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Novel IS*711*-Specific Chromosomal Locations Useful for Identification and Classification of Marine Mammal *Brucella* Strains[▽]

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We report five new IS*711* chromosomal locations that are specific for marine mammal *Brucella* groups of strains and useful for their identification and classification. Our data support their current classification into two species, *Brucella ceti* and *B. pinnipedialis*, with subgroups in each, but also the possibility of additional species.

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect many species of animals and humans. Six species were initially recognized within the genus *Brucella*: *Brucella abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (13, 20, 27). This classification is based mainly on differences in pathogenicity, host preference, and phenotypic characteristics. Four additional species have been included in the genus *Brucella* since 2007. *B. ceti* and *B. pinnipedialis* have been isolated from marine mammals, with cetaceans (dolphin, porpoise, and whale species) and pinnipeds (various seal species), respectively, as the preferred hosts (11, 12). *B. microti*, first described in 2008, was isolated initially from the common vole but later also from the red fox and from soil (21–23). The latest species is *B. inopinata*, isolated from a human breast implant infection, representing the *Brucella* species most distant from the others at the phenotypic and molecular levels (9, 24). The animal or environmental reservoir of *B. inopinata* is not known.

Molecular and phenotypic typing of marine mammal *Brucella* strains has led to their classification into two species, i.e., *B. ceti* and *B. pinnipedialis* (12). However, several subgroups within each species have been identified by molecular typing methods, such as multilocus sequence analysis (MLSA), multilocus variable-number tandem-repeat (VNTR) analysis (MLVA), and *omp2a* and *omp2b* porin gene diversity analysis (1, 3, 5, 7, 8, 14, 17, 26, 27). Among these subgroups, one within *B. ceti*, which is composed exclusively of strains isolated from various dolphin species, has been proposed to constitute a separate species with the name *B. delphini* (1, 14, 27). The isolates from cetaceans from the Pacific may also constitute a separate species (17). Three human cases with naturally acquired infection by *Brucella* strains presumably of marine origin have been reported, one case of spinal osteomyelitis from a patient in New Zealand (19) and two neurobrucellosis cases from Peruvian patients (25). Interestingly, these human isolates presented the same genotype as strains from cetaceans from the Pacific (28).

The presence of the mobile genetic element IS*711* (GenBank accession no. M94960) has been a useful target for molecular characterization of classical terrestrial mammal *Brucella* species and biovars based on the number and distribution of IS*711* copies within the bacterial genomes. Among classical *Brucella* species, IS*711*-based fingerprints are stable, species specific (except *B. canis*), and to some extent biovar specific (2). Marine mammal *Brucella* strains have been shown to carry a higher number of the IS*711* element in their genomes than terrestrial mammal *Brucella* species and biovars, with the exception of *B. ovis* (2, 3, 8, 18). Consequently, infrequent restriction site PCR (IRS-PCR) methods were applied, taking into account this higher number of IS*711* elements, to study the genomic diversity of marine mammal strains (5, 16). These studies confirmed the classification into two marine mammal *Brucella* species, with subgroups in each. In addition, three specific IS*711*-containing DNA fragments were detected, allowing the molecular identification of *B. ceti* and its subgroup composed exclusively of dolphin isolates (5, 8, 16).

In this study, to further analyze the distribution of IS*711* elements in the genomes of *Brucella* strains of marine origin, we applied ligation-mediated PCR (LM-PCR) specifically targeting IS*711*. This method aimed to determine other specific chromosomal locations of IS*711* that would allow molecular identification of the different *B. ceti* and *B. pinnipedialis* subgroups or presumed new species.

