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Diversity of *Listeria* Species in Urban and Natural Environments

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A total of 442 *Listeria* isolates, including 234 *Listeria seeligeri*, 80 *L. monocytogenes*, 74 *L. welshimeri*, 50 *L. innocua*, and 4 *L. marthii* isolates, were obtained from 1,805 soil, water, and other environmental samples collected over 2 years from four urban areas and four areas representing natural environments. *Listeria* spp. showed similar prevalences in samples from natural (23.4%) and urban (22.3%) environments. While *L. seeligeri* and *L. welshimeri* were significantly associated with natural environments ($P \leq 0.0001$), *L. innocua* and *L. monocytogenes* were significantly associated with urban environments ($P \leq 0.0001$). Sequencing of *sigB* for all isolates revealed 67 allelic types with a higher level of allelic diversity among isolates from urban environments. Some *Listeria* spp. and *sigB* allelic types showed significant associations with specific urban and natural areas. Nearest-neighbor analyses also showed that certain *Listeria* spp. and *sigB* allelic types were spatially clustered within both natural and urban environments, and there was evidence that these species and allelic types persisted over time in specific areas. Our data show that members of the genus *Listeria* not only are common in urban and natural environments but also show species- and subtype-specific associations with different environments and areas. This indicates that *Listeria* species and subtypes within these species may show distinct ecological preferences, which suggests (i) that molecular source-tracking approaches can be developed for *Listeria* and (ii) that detection of some *Listeria* species may not be a good indicator for *L. monocytogenes*.

Members of the genus *Listeria* have traditionally been classified into three typically hemolytic species (*Listeria monocytogenes*, *L. ivanovii*, and *L. seeligeri*) and two typically nonhemolytic species (*L. innocua* and *L. welshimeri*) (59). While *L. seeligeri* is considered nonpathogenic, it includes both hemolytic and nonhemolytic isolates (15, 67), with hemolytic isolates of this species containing a homologue of the main virulence gene cluster (i.e., the *prfA* cluster), which carries key virulence genes in *L. monocytogenes* and *L. ivanovii* (21). An additional nonhemolytic species, *Listeria grayi*, has not been formally excluded as a member of the genus *Listeria* but has been shown to be very different from the other *Listeria* spp. (4, 59, 65). Although proposed at one time to represent a new genus, *Murraya* (61, 62), *L. grayi* is currently considered a *Listeria* species (11). Two new nonhemolytic *Listeria* species (i.e., *Listeria rocourtii* and *L. marthii*) were reported in 2010 (24, 38). Due to the importance of *L. monocytogenes* as a human food-borne and animal pathogen, there has been considerable effort to understand the epidemiology and distribution of *L. monocytogenes* and other *Listeria* spp. in human and animal disease, foods, and food-processing plants. Only limited information is available, though, on the occurrence of different *Listeria* spp. outside food processing plants and in natural environments. Many have demonstrated that *Listeria* spp. can be isolated from various different environments, including soil, vegetation, surface water, sewage, animal feeds, farm environments, and food-processing environments (58). Most studies on *Listeria* prevalence in the natural environment have focused on farm environments and associated croplands (8, 16, 30, 35, 43, 45), and only limited data on *Listeria* prevalence in nonagricultural environments (usually urban and suburban environments) are available (28, 29, 40, 47, 48, 71). A number of reports indicate a fairly high prevalence of *Listeria* spp. (often >20%) in various environments (58). For example, in 1975 Weis and Seeliger (69) reported *Listeria* species prevalences in vegetation samples ranging from 9.7 to 44% for samples from agricultural areas and from 21.3 to 23.1% for samples from nonagricultural areas. The same study reported even higher prevalences of *Listeria* spp. in soil samples, ranging from 8.7 to 51.4% for agricultural sites and from 15.2 to 43.2% for nonagricultural sites. Primarily due to subsequent changes in the taxonomy of *Listeria* (51), many of the earlier studies (e.g., reference 69) did not include reliable information on the diversity of *Listeria* spp. present in different environments. Some smaller, more-recent studies indicate considerable *Listeria* species diversity in samples collected from various environments. For example, a survey of urban environments in the United Kingdom showed that *L. ivanovii* and *L. seeligeri* represented the most-common *Listeria* spp. isolated from soil samples (40). Studies of surface water samples in different countries have identified *L seeligeri*, *L. innocua*, *L. welshimeri*, *L. ivanovii*, *L. monocytogenes*, and *L. grayi* (2, 19), with *L. seeligeri* (19) and *L. monocytogenes* reported as the most-prevalent species. In contrast, most studies on foods and food-processing environments seem to find *L. monocytogenes* and *L. innocua* as the most prevalent *Listeria* spp. (40).

Even though different studies have provided evidence that *Listeria* spp. are broadly distributed through the natural environment, our understanding of the ecology and reservoirs of *Listeria* species and *L. monocytogenes* is fairly limited. Even though molecular subtyping and characterization methods are now commonly used to characterize *Listeria* isolates (58), stud-

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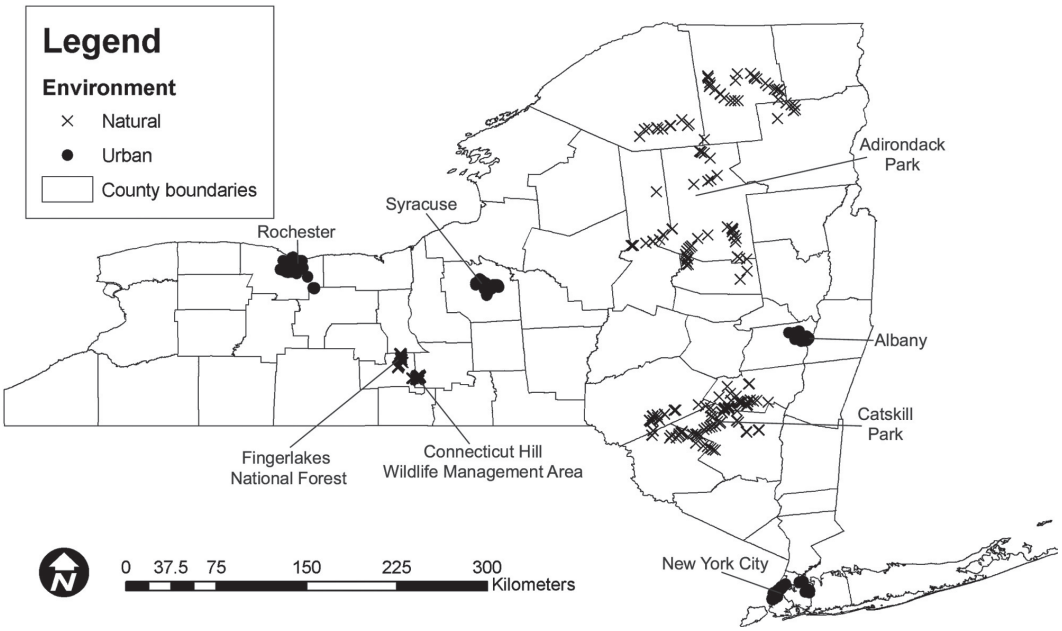

FIG 1 Distribution of sample locations across New York State. The geographic distribution of all locations sampled in four urban and four natural areas across New York State in 2001 and 2002 is shown. Northerly direction and distance scale (km) as indicated.

ies that include subtyping methods to characterize the ecology of *Listeria* spp. outside farm and food-processing environments are limited. Many studies on the distribution of *Listeria* in natural environments were actually conducted before molecular subtyping methods were available. Serotyping was most often used for strain discrimination in these studies but has been shown to have low discriminatory power and does not provide for reliable species-level identification (25). This study was designed to provide a better understanding of the occurrence of the genus *Listeria* outside food-processing and production and farm systems. A better understanding of the ecology of this genus not only will provide the basis for understanding the population genetics and natural history of closely related pathogenic and nonpathogenic *Listeria* species but is also needed to critically evaluate the validity of using the presence of any *Listeria* species as an indicator for *L. monocytogenes* contamination.

