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Molecular Characterization of *Mycoplasma agalactiae* Reveals the Presence of an Endemic Clone in Spain

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***Mycoplasma agalactiae* isolates from Spain were genetically characterized to investigate their genomic diversity and to better understand their relationship to isolates from other countries. Molecular typing revealed a high genomic homogeneity in Spanish *M. agalactiae* isolates, which clearly shows the circulation of one endemic clonal population.**

Mycoplasma agalactiae is the main etiologic agent of contagious agalactia (CA), a serious syndrome affecting small ruminants; the World Organization for Animal Health must be notified of its occurrence because of its high economic significance worldwide. The first genomic studies showed little genomic diversity within *M. agalactiae* species, apart from that provided by antigenic variation (1, 2). Recently, the development of new sequence-based typing systems has revealed more genetic heterogeneity than previously thought (3–5). To investigate the genomic diversity of Spanish *M. agalactiae* isolates and to elucidate their relationship to those from other geographic areas, we analyzed isolates from Spain using pulsed-field gel electrophoresis (PFGE), which has been demonstrated to be robust and discriminative for typing different species of mycoplasmas (6–9), including *M. agalactiae* (3, 10); we also used the most recently developed sequence-based typing techniques, such as multilocus variable-

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TABLE 1 MLST results at 5 housekeeping loci obtained with a subset of 48 Spanish *Mycoplasma agalactiae* field isolates and strains PG2 and Teramo from 2008 to 2010[a]

Isolate	Province of origin	Yr of isolation	PFGE profile	MLST allelic profile for:					
				dnaA	*gltX*	*gyrB*	*metS*	*tufA*	ST
24a	Salamanca	2008	II	1	1	2	2	2	5
26a	Zamora	2008	II	1	1	2	2	2	5
262a	León	2008	I	1	1	2	2	2	5
276a	Valladolid	2008	I	1	1	2	2	2	5
283a	Palencia	2008	I	1	1	2	2	2	5
286a	Cantabria	2008	I	1	1	2	2	2	5
286b	Cantabria	2008	I	1	1	2	2	2	5
286c	Cantabria	2008	I	1	1	2	2	2	5
287c	Burgos	2008	I	1	1	2	2	2	5
423d	Zamora	2008	I	1	1	2	2	2	5
472d	Salamanca	2008	I	1	1	2	2	2	5
513a	Salamanca	2009	I	1	1	2	2	2	5
651a	Zamora	2009	I	1	1	2	2	2	5
653a1	Zamora	2009	I	1	1	2	2	2	5
653a2	Zamora	2009	I	1	1	2	2	2	5
657c	Valladolid	2009	I	1	1	2	2	2	5
787a	Valladolid	2009	IV	1	1	2	2	2	5
787b	Valladolid	2009	IV	1	1	2	2	2	5
787c	Valladolid	2009	IV	1	1	2	2	2	5
793a	Valladolid	2009	V	1	1	2	2	2	5
793b	Valladolid	2009	V	1	1	2	2	2	5
799a	Valladolid	2009	V	1	1	2	2	2	5
799b	Valladolid	2009	V	1	1	2	2	2	5
799c	Valladolid	2009	V	1	1	2	2	2	5
1021a	Palencia	2009	I	1	1	2	2	2	5

TABLE 1 (Continued)

Isolate	Province of origin	Yr of isolation	PFGE profile	MLST allelic profile for:					
				dnaA	*gltX*	*gyrB*	*metS*	*tufA*	ST
1026b	Zamora	2009	I	1	1	2	2	2	5
1032b	León	2009	I	1	1	2	2	2	5
1033a	León	2009	III	1	1	2	2	2	5
1033b	León	2009	III	1	1	2	2	2	5
1033c	León	2009	III	1	1	2	2	2	5
1033d	León	2009	III	1	1	2	2	2	5
1033e	León	2009	III	1	1	2	2	2	5
1043a	Valladolid	2009	I	1	6	2	2	2	18
1058b	Valladolid	2009	I	1	1	2	2	2	5
1086a	León	2010	I	1	1	2	2	2	5
1114a	Segovia	2010	I	1	1	2	2	2	5
1132a	Segovia	2010	I	1	1	2	2	2	5
1160a	Palencia	2010	I	1	1	2	2	2	5
1423a	Valladolid	2010	VI	1	6	2	2	2	18
1423c	Valladolid	2010	VI	1	6	2	2	2	18
1506a	Valladolid	2010	I	1	1	2	2	2	5
1668a	Zamora	2010	I	1	1	2	2	2	5
1680a	Burgos	2010	I	1	6	2	2	2	18
1700a	León	2010	II	1	1	2	2	2	5
1700b	León	2010	II	1	1	2	2	2	5
1700c	León	2010	II	1	1	2	2	2	5
1703a	Valladolid	2010	II	1	1	2	2	2	5
1704b	Salamanca	2010	I	1	1	2	2	2	5
Teramo	Italy	Unknown	IV	1	1	1	1	1	1
PG2	Spain	1959	IV	1	1	1	1	1	1

[a] PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type.

