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Substructure within *Salmonella enterica* subsp. *enterica* Isolates from Australian Wildlife[▽]

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Multilocus sequence typing of 56 *Salmonella enterica* subsp. *enterica* strains isolated from Australian wildlife hosts was performed. The results of population assignment algorithms revealed that the 56 strains could be subdivided into two distinct clades. Strains belonging to the two clades were further distinguished phenotypically, genotypically, and with respect to host distribution.

Salmonella enterica subsp. *enterica* is responsible not only for the majority of *Salmonella* infections in humans (3) but also for infections in domesticated animals (2). Members of *S. enterica* subsp. *enterica* are capable of causing a variety of disease syndromes in humans and domesticated animals, such as enteric fever, diarrhea, bacteremia, and septicemia (5).

Falush et al. (9) analyzed 207 *S. enterica* strains using multilocus sequence typing (MLST) (14) and found that *S. enterica* subsp. *enterica* could be further subdivided. The use of an unsupervised population assignment algorithm revealed the existence of two clades within *S. enterica* subsp. *enterica* that the authors designated clades A and B. However, Falush et al. (9) did not further investigate the genetic or phenotypic properties of strains from these two clades.

In this study, we reanalyzed the publicly available data in the *Salmonella* MLST database (http://mlst.ucc.ie/) and included an additional 56 strains of *S. enterica* subsp. *enterica* collected from Australian vertebrates and typed using the same MLST scheme. Here we confirm the existence of two clades within *S. enterica* subsp. *enterica* and demonstrate that Australian strains belonging to clades A and B differ in their host distributions, as well as their phenotypic and virulence characteristics.

Strain characterization. The 56 *S. enterica* subsp. *enterica* isolates examined in this study were collected from Australian reptiles and mammals as part of a broader study of the *Enterobacteriaceae* and as part of a study of *S. enterica* in a South Australian population of the skink *Tiliqua rugosa* (10, 11, 15). The biochemical profile of these isolates was determined using the BBL Crystal enteric/nonfermenter identification systems (BD) according to the manufacturer's protocol.

DNA was extracted using DNAzol (Invitrogen) according to the manufacturer's protocol. Multilocus sequence typing was performed using the PCR and sequencing primers that were described previously (14). The resulting PCR products were purified using 1 to 2 μl of ExoSAP-IT (USB) according to the manufacturer's protocol. Sequencing was performed using BigDye Terminator chemistry (Applied Biosystems, Inc.) and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Inc.). Sequences were submitted to the *Salmonella* MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica) to determine the allele and sequence type designations for these isolates.

The nucleotide sequence data for the seven housekeeping genes (from this study) and the publicly available data in the *S. enterica* MLST database were concatenated. Noninformative sites were removed, and these data were analyzed using BAPS 4.1 (6, 7) and Structure 2.0 (8, 17).

The *S. enterica* isolates were screened for the presence of nine virulence genes: *fliC*, *sipB*, *sipC*, *sopB*, *spvR* (4), *sopE1* (16), *lpfC*, *sifA*, and *ctdB* (18). The annealing temperatures were 60°C for *lpfC* and *sifA*, 63°C for *cdtB*, and 55°C for all other virulence genes.

The serotype of one example of each sequence type (ST) was determined by the Microbiological Diagnostic Unit, University of Melbourne and Salmonella Reference Laboratory, Adelaide, Australia.

Characteristics of clade A and B strains. MLST analysis revealed that the 56 Australian *S. enterica* subsp. *enterica* isolates collected for this study were represented by 28 sequence types (Table 1).

Unsupervised population assignment analysis of the nucleotide sequence data for 137 STs (28 STs from this study and 115 *S. enterica* subsp. *enterica* STs represented in the MLST database) was conducted using the software programs BAPS and Structure. Both algorithms partitioned the STs into three clades, corresponding to clades A, B, and *Salmonella typhi*. Of the 115 STs in the database, only 16 were assigned to clade B (14%). In the newly collected Australian data, 16 of the 28 STs were assigned to clade B (57%).

Isolates recovered from Australian mammals or reptiles could be members of either clade A or B. However, isolates from reptiles were significantly overrepresented in clade B. Of the 41 isolates recovered from reptiles, 33 (80%) were members of clade B, while only 5 (33%) of the 15 isolates recovered from mammals were assigned to clade B [likelihood ratio, $\chi^{2(1)} = 10.76$, $P = 0.0010$].

