowtie2 and binned according to alignment
1012 score (110); the number of paired end reads assigning to each reference are then counted. To build a
1013 comprehensive reference database of *Leptospira* species to be used for read mapping and
1014 classification, we implemented two strategies. First, we downloaded reference sequences from
1015 GenBank for the thirteen previously published loci and removed redundant sequences. Second, for
1016 the 29 new loci we bioinformatically extracted each locus from the reference set of 69 *Leptospira*
1017 spp. genome assemblies described above. We first amplified each locus separately (using the same
1018 PCR conditions above for the AmpSeq multiplexes) using gDNA from *L. interrogans* serovar
1019 Copenhageni strain Fiocruz L1-130. PCR products were visualized on a 2% agarose gel to ensure
1020 robust amplification and treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, US) using 1 µL of
1021 ExoSAP-IT and 7 µL of PCR product under the following conditions: 37°C for 15 minutes followed
1022 by 80°C for 15 minutes. Treated products were then diluted (based on amplicon intensity) and
1023 sequenced using forward primer UT1F-acccaactgaatggagc and reverse primer UT2R-
1024 acgcacttgacttgtcttc (universal sequences incorporated into each unique primer pair, **see S8 Table**) in a
1025 BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA, US). We
1026 used 10 µL volumes for sequencing reactions containing the following reagents (given in final
1027 concentrations): 5x Sequencing Buffer, 1 µL BigDye® Terminator v3.1 Ready Reaction Mix, 1 µM

1028 primer, and 5 µL diluted PCR product. Thermocycling conditions were: 96°C for 20 seconds,
1029 followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Sanger
1030 sequences were assembled, trimmed, and converted to fasta format using SeqMan Pro (DNASTAR
1031 Lasergene, Madison, WI, USA), which were used to extract each locus (if present) from the 69
1032 *Leptospira* spp. genome assemblies using BLASTN (111) and a custom python script
1033 (https://gist.github.com/jasonsahl/2a232947a3578283f54c).
1034

1035 **Whole genome versus AmpSeq phylogenetic resolution**

1036 We compared phylogenetic resolution and tree topology among two maximum likelihood
1037 phylogenies generated, respectively, from whole genome reads and AmpSeq reads. Using 24 genome
1038 assemblies representing 18 P1 clade pathogenic *Leptospira* spp. (GenBank accession numbers
1039 provided in the phylogeny), we conducted a whole genome phylogenetic analysis using NASP v1.2.1
1040 and IQ-TREE v2.2.0.3 as described above with 1000 bootstrap replicates and *L. interrogans* serovar
1041 Copenhageni strain L1-130 as the reference. We then conducted an identical analysis but also
1042 included the AmpSeq generated reads from the nine *Leptospira* isolate genomic DNAs in our
1043 validation set. Inclusion of AmpSeq generated reads reduced the core genome to only nucleotide
1044 positions 1) included in the 42 loci, and 2) shared among the genome (n=24) and AmpSeq (n=9)
1045 reads. Both phylogenies were midpoint rooted.
1046

1047 **Species id and within species genotyping**

1048 Rat and human DNAs that amplified at ≥7 loci (out of 42) with >10x
1049 coverage were assigned a *Leptospira* species classification. To facilitate genotyping within
1050 species for samples with high breadth of coverage (*i.e.,* 25-42 loci amplified with >10x coverage),
1051 SNPs were identified from AmpSeq reads and a reference set of 22 publicly available *L. interrogans*

complete genome assemblies (GenBank accession numbers provided in the phylogeny). AmpSeq reads contain Illumina adaptors, universal tail sequences, and locus specific primers, all of which must be removed prior to SNP discovery if precise phylogenetic placement is the analysis goal; adaptors and universal tails were removed in ASAP and locus specific primers were trimmed using cutPrimers (https://github.com/aakechin/cutPrimers) (112). Trimmed AmpSeq reads and genome assemblies were aligned against reference genome *Leptospira kirschneri* serovar Grippotyphosa strain RedPanda1 (GenBank accession# GCA_027563495.1) using NASP v1.2.1 (82). A maximum likelihood phylogeny was then inferred on the concatenated SNP alignments using IQ-TREE v2.2.0.3 with the "-fast" option, default parameters (83), and the integrated ModelFinder method (84); the phylogeny was rooted with *Leptospira kirschneri* serovar Grippotyphosa strain RedPanda1.

