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MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*

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Abstract

Background: Since 1994, *Brucella* strains have been isolated from a wide range of marine mammals. They are currently recognized as two new *Brucella* species, *B. pinnipedialis* for the pinniped isolates and *B. ceti* for the cetacean isolates in agreement with host preference and specific phenotypic and molecular markers. In order to investigate the genetic relationships within the marine mammal *Brucella* isolates and with reference to terrestrial mammal *Brucella* isolates, we applied in this study the Multiple Loci VNTR (Variable Number of Tandem Repeats) Analysis (MLVA) approach. A previously published assay comprising 16 loci (MLVA-16) that has been shown to be highly relevant and efficient for typing and clustering *Brucella* strains from animal and human origin was used.

Results: 294 marine mammal *Brucella* strains collected in European waters from 173 animals and a human isolate from New Zealand presumably from marine origin were investigated by MLVA-16. Marine mammal *Brucella* isolates were shown to be different from the recognized terrestrial mammal *Brucella* species and biovars and corresponded to 3 major related groups, one specific of the *B. ceti* strains, one of the *B. pinnipedialis* strains and the last composed of the human isolate. In

the *B. ceti* group, 3 subclusters were identified, distinguishing a cluster of dolphin, minke whale and porpoise isolates and two clusters mostly composed of dolphin isolates. These results were in accordance with published analyses using other phenotypic or molecular approaches, or different panels of VNTR loci. The *B. pinnipedialis* group could be similarly subdivided in 3 subclusters, one composed exclusively of isolates from hooded seals (*Cystophora cristata*) and the two others comprising other seal species isolates.

Conclusion: The clustering analysis of a large collection of marine mammal *Brucella* isolates from European waters significantly strengthens the current view of the population structure of these two species, and their relative position with respect to the rest of the *Brucella* genus. MLVA-16 is confirmed as being a rapid, highly discriminatory and reproducible method to classify *Brucella* strains including the marine mammal isolates. The *Brucella2009* MLVA-16 genotyping database available at http://mlva.u-psud.fr/ is providing a detailed coverage of all 9 currently recognized *Brucella* species.

Background

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect many species of animals and man. Six species were classically recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* [1,2]. This classification is mainly based on differences in pathogenicity, host preference, and phenotypic characteristics [1-3]. Three additional species have been recently included in the genus *Brucella*, i.e. *B. ceti* and *B. pinnipedialis* isolated from marine mammals, with cetaceans (dolphin, porpoise, and whale species) and pinnipeds (various seal species) as preferred hosts respectively [4], and *B. microti* isolated from the common vole [5]. From a phenotypic point of view, *B. ceti* and *B. pinnipedialis* can be distinguished by their growth requirement for CO_2 and their oxidative metabolism [6,7].

The phylogenetic significance of this separation is supported by molecular analyses. At the molecular level, evidence for two distinct marine mammal *Brucella* subpopulations subsequently given species rank and designated *B. ceti* and *B. pinnipedialis* has been initially provided by study of DNA polymorphism at the porin-encoding *omp2* locus [8]. This was further confirmed by an infrequent restriction site-PCR (IRS-PCR) method, reflecting the higher number of IS*711* elements in the genome of marine mammal isolates compared to terrestrial mammal *Brucella* species [9-11]. IRS-PCR revealed six specific DNA fragments useful for the detection and identification of marine mammal *Brucella* isolates and the presence of a putative genomic island only in seal isolates except for hooded seal isolates [11,12]. Interestingly to date three human cases, one from New Zealand and two from Peru, with *Brucella* infections presumably of marine origin, have been described according to the specific molecular markers cited above, and may point towards a zoonotic potential of these marine mammal *Brucella* species [13,14]. One human case with laboratory acquired infection has also been reported [15].

In the past few years, polymorphic tandem repeat loci have been identified by analysing published genome sequences of *B. melitensis* 16 M, *B. suis* 1330, and *B. abortus* 9–941 [16-18]. Hundreds of *Brucella* strains have been typed to allow the development of an assay, called MLVA-16 assay (Multiple Locus VNTR Analysis) [5,17-23]. The sixteen loci have been grouped in 3 panels, called panel 1 (8 minisatellite loci), panel 2A (3 microsatellite loci) and panel 2B (5 microsatellite loci) [17,20]. Panel 1 has shown to be useful for species identification. Panel 2A and panel 2B increased the discriminatory power. Panel 2B was selected to contain the more highly variable markers, which is why this panel is often given a lower weight in clustering analysis [20,21]. Three of the five octamers in panel 2B have been initially evaluated by Bricker *et al.* [16]. The MLVA-16 assay provides a clustering of strains that is in accordance with the currently recognized *Brucella* species and biovars isolated from terrestrial mammals.

The aim of this study was to evaluate the MLVA-16 assay for the classification of marine mammal *Brucella* isolates, using 294 marine mammal *Brucella* strains obtained from 173 animals representing a wide range of marine mammal species from different European geographic origins (excluding the Mediterranean sea). This study also included the strain, presumably of marine origin, isolated from a patient in New Zealand [14]. A tentative overview of the global *Brucella* population structure was produced by comparison with published typing data.

Results

All strains could be typed at all loci, with few exceptions for panel 2B loci. At the loci bruce04, bruce09 and bruce16, multiple bands were observed in the PCR products of 12, 9 and 6 strains, respectively. This may suggest that in some occasions multiple alleles are present in the DNA preparation. Besides, two strains were negative in PCR either for bruce07 or bruce30. In 69 animals, strains were initially isolated from different organs, contributing

121 extra strains. In sixteen among these animals, more than one genotype was observed (in one animal 5 different genotypes were found). In most cases, these genotypes were also observed in at least one other animal. In five cases, at least one of the genotypes was unique in the present collection, suggesting that the presence of multiple genotypes could be the result of a mutation event that occurred in the course of infection. Three of these new genotypes were the result of one repeat unit changes at a single locus. The other two were a 2 repeat units change in bruce04 and a four repeat units change in bruce09. These observations suggest that occasionally the most highly mutable loci may vary in the course of infection. They also do not exclude the possibility that animals carrying multiple variants may have been infected by multiple strains present within the community.

