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# *Clostridium botulinum* Group I Strain Genotyping by 15-Locus Multilocus Variable-Number Tandem-Repeat Analysis▿†

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***Clostridium botulinum* is a taxonomic designation that encompasses a broad variety of spore-forming, Gram-positive bacteria producing the botulinum neurotoxin (BoNT). *C. botulinum* is the etiologic agent of botulism, a rare but severe neuroparalytic disease. Fine-resolution genetic characterization of *C. botulinum* isolates of any BoNT type is relevant for both epidemiological studies and forensic microbiology. A 10-locus multiple-locus variable-number tandem-repeat analysis (MLVA) was previously applied to isolates of *C. botulinum* type A. The present study includes five additional loci designed to better address proteolytic B and F serotypes. We investigated 79 *C. botulinum* group I strains isolated from human and food samples in several European countries, including types A (28), B (36), AB (4), and F (11) strains, and 5 nontoxic *Clostridium sporogenes*. Additional data were deduced from *in silico* analysis of 10 available fully sequenced genomes. This 15-locus MLVA (MLVA-15) scheme identified 86 distinct genotypes that clustered consistently with the results of amplified fragment length polymorphism (AFLP) and MLVA genotyping in previous reports. An MLVA-7 scheme, a subset of the MLVA-15, performed on a lab-on-a-chip device using a nonfluorescent subset of primers, is also proposed as a first-line assay. The phylogenetic grouping obtained with the MLVA-7 does not differ significantly from that generated by the MLVA-15. To our knowledge, this report is the first to analyze genetic variability among all of the *C. botulinum* group I serotypes by MLVA. Our data provide new insights into the genetic variability of group I *C. botulinum* isolates worldwide and demonstrate that this group is genetically highly diverse.***

The *Clostridium botulinum* taxon constitutes a polyphyletic cluster of anaerobic Gram-positive spore-forming species which share the feature of producing the dangerous botulinum neurotoxin (BoNT). BoNT is the etiological agent of botulism, a rare but severe neuroparalytic disease recognized in four natural forms (food-borne botulism, wound botulism, infant botulism, and adult intestinal colonization botulism) (15).

A historical subdivision of *C. botulinum* into four groups (I to IV) has been made on the basis of biochemical and biophysical criteria (fermentative and proteolytic capabilities, metabolic acids, spore heat resistance, optimal growth temperature, etc.) (4, 21).

On the basis of the toxin antigenic specificity of BoNT, *C. botulinum* strains are divided into seven serotypes (A to G) (4, 21). Serotypes A, B, E, and, rarely, F are responsible for human botulism cases. Cases associated with C and D serotypes are observed mainly in animals. No botulism cases linked to serotype G have been reported (1, 15). Group I includes serotype A strains and the proteolytic B and F strains. Group II includes all E serotype strains and the nonproteolytic B and F strains. Group III encompasses serotype C and D strains, while group IV is only composed of serotype G strains (4, 21). Differences in the BoNT gene nucleotide sequences lead to the definition of five subtypes (A1 to A5) for type A, five (proteolytic B1 to B3, bivalent B, and nonproteolytic B) for type B, four (E1 to E3 and E6) for type E, and seven (F1 to F7) for type F (2, 3, 6, 23). Two subtypes from BoNT/E-producing *Clostridium butyricum* strains (subtypes E4 and E5) have been described (3). Usually *C. botulinum* strains produce only a single serotype of BoNT, but "bivalent" strains producing two different serotypes of BoNT have been isolated (20, 21). Small-subunit rRNA (16S rRNA) sequence analysis has shown that *C. botulinum* is a taxonomic designation comprising diverse species of anaerobic spore-forming bacteria. Moreover,

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ribotyping has shown that the phenotypic groups correspond to genotypic units (4, 10, 26). rRNA 16S sequencing has also demonstrated that some groups of non-BoNT-producing clostridia, classified by current taxonomy as distinct species (*Clostridium sporogenes*, *Clostridium novyi*, *Clostridium proteolyticus*, and others), are indeed encompassed in one of four *C. botulinum* groups. In particular, *C. sporogenes* appears to be related to group I (10).

So far, several genotyping methods have been applied to *C. botulinum* strains for taxonomic, molecular epidemiology, and forensic purposes: pulsed-field gel electrophoresis (PFGE) (9, 18), amplified fragment length polymorphism (AFLP) (10, 14, 17), multilocus sequence typing (MLST) (13), and whole-genome DNA microarrays (2). The clustering of populations using these genotyping methods is only partially congruent with BoNT gene lineage, suggesting that BoNT genes have evolved separately from the bacterial chromosome through horizontal genetic transfer events (2, 4, 8, 10, 11). This hypothesis is supported by the discovery, in some *C. botulinum* strains, of transposable insertion sequences (IS) close to the BoNT genes and of BoNT gene sequences in plasmids (8, 24, 25, 29).

The reliability of multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) for high-resolution genotyping has already been shown for many pathogens (28, 22). A recent study highlighted the discrimination capability of a 10-locus MLVA (MLVA-10) scheme among *C. botulinum* serotype A strains (17).

High-resolution genetic characterization of *C. botulinum* strains still remains a challenge for epidemiological studies, as well as for forensic microbiology. In the past, some countries or terrorist groups were believed to have developed research programs evaluating BoNT as a biological weapon (1). These are reasons why additional efforts to improve MLVA capabilities are needed.

The aims of the present study are the improvement of genetic discrimination among group I *C. botulinum* strains and the setting up of an internet-based MLVA database useful in a terrorist attack, as well as in botulism outbreaks in human and wildlife. New VNTR markers were developed to better define genetic clustering among group I *C. botulinum* isolates (types A, B, bivalent, and F).

## MATERIALS AND METHODS

**Strains.** The 79 *C. botulinum* and 5 *C. sporogenes* strains examined in this study (Table 1) were provided by the Division of Analytical Microbiology, DGA CBRN Defense (Vert-Le-Petit, France), Bundeswehr Institute of Microbiology (Munich, Germany), Georg-August-University of Goettingen (Goettingen, Germany), Thueringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (Bad Langensalza, Germany), TNO Defense, Security, and Safety (Rijswijk, The Netherlands), Norwegian Defense Research Establishment, Division for Protection, (Kjeller, Norway), and National Reference Center for Botulism, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità (Rome, Italy). DNA was extracted by heating culture supernatants with Chelex resin solution at 95°C (7) or by cell lysis, followed by phenol-chloroform extraction and ethanol precipitation.

All strains were submitted to 16S rRNA gene analysis. Moreover, the 40 Italian strains were submitted to sugar fermentation and proteolytic activity tests. All of the *C. botulinum* strains belong to group I; 28 are BoNT/A strains, 36 BoNT/B, 4 BoNT/AB, and 11 BoNT/F (Fig. 1). In addition, five non-toxin-producing *C. sporogenes* strains were included to complete the *C. botulinum* group I genotypes.

All strains were from different botulism events, and no isolates were associated with one another.

The collection analyzed in this study includes clinical and food isolates, as well as reference strains whose whole-genome sequence data have been made public (Table 1). Their geographical origins include European (Italy, France, Germany, United Kingdom, Norway, and Spain) and non-European countries (New Zealand, United States, and Japan).

**VNTR identification.** By using the Microbial Tandem Repeats database (http://minisatellites.u-psud.fr) (5) on the Hall strain sequence (GenBank accession number NC_009698), 57 potential VNTR loci were detected, including 10 previously investigated by Macdonald and colleagues (17). Nine loci that have not been investigated previously were evaluated. Five were selected (Table 2). Consequently, a pool of 15 VNTR loci is now available to define an MLVA typing panel. For the 10 previously published VNTR loci, except for cbms10 (*Clostridium botulinum* mini satellite 10), new primers were designed, closer to the VNTR region than previous ones, for the purpose of optimizing allele sizes (Table 2). The primers were synthesized with a fluorescent Cy5.5 or Cy5 label on the forward primer (Eurofins MWJ, Edersberg, Germany).

**VNTR amplification and fragment separation.** Amplification was performed in seven duplex PCRs (cbms1-cbms11, cbms02-cbms08, cbms03-cbms04, cbms05-cbms12, cbms06-cbms14, cbms07-cbms15, and cbms10-cbms13) and a singleplex PCR (cbms09).