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Competing Interests

The authors declare they have no competing interests.

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Supporting Information

S1 Fig. Plots from the delta-*K* method. The hypothetical number of subpopulations (*K*) is plotted on the X-axis against A) delta-*K*, and B) The logarithm probability for each *K* [L(*K*)]. Ten independent runs in STRUCTURE were performed for each *K* value.

1394 **S2 Fig. Principle Coordinates Analysis plot.** Principle Coordinates Analysis plot of standardized
1395 genetic distances illustrating genetic similarity among 311 *Rattus norvegicus* collected in
1396 Boston (2016–2022). Each rat is color coded according to its probability
1397 of membership (Q ≥0.75) to one of 12 genetic groups assigned using
1398 STRUCTURE (**Table S3 and Fig 1**). The percentage of genetic variation explained by these two
1399 axes is indicated.
1400
1401 **S3 Fig. Spatial autocorrelation analysis graph.** Spatial autocorrelation analysis graph assessing the
1402 correlation between genetic and geographic distance (Y-axis) using twenty even distance classes
1403 corresponding to 100 m intervals (X-axis). The blue line is the correlation value (r), and the red
1404 dashed lines are the 95% confidence intervals (U=upper and L=lower) about the null hypothesis of
1405 no spatial structure as determined by bootstrap resampling. Values of r above the upper 95%
1406 confidence interval are significant.
1407
1408 **S4 Fig. *Leptospira interrogans* whole genome phylogeny.** A maximum likelihood midpoint rooted
1409 phylogeny of 28 *Leptospira interrogans* serogroup Icterohaemorrhagiae isolate genomes and 13
1410 enriched genomes from 40 rats and one human (bold text), together with 22 publicly available *L.*
1411 *interrogans* isolate genomes representing >9 serovars (GenBank accession numbers are provided in
1412 the figure annotations), based upon a concatenated SNP alignment of 27,678 positions out of a core
1413 genome size of 1,186,500 nucleotides (nts).
1414
1415 **S5 Fig. *Leptospira interrogans* serogroup Icterohaemorrhagiae WG-FAST phylogeny** A
1416 maximum likelihood phylogeny of 28 *Leptospira interrogans* serogroup Icterohaemorrhagiae isolate
1417 genomes from rats, together with two publicly available *L. interrogans* serogroup

1418 Icterohaemorrhagiae serovar Copenhageni complete genomes (GenBank accession#
1419 GCA_002073495.2 and GCA_010978155.1), and 13 enriched genomes (12 rats and 1 human), based
1420 upon a concatenated SNP alignment of 127 positions out of a core genome size of 2,123,028
1421 nucleotides (nts). The phylogeny was rooted with reference genome *L. interrogans* serogroup
1422 Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 (GenBank accession#
1423 GCA_000007685.1). The color of the genome ID corresponds to the rat genetic group in Fig 1; black
1424 text indicates that no genetic group was assigned. Two enriched samples (R289 and R292) had lower
1425 breadth and depth of coverage than the other enrichments (**see S5 Table**) and were inserted into the
1426 rat phylogeny based upon canonical SNPs with WG-FAST.
1427

1428 **S6 Fig. Pathogenic *Leptospira* whole genome and AmpSeq phylogenies.** A) Whole genome
1429 maximum likelihood midpoint rooted phylogeny including 18 P1 clade pathogenic *Leptospira* spp.
1430 B) compared to an AmpSeq phylogeny that also includes AmpSeq data generated from nine
1431 *Leptospira* isolate gDNAs (blue text). Species specific clades were maintained (color coded) and
1432 similar phylogenetic topologies were observed despite a large reduction in the core-genome of the
1433 AmpSeq tree (1,195 nts) compared to the whole genome tree (167,849 nts). Bootstrap values are
1434 indicated at specific nodes. The nine AmpSeq *Leptospira* isolate gDNAs grouped in their respective
1435 species clades.
1436

