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Fingerprinting of *Bacillus thuringiensis* Type Strains and Isolates by Using *Bacillus cereus* Group-Specific Repetitive Extragenic Palindromic Sequence-Based PCR Analysis

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A total of 119 *Bacillus thuringiensis* strains (83 type strains and 26 native isolates), as well as five *B. cereus* group species, were analyzed by repetitive extragenic palindromic sequence-based PCR analysis (Rep-PCR) fingerprinting. Primers Bc-REP-1 and Bc-REP-2 were specifically designed according to an extragenic 26-bp repeated sequence found in the six *B. cereus* group genomes reported. A total of 47 polymorphic bands were detected, and the patterns varied from 5 to 13 bands in number and from 0.2 to 3.8 kb in size. Virtually each type strain showed a distinctive *B. cereus* (Bc)-Rep-PCR pattern, except for *B. thuringiensis* serovars dakota (H serotype 15 [H15]) and sotto (H4a,4b), as well as serovars amagiensis (H29) and seoulensis (H35), which shared the same patterns. As expected, serovar entomocidus (H6) and its biovar subtoxicus showed an identical pattern; similarly, serovars sumiyoshiensis (H3a,3d) and fukuokaensis (H3a,3d,3e), which share two antigenic determinants, also showed identical Bc-Rep-PCR patterns. Interestingly, serovars israelensis (H14) and malaysiensis (H36), which share several phenotypic attributes, also showed identical Bc-Rep-PCR patterns. Native, coleopteran-active strains, including the self-agglutinated LBIT-74 strain, showed Bc-Rep-PCR patterns identical or very similar to that of the tenebrionis strain. Likewise, native mosquitocidal strains (including some self-agglutinated strains) also showed patterns identical or very similar to that of the serovar israelensis IPS-82 strain. Additionally, native β-exotoxin-producing strains from serovar thuringiensis showed patterns identical to that of the *B. thuringiensis* type strain. The *B. cereus* group-specific Bc-Rep-PCR fingerprinting technique was shown to be highly discriminative, fast, easy, and able to identify *B. thuringiensis* serotypes, including nonflagellar and self-agglutinated strains.

Bacillus thuringiensis is a gram-positive, flagellar, entomopathogenic bacterium that produces parasporal crystals constituted of insecticidal Cry proteins during the sporulation process (23, 52). Some strains also produce a thermostable adenine nucleotide analogue called β-exotoxin or thuringiensin (34). For decades, *B. thuringiensis* was developed and used as a control agent for lepidopteran pests, until the discovery of the mosquitocidal *B. thuringiensis* serovar israelensis in 1977 by Goldberg and Margalit (11) and the discovery of the coleopteran-active strain tenebrionis in 1983 by A. Krieg (31).

B. thuringiensis, *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus mycoides*, as well as the recently described *Bacillus pseudomycoides* (41) and *Bacillus weihenstephanensis* (33), constitute the so-called *B. cereus* group. Several authors (4, 8, 19) have suggested that these species should constitute only one species, due to their high genetic similarity. From these species, *B. thuringiensis* is the most diverse, and its strains have been classified in 84 serovars (serovarieties) (32), including the recently described serovar jordanica (H serotype 71 [H71]) (29). Serotyping is still the most widely accepted subspecific classification technique for varieties of *B. thuringiensis*, even if strains from the same serovar do not necessarily share the biochemical, genetic, or toxicological attributes (3).

While some serovars, such as serovar israelensis (H14), include strains with practically the same attributes (2), other serovars include strains with a wide diversity of features. This is the case of serovar morrisoni (H8a,8a), which includes some strains with toxicity toward mosquito larvae (44), others toward coleopteran larvae (22), and some others toward lepidopteran larvae (13). On the other hand, strains from different serovars may show high biochemical, genetic, and toxicological similarity, such as strains IMR 81-1 (serovar malaysiensis), 11S2-1 (serovar canadensis), B 175 (serovar thompsoni), K6 (self agglutinated), and B 51(self agglutinated), highly similar to serovar israelensis (50). Additionally, serotyping is useless for nonmotile strains as well as the so-called self-agglutinated strains, besides the agglutination found in some *B. cereus* strains with H antigens (32, 42).