The PCR amplifications were performed in a final volume of 15 μl containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 μM labeled forward and reverse primers, 1 U *Taq* polymerase, and 1 ng of template DNA. Identical thermocycling conditions were used for all VNTR assays, as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. PCR products were diluted 1:5 in water, and then 1-μl amounts were sized on a CEQ 8000 DNA sequencer (Beckman Coulter, Brea, CA) using 0.7 μl of MapMarker 1000 molecular-weight markers (BioVenture, Murfreesboro, TN) as described in Lista et al. (16). A comparison between expected and observed product sizes using the CEQ 8000 may be found in file S1 in the supplemental material.

All 15 loci were also amplified with unlabeled primers in singleplex assays, and amplicons were measured on a lab-on-a-chip Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). A comparison between observed and expected product sizes using the Agilent 2100 Bioanalyzer software may be found in file S2 in the supplemental material.

**Sequencing analysis.** Some representative PCR fragments resulting from VNTR amplifications were sequenced to verify fragment length and the corresponding number of repeat units (RU) (data not shown).

The PCR amplicons were purified and sequenced with a CEQ 8000 automatic DNA analysis system (Beckman Coulter, Fullerton, CA), using a commercial kit (GenomeLab DTCS [dye terminator cycle sequencing] quick start kit; Beckman Coulter) according to the manufacturer's instructions.

In order to confirm that all 84 samples used in this study belong to group I, 16S rRNA gene sequencing was performed (data available on request). Primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGA CTT) were used to amplify approximately 1,400 bases of this 1.5-kb gene. Then, the amplicons were purified using NucleoSpin extract II (Macherey-Nagel, Duren, Germany) and sequenced using the same primers (Genechron, Rome, Italy). The 16S rRNA gene phylogenetic dendrogram was created from an alignment of 16S rRNA gene sequences, some original from this study and others obtained from GenBank. Sequence data were imported into BioNumerics (version 6.5) (Applied Maths, Sint-Martens-Latem, Belgium) and aligned in a multiple alignment, and a dendrogram was calculated using the unweighted-pair group method with arithmetic mean (UPGMA) clustering algorithm (Fig. 1).

**VNTR genotyping.** Observed sizes were converted to repeat copy number by a macro (Openoffice Calc 3.2 by Oracle) developed by the Lista group (available on request to F.L.).

VNTR repeat unit profiles obtained from 15 loci were imported into BioNumerics version 6.5 (Applied Math, Sint-Martens-Latem, Belgium) as character values, and dendrograms were calculated using the categorical coefficient and the UPGMA clustering algorithm. A dendrogram was generated for the complete panel (MLVA-15) and also for two subsets: MLVA-10, including only previously published loci, and MLVA-7, including loci considered suitable for lab-on-a-chip analysis.

To better standardize the allele calling, the following *C. botulinum* group I genome sequences were also included in the analysis, using *in silico* analysis to deduce their MLVA data: ATCC 3502 A1 (GenBank accession number NC_009495), ATCC 19397 A1 (NC_009697), Hall A1 (NC_009698), Kyoto A2 (NC_012563), Loch Maree A3 (NC_010520), bivalent 657 BA4 (NC_012658), Okra B1 (NC_010516), Langeland F (NC_009699), H04402 065 A5/B3 (CP002011), and F strain 230613 (FR773526).

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TABLE 1. Strains used in this study

| Sample | Serologic profile | | Species | Type of botulism | Source | Yr of isolation | Geographic origin |
|---|---|---|---|---|---|---|---|
| | Type | Subtype[a] | | | | | |
| 45 | A | ND | *Clostridium botulinum* | Food-borne | Mushrooms in oil | 1986 | Italy (Lazio) |
| 46 | B | ND | *Clostridium botulinum* | Infant | Feces | 1991 | Italy (Friuli) |
| 50 | B | ND | *Clostridium botulinum* | Survey | Peppers in oil | 1990 | Italy (Puglia) |
| 53 | AB | A2B1 | *Clostridium botulinum* | Food-borne | Feces | 1993 | Italy (Lombardia) |
| 75 | B | ND | *Clostridium botulinum* | Food-borne | Feces | 2002 | Italy (Campania) |
| 79 | A | 2 | *Clostridium botulinum* | Infant | Feces | 2003 | Italy (Lazio) |
| 81 | B | ND | *Clostridium botulinum* | Food-borne | Feces | 2004 | Italy (Molise) |
| 85 | A | ND | *Clostridium botulinum* | Survey | Honey | 2004 | Italy (Emilia R.) |
| 87 | A | ND | *Clostridium botulinum* | Infant | Feces | 2004 | Italy (Lombardia) |
| 92 | F | ND | *Clostridium botulinum* | Food-borne | Asparagus | 2005 | Italy (Trentino) |
| 101 | A | ND | *Clostridium botulinum* | Food-borne | Feces | 2007 | Italy (Emilia R.) |
| 102 | B | ND | *Clostridium botulinum* | Food-borne | Canned tuna in oil | 2006 | Italy (Lazio) |
| 60 | A | 2 | *Clostridium botulinum* | Food-borne | Feces | 1996 | Italy (Basilicata) |
| 62 | A | ND | *Clostridium botulinum* | Food-borne | Feces | 1996 | Italy (Basilicata) |
| 66 | B | ND | *Clostridium botulinum* | Food-borne | Feces | 2000 | Italy (Friuli) |
| 68 | B | ND | *Clostridium botulinum* | Food-borne | Feces | 2000 | Italy (Campania) |
| ATCC 17843 | B | ND | *Clostridium botulinum* | | | | |
| 3/3 | B | ND | *Clostridium botulinum* | | | | |
| CEB94018 | B | ND | *Clostridium botulinum* | | | | |
| CEB94074 | B | ND | *Clostridium botulinum* | | | | |
| CEB94077 | B | ND | *Clostridium botulinum* | Survey | Food | | UK |
| CEB94078 | F | ND | *Clostridium botulinum* | | | | |
| 1/5 | A | ND | *Clostridium botulinum* | | | | |
| 2/5 | A | ND | *Clostridium botulinum* | | | | |
| 4/5 | A | ND | *Clostridium botulinum* | | | | |
| 5/5 | A | ND | *Clostridium botulinum* | | | | |
| CEB06128 | A | 1 | *Clostridium botulinum* | Infant | Feces | | France |
| CEB06129 | A | 1 | *Clostridium botulinum* | Food-borne | Canned peas | | France |
| CEB06173 | A | 2 | *Clostridium botulinum* | Food-borne | Asparagus | | France |
| 29 | A | 2 | *Clostridium botulinum* | Infant | Feces | 1988 | Italy (Lazio) |
| 31 | B | ND | *Clostridium botulinum* | Infant | Feces | 1989 | Italy (Friuli) |
| 90 | B | ND | *Clostridium botulinum* | Infant | Feces | 1995 | Italy (Lazio) |
| 103 | A | 2 | *Clostridium botulinum* | Infant | Feces | 1996 | Italy (Lombardia) |
| 127 | A | 2 | *Clostridium botulinum* | Infant | Feces | 1998 | Italy (Veneto) |
| 172 | B | ND | *Clostridium botulinum* | Infant | Feces | 2000 | Italy (Lazio) |
| 173 | B | ND | *Clostridium botulinum* | Infant | Feces | 2000 | Italy (Campania) |
| 181 | B | ND | *Clostridium botulinum* | Infant | Feces | 2000 | Italy (Emilia R.) |
| 186 | A | 2 | *Clostridium botulinum* | Infant | Feces | 2001 | Italy (Calabria) |
| 261 | B | ND | *Clostridium botulinum* | Infant | Feces | 2003 | Italy (Lazio) |
| 267 | B | 2 | *Clostridium botulinum* | Infant | Feces | 2003 | Italy (Lazio) |
| 275 | B | ND | *Clostridium botulinum* | Wound | Wound | 2004 | Italy (Sicilia) |
| 331 | B | 2 | *Clostridium botulinum* | Infant | Feces | 2004 | Italy (Lombardia) |
| 341 | B | ND | *Clostridium botulinum* | Infant | Feces | 2004 | Italy (Lombardia) |
| 361 | B | ND | *Clostridium botulinum* | Infant | Feces | 2005 | Italy (Campania) |
| 363 | A | ND | *Clostridium botulinum* | Infant | Feces | 2005 | Italy (Campania) |
| 380 | B | ND | *Clostridium botulinum* | Infant | Feces | 2006 | Italy (Campania) |
| 386 | AB | ND | *Clostridium botulinum* | Infant | Feces | 2006 | Italy (Veneto) |
| 389 | B | ND | *Clostridium botulinum* | Infant | Feces | 2006 | Italy (Lombardia) |
| 390 | A | ND | *Clostridium botulinum* | Infant | Feces | 2006 | Italy (Lazio) |
| 401 | B | ND | *Clostridium botulinum* | Infant | Feces | 2007 | Italy (Veneto) |
| 413 | B | ND | *Clostridium botulinum* | Infant | Feces | 2008 | Italy (Puglia) |
| 433 | B | ND | *Clostridium botulinum* | Infant | Feces | 2009 | Italy (Lazio) |
| 128 | B | ND | *Clostridium botulinum* | Infant | Feces | 1998 | Italy (Lombardia) |
| BM270 | A | ND | *Clostridium botulinum* | | Home-canned corn | 1928 | |
| BM271 | A | ND | *Clostridium botulinum* | | Canned peas | 1926 | |
| BM277 | F | ND | *Clostridium botulinum* | | Home-made liver paste | 1960 | |
| BM278 | A | ND | *Clostridium botulinum* | | | | |
| BM279 | A | ND | *Clostridium botulinum* | | | | |
| BM291 | F | ND | *Clostridium botulinum* | | | | |
| CIP104310T | A | ND | *Clostridium botulinum* | | | | |
| CIP104312 | F | ND | *Clostridium botulinum* | | | | |
| CIP60.10 | B | ND | *Clostridium botulinum* | | | | |
| CB745 | B | ND | *Clostridium botulinum* | | | | |
| A2 | F | ND | *Clostridium botulinum* | | | | |
| IBT 2268 | AB | ND | *Clostridium botulinum* | | | | Spain |