TABLE 2 DNA restriction fragments of 410 *Mycoplasma agalactiae* isolates generated by PFGE with restriction enzyme SmaI in Spain from 2008 to 2010[a]

DNA fragment	Size (kbp) for pulsotype:					
	I	II	III	IV	V	VI
A	427	427	**454**	427	427	427
B	159	159	159	159	159	**197**
C	124	124	124	124	124	124
D	83	**92**	83	83	**108**	83
E	56	56	56	**67**	56	56
F	9	9	9	9	9	9
G	5	5	5	5	5	5
Genome size	863	872	890	874	888	901

[a] All the values in the table are expressed in kbp. Boldface indicates restriction fragments showing size differences from those obtained for pulsotype I, which was the most frequently isolated (95% of isolates).

number tandem-repeat (VNTR) analysis (MLVA) (3) and multilocus sequence typing (MLST) (4). Typing systems were selected to obtain a comprehensive approach to the genomic diversity of Spanish *M. agalactiae* isolates and also to generate suitable data for evolutionary and population studies. Knowledge of the diversity and distribution of *M. agalactiae* clones will facilitate tracing the source of new international outbreaks as well as contribute to a better understanding of *M. agalactiae* population genetics and evolution.

TABLE 3 Spatial distribution of PFGE genomic profiles obtained for 410 Spanish *Mycoplasma agalactiae* field isolates from 2008 to 2010

Province of origin	PFGE pattern	No. of isolates	Total no. of isolates/province	% of isolates with PFGE pattern/province
Burgos	I	23	23	100
Cantabria	I	3	3	100
León	I	55	63	87.3
	II	3		4.8
	III	5		7.9
Palencia	I	56	56	100
Salamanca	I	28	29	96.6
	II	1		3.4
Segovia	I	2	2	100
Valladolid	I	115	127	90.6
	II	1		0.8
	IV	3		2.4
	V	5		3.9
	VI	3		2.4
Zamora	I	106	107	99.1
	II	1		0.9