The *Brucella* strains of marine mammal origin used in this study are listed in Table 1. They were from diverse marine mammal species (seal, otter, dolphin, porpoise, and whale species) and geographic origins (France, Norway, and Scotland). The human isolate from New Zealand presumably of marine origin was also included in this study. Bacterial culture and DNA extraction were performed as described previously (6). LM-PCR was performed on representative groups of *Brucella* strains, including terrestrial mammal *Brucella* control strains, classified previously by IS*711* Southern blot analysis (Table 1) (18). DNA was digested with a restriction enzyme cutting outside the IS*711* element (EcoRI, BglII, or BamHI), and an adapter specific for each enzyme was used with a primer specific for the IS*711* element. Thus, only DNA fragments containing part of the IS*711* element were amplified, and sequencing of these fragments enabled us to locate IS*711* copies in the genomes of marine mammal *Brucella* isolates. Primers and adapters used are listed in Table 2. Ligation of adapters and PCR amplifications were performed as described previously (10). The DNA fragments generated were run on agarose gels, and five fragments of interest that seemed to be specific to

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TABLE 1. *Brucella* strains of marine mammal origin used in this study

Species	Strain	Host or source	Latin name of host or source	Geographic origin of strain	MLVA-16 cluster (panel 1 genotype)[a]	MLSA ST[b]	*omp2b-omp2a* RFLP pattern[c]	IS*711* RFLP pattern[d] (cluster[e])	IS*711* copy no.[f]	Presence or absence, by fragment-specific PCR, of fragment[g]:				
										I	II	III	IV	V
B. pinnipedialis	9a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	17a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	22a-2	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	23a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (75)		P-I	C	19	+	−	−	−	−
	24a-2	Hooded seal	*Cystophora cristata*	Norway	C3 (75)		P-I	C	19	+	−	−	−	−
	25a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	30a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (74)		P-I	C	19	+	−	−	−	−
	37a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	38g-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	39a-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	53c-1	Hooded seal	*Cystophora cristata*	Norway	C3 (76)		P-I	C	19	+	−	−	−	−
	M163/99/10	Hooded seal	*Cystophora cristata*	Scotland	C3 (76)	ST25	P-I	C (4)	19	+	−	−	−	−
	M603/99/7	Hooded seal	*Cystophora cristata*	Scotland	C3 (76)	ST25	P-I	C (4)	19	+	−	−	−	−
	M2006/94/6	Hooded seal	*Cystophora cristata*	Scotland	C3 (76)	ST25	P-I	C (4)	19	+	−	−	−	−
	B2/94 (NCTC 12890; BCCN 94-73[h])	Common seal	*Phoca vitulina*	Scotland	C2 (25)		L-I	D (1)	23	+	+	−	−	−
	M13/01/1	Common seal	*Phoca vitulina*	Scotland	C2 (25)	ST25	L-I	D (1)	23	+	+	−	−	−
	M336/94/1	Common seal	*Phoca vitulina*	Scotland	C2 (25)		L-I	D (1)	23	+	+	−	−	−
	M339/94/1	Common seal	*Phoca vitulina*	Scotland	C2 (25)		L-I	D (1)	23	+	+	−	−	−
	M445/99/2	Common seal	*Phoca vitulina*	Scotland	C2 (25)	ST25	L-I	D (1)	23	+	+	−	−	−
	M514/96/4	Common seal	*Phoca vitulina*	Scotland	C2 (25)	ST25	L-I	D (1)	23	+	+	−	−	−
	M972/94/1	Common seal	*Phoca vitulina*	Scotland	C2 (25)		L-I	D (1)	23	+	+	−	−	−
	M621/99/2	Gray seal	*Halichoerus grypus*	Scotland	C2 (25)	ST25	L-I	D (1)	23	+	+	−	−	−
	M1771/94/1	Otter	*Lutra lutra*	Scotland	C2 (25)	ST25	L-I	D (1)	23	+	+	−	−	−
	M292/94/1	Common seal	*Phoca vitulina*	Scotland	C1 (71)	ST24	O-I	E (1)	24	+	+	−	−	−
	M449/02/2	Common seal	*Phoca vitulina*	Scotland	C1 (71)		O-I	E (1)	24	+	+	−	−	−
	M2466/93/4	Common seal	*Phoca vitulina*	Scotland	C2 (72)		L-I	E (1)	24	+	+	−	−	−
	M2533/93/1	Common seal	*Phoca vitulina*	Scotland	C1 (71)	ST24	O-I	E (1)	24	+	+	−	−	−
	M2375/94/3	Gray seal	*Halichoerus grypus*	Scotland	C2 (25)	ST25	L-I	E (1)	24	+	+	−	−	−
	M194/00/1	Gray seal	*Halichoerus grypus*	Scotland	C2 (25)		L-I	E (1)	24	+	+	−	−	−
	M192/00/1	Minke whale	*Balaenoptera acutorostrata*	Scotland	C1 (71)	ST24	O-I	E (1)	24	+	+	−	−	−
B. ceti	M490/95/1	Common seal	*Phoca vitulina*	Scotland	B (23)	ST23	M-J	F (2)	30	+	−	+	+	−
	05-0684-1144	Porpoise	*Phocoena phocoena*	France	B (23)		M-J	F	30	+	−	+	+	−
	M23/03/4	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M39/94/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	F (2)	30	+	−	+	+	−
	M51/04/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M58/05/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F	30	+	−	+	+	−
	M78/05/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	F	30	+	−	+	+	−
	M93/04/3	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M117/01/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M165/03/6	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M195/03/10	Porpoise	*Phocoena phocoena*	Scotland	B (70)		M-J	F (2)	30	+	−	+	+	−
	M234/05/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	F	30	+	−	+	+	−
	M291/03/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M499/99/10	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M515/96/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	F	30	+	−	+	+	−

