

24 **Abstract**

25 The recent range expansion of human babesiosis in the northeastern United States,
26 once found only in restricted coastal sites, is not well understood. This study sought to
27 utilize a large number of samples to examine the population structure of the parasites on a
28 fine scale to provide insights into the mode of emergence across the region. 228 *B.*
29 *microti* samples collected in endemic northeastern U.S. sites were genotyped using
30 published VNTR markers. The genetic diversity and population structure were analysed
31 on a geographic scale using Phyloviz and TESS. Three distinct populations were
32 detected in northeastern US, each dominated by a single ancestral type. In contrast to the
33 limited range of the Nantucket and Cape Cod populations, the mainland population
34 dominated from New Jersey eastward to Boston. Ancestral populations of *B. microti*
35 were sufficiently isolated to differentiate into distinct populations. Despite this, a single
36 population was detected across a large geographic area of the northeast that historically
37 had at least 3 distinct foci of transmission, central New Jersey, Long Island and
38 southeastern Connecticut. We conclude that a single *B. microti* genotype has expanded
39 across the northeastern U.S. The biological attributes associated with this parasite
40 genotype that have contributed to such a selective sweep remain to be identified..

41

42 **Author summary**

43 Babesiosis is a disease caused by a protozoan parasite, *Babesia microti*, related to
44 malaria. The disease is acquired by the bite of the deer tick, the same tick that transmits
45 Lyme disease. Although Lyme disease rapidly emerged over a wide range within the last
46 40 years, babesiosis remained rare with an extremely focal distribution. Within the last

47 decade, the number of reports of babesiosis cases has increased from an expanded area of
48 risk, particularly across the mainland of southern New England. We determined whether
49 the expanded risk may be due to local intensification of transmission as opposed to
50 introduction of the parasite. Historical fragmentation of the landscape suggests that sites
51 of *B. microti* transmission should have been isolated and thus evidence of multiple
52 genetically distinct populations should be found. By a genetic fingerprinting method, we
53 found that samples from the new mainland sites were all genetically similar. We
54 conclude that one parasite genetic lineage has recently expanded its distribution and
55 now dominates, suggesting that it has some phenotypic attribute that may confer a
56 selective advantage over others.

57

58 **Introduction**

59 Human babesiosis due to *Babesia microti* was first recognized on Nantucket
60 Island nearly 50 years ago [1], and a few years later the first cases of Lyme arthritis were
61 described from Old Lyme, Connecticut [2]. Both infections were found to be transmitted
62 by the deer tick (*Ixodes dammini*; American clade of *I. scapularis*), which had started to
63 be locally recognized as a human-biting pest [3]. In the 1970s and 80s, cases of either
64 were restricted to coastal New England sites, as well as foci in Wisconsin and Minnesota
65 [4–6]. Over the next 20 years, the number of Lyme disease cases significantly increased
66 and zoonotic risk spread rapidly across the northeastern United States. Lyme disease is
67 now endemic all the way north into Canada, west to Ohio, and south as far as Virginia.
68 Babesiosis, in contrast, lagged Lyme disease across these sites in time and in force of
69 transmission [7,8] and most cases were reported from coastal sites in the northeastern

70 U.S. However, in the last two decades, risk for babesiosis has intensified across the
71 northeastern U.S. [9,10].

72 The 20 year lag between the range expansion of Lyme disease and that of
73 babesiosis is not fully understood but in part relates to the difficulty with which *B.*
74 *microti* may be transported. The two key facts that pose a paradox for range expansion
75 are (1) only rodents and insectivores are known to be competent reservoirs of *B. microti*
76 (may pass infection to uninfected ticks; [11]; and (2) *B. microti* is not inherited by ticks
77 [11]. Larval ticks transported long distances by migratory birds, a critical mode of
78 introduction for the agent of Lyme disease (for which certain passerines are competent
79 reservoirs; [12]), do not develop into infected nymphs after they engorge on a bird
80 because birds are not likely to be reservoir competent for *B. microti*. A *B. microti*-
81 infected nymph (which acquired infection as a larva feeding on a mouse) transported by a
82 bird could develop into an infected adult tick, but because that stage feeds only on
83 medium to large sized mammals, especially deer, would not pass infection to a reservoir
84 competent animal during the adult bloodmeal; deer are not competent reservoirs and
85 carnivores are not likely to be competent. Hence, *B. burgdorferi* is said to travel on the
86 backs of birds but *B. microti* on mice. Mice or other small mammals are unlikely to
87 travel large distances. These considerations argue that the range expansion for *B. microti*
88 babesiosis is not due to introductions of infected ticks by migratory birds.

89 The existence of silent natural foci of transmission is suggested by early rodent
90 serosurveys for *B. microti* in Connecticut [13] and the detection of zoonotic clade
91 parasites from sites in Maine where human babesiosis had not been recorded [14].
92 However, ecological surveillance has not been conducted across the northeastern U.S.

93 with sufficient detail across the likely range to provide much data of utility in
94 understanding the tempo and mode of babesiosis risk. Longitudinal analyses of cases
95 reported to state departments of public health are useful because case reports are based on
96 a standard surveillance case definition and data are comparable between states. In Rhode
97 Island, risk diminished from south to north [15]. In New York, babesiosis case reports
98 gradually expanded from Long Island up the Hudson River valley. Similarly, in
99 Connecticut, case reports expanded through the years from the southeastern coast first
100 extending westward along the coast and then moving inland. [7,16–18]. The expansion
101 of risk has been limited and incremental, with no long-distance introduction events such
102 as those documented for Lyme disease, exemplified by its introduction into Canada. [19]
103 A recent model for the emergence of babesiosis in New England suggests a “stepping-
104 stone” model: a strong predictor of a town reporting babesiosis cases was the presence of
105 a neighboring town reporting cases and that Lyme disease risk was a prerequisite [8].
106 Two stepping stone scenarios might have been operating concurrently in the last 20 years.
107 (1) The force of *B. microti* transmission increased, slow and wave-like, across the
108 northeastern landscape with the coastal earliest known zoonotic sites seeding adjacent
109 more northerly sites. (2) Multiple cryptic enzootic sites (natural foci) with little zoonotic
110 risk existed across the region, with local intensification of the force of *B. microti*
111 transmission as tick densities increased to a threshold (estimated to be more than 20
112 nymphal deer ticks collected per hour; [20], and subsequent spread to adjacent areas.