MATERIALS AND METHODS

Sample collection. Samples were collected over 2 years (2001 and 2002) from multiple locations in four urban areas (Albany, New York City, Syracuse, and Rochester, NY) (Fig. 1) and four areas representing natural environments (Adirondack Park, Catskill Park, Connecticut Hill Wildlife Management Area [CT Hill], and Finger Lakes National Forest [FLNF]) (Fig. 1). The overall sampling scheme thus included two types of environment (which we designated "urban" and "natural"), four different areas for each type of environment, and multiple sampling locations within each area. While some samples collected in urban areas could also be considered "natural," we used this designation, as the rural natural environments sampled represented undeveloped areas that are relatively undisturbed by human activity (i.e., state parks and wildlife management areas).

In 2001, approximately even numbers of samples from urban (n = 295) and natural (n = 304) environments were obtained through 2 or 3 visits per area throughout spring, summer, and autumn. In 2002, every area was sampled once in each season (spring, summer, and autumn, yielding 594 and 612 samples from urban and natural areas, respectively). Samples were collected from different locations, and no exact location was sampled twice.

For samples collected in 2001, geographic location data were retrospectively obtained by plotting the sample locations in TopoUSA 4.0 (Delorme, Yarmouth, ME). In 2002, geographic location data were collected at the time of sampling using a Garmin Emap (Garmin International, Inc., Olathe, KS) handheld global positioning system (GPS) receiver and imported electronically into GPS Utility 4.04 (http://www.gpsu.co.uk). Geographic location data for each sample site were checked for accuracy by importing latitude and longitude coordinates into TopoUSA 4.0 and by comparing site locations with coordinate designations.

For areas representing natural environments, similar numbers of soil, vegetation, and surface water samples were collected. Soil and vegetation samples were collected from fields or forests; surface water samples were collected from standing water (pond/lake, swamp, or puddle) or flowing water (river/stream or runoff). Sample types collected in urban areas included soil and vegetation, surface water (standing or flowing), and sponge swipes of floors, sidewalks, and human contact surfaces (e.g., automated teller machines, benches, door handles, trash cans, mailboxes, parking meters, public telephones, picnic tables, railings, and vending machines). Soil and vegetation samples were taken from parks and playgrounds, surface water samples were taken from all available sources, and environmental sponges were taken from downtown areas and shopping malls. For soil and vegetation samples, approximately 50 to 100 g of material was collected, and for water samples, approximately 600 ml was collected. Sponges were rehydrated with 10 ml of sterile neutralizing buffer (Hardy Diagnostics, Santa Maria, CA) before the sampling. Samples were aseptically collected into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), using sterile gloves and/or presterilized disposable plastic spatulas or scoops, and held on wet ice up to 24 h before culture.

Isolation of *Listeria*. *Listeria* spp. and *L. monocytogenes* were isolated using selective enrichment in *Listeria* enrichment broth (LEB; Difco, Sparks, MD), followed by plating on Oxford medium (OX; Difco). Oxford agar was chosen because it is one of the selective agars specified by the U.S. FDA for the isolation of *Listeria* from foods. A 25-g aliquot of soil or vegetation was added to 225 ml of LEB in a Stomacher bag (Seward, Ltd.,

Norfolk, United Kingdom) and either mixed well by hand or mechanically stomached (model 400 Seward stomacher; Seward, Ltd.) for 2 min. For water samples, approximately 500 ml of each sample was filtered through at least three 150-ml Nalgene analytical filter (0.45-μm) units (Nalgene, Rochester, NY); some samples with high solid-content levels required more than three filters to achieve a filtered volume of 500 ml. Filters were subsequently placed into 100 ml of LEB and stomached for 2 min. For environmental sponges, 90 ml of LEB was added to each sponge, followed by manual mixing. After 24 and 48 h of incubation at 30°C, 100 μl of LEB was streaked onto OX as described previously (64). After incubation for 48 h at 30°C, up to four *Listeria*-like colonies (black, greenish, or gray colonies with esculin hydrolysis) were subcultured from OX to *L. monocytogenes* plating medium (LMPM; Biosynth International, Inc., Naperville, IL), a plating medium that differentiates *L. monocytogenes* and *L. ivanovii* (which appear as blue colonies due to phosphatidylinositol-specific phospholipase C activity) from other *Listeria* spp. (white colonies) (50). LMPM plates were incubated for up to 48 h at 37°C. Individual colonies on LMPM were classified as putative *L. monocytogenes*/*L. ivanovii* (blue), putative other *Listeria* spp. (white), or non-*Listeria* (atypical morphology and/or color).

Screening, confirmation, and phenotypic characterization. Putative *L. monocytogenes*/*L. ivanovii* isolates were confirmed as *L. monocytogenes* using an *L. monocytogenes*-specific PCR assay targeting *hly* (72); this assay does not amplify *L. seeligeri* or *L. ivanovii hly*. In 2001, all other putative *Listeria* isolates (i.e., white colonies on LMPM) were identified to the species level using Gram-stain, motility at 25°C, oxidase, catalase, and hemolysis production and API-*Listeria* test strips (3). Since analysis of the putative *Listeria* isolates from 2001 showed that none of the nonmotile isolates represented *Listeria*, we concluded that the motility test is an appropriate screening tool for eliminating isolates that are not *Listeria*. Putative *Listeria* isolates from 2002 were thus screened using the motility test only and subsequently identified to the species level and subtyped using *sigB* sequencing as described below.

Gene sequencing and phylogenetic analysis. All *Listeria* isolates were characterized by PCR amplification and sequencing of the partial open reading frame (ORF) of the stress response gene *sigB*. In addition, all isolates collected in 2001 were characterized by PCR amplification and sequencing of the housekeeping gene *gap*. PCR amplification and sequencing of *sigB* and *gap* were performed as described previously (46); for both genes, PCR products were directly sequenced in both directions. After sequences were assembled and proofread using Seqman (DNAStar, Madison, WI), they were aligned in Megalign (DNAStar) using the ClustalW algorithm and trimmed to consistent length for analysis. Allelic types (ATs) were assigned using DNAsp 4.06 (http://www.ub.es/dnasp); two gene sequences were classified as different allelic types if they differed by at least 1 nucleotide.

Phylogenetic analysis for *sigB* and *gap* allelic types was performed using a single isolate representing each unique allelic type. Phylogenetic trees were created in PAUP* (63) using the neighbor-joining (NJ) method (17). NJ trees were rooted with homologous gene sequences from *Bacillus subtilis* (http://genolist.pasteur.fr/SubtiList/) and bootstrapped for 2,000 replicates.

Simpson's index of discrimination. Simpson's index of diversity and 95% confidence intervals were calculated as previously described (27, 32).

Categorical analysis. Chi-square tests or a Fisher exact tests (if expected values were less than 5) were used to evaluate associations between *Listeria* spp. or *sigB* allelic types (representing subtypes within the different *Listeria* species) and (i) environmental source (urban or natural), (ii) individual areas (Albany, New York City, Syracuse, Rochester, Adirondacks, Catskills, CT Hill, or FLNF), (iii) specific sample sources (soil, vegetation, or surface water), (iv) and/or season (spring, summer, or fall). Since these tests determine whether the prevalences of different *Listeria* spp. differed among environments or sites, we used the total number of samples as a denominator (rather than the total number of isolates). Since one *L. monocytogenes* isolate and one other *Listeria* species isolate were identified in 30 samples, the overall chi-square analysis included two observations for each of these 30 samples. Thus, our denominator for each overall contingency table was 1,835 observations, while for individual 2-by-2 tables our denominator was the actual number of samples ($n = 1{,}805$).

All categorical analyses were performed using SAS 9.1 (SAS Institute, Inc., Cary, NC). P values of ≤ 0.05 were considered statistically significant and were not adjusted for the fact that multiple comparisons were made. Due to the large number of associations that were tested, it could be contended that the probability of a type 1 error was inflated and that the significance threshold should be adjusted. While we provide observed P values to avoid missing possible associations (caused by a very conservative P value), readers can evaluate the significance levels according to their preferred criteria (53).