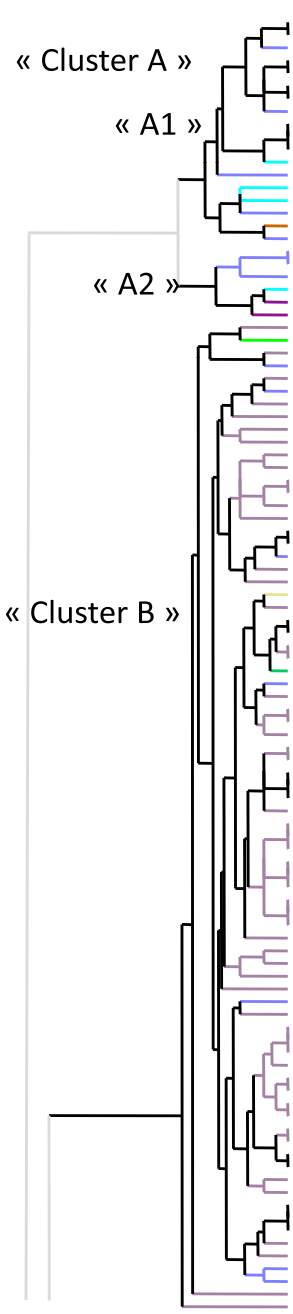
The 294 investigated marine mammal *Brucella* isolates which originated from 173 animals and one patient clustered in 117 different genotypes using the complete MLVA-16 assay. One representative for each genotype and animal was used for analysis, totalling 196 strains (Figures 1, 2, 3). Three main groups were identified, the *B. ceti* group, the *B. pinnipedialis* group and a third group comprising the human isolate from New Zealand. The 117 representative genotypes were compared with the 18 terrestrial mammal *Brucella* reference strains and published data (Figure 4). The 3 clusters were clearly separated from all the terrestrial mammal isolates.

The cetacean group composed of 102 strains presenting 74 genotypes (1–74) (Figure 1) could be separated into three major subclusters. The first two subclusters A1 and A2, from genotype 1 to 18 were only composed of dolphin isolates and one minke whale isolate (9 isolates from striped dolphins (*Stenella coeruleoalba*), 11 from Atlantic white sided dolphins (*Lagenorhynchus acutus*), 2 from bottlenose dolphins (*Tursiops truncatus*) and 1 from a common dolphin (*Delphinus delphis*)). These two subclusters correspond to sequence type ST26 [24], MLVA panel 1 genotype 24 (subcluster A1) and 77 (subcluster A2, Figure 1 and Figure 3), and together correspond to cluster A in [25] (Figure 3). The third subcluster, from genotype 19 to 74 corresponds to MLST sequence type 23, MLVA-16 panel 1 genotypes 23, 69 and 70, and is cluster B in [25] (Figure 1 and Figure 3). This subcluster was composed of 78 strains. Sixty-four were obtained from porpoises, 12 from 4 species of dolphins (9 from Atlantic white sided dolphin (*Lagenorhynchus acutus*), one from a white-beaked dolphin (*Lagenorhynchus albirostris*), one from a bottlenose dolphin (*Tursiops truncatus*), one from a common dolphin (*Delphinus delphis*), and one from a minke whale (*Balaenoptera acutorostrata*) isolated in Norway in 1995 [10] (Figure 1). An exception was the bmar111 (strain number M490/95/1), with the genotype 20, isolated in Scotland from a harbour (or common) seal (*Phoca vitulina*) and which belongs to the *B. ceti* group (Figure 1). This is, however, in agreement with previous observations, either phenotypic [26] or molecular, including MLVA typing [25]. This particular strain carries the two specific IRS-PCR fragments (II and III) of the *B. ceti* strains [11], and the PCR-RFLP pattern of the *omp2* genes is similar to that of *Brucella* strains isolated from porpoises [8].

The 93 representative *B. pinnipedialis* strains presented 42 different genotypes (75–116) (Figure 2) corresponding to cluster C in [25]. This group of isolates could similarly be further divided in three major subclusters. The first subcluster (genotype 75 to 101) was composed of several seal isolates (harbour seal and grey seal (*Halichoerus grypus*)) and the isolate from a European sea otter (*Lutra lutra*). It corresponds to MLST sequence type 25, MLVA panel 1 genotypes 25, 72, 73, and cluster C2 in [25]. The second subcluster (MLVA genotypes 102 to 107) corresponds to MLST sequence type 24, MLVA panel 1 genotypes 71 and 79 and is cluster C1 in [25]. Interestingly, the hooded seal isolates (15 strains) were exclusively clustered in 9 closely related genotypes, forming the third subcluster of the pinniped isolates (genotype 108 to 116) called C3 in [25]. Most of the hooded seal isolates analysed in this study were isolated in Norway in 2002 [27] and there were also 4 hooded seal isolates from Scotland that clustered with the Norwegian isolates. One of the 93 strains of the *B. pinnipedialis* group was obtained from a cetacean. This strain (M192/00/1), identified as bmar160 with the genotype107 in Figure 2, was isolated from a minke whale in Scotland in 2000. This strain was also demonstrated as a *B. pinnipedialis* strain by other molecular markers, as described by Maquart *et al*. [12] and Groussaud *et al*. [25].

The human isolate 02/611 (bmar265) from New Zealand (genotype 117 (Figure 2)), clearly separates from the clusters described above but fits in an intermediate position within the marine mammal groups of isolates in agreement with previous MLST analyses [28]. Figure 3 suggests a position closer to the *B. ceti* group in agreement with the phenotypic behaviour [14], but the typing of more strains from Pacific waters [29-31] will be needed in order to achieve a more conclusive cluster analysis.

