

1 **Novel *Rickettsia* spp. in two common overwintering North American passerines**

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16 **Keywords:** American robin; dark-eyed junco; rickettsiae; *Borrelia*; *Bartonella*; hemoplasmas;
17 migration; arthropod-borne disease; stable isotopes

18

19 **Running head:** Novel rickettsiae in migratory songbirds

20

21 **Abstract**

22

23 American robins and dark-eyed juncos migrate across North America, but their contributions to
24 arthropod-borne disease remain poorly characterized. We identified novel *Rickettsia* spp. in one
25 wintering migrant per bird species related to bellii, transitional, and spotted fever group
26 rickettsiae and suggest spring migration could disperse these pathogens hundreds-to-
27 thousands of kilometers.

28 **Main text**

29

30 Migratory birds play an important role in shaping risks of arthropod-borne diseases, given their
31 ability to disperse pathogens and vectors to new wintering or breeding locations. For pathogens
32 with high public health burdens, such as *Borrelia burgdorferi* and spotted fever group (SFG)
33 rickettsiae, migratory birds can disperse millions of infected vectors annually (1). Many
34 migratory birds are also competent for these pathogens and therefore can not only disperse
35 pathogens and arthropods but also transmit infections to naïve vectors following migration (2).

36

37 American robins (*Turdus migratorius*) and dark-eyed juncos (*Junco hyemalis*) are important
38 species to understand bird migration and arthropod-borne disease in North America. They are
39 competent for some pathogens and can have high ectoparasite intensities (2,3). Both have
40 diverse migratory behaviors, are widely distributed, and are common in suburban habitats (4,5),
41 which could facilitate pathogen dispersal to areas of high human exposure. Yet the contribution
42 of these common migratory bird species to arthropod-borne disease remains poorly understood.

43

44 Here, we sampled robins and juncos across North America for arthropod-borne pathogens of
45 public health significance. We focused on bacterial pathogens for which these birds are known
46 to be competent (*Borrelia* spp.) (2), for which detections have occurred in other passerines
47 (*Rickettsia* spp.) (6), and that are rarely or never seen in birds (*Bartonella* spp., hemoplasmas).

48

49 Robins were sampled monthly in southern Indiana (2020–2021), while juncos were sampled in
50 southern California (2006), Virginia’s Appalachian Mountains (2018, 2019), and northeastern
51 Ohio (2019). California and Ohio were sampled in the breeding season, while Virginia was
52 sampled in winter (7). We captured birds with mist nets, applied USGS bands, and collected
53 blood stored in 96% ethanol or Longmire’s buffer at –20°C, on Whatman FTA cards, or frozen
54 directly at –20°C. We also collected the first secondary feather for stable isotope analyses.
55 Sampling was approved by the Indiana University IACUC (06-242, 18-028), Federal Bird
56 Banding Permit 20261, and state permits. Table 1 shows sample sizes per site and month.

57

58 DNA was extracted from blood with Maxwell RSC Whole Blood DNA kits or Qiagen DNeasy 96
59 Blood and Tissue kits. We then used published PCR protocols to screen avian DNA for
60 *Bartonella* spp. (partial *gltA* gene), *Borrelia* spp. (partial 16S rRNA gene), and *Rickettsia* spp.
61 (23S-5S rRNA intergenic spacer [ITS]). A subset of robins was also tested for hemoplasmas
62 (partial 16S rRNA gene). Table S1 provides PCR primers and thermocycler conditions.

63

64 Of 675 samples, we detected rickettsiae in one robin (0.26%, 1/391) and one junco (0.35%,
65 1/284), representing the first reports of rickettsiae in these bird species. No other target
66 pathogens were detected (Table 1). Both sequences (GenBank accessions ON773823 and
67 ON773824) shared only 82.61% partial identity of the 23S and 5S rRNA flanking sequences of
68 their ITS to one another, indicating distinct rickettsiae. We used NCBI BLASTn to identify related
69 rickettsiae 23S-5S rRNA ITS and type strain sequences, followed by MUSCLE for alignment
70 and MrBayes for phylogenetics (10,000,000 generations, GTR+G+I) via NGPhylogeny.fr (8).