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TABLE 1—*Continued*

| Sample | Serologic profile | | Species | Type of botulism | Source | Yr of isolation | Geographic origin |
|---|---|---|---|---|---|---|---|
| | Type | Subtype[a] | | | | | |
| IBT 2295 | AB | ND | *Clostridium botulinum* | | | | Germany |
| 2/2F | F | ND | *Clostridium botulinum* | | | | |
| REB 1955 | F | ND | *Clostridium botulinum* | | | | Germany |
| IBT 2272 | F | ND | *Clostridium botulinum* | | | | Spain |
| IBT 2297 | F | ND | *Clostridium botulinum* | | | | New Zealand |
| REB 1072 | F | ND | *Clostridium botulinum* | | | | Germany |
| IBT 2299 | B | ND | *Clostridium botulinum* | | | | Japan |
| REB 83 | B | ND | *Clostridium botulinum* | | | | Germany |
| IBT 2293 | B | ND | *Clostridium botulinum* | | | | New Zealand |
| 1/3 | B | ND | *Clostridium botulinum* | | | | |
| IBT 2269 | B | ND | *Clostridium botulinum* | | | | Spain |
| 3/5 | A | ND | *Clostridium botulinum* | | | | |
| IBT 2292 | A | ND | *Clostridium botulinum* | | | | New Zealand |
| IBT 2267 | A | ND | *Clostridium botulinum* | | | | Germany |
| DSM795 | | | *Clostridium sporogenes* | | | | |
| 885/05 | | | *Clostridium sporogenes* | | | | |
| 925/05 | | | *Clostridium sporogenes* | | | | |
| C1 | | | *Clostridium sporogenes* | | | | |
| C2 | | | *Clostridium sporogenes* | | | | |
| ATCC 3502 | A | 1 | *Clostridium botulinum* | | | | U.S. |
| ATCC 19397 | A | 1 | *Clostridium botulinum* | | | | U.S. |
| Kyoto | A | 2 | *Clostridium botulinum* | Infant | | 1978 | Japan |
| Okra | B | | *Clostridium botulinum* | Food-borne | | | U.S. |
| Hall | | | *Clostridium botulinum* | | | | U.S. |
| 657 | Ba | Ba4 | *Clostridium botulinum* | Infant | | 1976 | U.S. |
| Loch Maree | A | 3 | *Clostridium botulinum* | Food-borne | Liver paste | 1922 | U.S. |
| Langeland | F | | *Clostridium botulinum* | Food-borne | Liver paste | 1958 | UK |
| H04402 065 | AB | A5B3 | *Clostridium botulinum* | Wound | | 2004 | Denmark |
| F str. 230613 | F | | *Clostridium botulinum* | | | | UK |

[a] ND, not determined.

## RESULTS

**Identification of new alleles.** Extensive variety in repeat unit (RU) sizes and number of alleles is observed among the 15 loci. When compared to previous *C. botulinum* MLVA-10 results (17), new alleles were found. Five new alleles (5.5, 8.5, 13, 14, and 15 RU) were observed for cbms01; nine new alleles (1, 3, 6, 10, 12.5, 13.5, 15, 19, and 21 RU) were observed for cbms03; seven new alleles (2, 10, 14, 23, 25, 26, and 35 RU) were observed for cbms04; four new alleles (7, 8, 13, and 15 RU) were observed for cbms05; three new alleles (3, 3.5, and 5 RU) were observed for cbms07; one new allele (2 RU) was observed for cbms11; and four new alleles (3, 10, 11, and 12 RU) were observed for cbms14.

The amplification of several VNTR loci resulted in "intermediate" allele fragment sizes (Table 3), probably due to insertions and/or deletions occurring outside or even within the repeat unit sequence, as observed in fully sequenced genomes. In cbms03, the intermediate allele, 12.5 RU, is the result of a 5-bp insertion located in the 5′ VNTR flanking region (data not shown). Moreover, some cbms03 fragments showed a shorter length than the 1-RU allele expected size. Most likely, as observed in the fully sequenced Langeland (NC_009699) and Loch Maree genomes (NC_010520), large deletions are scattered over this locus.

Locus cbms10, alias VNTR6 (17) (18-bp RU), is characterized by deletions/insertions in the 5′ and 3′ repeat regions, as shown by the comparison of eight fully sequenced strains (Table 4). Although the RU is 18 bp long, the resulting observed differences between fragment sizes mimic a 6-bp repeat unit variation. These data suggest that, for convenience, cbms10 could be considered a hexamer.

**Clustering of strains.** Analysis of 94 strains by use of the MLVA-15 scheme (84 analyzed strains plus 10 typed by *in silico* analysis) resulted in 86 genotypes with a Hunter and Gaston index of diversity (HGDI) (12) of 0.998.

The genotypes are distributed in seven main groups (Fig. 2) and 28 isolated strains (cluster cutoff value, 50%). Among the main groups, one, the "F group," contains nine strains, including the Langeland and 230613 F strains (cluster 1). Three groups are composed of serotype B strains only—the "B1 Okra group" (cluster 2), comprising Okra together with seven strains mainly originating from Northern Europe, and the "first Italian B group" (cluster 3) and "second Italian B group" (cluster 4) with seven and five strains, respectively, all originating from Italy. Two groups contain exclusively serotype A—the "A2 Kyoto group" (cluster 5), which contains the Kyoto strain plus eight serotype A strains, mainly A2, coming from Italy, and the "A1 ATCC 3502 group" (cluster 6) with 13 members, in which the ATCC 3502 A1, ATCC 19397 A1, and Hall A1 strains were included. One group contains different serotypes, being composed of both A and B members, i.e., the "A/B group" (cluster 7) with strains originating from Italy and France.

Twenty-eight strains appear to be genetically distinct from the seven established clusters.

MLVA-10 and MLVA-7 generated 82 and 52 distinct genotypes, respectively, with HGDI values of 0.996 and 0.971.