	M615/99/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M854/98/8	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M1570/94/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	F	30	+	−	+	+	−
	7763/2	Bottlenose dolphin	*Tursiops truncatus*	France	B (23)	ST23	M-J	F	30	+	−	+	+	−
	M452/97/2	Common dolphin	*Delphinus delphis*	Scotland	B (23)	ST23	M-J	F (2)	30	+	−	+	+	−
	M2/00/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M52/01/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)		M-J	F (2)	30	+	−	+	+	−
	M181/97/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)	ST23	M-J	F	30	+	−	+	+	−
	M187/00/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)	ST23	M-J	F (2)	30	+	−	+	+	−
	M2438/95/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)		M-J	F	30	+	−	+	+	−
	B202R	Minke whale	*Balaenoptera acutorostrata*	Norway	B (23)	ST23	M-J	F (2)	30	+	−	+	+	−
	B1/94 (NCTC 12891; BCCN 94-74[h])	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	M12/00/3	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	M38/04/3	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	M103/99/1	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	M199/04/2	Porpoise	*Phocoena phocoena*	Scotland	B (23)	ST23	M-J	G	29	+	−	+	+	−
	M1747/98/3	Porpoise	*Phocoena phocoena*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	M870/97/1	White-beaked dolphin	*Lagenorhynchus albirostris*	Scotland	B (23)		M-J	G	29	+	−	+	+	−
	M997/94/2	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (69)		M-J	G (2)	29	+	−	+	+	−
	M2788/97/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	B (23)		M-J	G (2)	29	+	−	+	+	−
	B14/94	Common dolphin	*Delphinus delphis*	Scotland	A1 (24)	ST26	N-K	H (3)	26	+	−	−	−	+
	M13/05/1	Striped dolphin	*Stenella coeruleoalba*	Scotland	A1 (24)	ST26	N-K	H	26	+	−	−	−	+
	M40/95/1	Striped dolphin	*Stenella coeruleoalba*	Scotland	A1 (24)	ST26	N-K	H (3)	26	+	−	−	−	+
	M642/99/2	Striped dolphin	*Stenella coeruleoalba*	Scotland	A1 (24)	ST26	N-K	H (3)	26	+	−	−	−	+
	M654/99/1	Striped dolphin	*Stenella coeruleoalba*	Scotland	A2 (77)	ST26	N-K	H (3)	26	+	−	−	−	+
	M656/99/1	Striped dolphin	*Stenella coeruleoalba*	Scotland	A1 (24)	ST26	N-K	H (3)	26	+	−	−	−	+
	M2194/94/1	Striped dolphin	*Stenella coeruleoalba*	Scotland	A1 (24)	ST26	N-K	H (3)	26	+	−	−	−	+
	M18/96/1	White-sided dolphin	*Lagenorhynchus acutus*	Scotland	A1 (24)		N-K	H	26	+	−	−	−	+
Brucella spp.	02/611	Human	*Homo sapiens*	New Zealand	(78)	ST27	Q-I	I	23	+	−	−	−	−

[a] According to Maquart et al. (17).