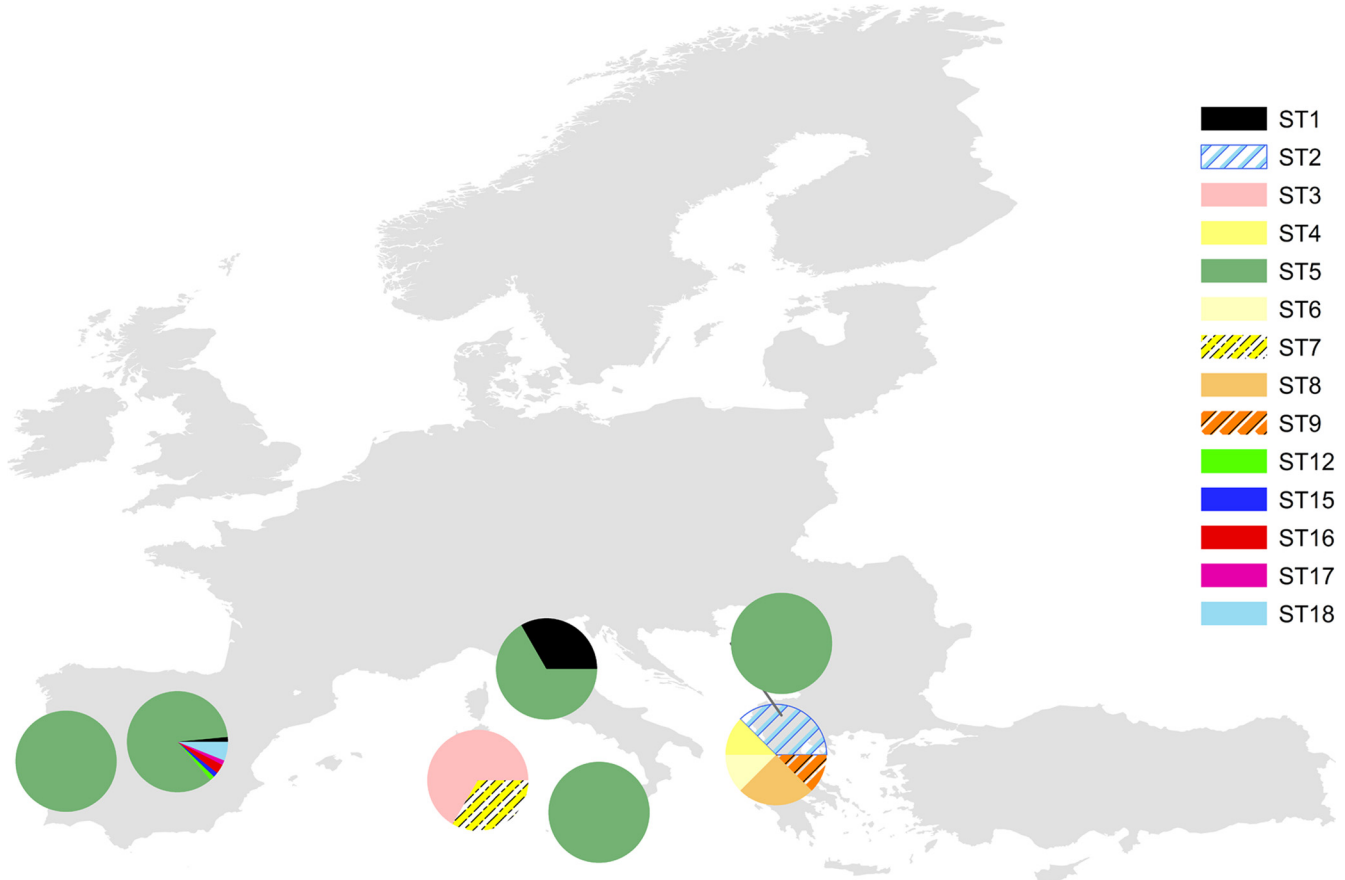
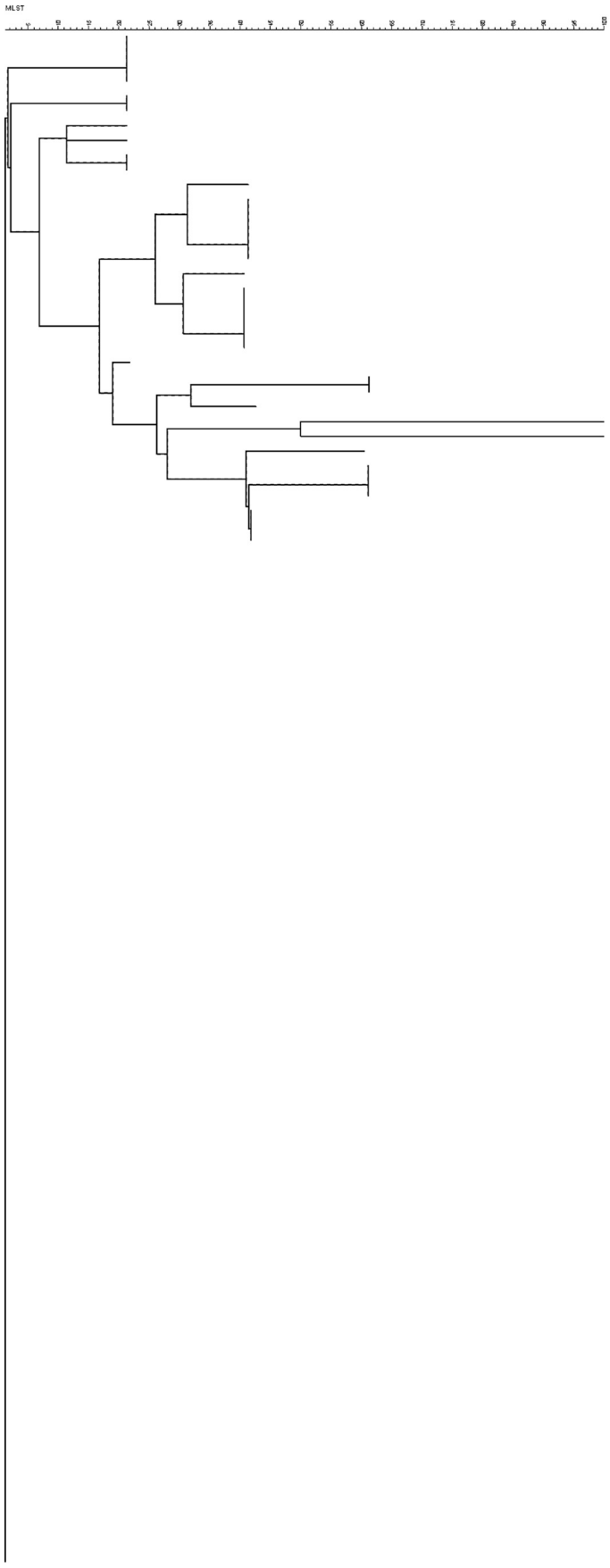