Of the 32 biochemical traits tested in the BBL Crystal enteric/nonfermenter identification kit, only the production of β-glucuronidase was found to differ between the two clades. All isolates in clade B were positive for β-glucuronidase pro-

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TABLE 1. Clade membership and characteristics of *S. enterica* subsp. *enterica* strains recovered from Australian vertebrates

Sequence type	Serotype[a]	Strain	Host family	Host species	Common name	Clade	Presence of virulence gene[b]				
							cdtB	*lpfC*	*sipB* or *sipC*	*sopE1*	*fliC*
50	Saintpaul	M873	Macropodidae	*Onychogalea fraenata*	Bridled nail-tail wallaby	A	0	1	1	0	0
82	Muenchen	R954	Crocodylidae	*Crocodylus porosus*	Saltwater crocodile	A	0	1	1	0	0
440	Adelaide	R572	Varanidae	*Varanus brevicauda*	Short-tailed monitor	A	0	0	1	0	0
462	Singapore	S02:9284:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	A	0	1	1	0	0
462	Singapore	S05:9365:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	A	0	1	1	0	0
578	Havana	MISC721	Macropodidae	*Macropus robustus*	Common wallaroo	A	0	1	1	0	0
578	Havana	MISC727	Macropodidae	*Macropus robustus*	Common wallaroo	A	0	1	1	0	0
821	Bonn	MISC352	Dasyuridae	*Antechinus flavipes*	Yellow-footed antechinus	A	0	1	1	1	0
821	Bonn	R596	Scincidae	*Egernia whitii*	White's skink	A	1	1	1	1	0
861	Hartford	MISC165	Dasyuridae	*Sminthopsis*	Dunnart	A	0	1	1	1	0
861	Hartford	MISC239	Muridae	*Rattus fuscipes*	Bush rat	A	0	1	1	1	0
863	Mississippi	MISC469	Dasyuridae	*Dasyurus viverrinus*	Eastern quoll	A	1	0	1	1	0
895	Emmastadt	MISC265	Dasyuridae	*Dasyrurus hallucatus*	Northern quoll	A	0	1	1	0	1
895	Emmastadt	MISC295	Potoroidae	*Potorous tridactylus*	Long-nosed potoroo	A	0	1	1	1	1
895	Emmastadt	MISC309	Muridae	*Rattus fuscipes*	Bush rat	A	0	1	1	0	1
904	Newport	R553	Pygopodidae	*Delma pax*	Legless lizard	A	0	1	1	0	0
905	Victoria	R159	Scincidae	*Pseudemoia entecasteauxii*	Tussock skink	A	1	0	1	1	0
1481	ND	R593	Agamidae	*Ctenophorus isolepis*	Military dragon	A	0	0	0	0	0
343	Chester	R571	Varanidae	*Varanus brevicauda*	Short-tailed monitor	B	1	0	1	0	0
343	Chester	MISC376	Dasyuridae	*Dasyrurus hallucatus*	Northern quoll	B	1	0	1	0	0
343	Chester	R295	Scincidae	*Ctenotus pantherinus*	Leopard ctenotus	B	1	0	1	1	0
343	Chester	R716	Varanidae	*Varanus acanthurus*	Spiny-tailed monitor	B	1	0	1	0	0
343	Chester	S12:9301:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	0	0
820	Rubislaw	S04:9369:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	0	1
820	Rubislaw	S04:9364:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	0	1
820	Rubislaw	S03:9369:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	0	1
820	Rubislaw	S10:9369:3	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	0	1
820	Rubislaw	MISC707	Potoroidae	*Bettongia lesueur*	Burrowing bettong	B	1	0	1	1	1
822	Brisbane	MISC421	Dasyuridae	*Dasyrurus hallucatus*	Northern quoll	B	1	0	1	0	0
859	Bahrenfeld	S03:9292:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S01:11852:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S02:10046:3	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S02:9363:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S03:10993:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S04:9277:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S04:9292:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S04:9363:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S05:11792:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	S05:11852:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
859	Bahrenfeld	R758	Scincidae	*Ctenotus pantherinus*	Leopard ctenotus	B	1	0	1	1	0
860	Onderstepoort	R707	Scincidae	*Ctenotus saxatilis*	Rock ctenotus	B	1	0	1	0	0
862	Charity	R350	Scincidae	*Tiliqua multifasciata*	Blue-tongue lizard	B	1	0	1	1	0
864	Oranienburg	S02:9365:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
864	Oranienburg	S01:9365:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	1	0	1	1	0
865	Treforest	R347	Varanidae	*Varanus gouldi*	Gould's monitor	B	1	0	1	0	0
866	Bukavu	M476	Dasyuridae	*Dasycercus cristicauda*	Crest-tailed mulgara	B	1	0	1	1	0
893	Blukwa	S02:11626:1	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	0	1	1	1	0
893	Blukwa	S02:9285:2	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	0	1	1	1	0
893	Blukwa	R308	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	0	1	1	1	0
894	Blukwa	R503	Scincidae	*Tiliqua rugosa*	Sleepy lizard	B	0	1	1	1	0
902	Urbana	MISC384	Dasyuridae	*Dasyurus hallucatus*	Northern quoll	B	1	0	1	0	0
903	Rottnest	R595	Scincidae	*Eremiascincus fasciolatus*	Sand-swimmer	B	1	0	1	0	0
906	ND	R292	Agamidae	*Ctenophorus reticulatus*	Western netted dragon	B	1	0	1	1	0
906	ND	R313	Agamidae	*Ctenophorus isolepis*	Military dragon	B	1	0	0	0	0
907	Bullbay	R291	Agamidae	*Pogonam minor*	Bearded dragon	B	1	0	1	0	0
908	ND	R343	Agamidae	*Diporiphora winneckei*	Cane grass dragon	B	1	0	1	1	0