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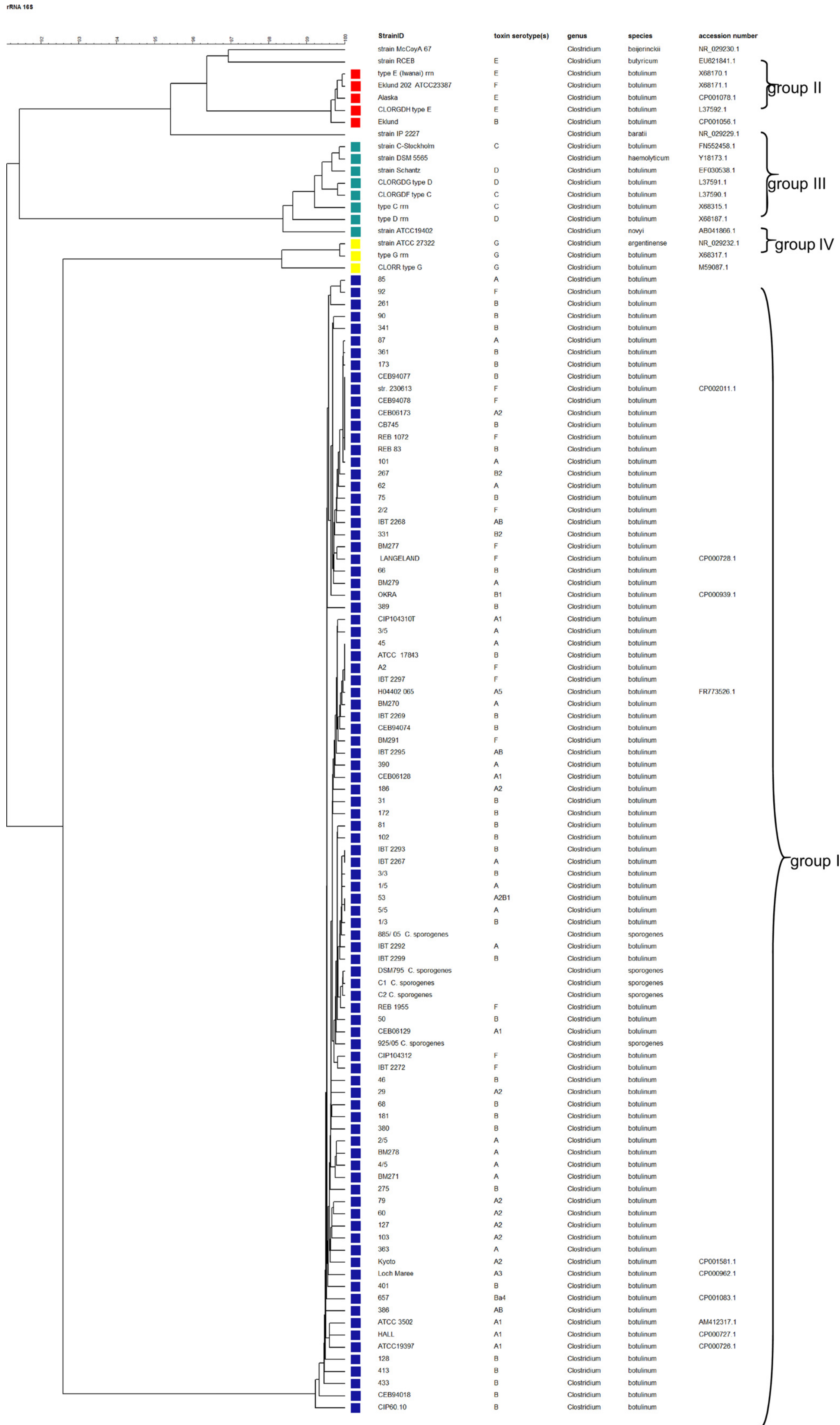


FIG. 1. UPGMA dendrogram generated by multiple alignment of rRNA 16S genes. To classify strains analyzed in the present report into groups I to IV, their rRNA 16S gene sequences were compared to available sequences of isolates from the four groups in GenBank.



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TABLE 2. VNTR characteristics

| Minisatellite | Correspondence[d] | Consensus size (bp) | No. of repeats[b] | Amplification product size[b] (bp) | Consensus sequence | Gene | Primer[a] | Genomic location[b] | Primer sequence (5′ → 3′) | DI[c] | No. of alleles |
|---|---|---|---|---|---|---|---|---|---|---|---|
| cbms01 | VNTR 1 | 15 | 8 | 250 | GAAGAAAATTTAAAT | DNA gyrase subunit A | F | 8684 | TGCTATGGGTGTAAAACTAAT GAGA | 86.7 | 15 |
| | | | | | | | R | 8933 | TGATACGTCTGGAAAAGTT GTTT | | |
| cbms02 | | 12 | 10 | 312 | GAAAAGCCTAAC | Hypothetical protein | F | 113273 | AGCGCCAATAAAGATAAC AAAG | 79.5 | 13 |
| | | | | | | | R | 113584 | TTCTAATCCAATAGGTATGTT TTGC | | |
| cbms03 | VNTR 2 | 7 | 8 | 305 | AGAGTAT | Amidase domain protein | F | 435411 | CAGTGGAACAAAAATTAGA TGAA | 94.8 | 29 |
| | | | | | | | R | 435715 | AAAATTTGGGCAATGTAAAACT | | |
| cbms04 | VNTR 3 | 6 | 13 | 212 | TAGAAC | Hypothetical protein | F | 589015 | TTATTATTAGATTCTGTATGGG CGTTT | 92.2 | 22 |
| | | | | | | | R | 589227 | AAAAATCATGAAAAAAAT GGTG | | |
| cbms05 | VNTR 10 | 24 | 5 | 209 | CTTCAGCTTCTTTTCT TTGAGCTT | Peptidoglycan hydrolase | F | 594191 | GTCTCCATGTGTTGCTGGTG | 85.4 | 10 |
| | | | | | | | R | 594399 | GAGAAAAATCATCTGAGCCTGT | | |
| cbms06 | VNTR 4 | 27 | 2 | 229 | GTAAAATAAATGTTA AAAAATAAGGAG | Hypothetical protein | F | 794406 | CTTTAATTCCTTGTGTTCCA | 29.9 | 2 |
| | | | | | | | R | 794634 | CGAATTGAAGCAAGAGTCCA | | |
| cbms07 | VNTR 5 | 18 | 4 | 258 | AAGATGTTGAAGAT GGTA | Putative glycosil hydrolase | F | 1192047 | AAGGGTTTGCACCAGATACG | 70.3 | 5 |
| | | | | | | | R | 1192304 | ACCCTTTTGTCCACCATTTG | | |
| cbms08 | | 9 | 8 | 328 | AAAAATAAA | Hypothetical protein glutamyl-tRNA synthetase | F | 1202258 | AAAATTAAGACCAGAAAAA GAAG | 79.5 | 13 |
| | | | | | | | R | 1202585 | AAAATAGGAATAGACAAACTTA AAGC | | |
| cbms09 | | 24 | 4 | 235 | AACGAGTGCCATGAA CACCATCAT | Hypothetical protein | F | 1329735 | GGAAGAAATGAGAGATTGT CACG | 59.6 | 4 |
| | | | | | | | R | 1329969 | TGGTCTTCTTACTGTTTGTGGTG | | |
| cbms10 | VNTR 6 | 6 | 27 | 459 | See text and Table 4 | Hypothetical protein | F | 1452407 | GAGGTGTAGTTATGAGAG ATGG | 90.6 | 25 |
| | | | | | | | R | 1452865 | CTTTCATATGCTTCTCTTTCA | | |
| cbms11 | VNTR 7 | 20 | 3 | 223 | TAGATCTATAATAAA GAATT | Methylcobalamide:CoM methyltransferas Na+/H+ antiporter family protein | F | 1620607 | AGGATGTCAAATCCCAATGC | 61.3 | 3 |
| | | | | | | | R | 1620829 | AAGAGCCTTTCCATTTCCTTT | | |
| cbms12 | VNTR 8 | 21 | 3 | 277 | CTTGAATTTTTACTAT CCTTA | Hypothetical protein | F | 1996195 | TCTCTCCATCTCAGACATTA GCTG | 46.8 | 3 |
| | | | | | | | R | 1996471 | ACAAGGACCGGGGAGATATT | | |
| cbms13 | | 18 | 22 | 549 | AGAAGGCGGAAGCT ATTG | Glycosyl transferase | F | 2189357 | CATTGAAAGGAATGATATTG TGGA | 87.4 | 17 |
| | | | | | | | R | 2189905 | TCTACCACTTCCGCTGGATT | | |
| cbms14 | VNTR 9 | 18 | 6 | 179 | TTGACCCTGGTTTG TCTG | Hypothetical protein | F | 2271590 | GGAAGTTTCCATTTTCCAGC | 82.2 | 8 |
| | | | | | | | R | 2271768 | CCTATTTGGGTAGACATTGCAG | | |
| cbms15 | | 40 | 4 | 311 | TAAGTTGTGTATTAT TTTTAATTGTGTAT AAAGTTGTTTT | Hypothetical protein 30S ribosomal protein S4 | F | 3645821 | TTGTTATCCATATAGCATACC TCTT | 62.6 | 7 |
| | | | | | | | R | 3646131 | CCAATAGACGCAAATGAAACTC | | |