Alternative typing methods for *B. thuringiensis* strains have been tested, mostly based on molecular techniques, such as arbitrary primer-PCR technology (7, 18), ribosomal DNA restriction fragment length polymorphism (RFLP) (1, 48), and amplified fragment length polymorphism (AFLP) (45), among others (39, 58), most of them using a limited number of strains. Diversity of rRNA intergenic spacer sequences of 31 strains proved insufficient to discriminate between isolates (97 to 99% similarity) (6). On the other hand, ribotyping (16S, 23S, and 5S rRNA gene RFLP) of 80 serovars of *B. thuringiensis* showed a great diversity of patterns (27, 28), similar to the diversity found with fluorescent AFLP, when 34 *B. thuringiensis* serovars were analyzed along with strains of *B. cereus* and *B. anthracis* (21).

Repetitive extragenic palindromic sequence-based PCR analysis (Rep-PCR) is a DNA fingerprinting technique origi-

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TABLE 1. *B. thuringiensis* type strains from the IEBC, Institut Pasteur, Paris, France subjected to Bc-Rep-PCR fingerprinting

Serovar or biovar	H serotype	IEBC no.	Serovar or biovar	H serotype	IEBC no.
Serovar			toguchini	31	T31 001
thuringiensis	1	T01 001	cameroun	32	T32 001
finitimus	2	T02 001	leesis	33	T33 001
alesti	3a,3c	T03 001	konkukian	34	T34 001
kurstaki	3a,3b,3c	T03A 001	seoulensis	35	T35 001
sumiyoshiensis	3a,3d	T03B 001	malaysiensis	36	T36 001
fukuokaensis	3a,3d,3e	T03C 001	andaluciensis	37	T37 001
sotto	4a,4b	T04 001	oswaldocruzi	38	T38 001
kenyae	4a,4c	T04B 001	brasiliensis	39	T39 001
galleriae	5a,5b	T05 001	huazhongensis	40	T40 001
canadensis	5a,5c	T05A 001	sooncheon	41	T41 001
entomocidus	6	T06 001	jinghongiensis	42	T42 001
aizawai	7	T07 001	guiyangiensis	43	T43 001
morrisoni	8a,8b	T08 001	higo	44	T44 001
ostriniae	8a,8c	T08A 001	roskildiensis	45	T45 001
nigeriensis	8b,8d	T08B 001	chanpaisis	46	T46 001
tolworthi	9	T09 001	wratislaviensis	47	T47 001
darmstadiensis	10a,10b	T10 001	balearica	48	T48 001
londrina	10a,10c	T10A 001	muju	49	T49 001
toumanoffi	11a,11b	T11 001	navarrensis	50	T50 001
kyushuensis	11a,11c	T11A 001	xiaguangiensis	51	T51 001
thompsoni	12	T12 001	kim	52	T52 001
pakistani	13	T13 001	asturiensis	53	T53 001
israelensis	14	T14 001	poloniensis	54	T54 001
dakota	15	T15 001	palmanyolensis	55	T55 001
indiana	16	T16 001	rongseni	56	T56 001
tohokuensis	17	T17 001	pirenaica	57	T57 001
kumamotoensis	18a,18b	T18 001	argentinensis	58	T58 001
yosso	18a,18c	T18A 001	iberica	59	T59 001
tochigiensis	19	T19 001	pingluonsis	60	T60 001
yunnanensis	20a,20b	T20 001	sylvestriensis	61	T61 001
pondicheriensis	20a,20c	T20A 001	zhaodongensis	62	T62 001
colmeri	21	T21 001	bolivia	63	T63 001
shandongiensis	22	T22 001	azorensis	64	T64 001
japonensis	23	T23 001	pulsiensis	65	T65 001
neoleonensis	24a,24b	T24 001	graciosensis	66	T66 001
novosibirsk	24a,24c	T24A 001	vazensis	67	T67 001
coreanensis	25	T25 001	thailandensis	68	T68 001
silo	26	T26 001	pahangi	69	T69 001
mexicanensis	27	T27 001	sinensis	70	T70 001
monterrey	28a,28b	T28 001			
jegathesan	28a,28c	T28A 001	Biovars		
amagiensis	29	T29 001	dendrolimus	4a,4b	T04A 001
medellin	30	T30 001	subtoxicus	6	T06A 001