Spatial analysis. GPS data for all samples and isolates were imported into ArcGIS 9 (ESRI, Redland, CA) for spatial analyses. Nearest-neighbor analysis (68) as implemented in CrimeStat 2.0 (http://www.nedlevine.com/nedlevine17.htm) was used to test for spatial clustering of *Listeria* spp. and *sigB* allelic types that were significantly associated with specific sampling areas. Nearest-neighbor analysis provides an approximation as to whether points are more clustered or dispersed than would be expected by chance (68). Typically, this type of analysis compares the average observed distance of the nearest neighbor to a random distance by calculating the nearest-neighbor index (NNI), which is obtained by dividing the average observed nearest-neighbor distance by the expected random distance (39). To perform spatial clustering analysis, site-specific maximal dispersal boundaries were used to standardize comparisons to the same geographic area around each site. For each of the 8 sampling areas, a rectangular border was drawn around the maximum point dispersion in each geographic direction to approximate the size of a given area. Spatial coordinates for the sample locations that yielded the species or allelic type of interest in a specific site were subjected to nearest-neighbor analysis. In addition, equal numbers of randomly selected sample locations (chosen from all sample locations within a given site using SPSS 13.0 [SPSS, Inc., Chicago, IL]) were also subjected to the same nearest-neighbor analysis. Selection and nearest-neighbor analysis of random points were repeated 10 times, and the average mean nearest-neighbor distance (MNND) and its standard deviation were calculated. This random MNND was then compared using a 1-sample t test to the actual mean nearest-neighbor distance; P values of ≤ 0.05 were considered significant. If the observed MNND was significantly lower than the MNND for the randomly sampled locations, we concluded that there was evidence for clustering of the species or allelic types of interest.

Isolate and data curation. All *Listeria* isolates were frozen at −80°C in brain heart infusion (BHI; Difco) broth containing 15% glycerol; all isolate characterization, including subtyping, was performed within <2 years after isolation. Isolate source information and subtyping data from this study are freely available through the Pathogen Tracker 2.0 database (http://www.pathogentracker.net).

RESULTS

Isolation and identification of *Listeria* spp. and *L. monocytogenes*. Putative *Listeria* isolates were obtained from 525 (29%) of the 1,805 samples collected in 2001 and 2002. These samples yielded a total of 563 putative *Listeria* isolates, including 485 isolates that were white on LMPM and 78 isolates that were blue on LMPM. All 78 isolates that were blue on LMPM were confirmed as *L. monocytogenes* by *hly* PCR (72). Among the 485 isolates that were initially white on LMPM (indicating *Listeria* spp. other than *L. monocytogenes* or *L. ivanovii*), 370 were motile at 25°C. The *Listeria*-specific *sigB* PCR assay (46) yielded PCR products for 362 of these isolates, all of which were confirmed as *Listeria* spp. by sequencing of the *sigB* PCR product. Two of the putative *Listeria* species isolates from 2001 were identified as *L. monocytogenes*. In summary, we isolated and confirmed a total of 80 *L. monocytogenes* isolates (78 that were

TABLE 1 *sigB* and *gap* allelic-type diversity among a subset of 119 isolates used to validate a rapid method for identifying isolates of *Listeria*[a]

Species (no. of isolates)	No. of allelic types based on:	
	sigB	*gap*
L. marthii (1)	1	1
L. innocua (9)	8	2
L. monocytogenes (18)	3	7
L. welshimeri (20)	11	2
L. seeligeri (71)	12	8
Total (119)	35	20

[a] The method used morphology on *Listeria monocytogenes* plating medium (LMPM), motility at 25°C, and *sigB* sequencing.

initially blue on LMPM and 2 that were initially white) and 362 isolates representing other *Listeria* spp.

Species-level identification and *sigB* allelic-type characterization of *Listeria* isolates. In order to rapidly identify isolates to the species level and subtype them, we characterized all 119 *Listeria* isolates collected in 2001 (including 18 *L. monocytogenes*) by sequencing of *sigB* and *gap*. Preliminary phylogenetic analysis of the 119 *gap* sequences revealed clear clustering of all *L. seeligeri* (8 allelic types) and *L. welshimeri* (2 allelic types) *gap* sequences (with 31 phylogenetically informative sites). *L. innocua*, *L. monocytogenes*, and *L. marthii* did not form monophyletic clusters, probably due to the low number of phylogenetically informative sites ($n = 9$) among the *gap* sequences for these species. *sigB* sequencing provided more-discriminatory subtyping (35 *sigB* versus 20 *gap* allelic types) (Table 1) and more-reliable species identification. We thus chose *sigB* sequencing as a molecular species-level identification and subtyping method (allowing for differentiation within species) for the remainder of this study. *sigB* sequencing has also recently been validated, in a multilocus sequence typing (MLST) study, as a reliable method for identification of *Listeria* isolates to the species level (14).

A neighbor-joining tree based on a 660-bp *sigB* sequence alignment, which represented all allelic types found among the 442 *Listeria* isolates characterized, revealed five well-supported *sigB*

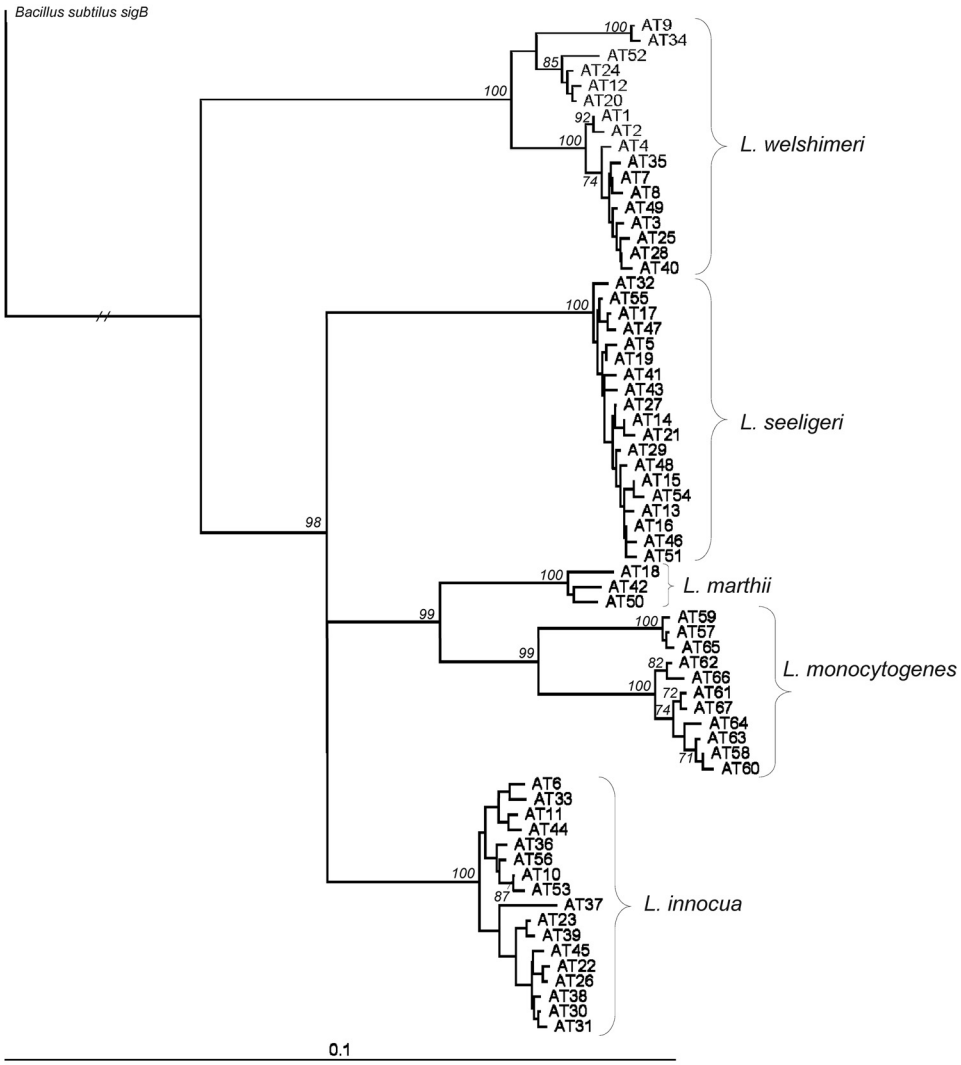

FIG 2 Phylogenetic tree of unique *sigB* allelic types. Neighbor-joining tree rooted with *Bacillus subtilis*; branch length for *B. subtilis* collapsed (//) by a factor of 100 for display purposes. Bootstrap values were obtained from 2,000 replicates; bootstrap values of >70 are shown.