[a] F, forward primer; R, reverse primer.
[b] Number of repeats, locus size, and location in sequenced genome of ATCC 3502 (NC_009495).
[c] DI, diversity index.
[d] Correspondence with previously described loci (17).

TABLE 3. MLVA allele profiles

| Sample | RU size of VNTR locus[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | cbms1 | cbms2 | cbms3 | cbms4 | cbms5 | cbms6 | cbms7 | cbms8 | cbms9 | cbms10 | cbms11 | cbms12 | cbms13 | cbms14 | cbms15 |
| 45 | 8 | 12 | 1 | 8 | 11 | 2 | 3.5 | 6 | 4 | 31 | 2 | 1 | 23 | 7 | 3 |
| 46 | 10 | 6 | 6 | 16 | 13 | 2 | 4 | 10 | 4 | 23 | 3 | 1 | 18 | 6 | 4 |
| 50 | 5 | 14 | 12 | 7 | 12 | 2 | 2 | 5 | 3 | 17 | 1 | 1 | 23 | 11 | 5 |
| 53 | 11 | 11 | del4 | 7 | 10 | 2 | 2 | 5.5 | 4 | 28 | 2 | 3 | 17 | 9 | 3 |
| 75 | 5 | 11 | 3 | 21 | 13 | 2 | 4 | 6 | 4 | 33 | 1 | 3 | 24 | 8 | 3 |
| 79 | 10 | 8 | 2 | 21 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 81 | 5 | 6 | 8 | 7 | 12 | 2 | 2 | 5 | 3 | 17 | 1 | 1 | 23 | 7 | 5 |
| 85 | 8 | 12 | 13.5 | 8 | 9 | 1 | 4 | 6 | 4 | 28 | 2 | 1 | 23 | 6 | 3 |
| 87 | 8 | 12 | 13.5 | 8 | 9 | 1 | 4 | 6 | 4 | 28 | 2 | 1 | 23 | 6 | 3 |
| 92 | 11 | 12 | 13 | 14 | 5 | 2 | 5 | 5 | 3 | 25.5 | 1 | 1 | 17 | 10 | 3 |
| 101 | 9 | 8 | 2 | 21 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 102 | 5 | 12 | 12.5 | 8 | 12 | 2 | 2 | 5 | 3 | 17 | 1 | 1 | 19 | 11 | 5 |
| 60 | 9 | 8 | 2 | 21 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 62 | 8 | 12 | 13.5 | 8 | 9 | 1 | 4 | 6 | 4 | 28 | 2 | 1 | 23 | 6 | 3 |
| 66 | 9 | 10 | 10 | 14 | 11 | 2 | 2 | 5 | 3 | 15 | 1 | 1 | 23 | 10 | 5 |
| 68 | 8 | 10 | 9 | 15 | 11 | 2 | 4 | 14 | 4 | 21 | 2 | 3 | 21 | 9 | 4 |
| ATCC 17843 | 15 | 10 | 15 | 17 | 11 | 2 | 4 | 6 | 2 | 17 | 1 | 1 | 20 | 11 | 3 |
| 3/3 | 13 | 10 | 3 | 12 | 11 | 2 | 5 | 7 | 3 | 17 | 1 | 1 | 16.5 | 3 | 4 |
| CEB94018 | 5 | 8 | del3 | 9 | 11 | 2 | 3.5 | 5 | 4 | 26 | 1 | 1 | 17 | 8 | 3 |
| CEB94074 | 9 | 10 | 3 | 16 | 11 | 2 | 5 | 7 | 3 | 25 | 1 | 1 | 14 | 3 | 4 |
| CEB94077 | 9 | 10 | 3 | 16 | 11 | 2 | 5 | 7 | 3 | 25 | 1 | 1 | 14 | 3 | 4 |
| CEB94078 | 10 | 9 | del5 | 12 | 15 | 2 | 3.5 | 5.5 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| 1/5 | 10 | 10 | 13 | 29 | 5 | 2 | 4 | 41 | 3 | 6 | 3 | 3 | 14 | 7 | 4 |
| 2/5 | 6 | 10 | 13 | 29 | 5 | 2 | 4 | 10 | 3 | 27 | 3 | 3 | 22 | 7 | 4 |
| 4/5 | 9 | 10 | 5 | 21 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| 5/5 | 9 | 10 | 5 | 21 | 5 | 2 | 4 | 6 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| CEB06128 | 6 | 10 | 14 | 14 | 10 | 1 | 4 | 6 | 2 | 17 | 1 | 1 | 21 | 7 | 2 |
| CEB06129 | 8 | 10 | 9 | 16 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| CEB06173 | 7 | 12 | 21.5 | 17 | 12 | 2 | 4 | 6 | 4 | 31 | 2 | 1 | 23 | 12 | 3 |
| CEB06173 | 10 | 12 | 2 | 14 | 12 | 2 | 3.5 | 6 | 3 | 59 | 3 | 1 | 22 | 9 | 3 |
| 31 | 12 | 4 | 9 | 25 | 10 | 1 | 4 | 7 | 3 | 30 | 3 | 3 | 23 | 9 | 4 |
| 90 | 7 | 14 | 16 | 31 | 11 | 2 | 2 | 5 | 3 | 15 | 1 | 1 | 23 | 11 | 5 |
| 103 | 9 | 10 | 2 | 15 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 29.5 | 9 | 3 |
| 127 | 9 | 6 | 2 | 21 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 172 | 8 | 6 | 6 | 18 | 13 | 2 | 4 | 10 | 4 | 23 | 3 | 1 | 18 | 6 | 4 |
| 173 | 14 | 14 | 3 | 1 | 9 | 2 | 3.5 | 6 | 4 | 28 | 2 | 1 | 23 | 6 | 3 |
| 181 | 9 | 10 | 9 | 11 | 11 | 2 | 4 | 6 | 4 | 24 | 2 | 3 | 21 | 9 | 4 |
| 186 | 9 | 10 | 2 | 14 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 261 | 13 | 15 | del4 | 26 | 13 | 2 | 3 | 9 | 4 | 26 | 1 | 3 | 19 | 11 | 5 |
| 267 | 6 | 12 | 11 | 16 | 11 | 2 | 3 | 6 | 2 | 17 | 1 | 1 | 23 | 7 | 3 |
| 275 | 6 | 10 | 8 | 23 | 11 | 2 | 5 | 6 | 4 | 32 | 1 | 3 | 20 | 9 | 34 |
| 331 | 5.5 | 14 | 12 | 18 | 11 | 2 | 2 | 5 | 3 | 15 | 1 | 1 | 23 | 11 | 5 |
| 341 | 5 | 14 | 7 | 8 | 12 | 2 | 2 | 5 | 3 | 17 | 1 | 1 | 23 | 7 | 5 |
| 361 | 12 | 14 | 3 | 1 | 9 | 2 | 3.5 | 6 | 4 | 28 | 2 | 1 | 23 | 6 | 3 |
| 363 | 9 | 4 | 2 | 22 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| 380 | 9 | 4 | 14 | 7 | 10 | 1 | 3.5 | 7 | 3 | 30 | 3 | 3 | 29 | 9 | 4 |
| 386 | 5 | 4 | 3 | 9 | 12 | 2 | 3 | 6 | 2 | 20 | 1 | 1 | 19 | 8 | 3 |
| 389 | 8 | 11 | 2 | 11 | 12 | 2 | 4 | 6 | 4 | 28 | 2 | 1 | 23 | 8 | 3 |
| 390 | 8 | 12 | 1 | 8 | 11 | 2 | 3.5 | 6 | 4 | 31 | 2 | 1 | 23 | 7 | 3 |
| 401 | 5 | 10 | 16 | 12 | 11 | 2 | 4 | 6 | 4 | 24 | 2 | 3 | 21 | 9 | 4 |
| 413 | 9 | 6 | 14 | 34 | 8 | 2 | 4 | 6 | 2 | 20 | 2 | 1 | 23 | 8 | 3 |
| 433 | 9 | 10 | 5 | 13 | 9 | 2 | 4 | 6 | 4 | 23 | 2 | 3 | 26 | 9 | 4 |
| 128 | 11 | 10 | 19 | 17 | 9 | 2 | 4 | 6 | 4 | 24 | 2 | 3 | 21 | 8 | 5 |
| BM270 | 7 | 10 | 5 | 7 | 11 | 2 | 4 | 6 | 2 | 34 | 3 | 1 | 23 | 6 | 3 |
| BM271 | 7 | 10 | 13 | 21 | 5 | 2 | 4 | 10 | 3 | 27 | 3 | 3 | 22 | 7 | 4 |
| BM277 | 9 | 9 | del5 | 12 | 15 | 2 | 3.5 | 5.5 | 3 | 27 | 1 | 1 | 21 | 8 | 3 |
| BM278 | 9 | 10 | 5 | 21 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| BM279 | 5 | 8 | 7 | 13 | 8 | 1 | 3.5 | 5.5 | 2 | 24 | 1 | 1 | 23 | 7 | 4 |
| BM291 | 6 | 12 | 12 | 13 | 9 | 2 | 5 | 5.5 | 3 | 25.5 | 1 | 1 | 17 | 10 | 3 |
| CIP104310T | 8 | 10 | 9 | 15 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| CIP104312 | 7 | 9 | 13 | 21 | 15 | 2 | 3.5 | 5.5 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| CIP60.10 | 8.5 | 9 | 5 | 21 | 15 | 1 | 4 | 16 | 3 | 5 | 1 | 3 | 22 | 8 | 7 |
| CB745 | 10 | 10 | del5 | 12 | 11 | 2 | 5 | 7 | 3 | 25 | 2 | 1 | 14 | 3 | 4 |
| A2 | 7 | 9 | del5 | 12 | 15 | 2 | 3.5 | 5.5 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| IBT 2268 | 7 | 10 | 5 | 7 | 12 | 2 | 4 | 6 | 2 | 34 | 3 | 1 | 23 | 6 | 3 |
| IBT 2295 | 9 | 10 | 16 | 18 | 5 | 2 | 4 | 9 | 4 | 27.5 | 3 | 3 | 24 | 8 | 4 |