FIG 1 Geographic distribution of 103 European *Mycoplasma agalactiae* isolates based upon their multilocus sequence typing allelic profiles. Isolates previously subjected to MLST have been included (2). Locations of pie charts indicate the geographic origins of the isolates, and their color reflects the different sequence types (STs).



Isolate	Country	Year of isolation	MLST ST
● 1680a	Spain	2010	18
● 1043a	Spain	2009	18
● 1423a	Spain	2010	18
● 1423c	Spain	2010	18
244F03	Italy	2003	3
247F03	Italy	2003	3
S4	Spain	2010	17
249F03	Italy	2003	7
H	Spain	2010	16
S1	Spain	2010	16
572	Mongolia	2006	14
356	Mongolia	2006	11
564	Mongolia	2006	11
659	Mongolia	2006	11
666	Mongolia	2006	11
12	Mongolia	2006	11
568	Mongolia	2006	13
14	Mongolia	2006	10
44	Mongolia	2006	10
334	Mongolia	2006	10
401	Mongolia	2006	10
752	Mongolia	2006	10
235F03	Greece	2003	4
224F03	Greece	2003	8
225F03	Greece	2003	8
234F03	Greece	2003	6
281F03	Spain	2003	12
L9	Spain	1993	15
227F03	Greece	2003	9
● Teramo	Italy	Unknown	1
● PG2	Spain	1959	1
10123	USA	1983	1
222F03	Greece	2003	2
233F03	Greece	2003	2
240F03	Greece	2003	2
● 1114a	Spain	2010	5
● 1132a	Spain	2010	5
● 286a	Spain	2008	5
● 286b	Spain	2008	5
● 286c	Spain	2008	5
● 287c	Spain	2008	5
● 513a	Spain	2009	5
● 1704b	Spain	2010	5
● 472d	Spain	2008	5
● 1032b	Spain	2009	5
● 1066a	Spain	2010	5
● 262a	Spain	2008	5
● 1021a	Spain	2009	5
● 1160a	Spain	2010	5
● 283a	Spain	2008	5
● 1026b	Spain	2009	5
● 1668a	Spain	2010	5
● 423d	Spain	2008	5
● 276a	Spain	2008	5
● 1506a	Spain	2010	5
● 24a	Spain	2008	5
● 1700b	Spain	2010	5
● 26a	Spain	2008	5
● 1700a	Spain	2010	5
● 1703a	Spain	2010	5
● 1700c	Spain	2010	5
● 657c	Spain	2009	5
● 1033a	Spain	2009	5
● 1033e	Spain	2009	5
● 1033b	Spain	2009	5
● 1033c	Spain	2009	5
● 1033d	Spain	2009	5
● 787a	Spain	2009	5
● 787b	Spain	2009	5
● 787c	Spain	2009	5
● 793a	Spain	2009	5
● 793b	Spain	2009	5
● 799a	Spain	2009	5
● 799b	Spain	2009	5
● 799c	Spain	2009	5
● 1058b	Spain	2009	5
● 651a	Spain	2009	5
● 653a1	Spain	2009	5
● 653a2	Spain	2009	5
13	Spain	2010	5
23	Spain	2010	5
27	Spain	2010	5
102	Spain	2010	5
1101	Spain	2010	5
1801	Spain	2010	5
2501	Spain	2010	5
14SR97	Macedonia	1997	5
1640/98	Italy	1998	5
165/99	Italy	1999	5
243F03	Italy	2003	5
250F03	Italy	2003	5
256F03	Italy	2003	5
259F03	Portugal	2003	5
263F03	Portugal	2003	5
269F03	Portugal	2003	5
271F03	Portugal	2003	5
287F03	Spain	2003	5
289F03	Spain	2003	5
501F06	Italy	2006	5
E	Spain	2010	5
I	Spain	2010	5
LoFaso	Italy	2000	5
S2	Spain	2010	5
10F03	unknown	2003	5