TABLE 2 Distribution of *sigB* allelic types among 442 *Listeria* isolates from 2001 and 2002 by species, environment, and sampling area

Species	*sigB* allelic type	No. of *Listeria* isolates from indicated site[a]										
		Natural					Urban					
		ADK	Catskills	CT Hill	FLNF	Subtotal	Albany	NYC	Rochester	Syracuse	Subtotal	Total
L. marthii	18	0	0	1	1	2	0	0	0	0	0	2
	42	0	0	1	0	1	0	0	0	0	0	1
	50	0	0	1	0	1	0	0	0	0	0	1
Subtotal		0	0	3 (+)*	1	4	0	0	0	0	0	4
L. innocua	6	0	0	0	0	0	3	2	0	2	7	7
	10	0	0	0	0	0	1	0	0	1	2	2
	11	0	0	0	0	0	1	2	0	1	4	4
	22	0	0	0	0	0	0	2	2	0	4	4
	23	0	0	0	0	0	1	2	2	1	6	6
	26	0	0	0	1	1	1	0	2	0	3	4
	30	0	0	0	0	0	0	0	0	1	1	1
	31	0	0	0	0	0	1	1	0	0	2	2
	33	0	0	0	0	0	0	0	1	0	1	1
	36	0	0	0	0	0	1	2	2	0	5	5
	37	0	0	0	0	0	1	3	1	0	5	5
	38	0	0	0	0	0	0	1	0	0	1	1
	39	0	0	0	0	0	0	2	0	0	2	2
	44	0	0	0	0	0	1	0	0	0	1	1
	45	0	0	0	0	0	2	1	0	0	3	3
	53	0	0	0	0	0	0	1	0	0	1	1
	56	0	0	0	0	0	1	0	0	0	1	1
Subtotal		0	0	0	1	1	14	19 (+)**	10	6 (−)***	49 (+)***	50
L. monocytogenes	57	4	4	1	2	11	9	6	4	11	30 (+)**	41
	58	0	0	1	0	1	11 (+)***	1 (−)*	3	3	18 (+)***	19
	59	0	1	0	0	1	1	0	0	1	2	3
	60	0	0	0	0	0	0	0	0	1	1	1
	61	0	0	0	0	0	0	2	0	1	3	3
	62	0	0	0	0	0	0	0	0	1	1	1
	63	0	0	0	0	0	0	1	0	0	1	1
	64	0	0	0	0	0	5	2	0	0	7	7
	65	0	0	0	0	0	0	1	0	0	1	1
	66	0	0	0	0	0	0	1	0	0	1	1
	67	0	0	0	0	0	1	1	0	0	2	2
Subtotal		4	5	2	2	13	27 (+)**	15	7 (−)*	18	67 (+)***	80
L. seeligeri	1	8	6 (−)**	35 (+)***	15	64 (+)***	5	1	4	4	14	78
	2	0 (−)*	1 (−)*	4	11 (+)***	16 (+)**	0	0	0	3 (+)*	3	19
	3	3	11	6	8	28 (+)***	1	2	1	3	7	35
	4	0	2	2	4	8	0	0	2	2	4	12
	7	0	2	0	0	2	3	3	2	5	13 (+)**	15
	8	0	0	0	0	0	0	0	0	3	3	3
	9	0	0	0	0	0	0	1	0	2	3	3
	12	3	4	1 (−)*	10 (+)*	18	8 (+)*	2	2	3	15	33
	20	0	0	0	1	1	3	0	2	1	6	7
	24	0	0	0	0	0	0	3	3	1	7	7
	25	0	0	0	0	0	1	0	2	0	3	3
	28	1	1	1	1	4	3	2	1	1	7	11
	34	0	0	0	0	0	0	0	0	1	1	1
	35	0	0	0	0	0	0	3	0	0	3	3
	40	0	1	0	0	1	0	0	0	0	0	1
	49	0	0	1	1	2	0	0	0	0	0	2
	52	0	0	0	0	0	0	1	0	0	1	1

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TABLE 2 (Continued)

Species	*sigB* allelic type	No. of *Listeria* isolates from indicated site[a]										
		Natural					Urban					
		ADK	Catskills	CT Hill	FLNF	Subtotal	Albany	NYC	Rochester	Syracuse	Subtotal	Total
Subtotal		15 (−)**	28 (−)*	50 (+)***	51 (+)*	144 (+)***	24	18	19	29	90	234
L. welshimeri	5	0	0	0	0	0	0	0	0	1	1	1
	13	0	0	0	0	0	0	0	0	1	1	1
	14	3	8 (+)**	1	0 (−)*	12 (+)***	0	0	0	0	0	12
	15	2	3	0	0	5	0	0	0	0	0	5
	16	2	5	3	0 (−)*	10	1	1	1	0	3	13
	17	0	2	1	2	5	0	0	0	0	0	5
	19	0	1	0	1	2	0	0	0	0	0	2
	21	0	8 (+)***	0	1	9 (+)*	0	0	0	2	2	11
	27	1	1	2	0	4	3	1	1	0	5	9
	29	0	0	0	0	0	0	0	0	1	1	1
	32	0	0	0	0	0	1	0	0	0	1	1
	41	0	0	0	0	0	2	0	0	0	2	2
	43	0	0	0	0	0	1	0	0	1	2	2
	46	0	0	0	0	0	0	0	1	0	1	1
	47	0	0	0	0	0	0	0	1	0	1	1
	48	0	0	0	0	0	0	0	1	0	1	1
	51	1	0	0	0	1	0	0	0	0	0	1
	54	0	1	0	0	1	0	0	0	0	0	1
	55	0	3	0	0	3	1	0	0	0	1	4
Subtotal		9	32 (+)***	7	4 (−)**	52 (+)***	9	2	5	6	22	74
Total		28	65	62	59	214	74	54	41	59	228	442

[a] *Listeria* species or *sigB* allelic-type prevalences that were significantly higher (+) or lower (−) for a specific environment (urban/pristine) or site as determined by categorical analyses were marked "*" ($P \leq 0.05$), "**" ($P \leq 0.005$), or "***" ($P \leq 0.0005$). ADK, Adirondacks; CTHill, Connecticut Hill Wildlife Management Area; FLNF, Finger Lakes National Forest; and NYC, New York City.

clusters (Fig. 2). Clustering of *sigB* sequences of the respective isolates was used to confirm and assign isolates to genospecies, including 234 *L. seeligeri*, 80 *L. monocytogenes*, 74 *L. welshimeri*, 50 *L. innocua*, and 4 *L. marthii* isolates (24). The 442 isolates represented 67 *sigB* allelic types (Table 2), including 19, 17, 17, 11, and 3 allelic types for *L. welshimeri*, *L. seeligeri*, *L. innocua*, *L. monocytogenes*, and *L. marthii*, respectively.

Prevalence of *Listeria* species in natural and urban environments. Among the 907 and 898 samples collected, over 2 years, from urban and natural environments, 23.4% and 22.3% were positive for *Listeria*, respectively (Table 3; see also Tables S1 and S2 in the supplemental material). Among the 412 *Listeria*-positive samples, 30 samples (2 and 28 from natural and urban areas, respectively) allowed for isolation of *L. monocytogenes* as well as another *Listeria* species; the 412 positive samples thus yielded 442 *Listeria* isolates (Tables 2 and 3). An initial overall 2-by-6 chi-square test clearly showed that the different *Listeria* spp. were not randomly distributed among urban and natural environments ($P \leq 0.0001$) (Table 2). Subsequent 2-by-2 chi-square tests showed that *L. seeligeri* and *L. welshimeri* were significantly more common ($P \leq 0.0001$) in natural environments, while *L. monocytogenes* and *L. innocua* were significantly more common ($P \leq 0.0001$) in urban environments (Table 2).