71

72 Both sequences had ≤91% identity to other *Rickettsia* spp. The robin sequence was related
73 (83.85–91.01% identity with 91% sequence coverage) to uncultured rickettsiae from *Ixodes*
74 *auritulus* in Argentina (MW824654), *Amblyomma americanum* and *Ixodes scapularis* in the USA
75 (KJ796407 and KJ796403), humans in Ethiopia (MK693112) and India (OK077732–OK077742),
76 and to *R. monacensis* (e.g., JQ796867), *R. tillamookensis* (CP060138), and *R. felis* (e.g.,
77 DQ139799). The junco sequence was only partially related (87.63–88.66% identity with 49%

78 sequence coverage) to the above sequences, and the main internal part (146 bp) of this ITS
79 sequence (without 23S and 5S rRNA flanking sequences) was unique with no identity to any
80 rickettsiae ITS sequences in GenBank. These sequences thus belong to two novel but not yet
81 cultured *Rickettsia* spp., and our 23S-5S ITS phylogeny suggested they are most similar to
82 rickettsiae within the bellii group (BG), transitional group (TRG), and SFG (9) (Fig. 1A).

83
84 *Rickettsia*-positive robins and juncos were sampled in winter (12/2020 and 11/2018). Most
85 robins wintering in Indiana remain year-round or migrate elsewhere to breed, while wintering
86 juncos in the Appalachians include migrant and resident subspecies; we earlier determined this
87 positive bird as migratory (*J. h. hyemalis*) (7). Using feather hydrogen isotopes and geographic
88 assignment (Figure S1) (10), we estimated the most likely breeding site of the robin to be the
89 Great Lakes (e.g., Wisconsin; 459 km median migration distance), whereas that of the junco
90 ranged from the western USA to Manitoba in Canada (2,377 km median migration distance; Fig.
91 1B). Positive birds were thus short- or long-distance migrants and could spread their novel
92 rickettsiae during spring migration. Further study of rickettsiae in these common birds will be
93 important to characterize their evolutionary history, whether passerines are competent hosts,
94 and dispersal potential in relation to bird migration.

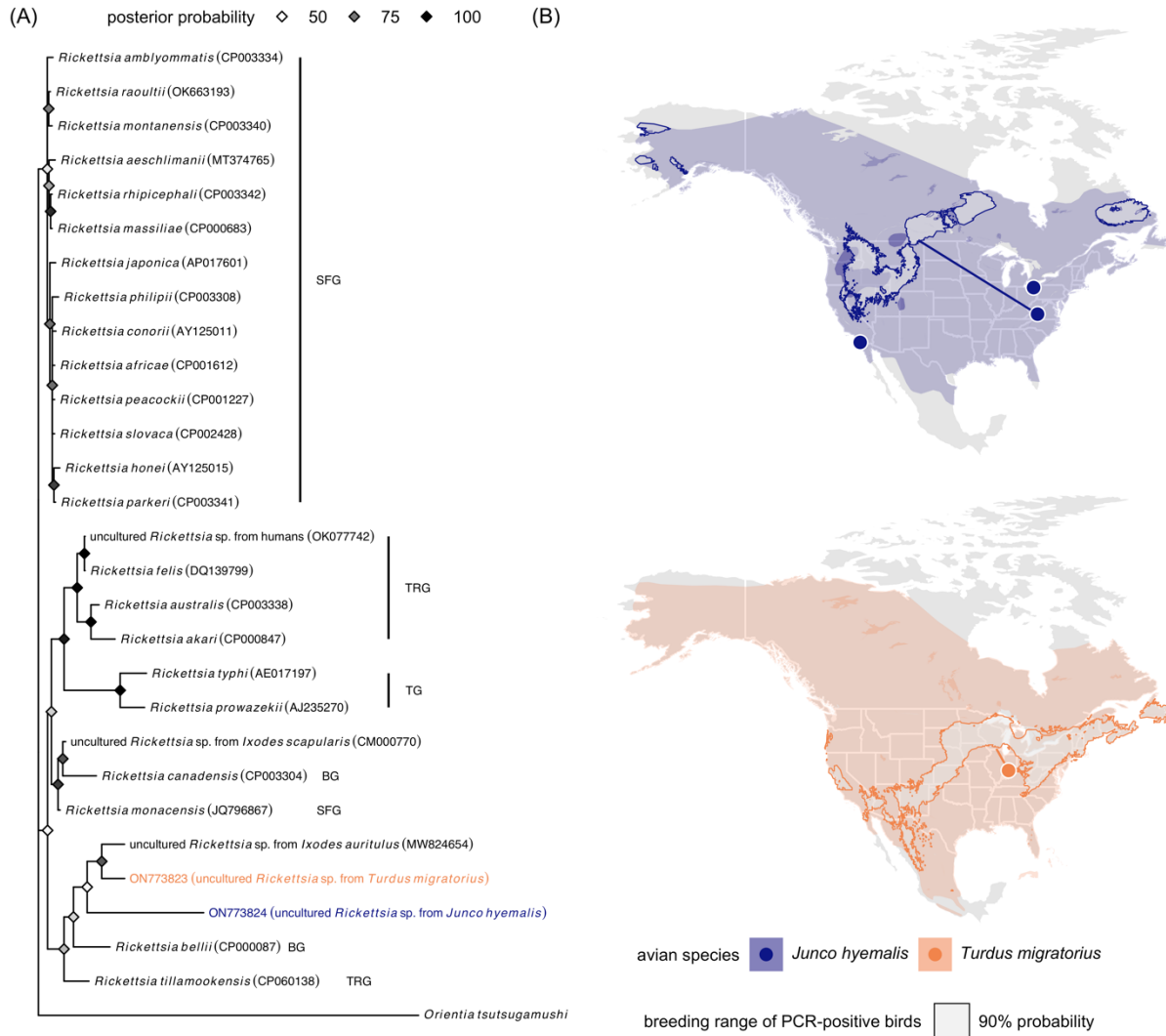
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102 **Table 1.** Sample sizes for robins and juncos per month, year, and region. All samples were
 103 tested for *Borrelia* spp, *Rickettsia* spp., and *Bartonella* spp.; parentheses indicate samples
 104 tested for hemoplasmas. Asterisks indicate the single *Rickettsia*-positive per bird species.
 105