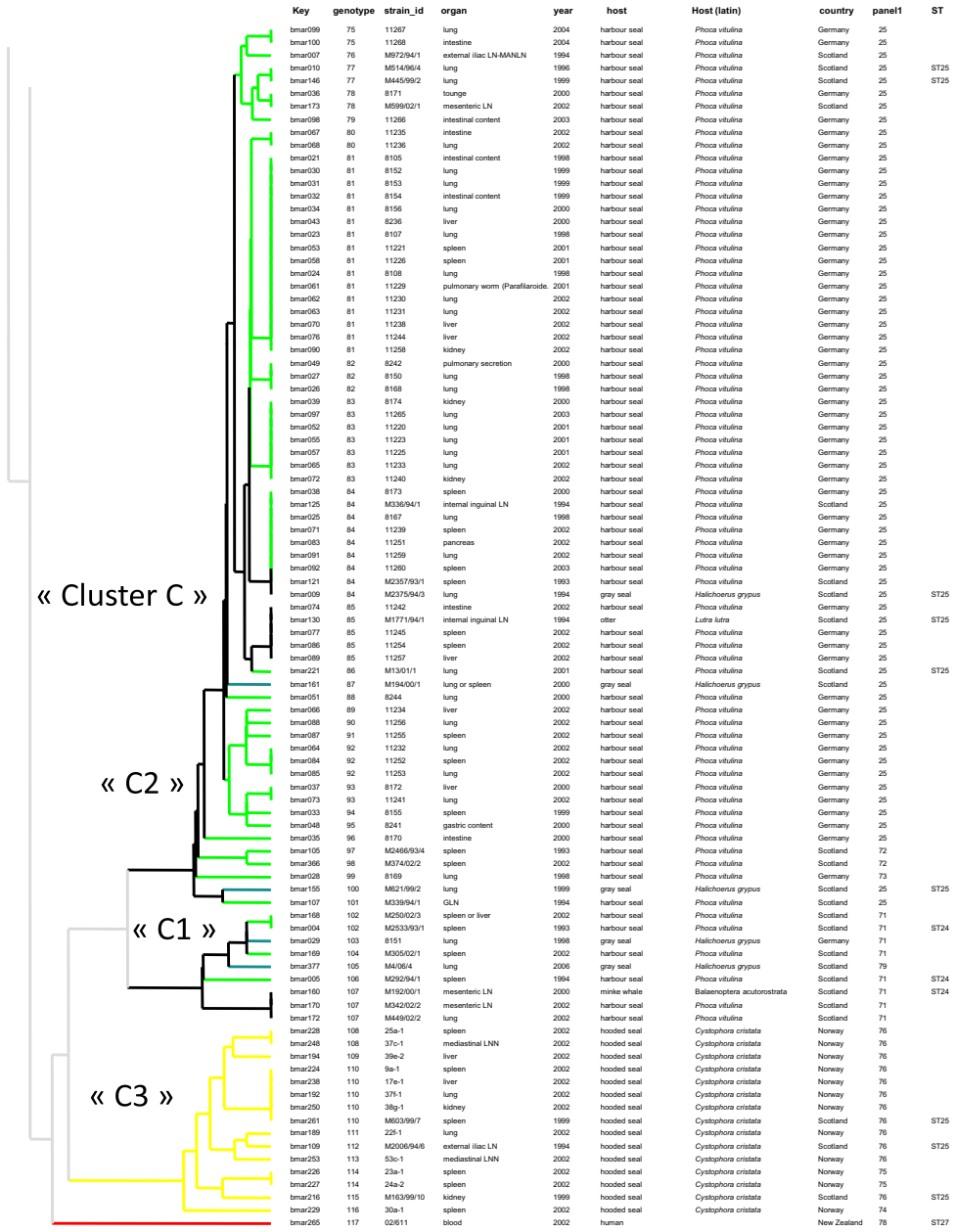
Owing to the inclusion of 40 representative strains in duplicate, the results described above could be compared to those recently described by Groussaud *et al*. who studied 74 marine mammal isolates by multilocus sequence typing, multilocus sequence analysis (MLST, MLSA) and MLVA. Duplicate typing was useful since Groussaud *et al*. used a partially different set of 21 VNTRs [25] (9 loci are common, including three loci from panel 1 (Bruce 08, 45, 55), one from panel 2A (Bruce18) and the whole panel 2B).