[b] According to Groussaud et al. (14). ST, sequence type.

[c] According to Cloeckaert et al. (7), Dawson et al. (8), and results of this study.

[d] According to Maquart et al. (18) and results of this study.

[e] According to Dawson et al. (8) for the strains indicated.

[f] According to Maquart et al. (18) and results of this study.

[g] Fragments as numbered in Fig. 1.

[h] Reference strains.

TABLE 2. Primers and adapters used in this study

Procedure and adapter/primer name	Adapter/primer location[c]	Fragment no.	Fragment size (bp)	Nucleotide sequence (5′–3′)
Ligation-mediated PCR				
Eco1	NA[d]	NA	NA	GAGTCGTGTTACTCCCATCG
Eco2	NA	NA	NA	AATTCGATGGGAGTAACACG
ABg1[a]	NA	NA	NA	GAGTCGTGTTACTCCCATCG
ABg2[a]	NA	NA	NA	GATCGATGGGAGTAACACGACTC
IS1[b]	IS*711*	NA	NA	CGGTTCCATTGCTCATCTGT
Fragment-specific PCR				
I1	BR1657	I	253	CTACATAGGCATCGCCAGAG
II1	BRA0655	II	750	TGCCATAGGGCACATTGTTC
III1	BR1612	III	523	TTATATGATGCGCCCGGAGG
IV1	BR0613	IV	670	GAAGGTAGAGCCGGAATATC
V1	BR0625	V	195	GCTTTCGCATTGGAGCTTAC
IS2	IS*711*	I through V	NA	GCTCACGGCTGTTCTCCTTT

[a] The same adapters and primers were used for LM-PCR with restriction enzymes BglII and BamHI.
[b] All LM-PCRs were performed with primer IS1.
[c] According to the genome sequence of *B. suis* 1330 chromosome I or II.
[d] NA, not applicable.

marine mammal *Brucella* strains were sequenced at Genome Express (Meylan, France) (data not shown). Specific PCRs were then performed by using a forward primer in the sequence identified upstream of IS*711* and a reverse primer designed for the IS*711* element (Fig. 1). These primers are listed in Table 2.

The IS*711*-specific locations identified relative to the *B. suis* 1330 genome sequences of chromosomes I and II (GenBank accession numbers NC_004310 and NC_004311, respectively) are shown in Fig. 1. Specific PCRs performed on the strains listed in Table 1 and the reference strains of terrestrial mammal *Brucella* species and biovars showed that all of these locations are specific to marine mammal *Brucella* strains. Among the novel IS*711* locations identified in this study, there was one near a Bru-RS1-like element (86% nucleotide identity) (in fragment III [Fig. 1]), previously described as being a hot spot for IS*711* insertion (15). The novel specific IS*711* locations were distributed as follows according to the PCR-amplified DNA fragments. (Interestingly, they correlated well with other molecular classification methods and, in particular, as expected