113 The population structure of *B. microti* may provide evidence for tempo and mode
114 of the expansion of babesiosis risk across the northeast. At the very basic level, new
115 demes will be related genetically to their parent populations. In expanding populations,

116 genetic diversity may be low be due to bottlenecks and founder effects at the expanding
117 front [21,22]. In fact, observed patterns of diversity will vary depending on the process
118 of population expansion, viz., whether the population is being "pushed" or
119 "pulled"[21,23]. A "pulled" expansion occurs when pioneers are seeding new
120 populations ahead of the source population, such as would occur if individual infected
121 ticks are being introduced into a new site. This causes the genetic diversity to be lower at
122 the edge than the main body of the population due to successive founder effects. By
123 contrast, a "pushed" expansion occurs when a population expands at the edges of the
124 source location due to population growth. This expansion is usually slower and allows for
125 diversity in the source population to keep pace with geographical spread. A skewed
126 population diversity can occur near the expanding front of the population due to "allele
127 surfing", that is high rates of reproduction can increase mutation and allow an allele to
128 surf the wave of population growth and become prevalent when it might not have become
129 fixed in a stationary population [21,24–26].

130 We have previously described variable number tandem repeat (VNTR) markers
131 for analyzing the population structure of *B. microti* and detected 3 distinct populations in
132 ticks and rodents across New England [27]. Whole genome sequencing of ecological
133 and clinical samples determined that these *B. microti* populations were strongly
134 differentiated, suggesting that they were geographically isolated [28]. However, neither
135 study analyzed sufficient samples to provide detail on the mode of expansion of the range
136 of *B. microti* in the northeastern U.S. Accordingly, we leveraged >200 diagnostic blood
137 samples from patients suspected of having acute babesiosis presenting to several clinical
138 practices across the northeastern U.S. and analyzed them with the VNTR assay. In

139 particular, we sought to determine the population structure of these parasites, and whether
140 range expansion was best represented by a “pulled” expansion model by introductions
141 into small founder populations, or a “pushed” model consistent with stepping stone
142 expansion.

143

144 **Materials and methods**

145 ***B. microti* blood samples**

146 De-identified discarded blood samples were collected from specimens that had
147 been sent to Imugen, Inc. for diagnosis of *B. microti* infection during the transmission
148 season of 2015. The town of the submitting doctor's office or hospital was associated
149 with each sample but no other data was available. Samples with a Ct>34 on the
150 diagnostic real time PCR performed at Imugen were excluded from the analysis because
151 they would not have had enough parasite DNA to yield reliable VNTR typing results.

152

153 **Ethics Statement**

154 This study was considered not to comprise human subjects research by the Tufts
155 University institutional review board.

156

157 **Genotyping**

158 DNA was extracted using a commercial spin column method (Qiagen Inc.). *B.*
159 *microti* was typed as described [27], with the exception that the hypervariable locus,
160 BMV4, was excluded. Samples were excluded from the final analysis if more than 1
161 locus failed to amplify. To avoid erroneously scoring stutter peaks, multiple peaks were

162 scored only if the size of the minor peak was almost equal to that of the major peak. *B.*
163 *microti* merozoites infecting humans are haploid [29]; so all analyses were done under
164 the assumption of haploidy. Samples that had multiple peaks in more than one locus
165 were excluded, as it was impossible to determine the individual haplotypes needed for
166 assigning a haplotype to a population using Phyloviz (see below). Samples that had
167 multiple peaks in only a single locus were retained in the analysis and treated as two
168 separate haplotypes.

169

170 **Data analysis**

171 VNTR haplotypes were analyzed with two programs (Phyloviz [30] and TESS
172 [31]) that utilize different algorithms for assigning them to a population. Phyloviz
173 determines mutually exclusive related groups by using the eburst algorithm on haplotype
174 data to identify founder haplotypes and then predicts the descent from the founder to the
175 other haplotypes without any predefined assumptions of populations or geographic
176 location. TESS uses a Bayesian clustering algorithm to determine population structure
177 from geographically defined haplotypes without assuming predefined populations.

178 TESS requires that a unique geographic location be associated with each sample.
179 Because samples were de-identified and only the location of the contributing clinical
180 practice was known, we created random locations for each sample within a standard
181 deviation of 0.05 degrees longitude and 0.025 degrees latitude from the town associated
182 with each sample using the tool provided by TESS. To ensure that nearest neighbor
183 connections could not occur over the ocean, 23 dummy points, i.e. points at which
184 sampling cannot occur, were added in the Atlantic Ocean along the shoreline. In

185 addition, the spatial network was altered to remove any remaining nearest neighbor
186 connections that spanned the ocean. Geographic distances between each sample point
187 were calculated using TESS. The program was then run for 10 permutations for K
188 populations, from 2 to 8, with allowance for admixture. The mean deviance information
189 criterion (DIC) was calculated across runs for each K population in order to choose the
190 best fit among alternate models. The output from the 10 individual runs of the chosen K
191 was downloaded into CLUMPP [32] which compiled them together. The resulting
192 ancestry coefficients were displayed as a bar graph. An ancestry coefficient of 0.80 or
193 greater for a single population was determined to be a member of that population. Any
194 sample with a coefficient less than 0.80 for any single population was determined to have
195 significant admixture from more than 1 source population. The ancestry coefficients
196 were spatially interpolated onto a map of New England using R [33]. Fst estimates were
197 calculated with Genepop on the web [34,35], PhiPT estimates were calculated using
198 GenAlEx [36], and the Shannon Index of Diversity was calculated using PAST [37] on
199 samples grouped by region. The Outline map of the northeastern United States was
200 downloaded from dmmaps.com (http://dmmaps.com/carte.php?num_car=3895&lang=en)
201