Key	genotype	strain_id	organ	year	host	Host (latin)	country	panel1	ST
bmar156	1	M642/99/2	brain	1999	striped dolphin	*Stenella coeruleoalba*	Scotland	24	ST26
bmar375	1	M105/06/8	liver	2006	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar283	2	M42/07/1	liver	2007	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar312	3	M57/07/1	spleen	2007	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar259	3	CITA	spleen	2004	striped dolphin	*Stenella coeruleoalba*	Spain	24	
bmar382	4	M260/03/1	brain	2003	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar167	4	M22/02/1	mesenteric LN	2002	striped dolphin	*Stenella coeruleoalba*	Scotland	24	
bmar113	5	M18/96/1	sub-cutaneous lesion	1996	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar011	6	M644/93/1	sub-cutaneous lesion	1993	common dolphin	*Delphinus delphis*	Scotland	24	ST26
bmar166	6	M9/02/01	sub-blubber abscess	2002	striped dolphin	*Stenella coeruleoalba*	Scotland	24	
bmar110	6	M40/95/1	sub-cutaneous lesion	1995	striped dolphin	*Stenella coeruleoalba*	Scotland	24	ST26
bmar255	7	M13/05/1	brain	2005	striped dolphin	*Stenella coeruleoalba*	Scotland	24	ST26
bmar292	8	M222/07/1	liver	2007	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar262	9	M656/99/1	brain	1999	striped dolphin	*Stenella coeruleoalba*	Scotland	24	ST26
bmar014	10	M2194/94/1	spleen	1994	striped dolphin	*Stenella coeruleoalba*	Scotland	24	ST26
bmar290	11	M158/06/1	brain	2006	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar367	12	M35/08/2	kidney	2008	minke whale	*Balaenoptera acutorostrata*	Scotland	24	
bmar322	13	M52/07/1	spleen	2007	AWSD	*Lagenorhynchus acutus*	Scotland	24	
bmar329	14	M66/06/1	kidney	2006	AWSD	*Lagenorhynchus acutus*	Scotland	77	
bmar284	14	M83/07/1	kidney	2007	AWSD	*Lagenorhynchus acutus*	Scotland	77	
bmar363	15	M267/05/1	colorectal LN	2005	AWSD	*Lagenorhynchus acutus*	Scotland	77	
bmar153	16	M654/99/1	brain	1999	striped dolphin	*Stenella coeruleoalba*	Scotland	77	ST26
bmar368	17	M231/07/2	brain	2007	bottlenose dolphin	*Tursiops truncatus*	Scotland	77	
bmar298	18	M231/07/3	kidney	2007	bottlenose dolphin	*Tursiops truncatus*	Scotland	77	
bmar103	19	M1068/91/2	sub-cutaneous lesion	1991	porpoise	*Phocoena phocoena*	Scotland	23	
bmar111	20	M490/95/1		1995	harbour seal	*Phoca vitulina*	Scotland	23	ST23
bmar187	21	M89/05/1	spleen	2005	porpoise	*Phocoena phocoena*	Scotland	69	ST23
bmar108	22	M997/94/2		1994	AWSD	*Lagenorhynchus acutus*	Scotland	69	
bmar371	23	M85/06/3	atlanto-occipital pus	2006	porpoise	*Phocoena phocoena*	Scotland	23	
bmar158	24	M2/00/1	internal inguinal LN	2000	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar232	25	M130/03/2	lung	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar220	26	M12/00/3	lung	2000	porpoise	*Phocoena phocoena*	Scotland	23	
bmar157	27	M575/99/4	brain	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar359	28	M311/03/5	liver abscess or uterine swab	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar117	29	M854/98/8	kidney	1998	porpoise	*Phocoena phocoena*	Scotland	23	
bmar223	30	M130/02/2	lung	2002	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar270	31	M55/07/1	kidney	2007	porpoise	*Phocoena phocoena*	Netherlands	23	
bmar338	31	M67/07/3	lung	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar152	32	M563/99/2	lung	1999	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar114	33	M515/96/2	spinal lesion	1996	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar306	34	M99/07/1	spleen	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar164	34	M49/01/1	spleen	2001	porpoise	*Phocoena phocoena*	Scotland	23	
bmar112	35	M2438/95/1		1995	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar185	36	M51/04/2	mammary gland	2004	porpoise	*Phocoena phocoena*	Scotland	23	
bmar118	37	M1747/98/3	spleen or mesenteric LN	1998	porpoise	*Phocoena phocoena*	Scotland	23	
bmar016	38	M870/97/1	liver	1997	white-beaked dolphin	*Lagenorhynchus albirostris*	Scotland	23	
bmar174	39	M23/03/4	liver	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar257	40	M120/05/1	mesenteric LN	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar015	40	M181/97/1	spleen lesion	1997	AWSD	*Lagenorhynchus acutus*	Scotland	23	ST23
bmar182	41	M58/05/1	kidney	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar346	41	M186/07/12	spleen	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar115	42	M452/97/2	spleen	1997	common dolphin	*Delphinus delphis*	Scotland	23	ST23
bmar162	43	M187/00/1	spleen	2000	AWSD	*Lagenorhynchus acutus*	Scotland	23	ST23
bmar281	44	M70/02/1	spinal lesion	2002	porpoise	*Phocoena phocoena*	Scotland	23	
bmar256	45	M100/04/6	lung	2004	porpoise	*Phocoena phocoena*	Scotland	23	
bmar297	45	M137/05/5	kidney	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar150	46	M610/99/1	liver	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar175	47	M89/03/5	brain	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar176	47	M165/03/6	spleen	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar102	48	7763/2		1997	bottlenose dolphin	*Tursiops truncatus*	France	23	ST23
bmar159	48	M499/99/5	spleen	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar012	48	M1570/94/1	blood	1994	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar280	49	M83/01/4	spleen	2001	porpoise	*Phocoena phocoena*	Scotland	23	
bmar286	50	M39/06/6	lung	2006	porpoise	*Phocoena phocoena*	Scotland	23	
bmar381	50	M99/07/2	kidney	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar214	50	M103/99/1	thoracic fluid	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar323	51	M24/08/1	lung	2008	porpoise	*Phocoena phocoena*	Scotland	23	
bmar266	51	M245/05/2	testicular abscess	2005	porpoise	*Phocoena phocoena*	Scotland	23	
bmar013	51	M1661/94/2	uterus	1994	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar151	52	M615/99/2	epididymis	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar379	52	M245/05/4	liver	2005	porpoise	*Phocoena phocoena*	Scotland	23	
bmar230	52	M108/03/1	lung	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar294	53	M181/07/7	MLN	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar231	54	M116/03/2	spleen	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar178	55	M78/05/2	vertebral lesion	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar311	56	M49/01/2	liver	2001	porpoise	*Phocoena phocoena*	Scotland	23	
bmar233	57	M150/03/3	spleen	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar291	58	M52/01/2	spleen	2001	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar365	59	M234/05/2	lung	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar314	60	M12/04/8	spinal abscess	2004	porpoise	*Phocoena phocoena*	Scotland	23	
bmar269	60	M90/06/4	spleen	2006	porpoise	*Phocoena phocoena*	Scotland	23	
bmar183	60	M291/03/2	blubber	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar147	61	M493/99/1	liver	1999	porpoise	*Phocoena phocoena*	Scotland	23	
bmar360	62	M54/07/11	lung	2007	porpoise	*Phocoena phocoena*	Scotland	23	
bmar163	62	M46/01/2	sub-blubber abscess	2001	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar186	63	M93/04/3	lung	2004	porpoise	*Phocoena phocoena*	Scotland	23	
bmar378	63	M97/06/1	MLN	2006	porpoise	*Phocoena phocoena*	Scotland	23	
bmar177	64	M74/05/2	kidney	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar332	64	M137/05/4	liver	2005	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar195	65	B202R-F7		1992	minke whale	*Balaenoptera acutorostrata*	Norway	23	ST23
bmar260	65	AFSSA 05-068.		2005	porpoise	*Phocoena phocoena*	France	23	
bmar301	66	M217/03/4	mammary gland	2003	porpoise	*Phocoena phocoena*	Scotland	23	
bmar184	67	M38/04/3	thymus lesion	2004	porpoise	*Phocoena phocoena*	Scotland	23	
bmar180	68	M61/05/1	meninges	2005	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar296	68	M147/08/2	kidney	2008	porpoise	*Phocoena phocoena*	Scotland	23	
bmar374	68	M199/04/3	spinal lesion	2004	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar106	69	M39/94/1	mammary	1994	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar181	70	M199/04/2	liver	2004	porpoise	*Phocoena phocoena*	Scotland	23	ST23
bmar116	71	M2788/97/1	spleen	1997	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar213	72	M2788/97/2		1997	AWSD	*Lagenorhynchus acutus*	Scotland	23	
bmar222	73	M117/01/1	epididymis	2001	porpoise	*Phocoena phocoena*	Scotland	23	
bmar254	74	M195/03/10	milk of left mammary gland	2003	porpoise	*Phocoena phocoena*	Scotland	70	

Figure 1

MLVA-16 clustering analysis of 102 *B. ceti* strains defines three groups of strains. All *B. ceti* isolates cluster into a first part (genotypes 1 to 74) of the dendogram constructed from MLVA-16 testing of 294 *Brucella* strains obtained from 173 marine mammals (pinnipeds, otter and cetaceans) and one human patient from New Zealand. One strain per genotype and per animal is included (consequently some animals are represented by more than one strain), 196 entries are listed corresponding to 117 genotypes. In the columns, the following data are presented: DNA batch (key), genotype, strain identification, organ, year of isolation, host (AWSD: Atlantic White Sided Dolphin), host (Latin name), geographic origin, MLVA panel 1 genotype, sequence type when described by Groussaud *et al.* [25]. The colour code reflects the host species (see Figure 3 for detailed correspondence). No colour was used when identical genotypes were observed in different host species. The letter nomenclature proposed by Groussaud *et al*. is used (*B. ceti*, cluster A (ST26) further subdivided into A1 and A2 and cluster B (ST23)).