***Listeria* species prevalence by sample type (soil, plant, and vegetation) and season.** Overall *Listeria* prevalences in samples from natural environments ranged from 16% for surface water to 19% for soil and 34% for vegetation. Among samples from urban areas, *Listeria* prevalences were 33% for surface water, 30% for soil, 28% for vegetation, 19% for sidewalks/floors, and 3% for human contact surfaces. *Listeria* prevalences also differed considerably between sampling areas. For example, among natural areas, prevalences ranged from 5% in water from FLNF to 39% in vegetation from the Catskills, while among urban areas prevalences ranged from 0% (human contact surfaces in New York City and Rochester) to 40% (surface water in Albany) (Fig. 3).

The seasonal *Listeria* prevalences ranged from 16% (urban environments, spring) to 33% (urban environments, summer) (Fig. 4). Overall *Listeria* prevalences differed significantly by season among samples from urban and natural environments ($P = 0.000042$ and $P = 0.00017$, respectively). In natural environments, prevalences for overall *Listeria* as well as for each individual *Listeria* species were highest in the summer, while prevalences for all *Listeria* species as well as for individual *Listeria* species in urban areas were lowest in the summer (Fig. 4).

Distribution of *Listeria* spp. by sample environment. Separate 4-by-6 chi-square analyses for all urban and all natural environments showed that *Listeria* spp. were not independently distributed among individual natural ($P \leq 0.0001$) and urban ($P = 0.0015$) sampling areas. Subsequent 2-by-2 chi-square analyses showed significant associations between specific *Listeria* spp. and certain sampling areas, including seven significant associations between natural sampling areas and

TABLE 3 Distribution of *Listeria*-positive samples by species, environment, and sampling area among 1,805 environmental samples obtained in 2001 and 2002

Environment and area	Total no. of samples	No. of negative samples	No. of samples positive for indicated *Listeria* species[a]											
			Only 1 species						>1 species					
			LMa	LI	LM	LS	LW	Subtotal	LM+LMa	LM+LI	LM+LS	LM+LW	Subtotal	Total
Natural														
Adirondacks	187	159	0	0	4	15	9	28	0	0	0	0	0	28
Catskills	253	189	0	0	4	28	31	63	0	0	0	1	1	64
CT Hill	208	147	3	0	1	49	7	60	0	0	1	0	1	61
Finger Lakes	259	200	1	1	2	51	4	59	0	0	0	0	0	59
Subtotal	907	695	4	1	11	143	51	210	0	0	1	1	2	212
Urban														
Albany	214	150	0	13	17	17	7	54	0	1	7	2	10	64
New York City	204	157	0	14	8	17	1	40	0	5	1	1	7	47
Rochester	207	171	0	10	2	15	4	31	0	0	4	1	5	36
Syracuse	273	220	0	4	12	25	6	47	0	2	4	0	6	53
Subtotal	898	698	0	41	39	74	18	172	0	8	16	4	28	200
Total	1,805	1,393	4	42	50	217	69	382	0	8	17	5	30	412

[a] LMa, *L. marthii*; LI, *L. innocua*; LM, *L. monocytogenes*; LS, *L. seeligeri*; and LW, *L. welshimeri*. Detection of *L. monocytogenes* as well as another *Listeria* species was possible, as *L. monocytogenes* can be differentiated from other *Listeria* spp. on LMPM; the presence of two non-*L. monocytogenes Listeria* spp. in the same sample would not be detected with the approach used here, as only a single non-*L. monocytogenes Listeria* species colony was further characterized for each sample.

specific *Listeria* spp. and four significant associations between urban areas and specific *Listeria* spp. (Table 2). For example, *L. seeligeri* was positively associated with CT Hill ($P \leq 0.0005$) and FLNF ($P \leq 0.05$), while *L. welshimeri* was positively associated with the Catskills ($P \leq 0.0005$).

Spatial clustering of *Listeria*. *Listeria* spp. that were associated with a specific sample site were also analyzed for evidence of spatial clustering using nearest-neighbor analysis (Table 4). Among the natural areas, we identified three instances of spatial clustering, including clustering of (i) *L. welshimeri* in the Catskills (Fig. 5), (ii) *L. seeligeri* in CT Hill, and (iii) *L. seeligeri* in FLNF (Table 4). Among the urban areas, we only identified evidence for spatial clustering of *L. innocua* in New York City (Table 4). All spatial clusters included isolation of the same species on at least four separate sampling dates, often several months apart. *L. welshimeri* was isolated from the Catskills on 5 out of 5 sampling dates, *L. seeligeri* was isolated from each CT Hill and FLNF on 5 out of 5 sampling dates, and *L. innocua* was isolated from New York City on 4 out of 6 sampling dates.

Distribution of *Listeria sigB* allelic types by sample environment (natural and urban). For categorical analysis of associations between *Listeria sigB* allelic types and urban and natural environments, individual allelic types with fewer than 10 occurrences were combined into a single category to avoid multiple categories with expected values less than 5. An initial overall 2-by-14 chi-square analysis showed that allelic types were not independently distributed between urban and natural environments ($P \leq 0.0001$). Subsequent 2-by-2 chi-square analyses showed that three and five allelic types were significantly more common among urban and natural environments, respectively (Table 2).

Distribution of *Listeria sigB* allelic types by sampling environment. Overall 4-by-14 chi-square tests showed that individual *sigB* allelic types were not independently distributed among either natural ($P \leq 0.0001$) or urban ($P \leq 0.0001$) sampling areas. Subsequent 2-by-2 chi-square analyses showed a significant positive association of five allelic types with specific natural areas (Table 2). For example, *L. seeligeri* allelic type 1 was significantly more likely to be isolated from samples collected in CT Hill ($P \leq 0.0005$). In addition, three allelic types showed a significant positive association with specific urban areas (Table 2).

Spatial clustering of individual *Listeria sigB* allelic types. *Listeria sigB* allelic types (ATs) that were specifically associated with individual sample areas were also analyzed for evidence of spatial clustering (Table 4). Among the natural areas, three instances of spatial clustering of *sigB* ATs were observed, including clustering of *L. welshimeri* ATs 14 and 21 in the Catskills (Table 4 and Fig. 5) and clustering of *L. seeligeri* AT 1 in CT Hill (Table 4). Among the urban areas, only one instance of spatial clustering was observed (allelic type 58 in Albany) (Table 4). All spatial clusters included isolation of the same AT on at least two separate sampling dates. ATs 14 and 21 were isolated from the Catskills on 3 out of 5 and 2 out of 5 sampling dates, respectively, AT 1 was isolated from CT Hill on 5 out of 5 sampling dates, and AT 58 was isolated from Albany on 3 out of 4 sampling dates.

***Listeria sigB* allelic-type diversity.** *sigB* AT data were used to evaluate overall subtype diversity as well as subtype diversity stratified by species and environment (urban or natural) (Table 5). The overall Simpson index of diversity (D), based on *sigB* ATs, was 0.939; D was higher for isolates from urban environments (0.958) than for isolates from natural environments (0.871). For *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*, the overall D was also higher for isolates from urban environments than for isolates from natural environments; these differences were significant for