202 **Results**

203 *B. microti* was typed from 234 specimens from 24 towns throughout New
204 England during 2015 (Fig 1 and Table 1). Of these samples, 42 had multiple alleles in
205 one locus and 6 had multiple alleles for more than 1 locus. The latter were excluded from
206 the analysis because we were unable to accurately determine the haplotype necessary for
207 analysis by PhyloviZ. From the 228 samples used in the study, 113 unique haplotypes

208 were obtained. The samples were grouped by geographic region (Table 1) and the
209 Shannon Index (H) was calculated for each region (Fig 2). The diversity for most regions
210 ranged from 1.8-2.5 and was not significantly different from each other. However, the
211 diversity from the New Jersey (NJ) samples was significantly lower ($H=0.9$, $p=0.02$) and
212 the diversity from southeastern Massachusetts (SeMA) samples was significantly higher
213 ($H=3.3$, $p<0.001$) than the rest. Population differentiation estimates, PhiPT, suggest
214 isolation between some regions and almost none between others (Table 2). Samples from
215 Nantucket (N) and Cape Cod (CC) have significant amounts of population differentiation
216 between each other and each of the other geographic groups. (Table 2) In contrast, there
217 is no evidence of any population differentiation between samples from NJ, Long Island
218 (LI), Connecticut (CT) and Rhode Island (RI). Samples from SeMA and western
219 Massachusetts (WMA) show moderate amounts of population differentiation between
220 each other and those from NJ, LI, CT and RI (Table 2).

221

222 **Table 1. Sites from which samples were collected and the number of haplotype**
223 **identified from each site.**

224

Region	No. Samples	No. per region	No. haplotypes
<u>Boston (Bos)</u>		19	11
Acton, MA	6		
Beverly, MA	1		
Boston, MA	3		

Norwood, MA	6		
Norwell, MA	1		
<u>Southeastern MA (SEMA)</u>		40	36
Fall River, MA	13		
Plymouth, MA	8		
New Bedford, MA	11		
Wareham, MA	4		
Dartmouth, MA	4		
<u>Western MA (WMA)</u>		7	7
Great Barrington, MA	1		
Pittsfield, MA	6		
<u>Cape Cod (CC)</u>		23	18
Falmouth, MA	6		
Hyannis, MA	17		
<u>Nantucket (N)</u>		16	13
Nantucket, MA	16		
<u>Rhode Island (RI)</u>		22	15
Providence, RI	4		

Wakefield, RI	18		
<u>Connecticut (CT)</u>		27	16
Putnam, CT	8		
Norwich, CT	19		
<u>Long Island (LI)</u>		46	19
Greenport, NY	2		
Hicksville, NY	15		
Riverhead, NY	12		
Southampton, NY	17		
<u>New Jersey (NJ)</u>		15	5
Flemmington, NJ	15		
Total:	228		113

225

226

227 **Table 2: PhiPT estimates for *B. microti* from human patients by region. ^a**

228

Region	Boston	Cape Cod	Long Island	Nantucket	Connecticut	New Jersey	Rhode Island	SeMA ^b
Cape Cod	0.51							
Long Island	0.05	0.64						

Nantucket	0.49	0.62	0.59					
Connecticut	0.07	0.62	0.02	0.54				
New Jersey	0.07	0.66	0.01	0.67	0.04			
Rhode Island	0.03	0.58	0.04	0.47	0.01	0.05		
SeMA ^b	0.04	0.33	<u>0.15</u>	0.33	<u>0.13</u>	<u>0.14</u>	<u>0.10</u>	
WMA ^c	0.003	0.51	<u>0.12</u>	0.48	0.09	<u>0.17</u>	0.06	0.04

229 ^a Significant population structure $\Phi_{IPT} > 0.25$ are shown in bold. Moderate population
230 structure $\Phi_{IPT} = 0.1-0.25$ is underlined.

231 ^b Southeastern Massachusetts

232 ^c Western Massachusetts

233

234

235 The eBurst algorithm of Phyloviz grouped the samples into 3 main clusters
236 consisting of samples primarily from Nantucket (N population), samples primarily from
237 Cape Cod (CC population) and those from all other sites except for SEMA (Mainland
238 population) (Fig 3). Samples from SEMA were divided among all 3 populations. About
239 6% of the samples remained unresolved and were not connected to any of the 3 major
240 groups; the majority of these (>75%) were from SEMA and RI.

241 By plotting the mean DIC for K populations from 2-8, we determined that 3
242 populations, K=3, best fit the data from TESS (Fig 4). Ancestry coefficients from 10
243 runs for K=3 were estimated for each sample, and the CLUMPP algorithm was used to
244 combine the data from all the runs (Fig 5). These coefficients indicate the probability of
245 membership into each of the 3 populations and corresponded well with the results from

246 Phyloviz (Fig 3). Many samples that remained unresolved with Phyloviz showed
247 significant amount of admixture, which would explain the inability of that algorithm to
248 decisively place them into any single cluster (Table 3 and Fig 3 inside pink circle).
249 However, the agreement between the two methods was not unanimous. There were a few
250 samples that Phyloviz was unable to assign to a cluster that TESS had >85% certainty of
251 inclusion into one of the populations (see unconnected bubbles inside larger circles Fig
252 3), as well as samples that Phyloviz connected to major populations that TESS could not
253 determine to >85% probability (see bubbles with grey connections stretched to fit into
254 pink circle Fig 3 and Table 3).