Key	genotype	strain_id	organ	year	host	Host (latin)	country	panel1	ST
bmar099	75	11267	lung	2004	harbour seal	*Phoca vitulina*	Germany	25	
bmar100	75	11268	intestine	2004	harbour seal	*Phoca vitulina*	Germany	25	
bmar007	76	M972/94/1	external iliac LN-MANLN	1994	harbour seal	*Phoca vitulina*	Scotland	25	
bmar010	77	M514/96/4	lung	1996	harbour seal	*Phoca vitulina*	Scotland	25	ST25
bmar146	77	M445/99/2	lung	1999	harbour seal	*Phoca vitulina*	Scotland	25	ST25
bmar036	78	8171	tounge	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar173	78	M599/02/1	mesenteric LN	2002	harbour seal	*Phoca vitulina*	Scotland	25	
bmar098	79	11266	intestinal content	2003	harbour seal	*Phoca vitulina*	Germany	25	
bmar067	80	11235	intestine	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar068	80	11236	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar021	81	8105	intestinal content	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar030	81	8152	lung	1999	harbour seal	*Phoca vitulina*	Germany	25	
bmar031	81	8153	lung	1999	harbour seal	*Phoca vitulina*	Germany	25	
bmar032	81	8154	intestinal content	1999	harbour seal	*Phoca vitulina*	Germany	25	
bmar034	81	8156	lung	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar043	81	8236	liver	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar023	81	8107	lung	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar053	81	11221	spleen	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar058	81	11226	spleen	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar024	81	8108	lung	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar061	81	11229	pulmonary worm (Parafilaroide.	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar062	81	11230	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar063	81	11231	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar070	81	11238	liver	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar076	81	11244	liver	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar090	81	11258	kidney	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar049	82	8242	pulmonary secretion	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar027	82	8150	lung	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar026	82	8168	lung	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar039	83	8174	kidney	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar097	83	11265	lung	2003	harbour seal	*Phoca vitulina*	Germany	25	
bmar052	83	11220	lung	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar055	83	11223	lung	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar057	83	11225	lung	2001	harbour seal	*Phoca vitulina*	Germany	25	
bmar065	83	11233	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar072	83	11240	kidney	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar038	84	8173	spleen	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar125	84	M336/94/1	internal inguinal LN	1994	harbour seal	*Phoca vitulina*	Scotland	25	
bmar025	84	8167	lung	1998	harbour seal	*Phoca vitulina*	Germany	25	
bmar071	84	11239	spleen	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar083	84	11251	pancreas	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar091	84	11259	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar092	84	11260	spleen	2003	harbour seal	*Phoca vitulina*	Germany	25	
bmar121	84	M2357/93/1	spleen	1993	harbour seal	*Phoca vitulina*	Scotland	25	
bmar009	84	M2375/94/3	lung	1994	gray seal	*Halichoerus grypus*	Scotland	25	ST25
bmar074	85	11242	intestine	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar130	85	M1771/94/1	internal inguinal LN	1994	otter	*Lutra lutra*	Scotland	25	ST25
bmar077	85	11245	spleen	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar086	85	11254	spleen	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar089	85	11257	liver	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar221	86	M13/01/1	lung	2001	harbour seal	*Phoca vitulina*	Scotland	25	ST25
bmar161	87	M194/00/1	lung or spleen	2000	gray seal	*Halichoerus grypus*	Scotland	25	
bmar051	88	8244	lung	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar066	89	11234	liver	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar088	90	11256	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar087	91	11255	spleen	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar064	92	11232	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar084	92	11252	spleen	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar085	92	11253	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar037	93	8172	liver	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar073	93	11241	lung	2002	harbour seal	*Phoca vitulina*	Germany	25	
bmar033	94	8155	spleen	1999	harbour seal	*Phoca vitulina*	Germany	25	
bmar048	95	8241	gastric content	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar035	96	8170	intestine	2000	harbour seal	*Phoca vitulina*	Germany	25	
bmar105	97	M2466/93/4	spleen	1993	harbour seal	*Phoca vitulina*	Scotland	72	
bmar366	98	M374/02/2	spleen	2002	harbour seal	*Phoca vitulina*	Scotland	72	
bmar028	99	8169	lung	1998	harbour seal	*Phoca vitulina*	Germany	73	
bmar155	100	M621/99/2	lung	1999	gray seal	*Halichoerus grypus*	Scotland	25	ST25
bmar107	101	M339/94/1	GLN	1994	harbour seal	*Phoca vitulina*	Scotland	25	
bmar168	102	M250/02/3	spleen or liver	2002	harbour seal	*Phoca vitulina*	Scotland	71	
bmar004	102	M2533/93/1	spleen	1993	harbour seal	*Phoca vitulina*	Scotland	71	ST24
bmar029	103	8151	lung	1998	gray seal	*Halichoerus grypus*	Germany	71	
bmar169	104	M305/02/1	spleen	2002	harbour seal	*Phoca vitulina*	Scotland	71	
bmar377	105	M4/06/4	lung	2006	gray seal	*Halichoerus grypus*	Scotland	79	
bmar005	106	M292/94/1	spleen	1994	harbour seal	*Phoca vitulina*	Scotland	71	ST24
bmar160	107	M192/00/1	mesenteric LN	2000	minke whale	Balaenoptera acutorostrata	Scotland	71	ST24
bmar170	107	M342/02/2	mesenteric LN	2002	harbour seal	*Phoca vitulina*	Scotland	71	
bmar172	107	M449/02/2	lung	2002	harbour seal	*Phoca vitulina*	Scotland	71	
bmar228	108	25a-1	spleen	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar248	108	37c-1	mediastinal LNN	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar194	109	39e-2	liver	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar224	110	9a-1	spleen	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar238	110	17e-1	liver	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar192	110	37f-1	lung	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar250	110	38g-1	kidney	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar261	110	M603/99/7	spleen	1999	hooded seal	*Cystophora cristata*	Scotland	76	ST25
bmar189	111	22f-1	lung	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar109	112	M2006/94/6	external iliac LN	1994	hooded seal	*Cystophora cristata*	Scotland	76	ST25
bmar253	113	53c-1	mediastinal LNN	2002	hooded seal	*Cystophora cristata*	Norway	76	
bmar226	114	23a-1	spleen	2002	hooded seal	*Cystophora cristata*	Norway	75	
bmar227	114	24a-2	spleen	2002	hooded seal	*Cystophora cristata*	Norway	75	
bmar216	115	M163/99/10	kidney	1999	hooded seal	*Cystophora cristata*	Scotland	76	ST25
bmar229	116	30a-1	spleen	2002	hooded seal	*Cystophora cristata*	Norway	74	
bmar265	117	02/611	blood	2002	human		New Zealand	78	ST27

Figure 2

MLVA-16 clustering analysis of 93 *B. pinnipedialis* strains defines 3 groups of strains. All *B. pinnipedialis* isolates cluster together in the second part (genotypes 75 to 117) of the dendogram constructed from MLVA-16 testing of 294 *Brucella* isolates obtained from 173 marine mammals (pinnipeds, otter and cetaceans) and one human patient from New Zealand. In the columns, the following data are presented: DNA batch (key), genotype, strain identification, organ, year of isolation, host (AWSD: Atlantic White Sided Dolphin), host (Latin name), geographic origin, MLVA panel 1 genotype, sequence type when described by Groussaud *et al*. [25]. The colour code reflects the host species (see Figure 3 for detailed correspondence). No colour was used when identical genotypes were observed in different host species. The red branch (genotype 117) corresponds to the human isolate (ST27). The letter nomenclature proposed by Groussaud *et al*. is used (*B. pinnipedialis*, cluster C, including C1 (ST24), C2 (ST25) and C3 (ST25)).