1437 **S7 Fig. *Leptospira interrogans* AmpSeq phylogeny.** Maximum likelihood phylogeny based upon an
1438 alignment of 4,260 shared nucleotide positions using *Leptospira* AmpSeq data generated from nine
1439 *lipL32* qPCR positive *Rattus norvegicus* (bold text) along with 22 *Leptospira interrogans* isolate
1440 genomes (GenBank accession numbers are provided in the figure annotations). The phylogeny is

1441 rooted with *Leptospira kirschneri* serovar Grippotyphosa strain RedPanda1 (GenBank accession#
1442 GCA_027563495.1).
1443
1444 **S1 Table. *Rattus norvegicus* collections.**
1445 **S2 Table. Analyses summary and results for all rodent and human samples.**
1446 **S3 Table. *Rattus norvegicus* population assignment.**
1447 **S4 Table. *Rattus norvegicus* FSTAT results**
1448 **S5 Table. DNA capture and enrichment setup and results.**
1449 **S6 Table. *Leptospira* AmpSeq results**
1450 **S7 Table. *Rattus norvegicus* trapping locations.**
1451 **S8 Table. *Leptospira* AmpSeq primers and conditions**
1452 **S9 Table. *Leptospira* AmpSeq primer design genomes**
1453 **S1 File: Predicted amplification of 42 AmpSeq loci against 69 *Leptospira* spp.**
1454

1455 Data Availability Statement

1456 MSAT multi-locus genotypes for 311 *R. norvegicus* are provided in **S2 Table**. Whole genome
1457 sequencing reads have been deposited in GenBank under BioProject PRJNA1080226 with SRA
1458 accession numbers SRR28374870-SRR28374875, SRR28374877-SRR28374878, SRR28374880,
1459 SRR28374882-SRR28374900 for isolate genomes and SRR28374876, SRR28374879,
1460 SRR28411353-SRR28411357, SRR28411359-SRR28411365, SRR28411370 for enriched genomes.
1461 Raw AmpSeq data for up to 42 loci are also provided in GenBank under the same BioProject with
1462 SRA accession numbers SRR28374881, SRR28411338-SRR28411352, SRR28411358,
1463 SRR28411366-SRR28411369 and SRR28765937- SRR28765945.