Four hundred ten *M. agalactiae* isolates collected from 171 Spanish sheep flocks from 2008 through 2010, type strain PG2 (Institut Pasteur, Paris, France), and strain Teramo (Mycoplasma Experience, Ltd., Reigate, United Kingdom) were subjected to extensive genomic characterization (see Table S1 in the supplemental material). All the information regarding the sampling and the isolation procedure is detailed in the work of Ariza-Miguel et al. (11). The species designation of the isolates was confirmed by real-time PCR targeting the *p40* gene (12, 13). All isolates were analyzed by PFGE with the restriction enzyme SmaI and by MLVA at 4 highly variable VNTR loci (i.e., MagaI VNTR 5, MagaI VNTR 14, MagaI VNTR 17, and MagaI VNTR 19) as previously described (3). MLST analyses (4) were carried out on a subset of 48 field isolates which showed different genomic profiles in the previous analyses, as well as on isolates from different geographic origins and times of isolation selected to yield the highest genetic variability (Table 1). A neighbor-joining dendrogram showing relatedness among isolates on the basis of their MLST allelic profiles was constructed by using BioNumerics v.6.6 software, and BURST analysis was performed with eBURST v3 (http://eburst.mlst.net/). Information about the isolates analyzed, as well as a new allelic profile, was submitted to the PubMLST database (http://pubmlst.org/magalactiae/).

We detected a high genomic homogeneity in *M. agalactiae* isolates from Spain using three different genotyping tools (i.e., PFGE, MLVA, and MLST). PFGE provided the highest discriminative power and was capable of distinguishing between some isolates which were largely indistinguishable by MLVA or MLST (see Table S1 in the supplemental material). Genomic characterization by PFGE identified 6 different pulsotypes which were closely related and showed very similar fingerprint patterns, with only small size differences in one band among pulsotypes (Table 2). Ninety-five percent of the isolates belonged to the same genomic profile, named pulsotype I, resulting in a Simpson's index of diversity of 0.104. Pulsotype I was found widely distributed in all the provinces sampled; it was the pulsotype of from 87% to 99% of the isolates analyzed per province. The rest of the genomic profiles were found disseminated in 3 neighboring provinces (Table 3). PG2 and Teramo strains belonged to pulsotype IV and clustered with 3 field isolates. Surprisingly, genetic profiles obtained by MLVA were largely indistinguishable, with all field isolates showing the same genetic profile at the 4 highly variable VNTR loci. Moreover, only MLVA at the MagaI VNTR 17 locus was capable of distinguishing between field isolates showing a band at 285 bp and between PG2 and Teramo strains showing a band at 169 bp. The Simpson's index of diversity was determined to be 0.005. Finally, MLST analyses of the 48 field isolates revealed 2 different sequence types (STs). Forty-four out of 48 field isolates (92%) belonged to ST 5 (allelic profile 11222). The other ST was not described at that moment and, after its submission to the PubMLST database, was designated ST 18 (allelic profile 16222) (Table 1).

Overall, molecular typing revealed a high genomic homogeneity in Spanish *M. agalactiae* isolates, which clearly show the circulation of one endemic clonal population. A similar finding was recently observed in the French western Pyrenees region by Nouvel et al. (5), who reported that the endemic CA repeatedly observed over the past 30 years in that region has been caused by a unique subtype of *M. agalactiae*. MLVA placed all the French isolates in the same genotype, designated ST 10. Interestingly, all 410 Spanish *M. agalactiae* isolates analyzed in this study were placed in the same MLVA type, suggesting that the same highly successfully adapted strain has been circulating in Spain and France during the last 2 years. To obtain further information about the endemic clone, a representative isolate, namely, 1668a, was fully sequenced, and future studies will help to clarify the molecular mechanisms involved in the evolutionary success of this clone as well as to provide new insights into the genomic diversity and evolution of the species. In contrast, several studies have reported an unexpectedly high diversity in *M. agalactiae* Spanish isolates recovered from goats (3, 4, 14). Further studies are necessary to test whether this is because various CA-causing mycoplasmas have been detected in Spanish goat herds (15–17), while *M. agalactiae* has been the only species detected in sheep, limiting the possibility of genetic exchange (11).