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TABLE 3—*Continued*

| Sample | RU size of VNTR locus[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | cbms1 | cbms2 | cbms3 | cbms4 | cbms5 | cbms6 | cbms7 | cbms8 | cbms9 | cbms10 | cbms11 | cbms12 | cbms13 | cbms14 | cbms15 |
| 2/2F | 7 | 9 | del5 | 12 | 15 | 2 | 3.5 | 5.5 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| REB 1955 | 7 | 10 | del7 | 21 | 11 | 2 | 4 | 6 | 3 | 26 | 1 | 1 | 21.5 | 7 | 4 |
| IBT 2272 | 7 | 9 | del5 | 12 | 15 | 2 | 3.5 | 2 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| IBT 2297 | 7 | 9 | del8 | 12 | 9 | 1 | 3.5 | 5 | 4 | 28 | 1 | 1 | 21 | 7 | 4 |
| REB 1072 | 7 | 9 | del5 | 12 | 15 | 2 | 3.5 | 2 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| IBT 2299 | 7 | 10 | del3 | 12 | 8 | 2 | 5 | 5 | 3 | 17 | 1 | 1 | 14 | 6 | 4 |
| REB 83 | 6 | 12 | 12.5 | 13 | 11 | 2 | 4 | 6 | 3 | 17 | 1 | 1 | 27.5 | 8 | 6 |
| IBT 2293 | 11 | 9 | del2 | 11 | 7 | 2 | 4 | 5.5 | 3 | 17 | 1 | 3 | 22 | 11 | 3 |
| 01/03/11 | 7 | 10 | del3 | 12 | 8 | 2 | 5 | 7 | 3 | 25.5 | 1 | 1 | 14 | 6 | 4 |
| IBT 2269 | 7 | 10 | 3 | 12 | 11 | 2 | 5 | 7 | 3 | 17 | 1 | 1 | 14 | 3 | 4 |
| 3/5 | 8 | 10 | 5 | 21 | 5 | 1 | 3.5 | 16 | 4 | 27.5 | 1 | 3 | 22 | 6 | 4 |
| IBT 2292 | 7 | 10 | del2 | 15 | 5 | 1 | 3 | 11 | 4 | 24.5 | 1 | 3 | 24 | 8 | 4 |
| IBT 2267 | 9 | 10 | del1 | 9 | 5 | 2 | 4 | 5.5 | 4 | 27.5 | 3 | 3 | 22 | 7 | 4 |
| DSM795 | 7 | 7 | del8 | 33 | 9 | 1 | 3.5 | 5 | 3 | 23 | 1 | 3 | 22 | 7 | 4 |
| 885/05 | 7 | 7 | 3 | 12 | 9 | 1 | 4 | 5 | 4 | 25 | 1 | 3 | 23 | 7 | 4 |
| 925/05 | 11 | 10 | del5 | 13 | 13 | 1 | 3.5 | 6 | 4 | 45 | 1 | 4 | 24 | 9 | 4 |
| C1 | 8 | 2 | 1 | 7 | 6 | 1 | 3.5 | 5 | 4 | 31 | 1 | 1 | 14 | 7 | 4 |
| C2 | 8 | 2 | 5 | 5 | 6 | 1 | 3.5 | 5 | 4 | 44 | 1 | 1 | 24 | 7 | 4 |
| ATCC 3502 | 8 | 10 | 8 | 13 | 5 | 2 | 4 | 8 | 4 | 27 | 3 | 3 | 22 | 6 | 4 |
| ATCC 19397 | del9 | 10 | 5 | 21 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| Kyoto | 10 | del1 | 2 | 21 | 12 | 2 | 3.5 | 6 | 3 | 17 | 3 | 1 | 28 | 9 | 3 |
| Okra | 13 | 10 | 3 | 12 | 11 | 2 | 5 | 7 | 3 | 25 | 1 | 1 | 14 | 3 | 4 |
| Hall | 9 | 10 | 4 | 29 | 5 | 2 | 4 | 10 | 4 | 27 | 3 | 3 | 22 | 7 | 4 |
| 657 | 6 | del1 | 7 | 11 | 9 | 1 | 4 | 6 | 2 | del13 | 1 | 1 | 18 | 9 | 4 |
| Loch Maree | del12 | del2 | del6 | 17 | 11 | 2 | 3 | 5.5 | del2 | 28 | 1 | 3 | 22 | 7 | del2 |
| Langeland | 7 | 9 | del5 | 13 | 15 | 2 | 3.5 | 5.5 | 3 | 28 | 1 | 1 | 21 | 8 | 3 |
| H004402 065 | 7 | 12 | 6.5 | 8 | 5 | 2 | 4 | 6.5 | 4 | 26 | 1 | 3 | 15 | 9 | 3 |
| str. 230613 | 7 | 9 | del5 | 12 | 15 | 2 | 3.5 | 5.5 | 3 | 22 | 1 | 1 | 21 | 8 | 3 |

[a] "del" indicates a fragment whose length is less than the expected size of a 1-RU allele amplicon or, if referring to a fully sequenced genome, a locus with deletions with respect to the corresponding sequence in ATCC 3502. "ins" indicates a locus in a fully sequenced genome with insertions with respect to the corresponding sequence in ATCC 3502.