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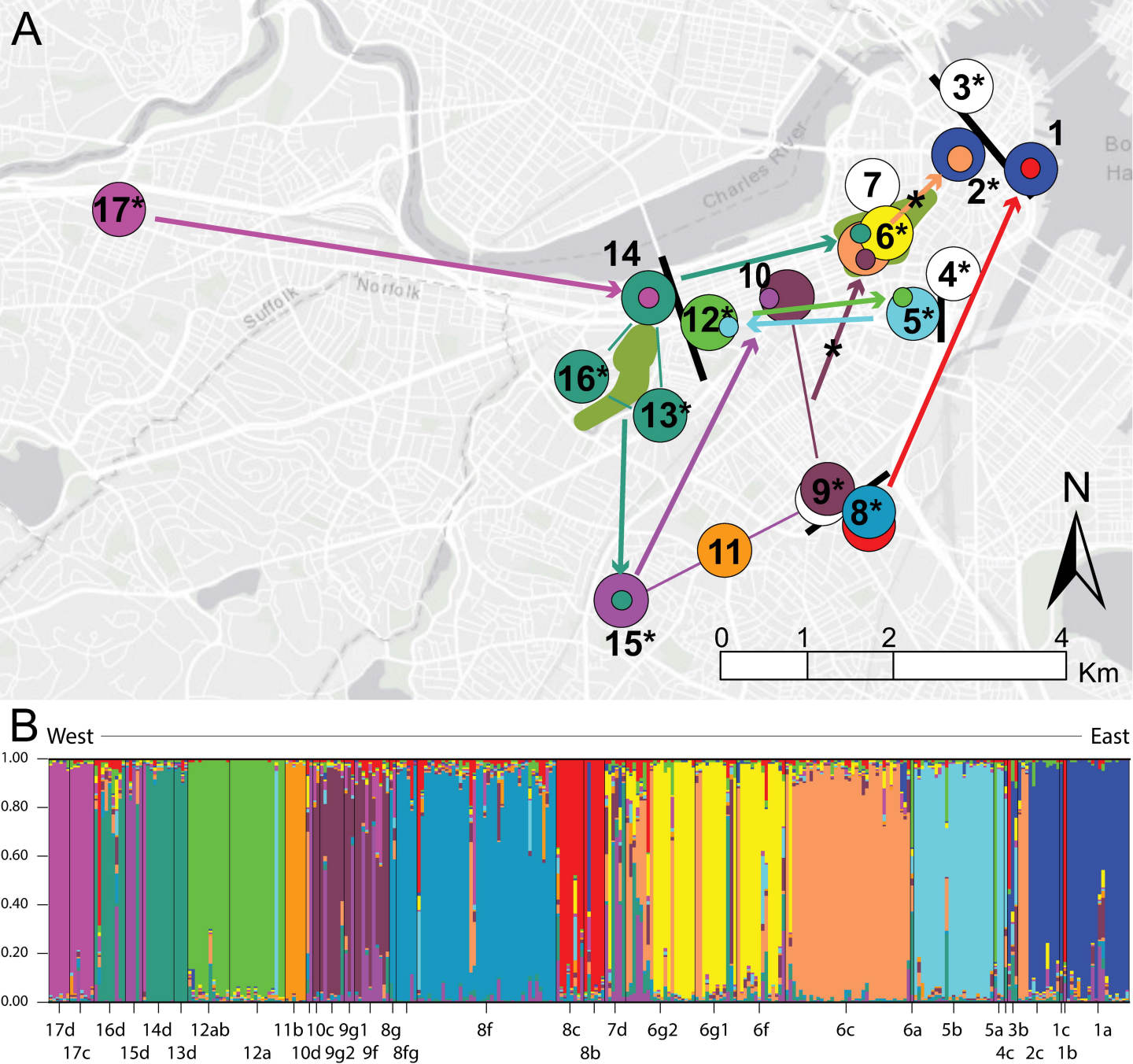
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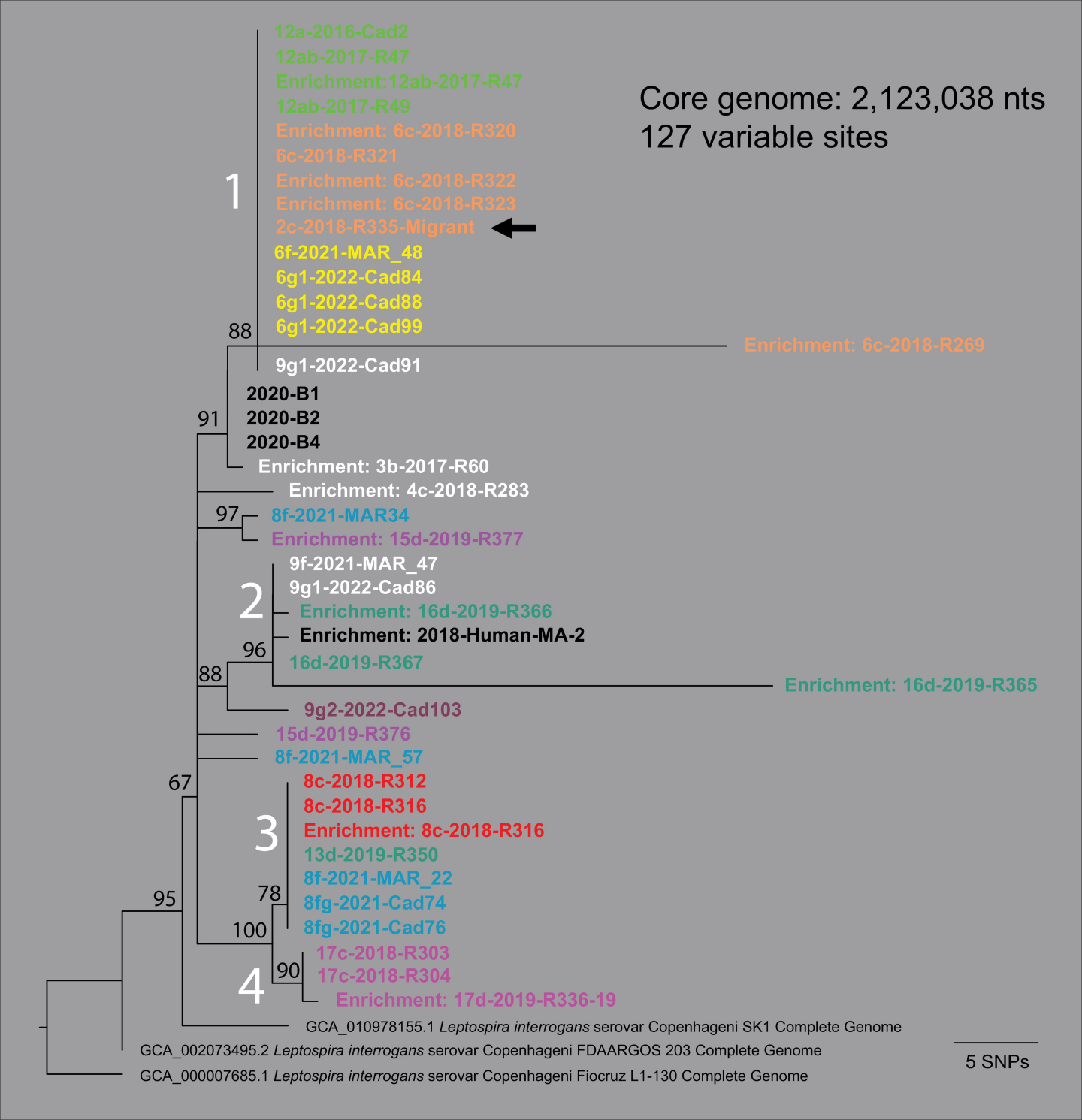
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Enrichment: 6c-2018-R323
2c-2018-R335-Migrant
6f-2021-MAR_48
6g1-2022-Cad84
6g1-2022-Cad88
6g1-2022-Cad99
88
Enrichment: 6c-2018-R269
9g1-2022-Cad91
2020-B1
91
2020-B2
2020-B4
Enrichment: 3b-2017-R60
Enrichment: 4c-2018-R283