nally based on the design of PCR primers from Rep sequences found in the *Escherichia coli* and *Salmonella typhimurium* genomes (56). Amplicons obtained from contiguous Rep sequences generate distinctive electrophoretic patterns among different strains. Similar approaches use other repetitive sequences, such as the so-called ERIC and BOX sequences, developed for *E. coli* and *S. typhimurium* (24) and for *Streptococcus pneumoniae*, respectively (37). Rep-PCR fingerprint analysis of strains has proved to be simple, fast, and reproducible in a great variety of organisms (14, 36). However, this technique has been applied to organisms with little (if any) relationship with enterobacteria, that is, organisms with no homology whatsoever with the Rep sequences of *E. coli*, including some eukaryotic organisms (15, 38), which may indicate that these Rep-PCR analyses are arbitrary primer-PCR analyses, in those cases. This is the case of the Rep-PCR analysis of 28 *B. thuringiensis* serovars, using primers from the *E. coli* Rep sequence (46). We know now that this sequence is not found in the *B. cereus* group genomes.

This report presents the *B. cereus* (Bc)-Rep-PCR analysis of 125 *B. thuringiensis* strains, including 83 serovars, two biovars, and 26 native isolates, with primers specifically designed from a 26-bp Rep sequence found in the *B. cereus* group genomes.

MATERIALS AND METHODS

Bacterial strains. Type and biotype strains of *B. thuringiensis* were kindly donated by the International Entomopathogenic *Bacillus* Center (IEBC), Pasteur Institute, France (Table 1), as well as other non-type strains, such as serovar Morrisoni strain tenebrionis (T08 017), serovar morrisoni PG14 (T08 018), the standard serovar israelensis (IPS-82), serovar canadensis 11S2.1 (T05A030), serovar thompsoni B175 (T12007), the autoagglutinated K6 (AAT028), and B51 (AATO21). *B. cereus* DSM31 (species type strain), *B. cereus* subsp. *moritai* (CER 081), *B. cereus* CER 183, *B. mycoides* IP-M 001 (species type strain), *B. anthracis* 7702, and the type strain of *B. subtilis* (IP-S 001) were donated by the Pasteur Institute. The *B. thuringiensis* serovar morrisoni strain san diego was directly

TABLE 2. Three groups of native *B. thuringiensis* isolates from the CINVESTAV-Irapuato stock collection subjected to Bc-Rep-PCR fingerprinting

Group and isolate	H serotype[a]	Attribute
Group 1		
LBIT-13	NM	Toxic to lepidopterans
LBIT-18	8a,8b	Toxic to coleopterans
LBIT-24	8a,8b	Toxic to coleopterans
LBIT-73	8a,8b	Toxic to coleopterans
LBIT-74	SA	Toxic to coleopterans
LBIT-196	NSP	Toxic to coleopterans
LBIT-358	8a,8b	Toxic to coleopterans
LBIT-419	8a,8b	Toxic to coleopterans
Group 2		
LBIT-52	4a,4b	Toxic to dipterans
LBIT-58	6	Toxic to dipterans
LBIT-62	NST	Toxic to dipterans
LBIT-93	8a,8b	Toxic to dipterans
LBIT-94	6	Toxic to dipterans
LBIT-153	14	Toxic to dipterans
LBIT-163	NST	Toxic to dipterans
LBIT-201	NST	Toxic to dipterans
LBIT-388	SA	Toxic to dipterans
LBIT-393	14	Toxic to dipterans
LBIT-396	SA	Toxic to dipterans
LBIT-426	NST	Toxic to dipterans
LBIT-432	NST	Toxic to dipterans
Group 3		
LBIT-63	1	β-Exotoxin
LBIT-279	1	β-Exotoxin
LBIT-299	1	β-Exotoxin
LBIT-301	1	β-Exotoxin
LBIT-398	SA	β-Exotoxin

[a] NM, nonmotile; NSP, nonserotypable; SA, self agglutinated; NST, not serotyped.

isolated from the commercial product M-One (Mycogen Corp). Native strains (LBIT series) are part of the native *B. thuringiensis* stock collection at CINVESTAV-Irapuato, Mexico (Table 2).