255

256 **Table 3. Ancestry coefficients from TESS of samples that showed significant**
257 **admixture.**

258

Haplotype	Region	M	CC	N
197	N	0.59	0.01	0.39
272	WMA	0.44	0.18	0.38
286	WMA	0.69	0.19	0.12
315	SEMA	0.57	0.27	0.16
314	SEMA	0.51	0.37	0.13
327	SEMA	0.79	0.03	0.18
312	SEMA	0.67	0.17	0.16
307	SEMA	0.73	0.05	0.22
308	SEMA	0.62	0.15	0.23

232	RI	0.56	0.14	0.30
233	RI	0.43	0.17	0.40
289	Bos	0.56	0.14	0.30
290	Bos	0.43	0.17	0.40
331	SEMA	0.20	0.68	0.11
330	SEMA	0.41	0.49	0.10
310	SEMA	0.22	0.71	0.08
287	SEMA	0.22	0.71	0.06
235	RI	0.22	0.08	0.70
283	N	0.22	0.03	0.75
285	SEMA	0.20	0.09	0.70
234	SEMA	0.21	0.05	0.74

259

260 The geographically placed ancestry coefficients produced by TESS were spatially
261 interpolated onto a map of New England (Fig 6). Haplotypes from the Nantucket
262 population are primarily found on Nantucket. There has been some introduction into
263 southeastern MA. The CC population also has limited scope: these haplotypes are found
264 primarily on CC with some extending along the eastern coast of MA south of Boston.
265 Contrary to the limited range of the N and CC populations, the mainland population
266 dominates all of NJ, LI, CT, RI and MA, other than Cape Cod and Nantucket. It should
267 be noted that this study did not include any data from Martha's Vineyard; so it may be
268 that the predicted populations included in this figure are erroneous.

269 Each of the 3 populations has a dominant haplotype that is also the putative
270 ancestral type (as determined by PhyloViz), type 4 for mainland, type 49 for Nantucket,
271 and type 88 for Cape Cod (Table 4). Type 49 is present in 48% of Nantucket samples;
272 Type 88 is found in 37% of Cape Cod samples, and type 4 ranges from 33% to 75% in
273 the regions included in the mainland population (Figure 7). SEMA is the only region
274 with a mixture of the dominant types; type 4 was detected in 22% of samples and type 88
275 detected in 7%. All other haplotypes in this study are detected only once or twice from
276 any given region, with the exception of type 91 from LI which was found 4 times (8% of
277 the observed haplotypes). Type 91 differs from the dominant type 4 by only the BMV1
278 locus (335bp instead of 340bp) of type 4.

279

280 **Table 4. The microsatellite amplicon sizes of the 3 major haplotypes in base pairs.**

281

Haplotype	Pop	BMV1	BMV2	BMV5	BMV8	BMV10	BMV13	BMV23	BMV20
4	M	340	405	317	241	305	396	243	695
49	N	340	405	317	241	305	520	248	713
88	CC	346	398	389	271	305	351	243	713

282

283

284 **Discussion**

285 Our analysis provides data to help reconstruct the tempo and mode of the
286 processes that have led to the current epidemic population structure of *B. microti* in
287 northeastern US. There are at least 3 distinct populations of *B. microti* in New England,

288 as we suggested previously [27,28] in analyses of ecological as well as clinical samples,
289 with PhiPT ranging from 0.32-0.67 between them (Table 2). Each of the three
290 populations has a single dominant haplotype that is found in at least 30% of the samples
291 from each site and is the presumed ancestral strain; type 4 for mainland, type 49 for
292 Nantucket and type 88 for CC. Southeastern MA is currently experiencing a natural
293 experiment as the 3 populations, CC, N and M, are zoonotic in this area. The CC
294 population is moving northward and westward along the eastern coast of MA, the N type
295 is invading from the southern coast, and the mainland type is invading from the west.
296 The genetic signature from all 3 populations can be clearly detected in clinical samples
297 from this area, and significant admixture is occurring (Fig 6). For this reason, the
298 diversity of *B. microti* from SEMA is significantly greater than that from all other regions
299 in our study. Although we do not know when each of the *B. microti* populations were
300 first introduced into SEMA, nor which one arrived first, type 4 is found more often in this
301 area and the majority of samples harbor loci that originate from type 4. This dominance
302 is clearly represented in the map of the ancestry coefficients from TESS, and suggests
303 that type 4 parasites have some attribute that allows for greater amplification than do the
304 other *B. microti* populations. It may be that type 4 parasites are more transmissible.

305 If the expansion of *B. microti* in New England was caused by individual founders
306 “pulling” the population, we would have expected the diversity estimates from ancestral
307 sites (Nantucket; Cape Cod; Long Island; [11], where cases have been diagnosed since
308 the 1970s, to be greater than those from incipient sites with more recent emergence of
309 cases. However, this was not the case; the diversity estimates of *B. microti* from the
310 regions we sampled across the northeastern United States were not significantly different.

311 In fact, the diversity of *B. microti* from ancestral sites, such as Nantucket and Long
312 Island, were no greater than those from more newly established sites. Furthermore, the
313 diversity from coastal CT was not significantly different than that from northern CT
314 where babesiosis cases were first detected 15 years later. The maintenance of diversity
315 across New England supports the theory that expansion was the result of a “pushing”
316 population expansion, consistent with the stepping-stone hypothesis inferred by Walter
317 and colleagues [8]. Notably different, however, were samples from NJ; their diversity
318 was significantly less than those from every other site in our study; more than 70% of the
319 parasite samples comprised the dominant type 4. The lack of genetic diversity is
320 consistent with the New Jersey foci representing newly established populations that have
321 experienced significant founder effects. However, *B. microti*-infected ticks were
322 documented from northern New Jersey in the early 1990s [38] and human cases shortly
323 thereafter [39]. New Jersey became endemic for babesiosis at the same time as northern
324 CT and northern RI, but the diversity of *B. microti* from those states are similar to those
325 from the rest of the study populations. The biological basis for the limited diversity
326 found in New Jersey *B. microti* samples remains to be described.