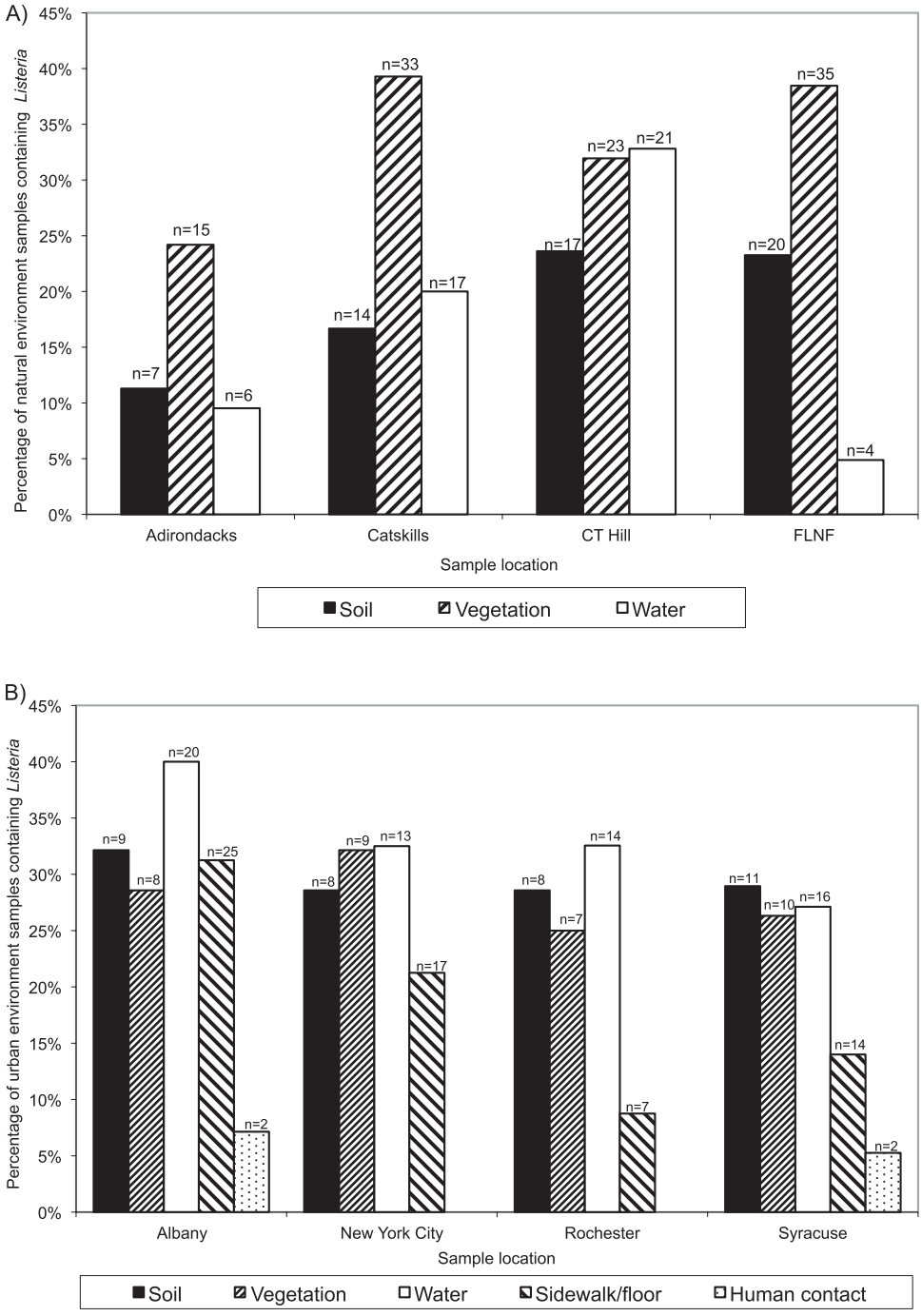

FIG 3 *Listeria* species isolation frequency by sample type. Prevalence of *Listeria* spp. (percentage of positive samples) in different sample types (soil, vegetation, water, sidewalk/floors, and human contact surfaces) collected from (A) natural and (B) urban environments (2001 and 2002).

L. seeligeri and *L. monocytogenes* (as demonstrated by nonoverlapping 95% confidence intervals).

DISCUSSION

Testing of more than 1,800 samples collected from urban and natural environments, in conjunction with phenotypic and molecular characterization of isolates, allowed us to assess the diversity and distribution of the genus *Listeria* in a well-defined geographic region (New York State) in areas other than farm environments and food-processing and production facilities. Our data show that even though *Listeria* represents a broadly disseminated genus, *Listeria* spp. as well as some specific subtypes within the species appear to show distinct ecological preferences. In addition, most *Listeria* spp. appear to include a proportion of subtypes that can establish persistent yet geographically dispersed populations outside food-processing and farm-associated environments, a trait likely to contribute to the wide geographical distribution of the genus *Listeria*.

PCR amplification and sequencing of *sigB* allow for rapid

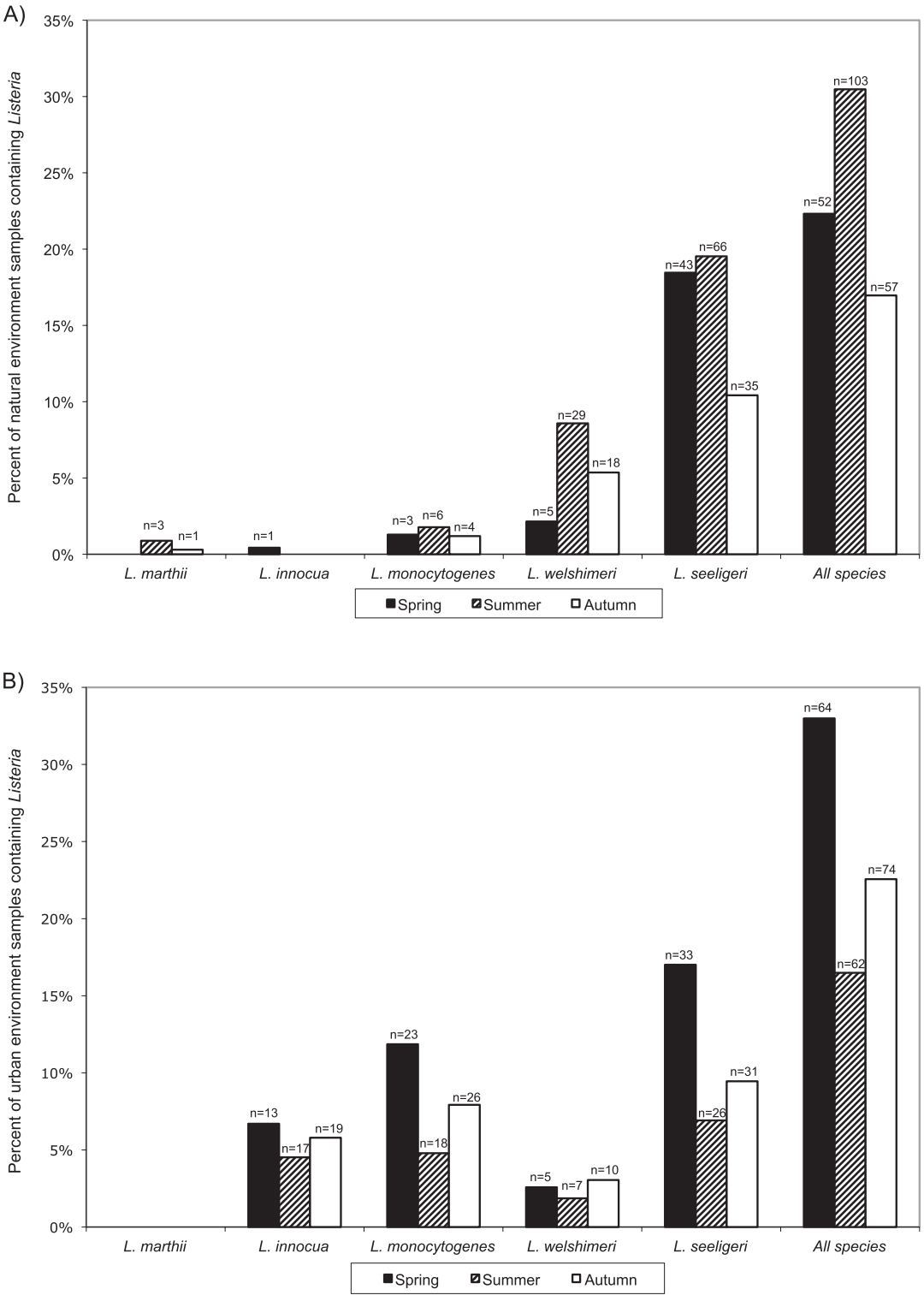

FIG 4 *Listeria* species isolation frequency by season. Seasonal prevalence (average of 2001 and 2002 data) of each *Listeria* species expressed as the percentage of positive samples from natural (A) and urban (B) environments with the corresponding number of positives samples indicated. A total of 233, 338, and 336 samples from natural environments and 194, 376, and 328 samples from urban environments were collected during spring, summer, and autumn, respectively.