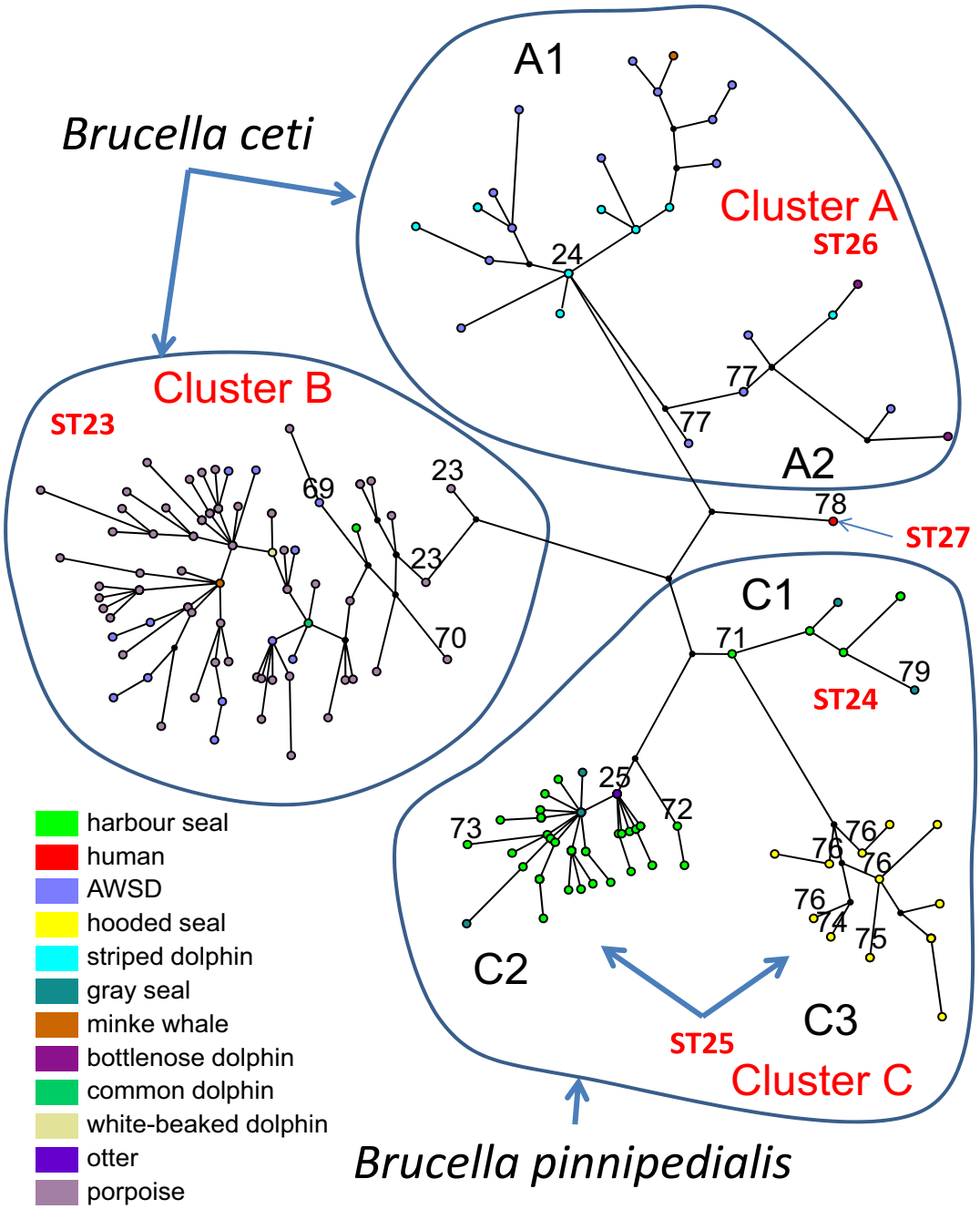


Figure 3

Maximum parsimony analysis on 117 marine mammal *Brucella* genotypes. Each coloured circle corresponds to one MLVA-16 genotype from a marine mammal species. Numbers in black (23, 24, 25, 69 to 79) indicate the MLVA the panel 1 genotype for the colour circle below. The panel 1 genotype along daughter branches is indicated only when it is different from the proposed parent node (i.e. in cluster A, all strains are panel 1 genotype 24 in subcluster A1 or 77 in subcluster A2). The tentative MLST sequence type (ST23 to ST27) as predicted from strains shared between this study and [25] is indicated, together with species assignment. The host species colour code indicated is the same as in Figures 1 and 2 (AWSD: Atlantic White Sided Dolphin).