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8f-2021-MAR34
Enrichment: 15d-2019-R377
2
9f-2021-MAR_47
9g1-2022-Cad86
Enrichment: 16d-2019-R366
Enrichment: 2018-Human-MA-2
96
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78
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100
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4
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17c-2018-R304
Enrichment: 17d-2019-R336-19
GCA_010978155.1 Leptospira interrogans serovar Copenhageni SK1 Complete Genome
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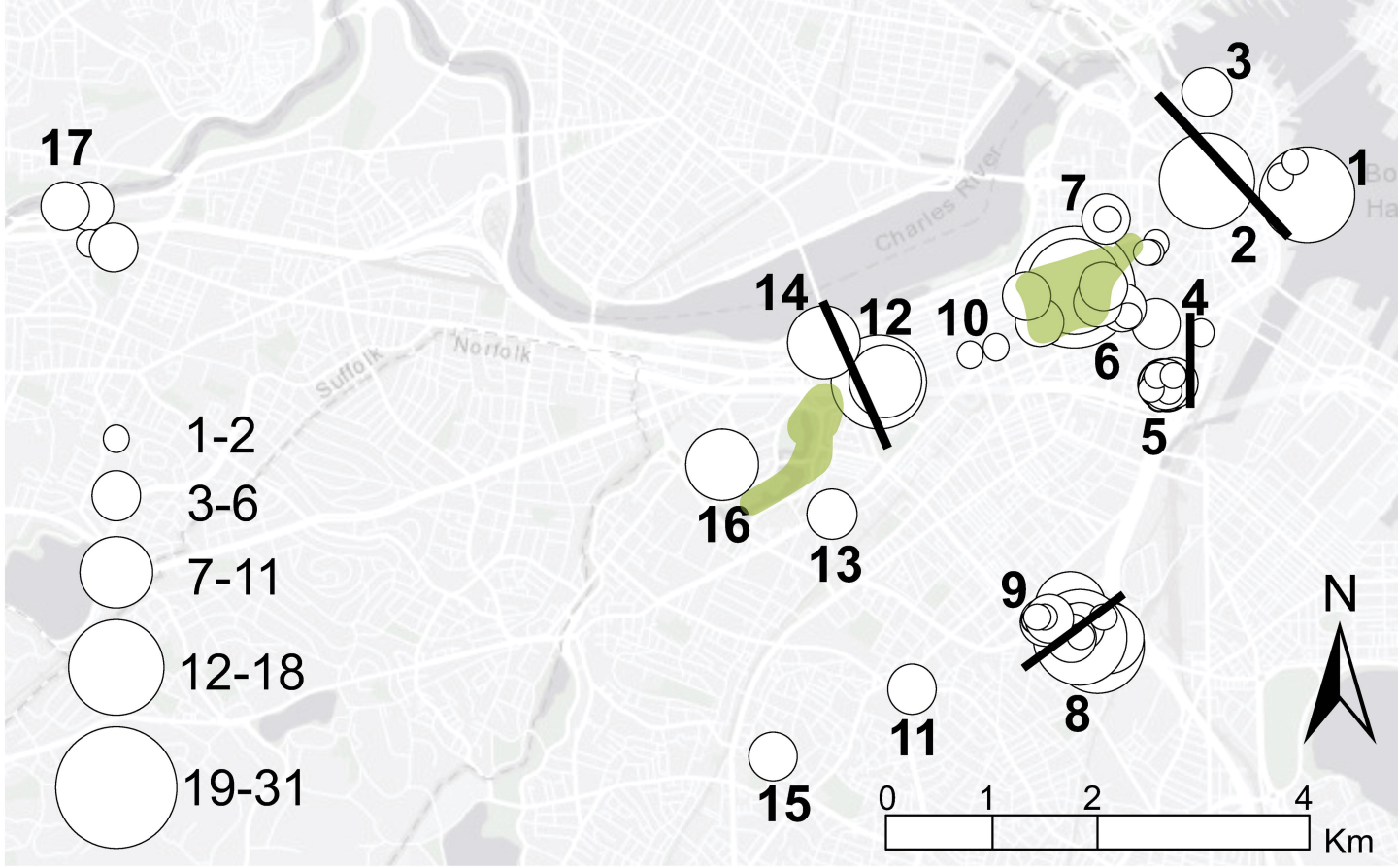
5 SNPs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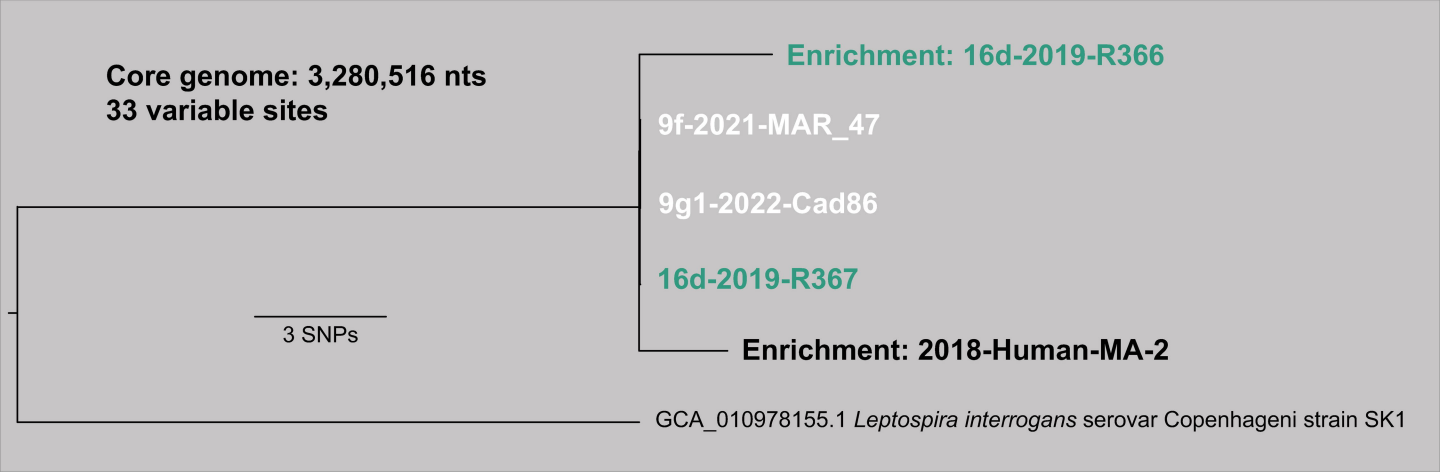
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16d-2019-R367
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3 SNPs
GCA_010978155.1 Leptospira interrogans serovar Copenhageni strain SK1