[a] ND, not determined.
[b] 0, absent; 1, present.

duction, while all of the 18 clade A isolates were negative [likelihood ratio, $\chi^{2(1)} = 70.33$, $P < 0.0001$].

Isolates belonging to the two clades were further characterized by the presence of the virulence genes *sipB*, *sipC*, *sopE1*, *sopB*, *fliC*, *stn*, *sifA*, *lpfC*, *cdtB*, and *spvR*. Among the 56 isolates, 13 different virulence profiles were observed (Table 1). The genes *sopB*, *stn*, and *sifA* were detected in all isolates. The gene *spvR*, which occurs on a plasmid, was detected in only three isolates recovered from mammals, representing one ST (895). The gene *lpfC* was significantly less common in isolates belonging to clade B (11% of 38 isolates) than in clade A isolates (78% of 18 isolates) [likelihood ratio, $\chi^{2(1)} = 29.5$, $P < 0.0001$]. In contrast, the gene *cdtB* was significantly more common among clade B isolates (89%) than among clade A isolates (20%) [likelihood ratio, $\chi^{2(1)} = 25.69$, $P < 0.0001$]. There were no detectable differences between clade A and B isolates in the frequencies of any other virulence traits.

Using MLST data, Falush and colleagues (9) were the first to demonstrate that *S. enterica* subsp. *enterica* could be subdivided into two clades. Using an expanded MLST data set, this study has confirmed the existence of these subdivisions. The Australian *S. enterica* subsp. *enterica* isolates collected in this study that were members of clade B could be distinguished phenotypically, as members of clade B were positive for the production of β-glucuronidase, while all clade A isolates were negative. Strains of the two clades also differed in their viru-

lence gene profiles. The data also indicate that strains of the two clades have different host distributions, with clade B strains being more likely to be recovered from reptiles than clade A strains.

Of the 10 virulence genes examined in this study, only the virulence genes *lpfC* and *cdtB* differed between the two clades. Both *lpfC* and *cdtB* are thought to aid in host recognition/invasion (1, 13). Thus, these genes may be, in part, responsible for the difference in host preference exhibited by strains of the two clades.

There are many levels at which the diversity of a species may be considered: strain, sequence type, sequence complex, etc. Strains of the *Escherichia coli* phylogroups (A, B1, B2, D, and E) have been shown to differ in their phenotypic and genotypic characteristics, as well as in their ecological niches and abilities to cause disease (12). The results of this study indicate that partitioning strains of *S. enterica* subsp. *enterica* into clades is a biologically meaningful exercise. The factors governing this apparent "host specificity" are yet to be elucidated, as is the epidemiological and clinical significance of the differences exhibited by members of the different clades.

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