327 Some patient samples may have been mistakenly assigned to location because we
328 used convenience samples that were de-identified other than for site of the contributing
329 clinical practice. We assumed that a case became exposed near the healthcare provider
330 who provided the sample to Imugen for analysis. Residents of any of our sites are likely
331 to travel within the northeast, and may vacation or visit in sites where risk is similar to
332 where they live. We are confident, for example, that two samples from our Nantucket
333 cohort acquired infection elsewhere. Each of these samples contained parasite haplotypes

334 that grouped with the mainland population. We have analyzed sufficient numbers of
335 ecological samples from Nantucket Island and have never detected the other lineages
336 [27]. Despite this clear example of mistaken assignment, the outcome of our analysis did
337 not appear to be effected; TESS correctly concluded that Nantucket Island is dominated
338 solely by the Nantucket population and the other sites by their respective parasite
339 populations. Accordingly, we believe that our analysis is robust enough to be unaffected
340 by other unknown errors in geographic assignment of samples and that our conclusions
341 about the population structure of *B. microti* in the northeastern U.S. are reasonable.

342 It is also possible that focusing our analysis solely on parasites derived from
343 presumably symptomatic patients (those presenting to a healthcare provider who in turn
344 requested analysis of a sample for confirmation of a diagnosis) does not capture variation
345 of all those that may be present in the enzootic cycle of the mainland parasites. There is
346 as yet no published evidence that the diversity of *B. microti* infectious for humans differs
347 from that in local mice or ticks, i.e., that only a subset of naturally occurring strains are
348 zoonotic. However, such an argument would need to apply across all sites and we note
349 that there is much variation evident in parasites from patients presenting to healthcare
350 providers on Nantucket, Cape Cod, or Southeastern Massachusetts.

351 Significant differentiation ($\Phi_{ST} > 0.36$) between each of the 3 populations
352 implies that they have been isolated from each other and remain so. We have previously
353 speculated that the microbial guild transmitted by *I. dammini* had been maintained in
354 relict or refugial foci during glaciation [11]. Then too, postcolonial deforestation likely
355 provided a fragmented landscape that only allowed for perpetuation of ticks and their
356 hosts in small less-disturbed natural foci. The lack of differentiation among parasites

357 from the mainland sites, from central NJ westward to RI, appears to be inconsistent with
358 a scenario of multiple relict foci across the mainland northeastern landscape, with
359 coalescence of the isolated demes occurring as a result of amplification and expansion of
360 the foci as successional habitat increased over the last 100 years. In the 1990s, babesiosis
361 was documented from 3 distinct sites within the area where the mainland population
362 parasites have been detected, viz., Long Island, southeastern CT and central NJ. Each of
363 these foci was isolated from the others; few cases were identified in areas between them.
364 Ecological sampling, where it was done, supports the inference that *B. microti* was indeed
365 absent or very rare [7,13,15,16,18,40]. We expected to detect a distinct genetic signature
366 of multiple small isolated foci within parasites from the mainland lineages but there is
367 little differentiation among LI, CT, RI and NJ, and our analyses group these sites
368 together into a single population. In fact, the mainland haplotype, type 4, dominates
369 from NJ eastward through NY, CT and RI and northward towards Boston, creating an
370 epidemic population structure.

371 It may be that these sites were not isolated for sufficient time for genetic drift to
372 operate, thereby explaining the lack of differentiation among mainland parasites. It is
373 also possible that the epidemic population structure occurred purely by chance, i.e.
374 genetic drift has occurred as *B. microti* has expanded leading to an overabundance of a
375 single haplotype. Some alleles may reach a high frequency because of repeated founder
376 events [22], a process called genetic surfing [26]. We assume that our VNTR loci are
377 neutral or are not linked with loci under selection and thus the observed lack of variation
378 is not due to selective constraints. The alternative hypothesis for the lack of diversity
379 among mainland *B. microti* is that there were no refugial or relictual sites within

380 fragments of forest, and that the parasite populations have not actually been isolated from
381 each other, allowing sufficient gene flow within the various sites comprising the
382 mainland. However, the population structure of *I. dammini* suggests otherwise. A
383 seminal study of the population structure of this vector tick and *B. burgdorferi* infecting
384 them [41] sampled 12 sites in the northeast from Massachusetts to Virginia; 5 of these
385 overlap with our area of study. Mitochondrial 16SrDNA haplotypes demonstrated that
386 the New York-CT region may have contained refugial tick populations that served as a
387 source for expansion of the range of *I. dammini*. Although tick populations that were
388 sampled were structured, this was not observed for *B. burgdorferi*, although the borrelial
389 genes that were analyzed were likely to have been influenced by balancing selection [41].
390 Additional studies are required to identify the relative contributions of selective and
391 demographic processes that serve as the basis for biogeographic variation in northeastern
392 populations of *B. microti*.

393 We believe the most likely scenario is that type 4 parasites have selectively swept
394 across the mainland landscape, replacing and erasing historic genetic signatures of other
395 lineages. Such a hypothesis is not without precedent with the microbial guild maintained
396 by *I. persulcatus*-like ticks. The population structure of *B. afzelii* (an Eurasian agent of
397 Lyme disease that appears restricted to rodent hosts) in Sweden is essentially clonal,
398 which may be the result of the epidemic spread of a single genotype [42]. Across
399 Europe, however, *B. afzelii* has significant population structure [43], similar to what we
400 have found in this study. There are likely public health implications of a specific *B.*
401 *microti* lineage that appears to be rapidly expanding its range.