## DISCUSSION

Genetic characterization of *C. botulinum* neurotoxin-producing strains is a challenging issue because of the great variability not only of toxin types and subtypes but of their host genetic background. Different genotyping methods, PFGE, AFLP, MLST, and MLVA, are suitable for epidemiological studies and forensic discrimination. Each one of these techniques shows advantages and limitations. PFGE and AFLP are more difficult to standardize among different laboratories because they produce patterns which cannot be conveniently coded. MLST, possibly the most informative method, appears to be more time consuming and difficult to perform. MLVA is easier to perform and less time consuming than MSLT and can be more easily standardized by different laboratories than AFLP and PFGE.

In the present study, in order to include all of the *C. botulinum* group I serotypes (A, B, F, and bivalent) and gain higher discriminating power and clustering robustness, we propose to introduce five new loci, resulting in an MLVA-15 scheme.

A previously described MLVA-10 scheme has been applied

TABLE 4. Comparison of cbms10 alleles in fully sequenced genomes[a]

| GenBank accession no. | Strain | Length of 5′ flanking region (bp)[b] | No. of RUs (18 bp) | Length of 3′ flanking region (bp)[b] | Length of PCR fragment (bp) |
|---|---|---|---|---|---|
| NC_009495 | ATCC 3502 A1 | 162 | 9 | 92 | 459 |
| NC_009697 | ATCC 19397 A1 | — | 9 | — | 459 |
| FR773526 | H04402 065 A5 | −6 | 9 | — | 453 |
| NC_009698 | Hall A1 | — | 9 | — | 459 |
| NC_012658 | 657 BA4 | −24 | 6 | — | 381 |
| NC_012563 | Kyoto A2 | −6 | 6 | — | 399 |
| NC_010520 | Loch Maree A3 | +3 | 9 | +3 | 465 |
| NC_010516 | Okra B1 | +3 | 8 | +3 | 447 |
| NC_009699 | Langeland F | +3 | 9 | +3 | 465 |
| CP002011 | 230613 F | +3 | 7 | +3 | 429 |

[a] The consensus sequence of the locus, considered as an 18-bp motif, could be TATGATGGGAATGGATGA.
[b] For ATCC 3502, the total length of the region is given, while for the following genomes, only differences in length from the corresponding region of ATCC 3502 are given; a dash indicates that the length is the same as in ATCC 3502.

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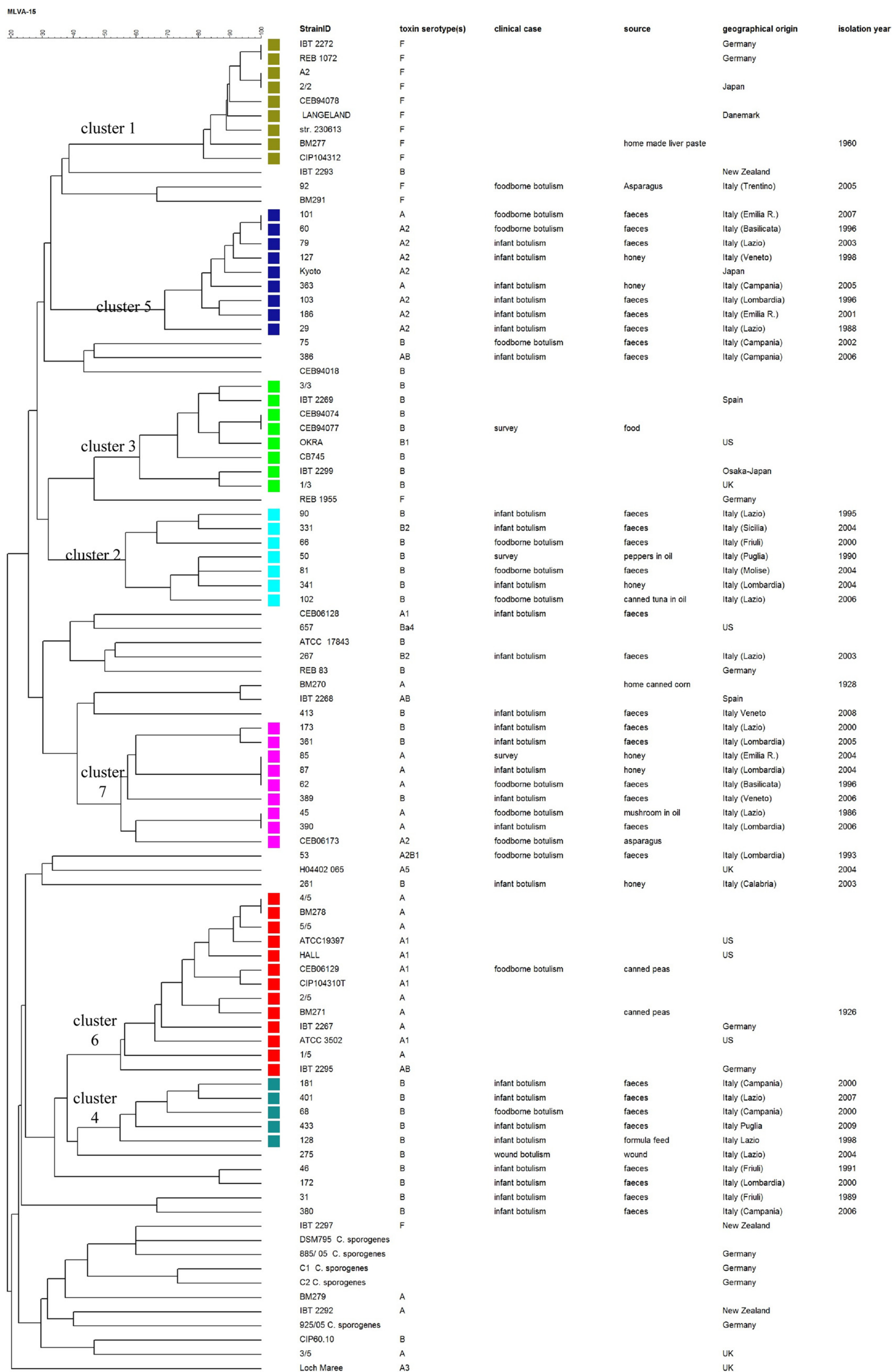


FIG. 2. MLVA-15 dendrogram. Clustering analysis was performed using the categorical coefficient and the UPGMA clustering algorithm. The groups described in the text are highlighted on the dendrogram with different colors as follows: olive for cluster 1, light green for cluster 2, light blue for cluster 3, turquoise for cluster 4, blue for cluster 5, red for cluster 6, and violet for cluster 7.