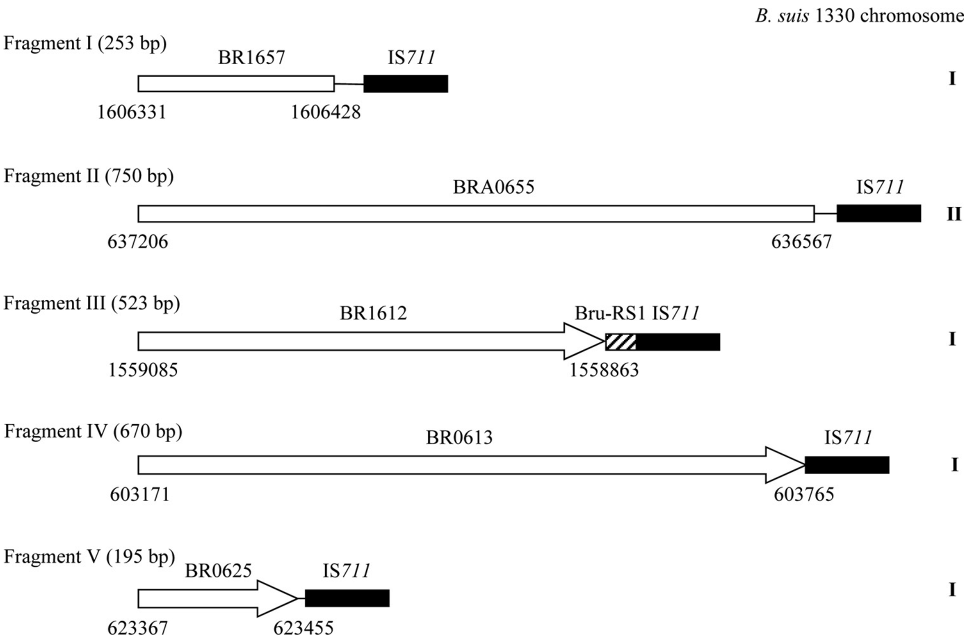

FIG. 1. Schematic representation of IS*711*-containing DNA fragments I to V identified by LM-PCR. The nucleotide positions relative to the genome sequences of *B. suis* 1330 chromosomes I and II (GenBank accession numbers NC_004310 and NC_004311, respectively) are indicated. Open reading frame BR1657 (in fragment I) codes for a putative two-component response regulator. BRA0655, or the *ugpB* gene (in fragment II), codes for a putative glycerol-3-phosphate ABC transporter. BR1612 (in fragment III) codes for a putative spermidine/putrescine ABC transporter. BR0613 (in fragment IV) codes for a putative sensor histidine kinase. BR0625 (in fragment V) codes for a hypothetical protein. The hatched region upstream from IS*711* in fragment III represents a Bru-RS1-like element.

with their IS*711* Southern blot restriction fragment length polymorphism [RFLP] profiles [Table 1].) Fragment I was detected in all marine mammal strains studied ($n = 74$) and also in the human isolate from New Zealand. Fragment II was found to be specific to *B. pinnipedialis* strains but was not detected in the hooded seal isolates constituting a particular group of pinniped strains with some distinct molecular characteristics (1, 16, 17, 18). These strains were previously shown to carry fewer IS*711* elements in their genomes, and this could explain why we were not able to detect specific IS*711*-containing DNA fragments in this group of strains (Table 1). They could be ancestral to the other *B. pinnipedialis* strains, as suggested previously (16). Fragments III, IV, and V were found to be specific to *B. ceti* strains, with III and IV on the one hand and V on the other hand corresponding to the two major groups of cetacean strains classified by other molecular methods (Table 1). As indicated above, the latter group of strains corresponds to that composed exclusively of dolphin isolates. Unfortunately, under our conditions, no specific IS*711*-containing DNA fragment could be detected in the human isolate from New Zealand studied, although it presented a distinct IS*711* RFLP pattern, likely with other specific IS*711* locations (18). Nevertheless, this finding as well as the distinct IS*711* locations in the dolphin group of strains and other molecular data supports the possibility of additional *Brucella* species in marine mammal *Brucella* isolates besides *B. ceti* and *B. pinnipedialis* (27). The specific IS*711* locations found in this study, together with others previously reported (4, 5, 29), may be of further usefulness for the molecular identification of *Brucella* isolates of marine origin.

Nucleotide sequence accession numbers. The nucleotide sequences of the five DNA fragments from this study have been deposited in GenBank under accession numbers HM352552 (fragment I), HM352553 (fragment II), HM352554 (fragment III), HM352555 (fragment IV), and HM352556 (fragment V).

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