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404

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409 study.

410

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532

533 **Figure Legends**

534 **Figure 1. Map of the Northeastern United States labeled with the sites from which**
535 **samples were collected.**

536

537 **Figure 2. Shannon's Index of diversity with standard error for *B. microti***
538 **haplotypes found in each region.** New Jersey (NJ), Long Island (LI), Western
539 Massachusetts (WMA), Connecticut (CT), Rhode Island (RI), southeastern
540 Massachusetts (SEMA), Boston (Bos), Cape Cod (CC) and Nantucket (N).

541

542 **Figure 3. Cluster analysis of *B. microti* samples using Phyloviz.** Each small bubble
543 represents a unique haplotype. Bubbles are colored to correspond with the region from
544 which the sample originated. The size is not directly correlated with the number of
545 samples. Haplotypes that differ by a single locus are connected with a gray line. The
546 large circles correspond with the population groupings calculated by TESS; blue is the
547 Nantucket population, red is the Cape Cod population, green is mainland population and
548 pink are the haplotypes that showed significant admixture and could not be placed solely
549 in any of the 3 populations. Bubbles that are unconnected to the major groups are placed
550 in the larger circles according to the ancestry coefficients from TESS.

551

552 **Figure 4. Graph of the mean DIC.** Mean DIC was calculated from 10 individual
553 TESS runs for population size 2-8. Three populations, $K=3$, best fit the data.

554

555 **Figure 5. Ancestry coefficients from TESS for $K=3$ populations.** Geographic
556 distances between each sample point were calculated using TESS. Green corresponds to
557 the mainland population, red is Cape Cod and blue is Nantucket. Lines beneath the bar
558 chart indicate the source of the sample. Black= Nantucket, light blue= RI, dark blue=
559 CT, purple= WMA, pink= Bos, red=CC, yellow= SeMA, dark green = NJ and light
560 green= LI

561

562 **Figure 6. Geographic interpolation of the ancestry coefficients showing the**
563 **distribution of each population of *B. microti*.** Cluster 1 (green) = mainland population,
564 cluster 2 (red) = Cape Cod population, and cluster 3 (Blue) = Nantucket population.

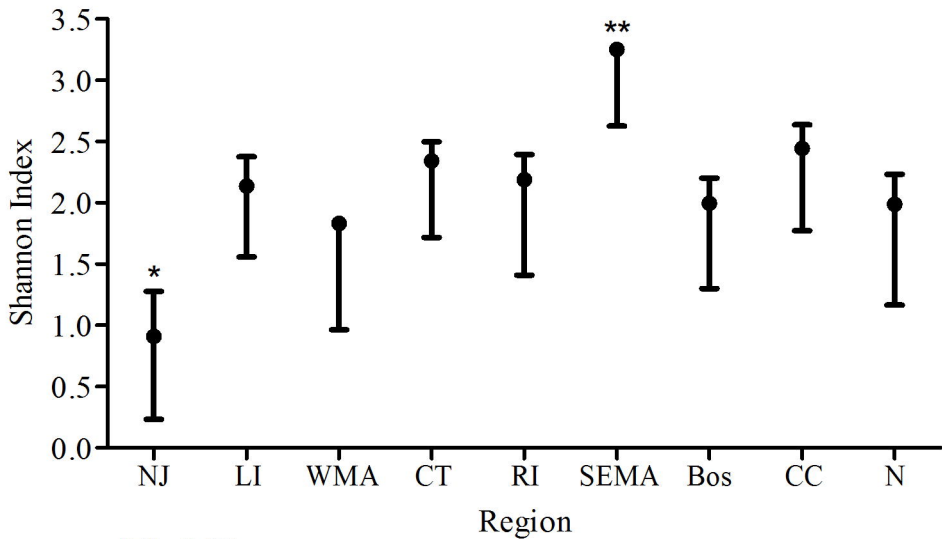
565 Areas with samples that have a high admixture coefficient, ie a high probability of
566 membership to that population, are shaded darker. Lighter shades indicate areas where
567 there the ancestry coefficients are lower, indicating areas where mixing is occurring. This
568 study did not include data from Martha's Vineyard; so the predicted populations on that
569 island may be erroneous.

570

571 **Figure 7. The percent of the total samples for each region for each of the main**
572 **haplotypes: type 4, type 88 and type 49.**