DNA extraction. DNA was extracted from each strain, following a modified protocol reported previously (51). Fresh 30-ml Luria-Bertani broth cultures (optical density at 600 nm, 1) were centrifuged at 3,000 × *g* for 5 min at 4°C, and the pellets were washed again in 10 ml of J buffer (1.0 M Tris-HCl, 0.1 M EDTA, 0.15 M NaCl [pH 8]). Pellets were resuspended in 4 ml of J buffer, and lysozyme was added to a final concentration of 4 mg/ml, followed by incubation at 37°C for 30 min. Then, 50 μl of RNase (10 mg/ml) was added, and suspensions were incubated for 15 min at 50°C. Next, 200 μl of 20% sodium dodecyl sulfate was added and incubated for 20 min at 70°C, followed by the addition of 120 μl of proteinase K (10 mg/ml) and incubation overnight at 55°C. A total of 1.15 ml of NaCl 6 M was then added, gently mixed in ice for 15 min, and centrifuged at 3,900 × *g* for 20 min at 4°C. The supernatant was mixed with an equal volume of isopropanol and centrifuged at 17,000 × *g* for 20 min at 4°C. The pellet was washed with 70% ethanol, air dried, and dissolved in 200 μl of Tris-EDTA buffer (pH 8). DNA was quantified by spectrophotometry, and samples were stored at −20°C until further use.

Search for REPs in the *B. cereus* genome and primer design. Due to the availability of the first *B. cereus* genome in 2003 (http://ergo.integratedgenomics.com/B_cereus.html), REP sequences were searched in this genome to design specific REP primers for the *B. cereus* group. All the extragenic sequences in the genome were analyzed with scripts written in Perl (http://www.perl.org/). Short REP sequences were combined to obtain larger ones until a highly conserved 26-bp sequence was found, showing the highest repeatability, in terms of both the number of repeats within the genome and the homology between the repeats. Its presence within the recently reported *B. cereus* group genomes was corroborated by searching the sequence in the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/fasta33/) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) data banks. Direct and reverse primers were designed according to this sequence to amplify inter-REP regions.

TABLE 3. Frequency of the Bc-REP and designed primer sequences in five reported genomes of the *B. cereus* group[a]

Frequency	Genome					
	Bc1	Bc2	Bc3	Ba1	Ba2	Bt
Bc-REP 100%	28	58	15	14	14	15
Bc-REP 96%	24	18	6	6	6	8
Direct	89	94	32	28	28	33
Reverse	37	71	20	18	18	19

[a] Bc1, *B. cereus* ATCC 14579 genome (NC 004722.1); Bc2, *B. cereus* ATCC 10987 genome (NC 003909.8); Bc3, *B. cereus* ZK genome (NC 006274); Ba1, *B. anthracis* strain Ames genome (NC 003997.3); Ba2, *B. anthracis* strain Sterne genome (NC 005945.1); Bt, *B. thuringiensis* strain 97.27 genome (NC 005957.1); Bc-REP 100%, frequency of sequences showing 100% homology with Bc-REP; Bc-REP 96%, frequency of sequences showing 96% homology with Bc-REP; Direct, frequency of sequences showing 100% homology with the designed direct primer; Reverse, frequency of sequences showing 100% homology with the designed reverse primer.

Rep-PCR amplification conditions. PCR mixtures were prepared as follows: 100 ng of template DNA, 300 ng of each primer, 5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate mixture, and 2.5 U of *Taq* DNA polymerase (Invitrogen) to a 25-μl final volume. PCR amplifications were performed under the following conditions: an initial denaturation of 5 min at 94°C, followed by 34 cycles each of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and polymerization at 72°C for 1.5 min. Amplification was finished with an extension step at 72°C for 7 min. All PCR amplifications were performed with a Perkin-Elmer GeneAmp PCR System 2400. Amplified samples were kept at −20°C until electrophoretic analysis was performed.

Electrophoretic analysis. Bc-Rep-PCR patterns were visualized by agarose gel electrophoresis. Aliquots of 10 μl each of the amplification products were loaded onto 1.2% agarose slabs (11 by 14 cm) and run in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer at 2 V/cm during 5 h. Slabs were stained with 0.4 μg of ethidium bromide/ml and documented with a Gel Doc 2000 gel system (Bio-Rad). Molecular weight analysis of patterns was performed with the Quantity One version 4.2.1 software (Bio-Rad), with the 1-kb DNA ladder (Invitrogen) as a molecular weight marker.