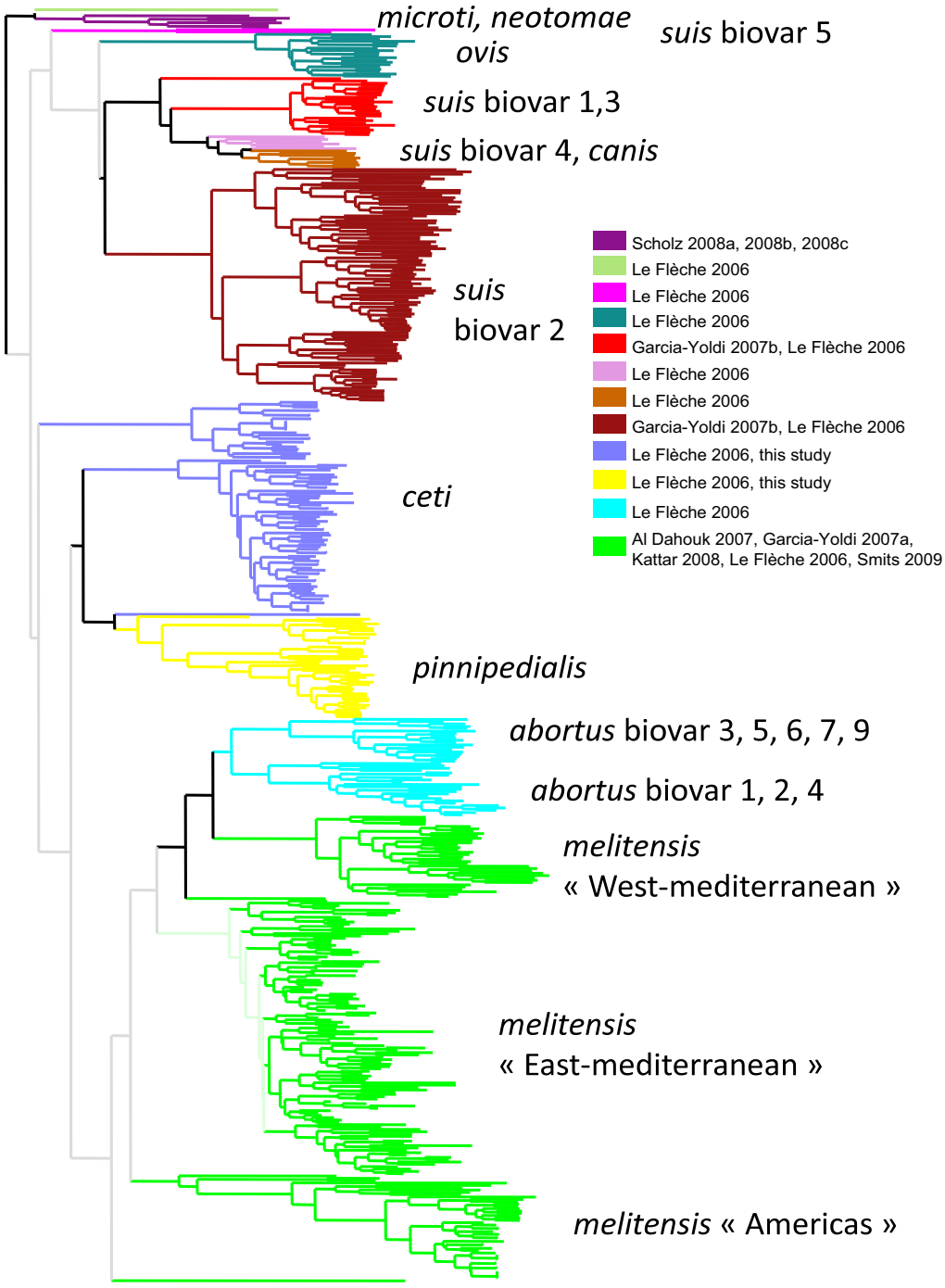


Figure 4

Current view of the global population structure of the *Brucella* genus. Clustering was done using the Neighbor Joining (NJ) algorithm. The microti/neotomae cluster was used to root the tree. The dendrogram is based upon more than 500 genotypes, observed by typing more than 750 strains [see Additional file 1]. The terrestrial mammal strains data were compiled from [5,17,19-23,37]. The colour code reflects the *Brucella* species (or some highly specific biovars). The publications from which the data were derived are indicated. The long blue branch close to the *B. pinnipedialis* cluster represents the human isolate from New Zealand (MLST ST27).

Discussion

Since 1994, marine mammal *Brucella* strains have been isolated and characterized, both phenotypically and by means of different molecular typing methods. This led to the division of the marine mammal *Brucella* strains in 2 species i.e. *B. ceti* on one side and *B. pinnipedialis* on the other side defined by oxidative metabolism patterns and CO_2 requirement for growth, and a number of subclusters defined by complementary molecular analysis methods.

This MLVA-16 study is, to date, the most important one in terms of number of strains analysed and number of animal species from which these strains have been isolated. These strains were isolated from animals stranded, caught or killed for scientific purposes in the waters surrounding Europe, from the Barents Sea, above the Arctic Circle to the Atlantic coast of Spain. For the 295 strains analysed, using the MLVA-16 assay, 117 genotypes were resolved and seven clusters were identified, (i) two clusters almost exclusively composed of dolphin isolates, (ii) the predominantly porpoise cluster of strains (which also includes several strains isolated from dolphins), (iii) two main seal species clusters, (iv) the hooded seal cluster, and (v) the human isolate. The last cluster might correspond to Pacific Ocean isolates [29-31], which are underrepresented in the present collection. The hooded seal cluster of strains was composed of strains from Scotland and Norway. The low level of genetic diversity between the hooded seal isolates from Scotland and from Norway could indicate that all the investigated hooded seals originated from the same population of animals. The population that was sampled between Svalbard and Greenland have their breeding area in the pack ice north of Jan Mayen (West Ice), but except for the few weeks on ice during birth, mating and moulting, the hooded seal is a typical pelagic and a migratory species with a huge geographical range [27]. It is thus likely that the hooded seals stranded in Scotland were originally from this area rather than from the more western bound populations of hooded seals that breed in the Davis Strait and at the east coast of Canada.

Groussaud *et al*. [25] analysed the diversity of marine mammal isolates by MLVA using another selection of VNTRs, including all 8 loci defining the HOOF-prints MLVA assay described by Bricker *et al*. [16] and 13 additional loci characterised by Le Flèche *et al*. [17] and Whatmore *et al*. [18]. This panel of 21 VNTR loci corresponded to a 21-locus MLVA scheme sharing 9 loci with MLVA-16 and also provides a high degree of diversity. In this previous study, multilocus sequence types (STs) were determined, allowing the clustering of marine mammal isolates in five groups labelled ST23 to ST27. The closely related ST24 and ST25 were composed of the pinniped isolates, forming the cluster C. The hooded seal isolates define subcluster C3. ST26 was exclusively composed of dolphin isolates and formed the cluster A. The other cetacean isolates all clustered in the cluster B (ST23) and consisted of strains isolated from porpoises and dolphins. ST27 was represented by only one isolate from an aborted bottlenose dolphin foetus originating from the Western coast of the United States (strain F5/99) [28]. Our results are thus in excellent accordance with those published by Groussaud *et al*. [25] showing that the previously identified population structure of marine mammal *Brucella* strains is not significantly modified by the inclusion of a large number of strains from European waters. MLVA-16 results are also in accordance with the recently reported genomic structures of 24 marine mammal *Brucella* isolates for which three subgroups were identified [32]. In that study, one separate group was identified for the *B. pinnipedialis* strains, another subgroup included dolphin isolates and a third subgroup comprised dolphin and porpoise isolates. The only hooded seal isolate analysed in that study clustered in the *B. pinnipedialis* group but revealed a separate pattern with a 62 kb missing fragment, specific for this group and relevant for a distinct genetic background [32].