The global relationship of *M. agalactiae* clones on the basis of

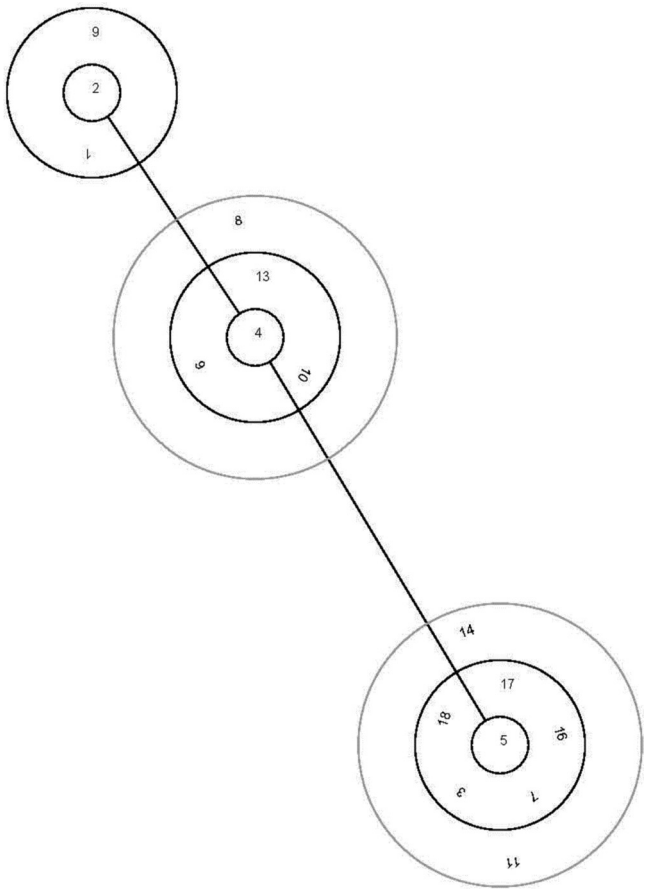


FIG 3 BURST analysis of 104 worldwide *Mycoplasma agalactiae* isolates based upon their multilocus sequence typing allelic profiles. Clonal complexes were defined as groups of multilocus genotypes in which every genetic profile shared at least 3 out of 5 loci with at least one other member of the group. Sequence type 5 was found to be the ancestral genotype. European isolates previously analyzed were added to the study (2).

FIG 2 Genetic relationships among 104 worldwide *Mycoplasma agalactiae* isolates based upon allelic differences at 5 housekeeping loci. Name of isolates, years of isolation, countries of origin, and sequence types (MLST) are specified to the right of the branches. Isolates previously analyzed were added to the study (2). Black dots indicate the isolates analyzed in this study. The dendrogram was produced by using the neighbor-joining method of the BioNumerics v.6.6 software.

available MLST allelic profiles also showed a high genetic homogeneity, with isolates belonging to ST 5 being widely distributed throughout many southern European countries analyzed so far (Fig. 1) (4). The 44 Spanish field isolates belonging to ST 5 examined in this study clustered with previously analyzed isolates from Spain and other southern European countries: Portugal, Italy (including Sicily and Sardinia), and Macedonia. The other 4 field isolates belonging to novel ST 18 clustered closely with the previous one, forming a new branch (Fig. 2). Interestingly, strain Teramo (Italy) and type strain PG2 (Spain) clustered with strain 10123 from the United States, suggesting an evolutionary relationship. We hypothesize that a highly adaptive genotype may have increased rapidly in frequency to produce an epidemic clone in southern Europe. Then, that clone diversified though recombination or mutation to produce minor clonal variants (18). BURST analysis supports this hypothesis, since it defined ST 5 as the adaptive ancestral genotype from which the minor clonal variants have arisen (Fig. 3). Further investigations are necessary to test that hypothesis, and the inclusion of new isolates from other geographic areas and times of isolation will help to clarify the evolution of this pathogen and its current population structure.

In conclusion, this study provides a genomic characterization of *M. agalactiae* in Spain and contributes to the better understanding of the global distribution of clones. Molecular typing revealed a high genomic homogeneity in Spanish *M. agalactiae* isolates, which clearly show the circulation of one endemic clonal population, and will facilitate the design of prophylactic measures.

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