and reliable species-level identification and subtyping of *Listeria* isolates. In addition to an MLST study that indicated that *sigB* sequence data allow for reliable species classification of *Listeria* isolates (14), another previous study (44), showed a good discriminatory power of *sigB* allelic typing based on 157 *L. monocytogenes* isolates. The data reported here further validate this assay as a

TABLE 4 Spatial clustering, using nearest-neighbor analysis, of *Listeria* spp. and *sigB* allelic types associated with a specific area among isolates from 2001 and 2002

Environment	Area	Size of area (km^2)	No. of sample locations	Species (allelic type)	No. of positive samples	Actual MNND[a] (km)	Random MNND (km)	P^b	Clustering
Natural	Catskills	5,743	253	*L. welshimeri*	32	1.967	2.853	0.001	Yes
				L. welshimeri (AT14)	8	5.787	8.887	0.007	Yes
				L. welshimeri (AT21)	8	3.185	7.448	0.003	Yes
	CT Hill	40	208	*L. marthii*	3	1.473	1.516	NS	No
				L. seeligeri	50	0.1357	0.1794	0.001	Yes
				L. seeligeri (AT1)	35	0.1822	0.2123	0.013	Yes
	FLNF	59	259	*L. seeligeri*	51	0.0711	0.1191	≤0.0001	Yes
				L. seeligeri (AT2)	11	0.4559	0.4963	NS	No
				L. seeligeri (AT12)	10	0.6856	0.5644	NS	No
Urban	Albany	151	214	*L. monocytogenes*	27	0.4579	0.4675	NS	No
				L. monocytogenes (AT58)	11	0.4863	0.749	≤0.0001	Yes
				L. seeligeri (AT12)	8	0.959	1.169	NS	No
	NYC	511	204	*L. innocua*	19	0.4868	1.013	0.003	Yes
	Syracuse	274	273	*L. seeligeri* (AT2)	3	1.968	3.087	NS	No

[a] MNND, mean nearest-neighbor distance.
[b] Value for a 1-sample *t* test comparing actual and random mean nearest-neighbor distances. NS, not statistically significant ($P > 0.05$).

low-cost discriminatory tool for initial subtype characterization of *Listeria* species isolates. In comparison, sequencing of the 16S rRNA, the 23S rRNA, and the 16S-23S rRNA intergenic regions has been shown to provide very limited subtype discrimination between *Listeria* species isolates (13, 22, 23, 54). One previous study also showed that *L. monocytogenes* isolates representing eight EcoRI ribotypes could be differentiated into 10 *sigB* allelic types (7), indicating that *sigB* sequencing provides discriminatory power similar to other, broadly used, but more-expensive subtyping methods. While it is likely that highly discriminatory molecular subtyping, such as pulsed-field gel electrophoresis (PFGE) or MLST, would provide further improved subtype differentiation of the isolates collected here, *sigB* sequencing allows for subtype discrimination within each *Listeria* species and provides a highly economical approach (<$15/isolate) for subtyping of large isolate sets. Further characterization of a subset of *L. monocytogenes* isolates reported here by MLST (including *sigB*, *gap*, *prs*, *ribC*, *purM*, *inlA*, and *actA* sequencing) and automated EcoRI ribotyping indeed showed improved subtype discrimination (55), but at a considerably higher cost per isolate.

While *Listeria* is ubiquitous and found in both urban and natural environments, individual *Listeria* spp. and *sigB* allelic types differ significantly in their prevalences between urban and natural environments and among specific sample sites and seasons. Seeliger noted in 1961 (60) that outbreaks of animal listeriosis on multiple continents "gave rise to the assumption of the organisms being globally spread. . . [and]. . . that neither geographical nor geomedical borders exist." Further studies by Seeliger and others subsequently confirmed that isolates classified as *L. monocytogenes* were indeed widespread among vegetation and soil samples collected from agricultural and nonagricultural sites (69–71); it is important to note, though, that the taxonomy of the genus *Listeria* has been considerably revised since these early studies were reported. For example, *L. innocua* and *L. seeligeri* were defined as separate species in 1977 and 1983, respectively (59). Consistent with previous studies that noted considerable prevalences (often >20%) of *Listeria* spp. in different environments (2, 10, 18, 19, 69, 71), we also found *Listeria* prevalences of >20% among samples collected from both urban and natural environments.

Even though there were similar overall prevalences of *Listeria* in natural and urban samples, the prevalences of individual *Listeria* spp. and *sigB* allelic types differed significantly between urban and natural environments. For example, while *L. seeligeri* and *L. welshimeri* were significantly overrepresented among isolates from natural environments, *L. monocytogenes* and *L. innocua* were overrepresented among isolates from urban environments. Some species also showed significant positive associations with specific sampling areas; these associations did not appear to drive the association between specific species and natural or urban environments, though. Prior to our study reported here, only very few studies (e.g., references 1, 5, 10, 20, and 58) on the distribution of different *Listeria* spp. in different environments were available. One report from the United Kingdom found that *L. ivanovii* and *L. seeligeri* were marginally more common in urban soils than other *Listeria* spp. (40). *Listeria grayi* and *L. rocourtiae* were not identified among our isolates; however, the fact that these species were not detected may reflect poor growth for these species in the standard selective and differential enrichment and plating media used or an inability to amplify these species with our *sigB* primers rather than an absence of these species in the samples collected (as the enrichment media used have not been validated for detection of these species). We also did not identify *L. ivanovii* among our isolates. Oxford agar, which we used for primary isolation, has been shown in at least one study (42) to be inhibitory to *L. ivanovii*.

In addition to significant associations between different *Listeria* spp. and urban and natural environments, we also found associations between some *Listeria sigB* allelic types and different environments, indicating the potential existence of ecotypes within a given *Listeria* species. This is consistent with previous studies indicating the presence of host- or niche-specific *L. monocytogenes* subtypes (34) as well as with a variety of studies that have identified ecotypes of other bacterial species and genera (9). Ivanek et al.