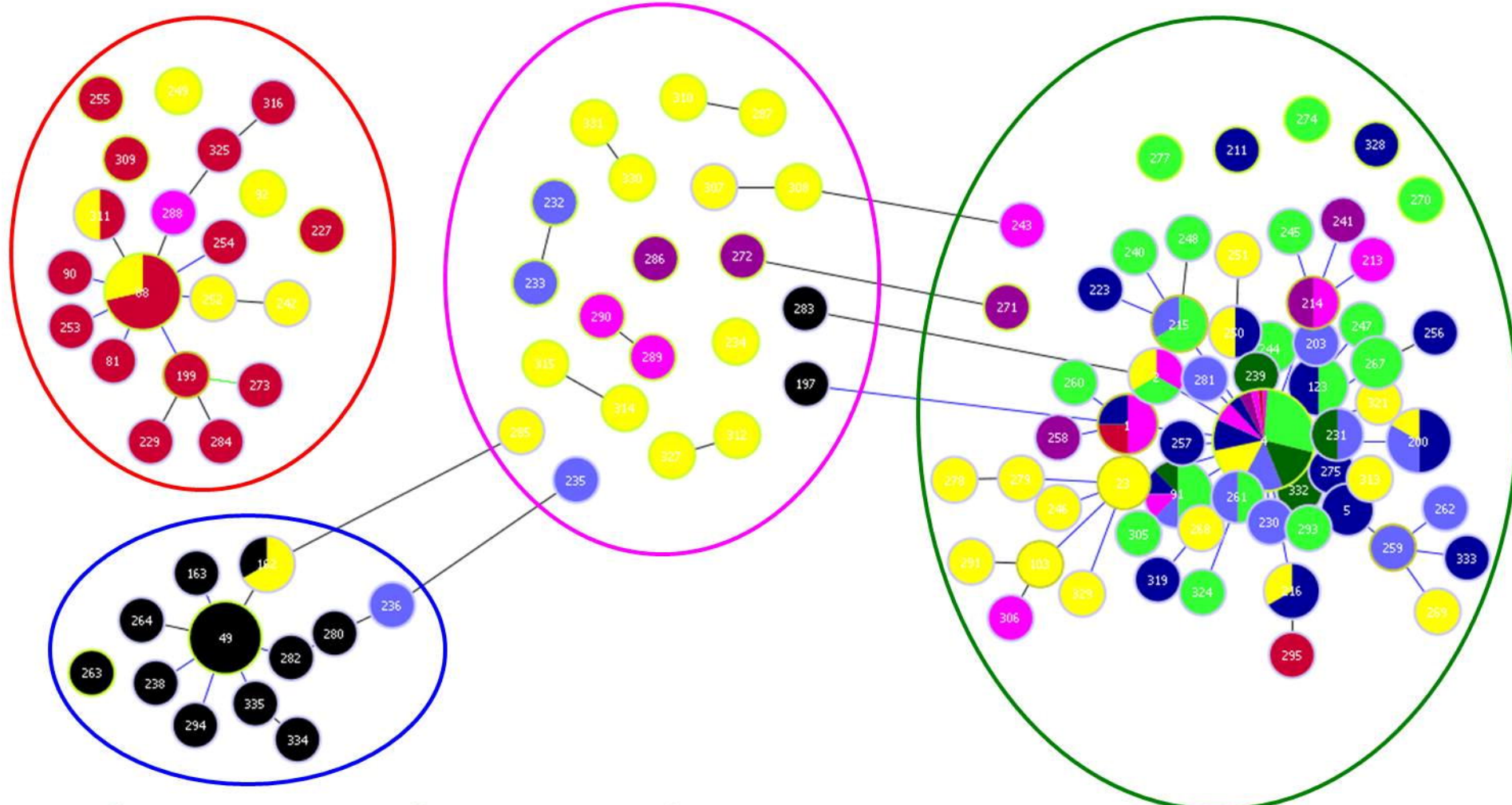
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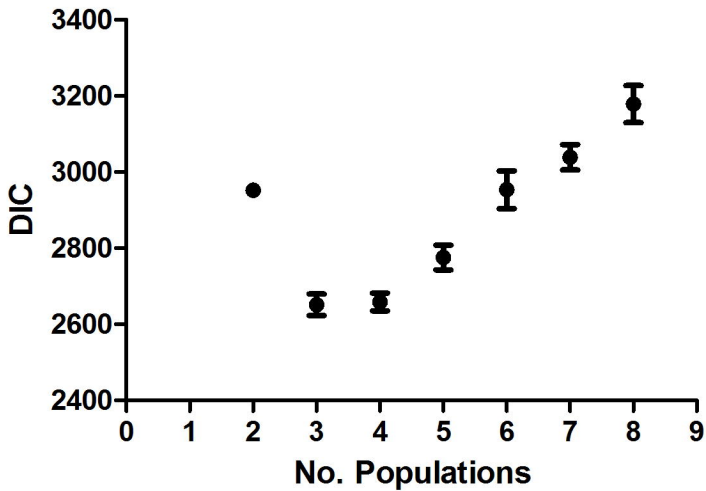


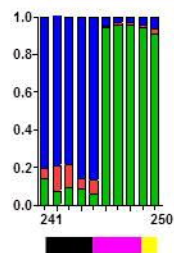
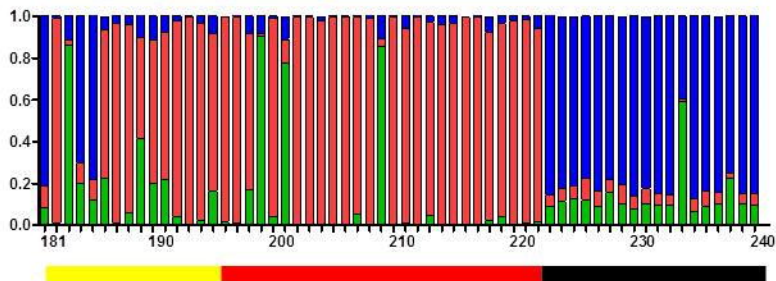
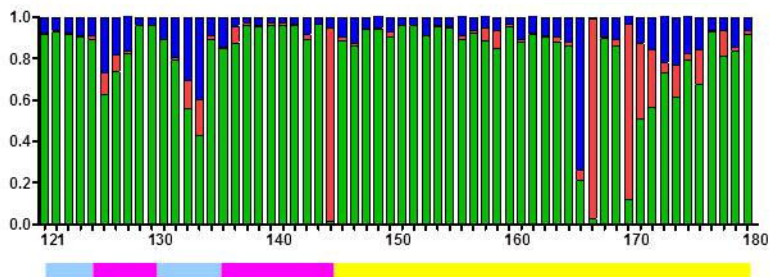
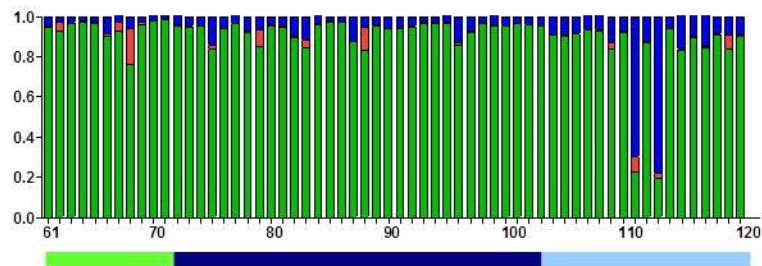
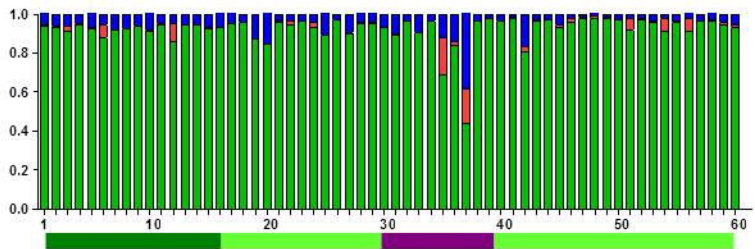
* P=0.02