Analysis of Bc-Rep-PCR patterns. Polymorphic bands from all the Rep-PCR patterns were individually identified by their specific migration rates in the electrophoretic analyses. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Jaccard's similarity coefficients were generated by the SIMQUAL subroutine from the NTSYS-pc 2.02j (Applied Biostatistics, Inc.) package. Cluster analyses along with their corresponding dendrograms were generated by the unweighted-pair group method using average linkages (UPGMA), with the SAHN and TREE subroutines from the NTSYS-pc package.

RESULTS

REP sequence in the *B. cereus* genome. The following 26-bp Bc-REP sequence was found in the *B. cereus* genome: CCCC ACTGATTAAAGTTTCACTTTAT. Bases 11 to 16 paired with bases 20 to 25, forming a palindromic sequence and a potential hairpin with a 6-bp stem and an estimated total secondary structure energy of −2.2 Kcal/mol. This section of the Bc-REP is highly conserved in the analyzed genomes (see below). The Bc-REP sequence was analyzed by the fasta3 program (http://www.ebi.ac.uk/fasta33) and BLAST (http://www.ncbi.nlm.nih.gov/), finding the sequence in both directions of the *B. cereus*, *B. anthracis*, and *B. thuringiensis* reported genomes (Table 3). No further significant matches were found in all the genomes and nucleotide sequences available at the EMBL and NCBI gene banks, including the poorly sequenced *B. mycoides* (see below).

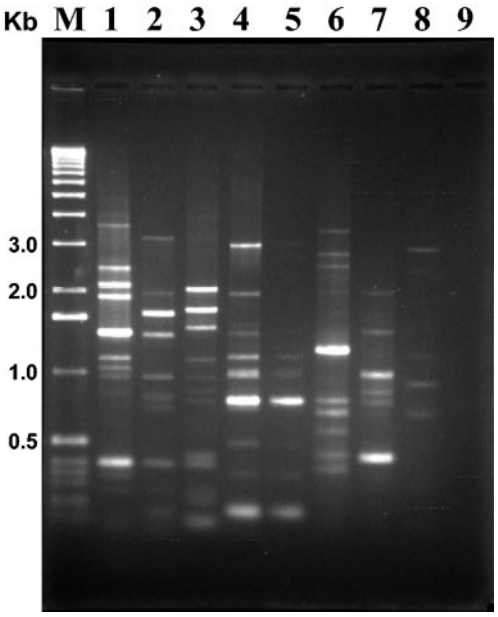

FIG. 1. Bc-Rep-PCR fingerprint patterns of the *B. cereus* group strains. Lane 1, *B. thuringiensis* serovar israelensis; lane 2, *B. thuringiensis* LBIT-13; lane 3, *B. cereus* DSM31; lane 4, *B. cereus* CER81; lane 5, *B. cereus* CER 183; lane 6, *B. mycoides* IP-M 001; lane 7, *B. anthracis* 7702; lane 8, *B. subtilis* IP-S 001; lane 9, negative control; lane M, molecular weight marker (1-kb DNA ladder; Invitrogen).

Primer design and PCR amplification. Two different primers were designed from the Bc-REP sequence, a direct 18-mer primer called Bc-REP-1 (5′-ATTAAAGTTTCACTTTAT-3′) and a reverse 14-mer primer called Bc-REP-2 (5′-TTTAATC AGTGGGG-3′), both with an estimated T_m of 42°C. These primers were frequently found in the *B. cereus* group reported genomes (Table 3). Primers were tested in combination and separately and under a series of Mg^{2+} and template DNA concentrations. Best amplification and defined patterns were obtained with the combination of primers, used with 5 mM Mg^{2+} concentration. No difference was detected when template DNA varied from 0.1 to 1 μg in the PCR mixture. Preliminary PCR tests with DNA from six different *B. cereus* group strains indicated the usefulness of those primers, as specific and reproducible patterns were obtained from *B. thuringiensis* serovar israelensis (mosquitocidal type strain), *B. thuringiensis* LBIT-13 (nonserotypable strain due to the lack of flagella), *B. cereus* DSM31 (species type strain), *B. cereus* subsp. *moritai* (CER 081), *B. cereus* CER 183, *B. mycoides* IP-M 001 (species type strain), and *B. anthracis* 7702. The type strain of *B. subtilis* (IP-S 001) was also included in the comparison, but only four faint (probably unspecific) bands were amplified (Fig. 1).