Avian host	Date	Region	Number sampled
<i>Turdus migratorius</i>	1/2020	Southern Indiana	21 (8)
<i>Turdus migratorius</i>	2/2020	Southern Indiana	15 (8)
<i>Turdus migratorius</i>	3/2020	Southern Indiana	77 (41)
<i>Turdus migratorius</i>	4/2020	Southern Indiana	67 (32)
<i>Turdus migratorius</i>	5/2020	Southern Indiana	8 (7)
<i>Turdus migratorius</i>	9/2020	Southern Indiana	18
<i>Turdus migratorius</i>	10/2020	Southern Indiana	20
<i>Turdus migratorius</i>	11/2020	Southern Indiana	25
<i>Turdus migratorius</i>	12/2020	Southern Indiana	24*
<i>Turdus migratorius</i>	1/2021	Southern Indiana	21
<i>Turdus migratorius</i>	2/2022	Southern Indiana	1
<i>Turdus migratorius</i>	3/2022	Southern Indiana	18
<i>Turdus migratorius</i>	4/2022	Southern Indiana	37
<i>Turdus migratorius</i>	7/2021	Southern Indiana	5
<i>Turdus migratorius</i>	9/2021	Southern Indiana	28
<i>Turdus migratorius</i>	10/2021	Southern Indiana	6
<i>Junco hyemalis</i>	2/2006	Southern California	25
<i>Junco hyemalis</i>	3/2006	Southern California	54
<i>Junco hyemalis</i>	4/2006	Southern California	32
<i>Junco hyemalis</i>	5/2006	Southern California	25
<i>Junco hyemalis</i>	6/2006	Southern California	24
<i>Junco hyemalis</i>	7/2006	Southern California	9
<i>Junco hyemalis</i>	7/2019	Northeastern Ohio	20
<i>Junco hyemalis</i>	11/2018	Appalachian Mountains	65*
<i>Junco hyemalis</i>	11/2019	Appalachian Mountains	30

106 **Figure 1.** (A) Bayesian phylogeny of the novel *Rickettsia* spp. with closely related and reference
 107 rickettsiae sequences from GenBank, including those in the bellii group (BG), typhus group
 108 (TG), transitional group (TRG), and spotted fever group (SRG). Nodes are colored by posterior
 109 probability. (B) Field sites relative to the American robin and dark-eyed junco distribution with
 110 the estimated breeding origins of the two PCR-positive birds. Geographic assignments were
 111 performed using feather hydrogen of previously established known-origin juncos, robins, and
 112 other passerines (Fig. S1). Paths display median migration distance, defined as kilometers
 113 between winter capture site and median coordinates in the 90% probability breeding ground.
 114



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