**P<0.001

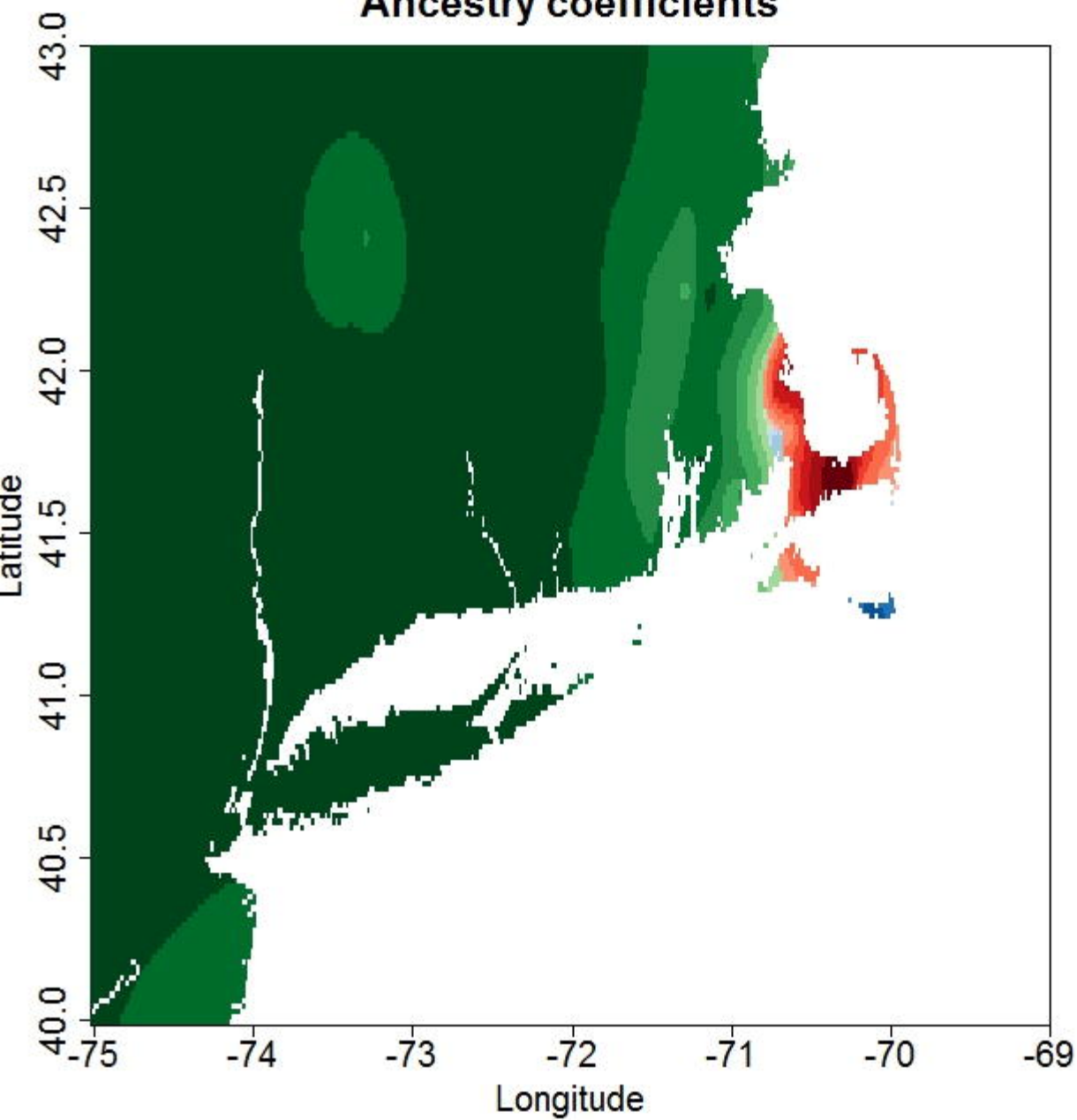


- | | | |
|---|--|---|
| ● Cape Cod | ● Boston | ● New Jersey |
| ● Southeastern MA | ● Western MA | ● Nantucket |
| ● Long Island | ● Connecticut | ● Rhode Island |

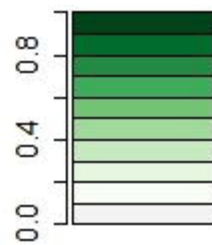




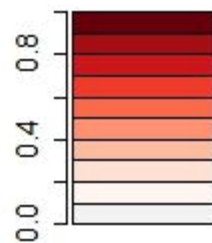
Ancestry coefficients



Cluster 1



Cluster 2



Cluster 3