MLVA-16 classification in the present report revealed some exceptions like the M490/95/1 strain, isolated from a common seal, which was clustered in the *B. ceti* group of strains. This exception suggests that transmission from cetaceans to pinnipeds may occur. Although the currently recognized terrestrial mammal *Brucella* species also have a preferred host, they can be isolated from different hosts in regions where brucellosis is endemic, e.g. *B. melitensis* which has been isolated from cattle in the southern part of France [33].

The human isolate from New Zealand formed a separate seventh MLVA-16 cluster. Whatmore *et al*. [28] have shown that the F5/99 strain, isolated from an aborted bottlenose dolphin fetus from the Western coast of the United States (together with three human isolates, one from New Zealand and two from Peru) shared the same MLST genotype (ST27). Interestingly, at the *omp2* level both strains were identical and also identical to isolates from the North Pacific minke whales. In all these strains the porin *omp2* genes were different from those from marine mammal strains isolated on European coasts [30]. Briefly, the *omp2* genes of these isolates from the Pacific share common features with both marine mammal (from Europe) and terrestrial mammal strains [29]. Another interesting observation is that all the Pacific isolates investigated so far (including the three reported human cases) carry fragment I identified by IRS-PCR which is part of a putative genomic island specific for *B. pinnipedialis* [12]. Since these cetacean isolates are quite distinct from European marine mammal isolates there might be a third

marine mammal *Brucella* species or subspecies found in Pacific waters. Owing to the simplicity of MLVA-16 typing, and in particular of panel 1 which can be typed on regular agarose gels and already provides a high informativity in classifying marine mammal strains (Figure 3), more typing information on Pacific Ocean strains (including the strains described in [29-31]) will likely be made available in a near future. The *Brucella*2009 genotyping database available at http://mlva.u-psud.fr/ and based upon the data provided in Additional file 1 can be used for this purpose. Figure 4 shows the global population structure of the nine species currently constituting the *Brucella* genus, as can be revealed by MLVA-16 typing using this dataset (the extended data set provided here may provide new opportunities to evaluate additional methods for *Brucella* MLVA data clustering recently proposed [34]).

Conclusion

MLVA-16 proved to be useful for molecular classification of a high number of marine mammal *Brucella* strains and allows the typing of large populations, while providing a clustering in agreement with all previously reported methods, together with a much higher discriminatory power. From the clustering achieved, a few representative strains can be selected for whole genome sequencing.

Methods

Brucella *strains*

MLVA analysis was performed on 294 isolates from 173 marine mammals and one human patient. The strains essentially originate from the Northern Atlantic, from three main sources, Scotland (216 isolates from 116 animals), Germany (58 isolates from 42 animals) [35] and Norway (18 isolates from 13 animals) [27]. Six additional strains from various geographic origins were analysed. Two strains were obtained from France (one strain from a bottlenose dolphin (*Tursiops truncatus*) and one from a harbour porpoise (*Phocoena phocoena*)), one from Spain (from a striped dolphin (*Stenella coeruleoalba*)) [36] and two from The Netherlands (two strains from one harbour porpoise (*Phocoena phocoena*)). The sixth strain was a human isolate from New-Zealand (strain 02/611 genotype 117) [14]. Strains (one strain per genotype and animal) are listed in Figures 1 and 2 and in Additional file 1.

MLVA analysis

The method for selecting appropriate VNTRs has been described previously by Le Flèche *et al.* [17], adapted by Al Dahouk *et al.* (the initial MLVA-15 assay was completed by bruce19) [20]. The results were compared with the MLVA-16 results obtained for the 18 terrestrial mammal *Brucella* reference strains published previously by Le Flèche *et al.* [17] and additional published data [5,19-23,37]. The sixteen loci have been classified in 3 panels, called panel 1 (8 minisatellite loci), panel 2A (3 microsatellite loci) and panel 2B (5 microsatellite loci) [20]. Panel 1 was composed of bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, bruce55, useful for species identification. Panel 2, showing a higher discriminatory power, was split into two groups, panel 2A and 2B, composed of three (bruce18, bruce19, bruce21) and five (bruce04, bruce07, bruce09, bruce16, bruce30) markers, respectively. Panel 2B contains the more variable loci, and this panel can be given a lower weight in clustering analysis, as described by Al Dahouk *et al.* [20] and Kattar *et al.* [21].

PCR amplification

Brucella DNA was prepared as previously described by Cloeckaert *et al.* [38]. PCR amplification was performed in a total volume of 15 μl containing 1 ng of DNA, 1× PCR reaction buffer, 1 U of Taq DNA polymerase (QBiogen, Illkirch, France), 200 μM of each deoxynucleotide triphosphate, and 0.3 μM of each flanking primer as described by Le Flèche *et al.* [17].

Amplifications were performed in a MJ Research PTC200 thermocycler. An initial denaturation step at 96°C for 5 minutes was followed by 30 cycles of denaturation at 96°C for 30 s, primer annealing at 60°C for 30 s, and elongation at 70°C for 1 min. The final extension step was performed at 70°C for 5 min.

Two to five microliters of the amplification product were loaded on a 3% standard agarose gel for analyzing tandem repeats with a unit length shorter than 10 bp (panel 2) and on a 2% standard agarose gel for all others (panel 1), and run under a voltage of 8 V/cm until the bromophenol blue dye had reached the 20 cm position. Gels were stained with ethidium bromide, visualized under UV light, and photographed (Vilber Lourmat, Marnes-la-Vallée, France). A 100-bp and a 20-bp ladder (EZ load 100 bp or 20 bp PCR Molecular Ruler, Biorad, Marnes-la-Coquette, France) were used as molecular size markers depending on the tandem repeat unit length. Gel images were managed using the BioNumerics software package (version 6.0, Applied-Maths, Belgium).

Data analysis

Band size estimates were converted to a number of units within a character dataset using the BioNumerics software and the previously published allele calling convention [17]. Clustering analyses used the categorical coefficient and the UPGMA (unweighted pair group method using arithmetic averages) or Neighbor Joining algorithm. The use of categorical parameter implies that the character states are considered unordered. The same weight is given to a large or a small number of differences in the number of repeats at each locus. Maximum parsimony was done using BioNumerics, running 200 bootstrap simulations

treating the data as categorical and giving the same weight to all loci.

Authors' contributions

JG and GV coordinated contributions by the different participants. IJ, MT, GF, BD, SAD, HN, FR, KW and JG isolated and/or maintained strains and/or produced DNA. PLF did the MLVA genotyping work. GV and PLF were in charge of the BioNumerics database, error checking, clustering analyses. MM, AC and GV wrote the report. IJ helped to draft the manuscript. All authors read, commented and approved the final manuscript.

Additional material

Additional file 1

MLVA-16 data*. The repeat copy numbers at each locus are indicated for each strain.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-145-S1.xls]

Acknowledgements

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