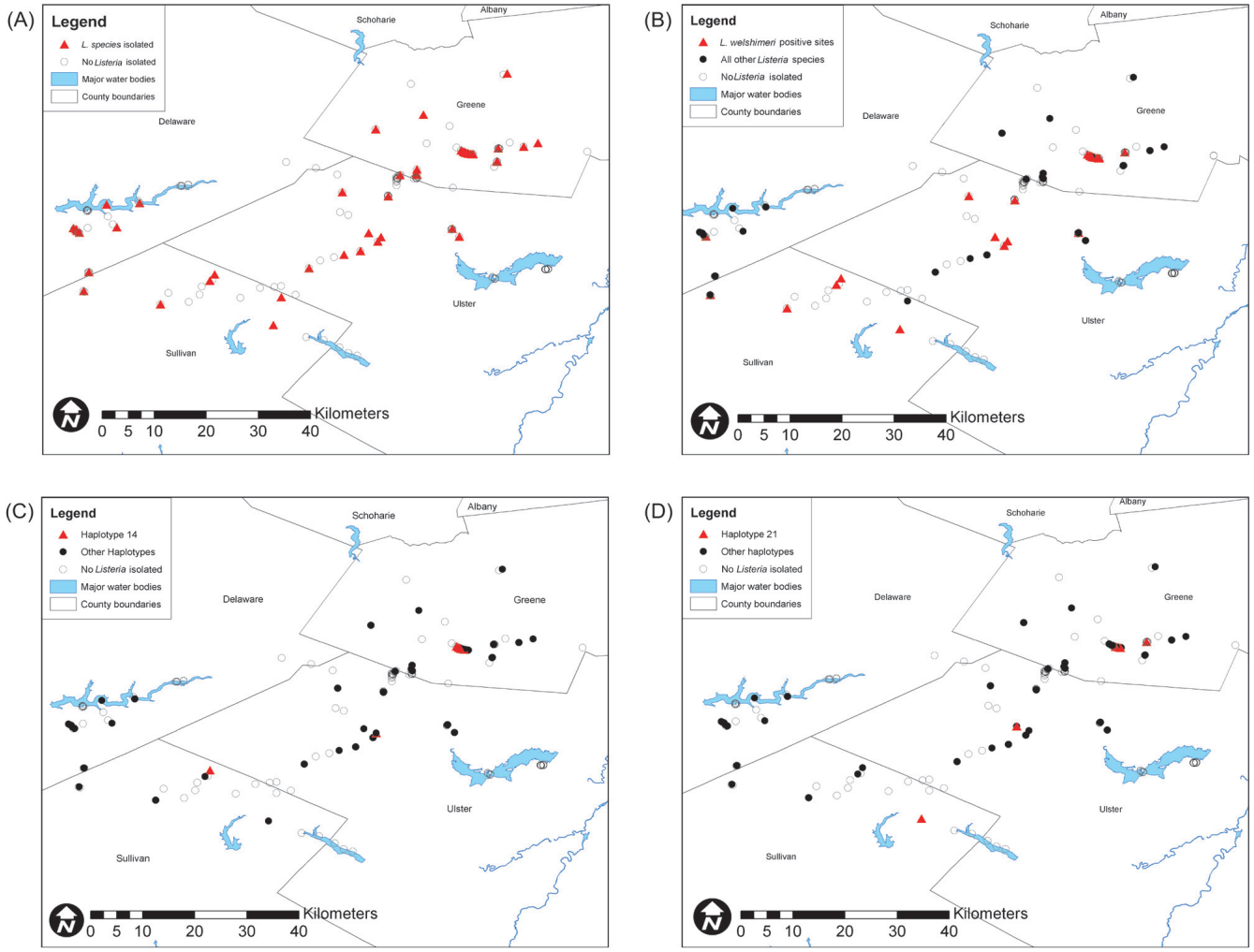

FIG 5 Spatial clustering of *L. welshimeri* and *L. welshimeri* allelic types AT14 and AT21 in the Catskill Mountains. Spatial distribution is shown for all sampling locations and all sample locations positive for *Listeria* spp. (A), sample locations positive for *L. welshimeri* and other *Listeria* spp. (B), sample locations positive for *L. welshimeri* allelic type 14 ("haplotype 14") and sample locations positive for other allelic types (regardless of *Listeria* species) (C), and sample locations positive for *L. welshimeri* allelic type 21 ("haplotype 21") and sample locations positive for other allelic types (D).

(33) recently, in a comprehensive retrospective analysis of ecological and biogeographic characteristics of the natural environment sample areas included in this study, showed that precipitation occurrence and alternating freezing and thawing temperatures prior to sample collection were key predictors for the isolation of *Listeria* spp. from a given location (33). Interestingly, analysis of seasonal data showed that *Listeria* prevalence in natural areas in the summer was the highest ($P = 0.000042$), while urban areas showed the lowest *Listeria* prevalence in the summer ($P = 0.00017$).

TABLE 5 *sigB* allelic-type diversity stratified by *Listeria* species and sample environment among isolates from 2001 and 2002

Species	Simpson index of diversity for isolates from indicated environment (no. of isolates)[a]		
	Natural	Urban	All
L. monocytogenes	0.295 (13)†	0.722 (67)†	0.678 (80)
L. seeligeri	0.737 (144)†	0.907 (99)†	0.832 (234)
L. marthii	0.833 (4)	NA (0)	0.833 (4)
L. welshimeri	0.867 (52)	0.931 (22)	0.902 (74)
L. innocua	NA (1)	0.935 (49)	0.935 (50)
Total	0.871 (214)†	0.958 (228)†	0.939 (442)

[a] "†" marks Simpson indices of discrimination for urban and natural areas that have nonoverlapping 95% confidence intervals. NA, not applicable (Simpson's index of discrimination cannot be determined for fewer than two isolates).

While these observations provide a starting point for further studies on potential physiological and genetic differences that might explain ecological preferences of different *Listeria* spp. and subtypes, future studies in other locations will also be needed to determine whether the findings reported here for New York State can be confirmed elsewhere.

The genus *Listeria* comprises a high level of genetic diversity, which appears to be higher among urban environments. Assessment of *Listeria sigB* allelic diversity showed that the overall level of diversity among *Listeria* spp. as well as the diversity stratified by species (with the exception of *L. marthii*) was higher among strains isolated from urban environments. Greater diversity among *Listeria* isolates from urban areas may suggest that urban environments allow for less-efficient dispersal of bacteria (e.g.,

due to landscape barriers), hence reducing the likelihood of isolation of the same strain from different sites. Natural environments, on the other hand, may allow for more-efficient bacterial dispersal (e.g., due to free movement of animals), thus increasing the chance of isolating the same strain at different sampling sites. Our findings furthermore could be interpreted as an indication that *Listeria* isolates from urban and natural environments represent two separate populations influenced by different ecological constraints (27). While we were not able to identify any other studies that evaluated *Listeria* diversity among isolates from different environments, our data are consistent with an emerging body of literature indicating that *L. monocytogenes* isolates found in different environments and hosts represent distinct populations (26). The true levels of *Listeria* diversity in different environments may even be higher than reported here, since traditional culture of *Listeria* from environmental samples, which requires the use of selective enrichment and plating media (12, 66), may favor recovery of certain *Listeria* spp. or lineages (6). In particular, even though most selective media designed for the recovery of *L. monocytogenes* are thought to be adequate for the recovery of other *Listeria* spp., this has not been thoroughly evaluated (12), and it cannot be excluded that the selective enrichment used here may have prevented or reduced the recovery of certain *Listeria sigB* allelic types. However, we feel that the use of a single enrichment procedure allowed an adequate initial assessment of *Listeria* diversity in different environments; selective enrichment procedures were deemed necessary to allow recovery of *Listeria* spp., which are likely to be found at low levels in environmental samples, which may contain high levels of competing background flora (2).

Certain *Listeria* spp. and *sigB* allelic types tend to cluster spatially within both natural and urban environments, and there is evidence that these species and allelic types can persist over time. Nearest-neighbor spatial analysis (68) showed that a number of *Listeria* species and allelic types showed clustering within a given area, indicating establishment of area-specific local populations in addition to the presence of widely distributed allelic types. All statistically significant spatial clusters of *Listeria* species and *sigB* allelic represented isolates from samples obtained at multiple and in some cases all sampling dates, further supporting that these clusters represent area-specific persistent populations. These findings are consistent with a number of studies that have indicated that specific *Listeria* subtypes can establish persistent populations in food-processing and retail environments (41, 52, 56), including some instances where a given subtypes seems to have persisted in a specific plant for at least 12 years (34). We propose that the ability of members of the genus *Listeria* to establish persistent populations in different environments is critical for its widespread and ubiquitous presence. Persistence of *Listeria* in urban and natural environments also has important implications for studies that use reisolation of a specific *Listeria* subtype in a processing plant or retail environment as evidence for *Listeria* persistence in that location (34, 37, 56, 57). Our data specifically indicate that reisolation of a specific *Listeria* subtype in a food-related environment that it is not well isolated from its surroundings (e.g., retail environments which cannot control outside traffic and install footbaths) may also, in some cases, present reintroduction of a subtype that persists in the surrounding environment.

Conclusions. Our data not only support that the genus *Listeria* is widely distributed and highly prevalent in many environments but also show that species and *sigB* allelic types within this genus appear to show distinct ecological preferences. The observation that *L. seeligeri*, which is nonpathogenic in mammals (31) but often carries a homologue of the *L. monocytogenes prfA* virulence gene cluster, is more common among samples from natural environments and is the most common *Listeria* species isolated there may indicate that possession of these virulence genes provides a selective advantage in these environments over other *Listeria* spp. (e.g., *L. innocua*), possibly by facilitating survival of protozoan predation as previously postulated (36). On a more practical level, our data showing species-specific prevalence patterns in different environments suggest that the use of *Listeria* spp. as an indicator for *L. monocytogenes* contamination may require refinement. For example, *L. innocua* may be a better indicator for *L. monocytogenes* contamination than *L. seeligeri* or *L. welshimeri*, which appear to occupy environmental niches distinct from that of *L. monocytogenes* (e.g., natural environments), while *L. monocytogenes* is typically associated with other environments (e.g., urban environments). The further characterization of different *Listeria* ecotypes may also ultimately facilitate development of molecular-subtyping-based source tracking approaches analogous to those developed for *Escherichia coli* source tracking (49).

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