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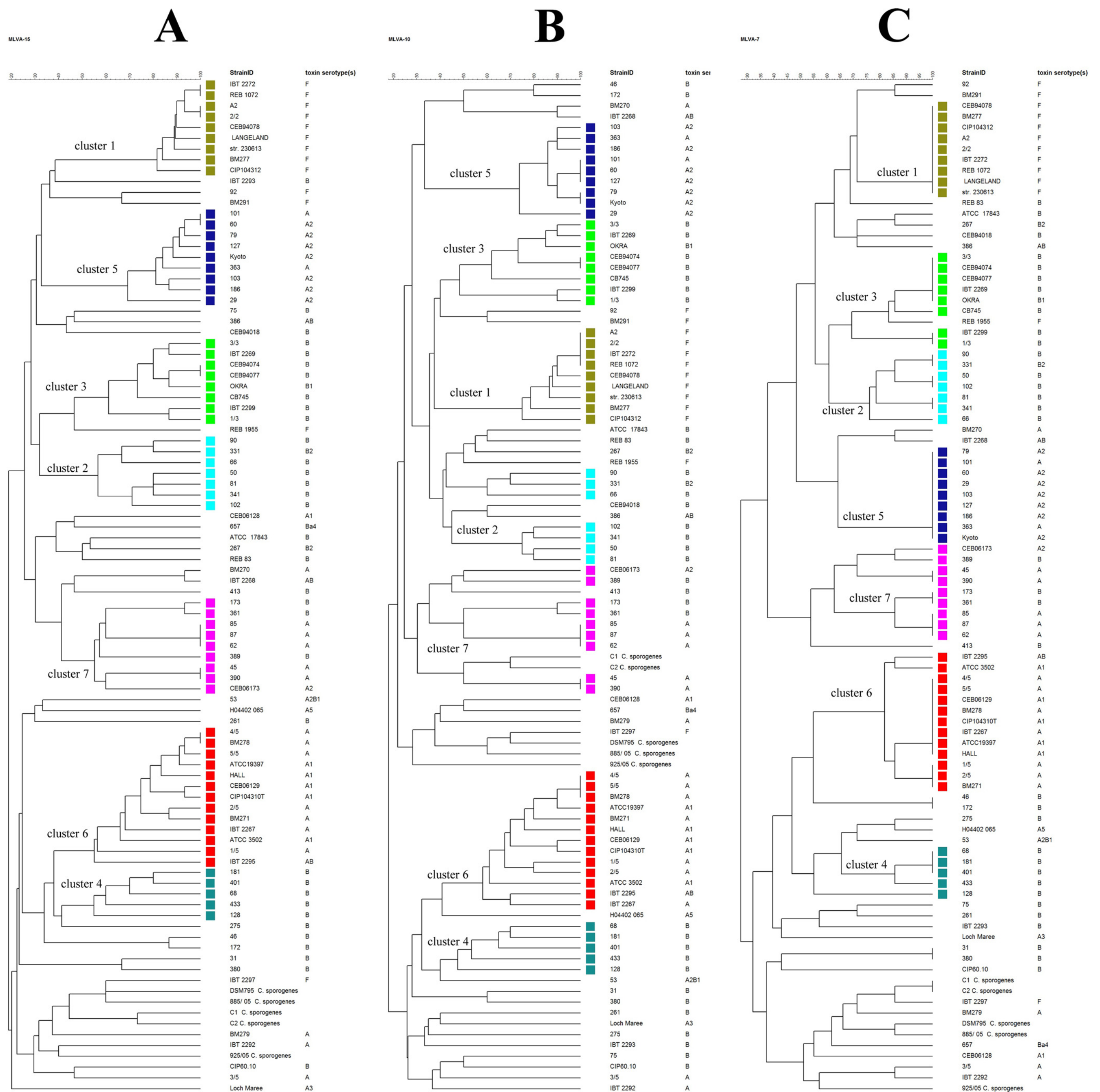


FIG. 3. Comparison of MLVA-15, MLVA-10, MLVA-7 dendrograms. Graphic representation of UPGMA clustering analysis using the three different MLVA panels, MLVA-15 (A), MLVA-10 (B), and MLVA-7 (C). The MLVA-15 groups (A) are highlighted with the same colors as in Fig. 2. In panels B and C, the groups are highlighted with reference to the colors used for MLVA-15 clustering, to highlight the differences with MLVA-10 and MLVA-7 clustering.

mainly to *C. botulinum* serotype A strains because, for the other subtypes analyzed (B to G), VNTR primers amplified only a few loci or the PCR products were not of the expected size (17). The amplification failure observed for most of the loci was probably due to the mutations within the primer binding sites. *In silico* analysis on fully sequenced genomes indicates the conservation of the primer sequences not only in A strains but also in B Okra and F Langeland (group I) strains (11, 17). In contrast, the selected primers for the 15 loci fail to match on the complete genomes of group II isolates (B Eklund and E3 Alaska), suggesting that these 15 loci are absent or highly mutated in *C. botulinum* group II strains. This finding further supports the idea that group I and group II species are well separated (4, 11, 26).

Eighty-nine *C. botulinum* strains analysis using MLVA-15 determined seven main clusters. Six of these clusters are char-

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acterized by homogeneity of toxin serotype (Fig. 2). Preliminary data from MLST (gyrase B, adenylate kinase, and transcription factor A, approximately 350 to 400 bp) performed in clusters 1, 2, and 6 confirm the genetic homogeneity of these clusters.

Most group I *C. botulinum* type B strains are represented in three distinct clusters (2, 3, and 4), one of which (group 4) appears genetically very distant from the other two B clusters. Cluster 6 (A1 subtypes) is closer to group 4 (B) than to the A2-producing group. Among serotype F strains, Reb 1955 appears very divergent from all other strains. This is a confirmation of previous studies that show a lack of congruence between the phylogenies of toxin genes and other chromosomal genes (4, 10, 11). A possible explanation could be horizontal gene transfer, a phenomenon already well known in different bacterial species, sometimes involving virulence factors (19). In support of this hypothesis, IS elements were discovered in the flanking regions of the BoNT gene in some genomes and/or the BoNT gene was found in plasmids in some *C. botulinum* strains. IS elements and plasmids are involved in the mobilization of DNA sequences among different strains (8, 11, 24, 25, 29). IS elements allow the transfer of genes between chromosomes and plasmids, and plasmids can mediate the exchange of genetic material between individuals (27).

*C. sporogenes* strains, as expected, show a genetic distribution far from *C. botulinum* cluster 7.

Comparing the MLVA results with the results of previous genotyping studies, there is a good accordance with AFLP (10, 14) and MLVA (17) results. In a *C. botulinum* AFLP study (14), proteolytic F strains showed a single cluster, whereas both A and proteolytic B strains formed different clusters, not often strictly related with the same serotype clusters, as shown in the dendrogram in Fig. 2. In a published MLVA report (17), strains are divided into two main groups, one including A1 and A3 strains and the other A2 and bivalent A1(B) strains. The ATCC 3502 strain falls into the first group, and the Kyoto strain falls into the second. Although not all toxin subtypes have been determined for strains investigated here, it is possible to identify the similarities between the two dendrograms because nine strains are shared. The two main groups described by Macdonald et al. (17) could correspond, respectively, to the present A1 ATCC 3502 group and the A2 Kyoto group. In Fig. 2, these two clusters represent two different main branches. This fact could be an element in favor of the phylogenetic reliability of the chosen MLVA-15 marker panel.

Even in the relatively limited and very diverse collection investigated here, an improvement of discrimination power is achieved by MLVA-15, since the HGDI values calculated for MLVA-10 and MLVA-15 were 0.996 and 0.998, respectively, which is an increase of 0.002 for the MLVA-15. MLVA-15 and MLVA-10 were also applied to a larger number of samples (up to 154 strains, data not shown), and in this case, the HGDI values were 0.991 and 0.986, which is a more evident increase (0.005). Moreover, in analyzing the cluster distribution with MLVA-10, two groups (3 and 7) are separated into further subgroups. From these observations, it can be concluded that the addition of five new loci determine a more compact and robust clustering (Fig. 3).

We explored the possibility of transferring the MLVA-15 scheme to a less expensive, laborious, and time-consuming platform, either agarose gel or lab-on-a-chip. This would enhance its use in a wider range of laboratories. Only seven markers (cbms05, cbms06, cbms09, cbms11, cbms12, cbms14, and cbms15) were suitable for such platforms. This is due to the less accurate reproducibility of fragment sizing with these separation techniques. The lab-on-a-chip Agilent 2100 bioanalyzer platform is more suitable for rapid typing of small collections of samples and could provide a point-of-care or field-compatible assay.

A UPGMA analysis was calculated for this subpanel (MLVA-7). The MLVA-7 dendrogram shows a clustering with few differences from that obtained using MLVA-15 (52 versus 86 profiles) (Fig. 3). The congruence between MLVA-15 and MLVA-7 as measured by UPGMA analysis, using BioNumerics version 6.5 with Pearson correlation, was 81.75. The MLVA-7 clustering tends to underestimate evolutive distances between groups, so at the cutoff chosen for MLVA-15 (50%), group 1, group 2, and group 3 are not separated. We conclude that MLVA-7 can be useful for discrimination at the cluster level but not at the strain level.

This study shows for the first time genetic variability among all of the *C. botulinum* group I serotypes by using a more refined and robust MLVA scheme. More-extended studies on a larger number of strains, coming from various geographic origins as well as encompassing a wider spectrum of neurotoxin subtypes, will be done in the future to gain new insights about the genetic diversity of *C. botulinum* worldwide.

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