Bc-Rep-PCR fingerprinting of *B. thuringiensis* type strains. Once PCR conditions were established and primers were tested, 83 *B. thuringiensis* type strains, the biovars subtoxicus and dendrolimus, and strains tenebrionis and morrisoni PG14 were analyzed. Practically all the Bc-Rep-PCR patterns obtained from the type strains varied from 5 to 13 bands in number and from 0.2 to 3.8 kb in band size. Only serovars graciosensis (H66) and muju (H49) showed three bands in their patterns. In all, 47 polymorphic bands were identified from all the Bc-Rep-PCR patterns; no common bands were detected for all of them. Figure 2 shows the Bc-Rep-PCR patterns of 11 type serovar strains of *B. thuringiensis*, showing all the polymorphic bands, as well as a schematic representation of them.

The overwhelming majority of the type strains showed distinctive Bc-Rep-PCR patterns. However, a few serovars shared the same pattern, such as the apparently unrelated serovars sotto (H4a,4b) and dakota (H15); the mosquitocidal (and highly related) serovars israelensis (H14) and malaysiensis (H36); also serovars sumiyoshiensis (H3a,3d) and fukuokaensis (H3a,3d,3e), which share two H-antigenic determinants; and the apparently unrelated serovars amagiensis (H29) and seoulensis (H35). The serovar entomocidus (H6) and its biovar subtoxicus (H6) also showed the same pattern, but the serovar sotto (H4a,4b) and its biovar dendrolimus (H4a,4b) showed a similar but not identical pattern, with the pattern of serovar leesis (H33) more similar to that of biovar dendrolimus (Fig. 3).

These results were corroborated once the binary matrix was analyzed and a dendrogram was generated by UPGMA (Fig. 4). It shows, for example, that the mosquitocidal strain serovar morrisoni PG14 (H8a,8b) was more related to other mosquitocidal strains, such as serovars israelensis (H14) and malaysiensis (H36), than to the other two serovar morrisoni (H8a,8b) strains (the type strain and tenebrionis). Interestingly, it also shows that all the strains that share H-antigenic determinants, such as serovars sotto (H4a,4b) and kenyae (H4a,4c), are widely separated in the dendrogram, just as happens with serovar alesti (H3a,3c) and serovar kurstaki (H3a,3b,3c), which are also separated in the dendrogram, and these from the serovar sumiyoshiensis-fukuokaensis complex. However, serovars kurstaki (H3a,3b,3c), galleriae (H5a,5b), and aizawai (H7) form a very tight group.

Bc-Rep-PCR fingerprinting of *B. thuringiensis* native isolates. A total of 26 native strains, from the CINVESTAV-Irapuato *B. thuringiensis* stock collection (LBIT series), were analyzed by Bc-Rep-PCR (Table 2). They included the LBIT-13 nonmotile strain, 7 native strains with coleopteran activity, 13 mosquitocidal strains, and 5 β-exotoxin-producing strains. Among these strains, four (LBIT-74, LBIT-388, LBIT-396, and LBIT-398) are self agglutinated (nonserotypable).

The mosquitocidal strains showed a Bc-Rep-PCR pattern identical to that of the mosquitocidal IPS-82 standard, including not only the serovar israelensis (H14) strains, but also one serovar kenyae strain (H4a,4c; strain LBIT-52) and one serovar entomocidus strain (H6; strain LBIT-58), as well as two self-agglutinated strains (LBIT-396 and LBIT-388) (Fig. 5). Additionally, another group of mosquitocidal strains, highly related to the first one, was represented by the serovar morrisoni PG-14 strain, along with strains LBIT-93 (serovar morrisoni; H8a,8b), LBIT-94 (entomocidus; H6), and LBIT-426 (not serotyped) (Fig. 5). Accordingly, other nonmosquitocidal strains, tested and characterized earlier (50), showed Bc-Rep-PCR patterns identical to that of *israelensis* (Fig. 6).

On the other hand, the seven strains with coleopteran activity showed Bc-Rep-PCR patterns identical to those of the reference serovar morrisoni tenebrionis strain and the san diego strain. They included not only serovar morrisoni (H8a,8b) strains but also one self-agglutinated strain (LIBT-74) and one

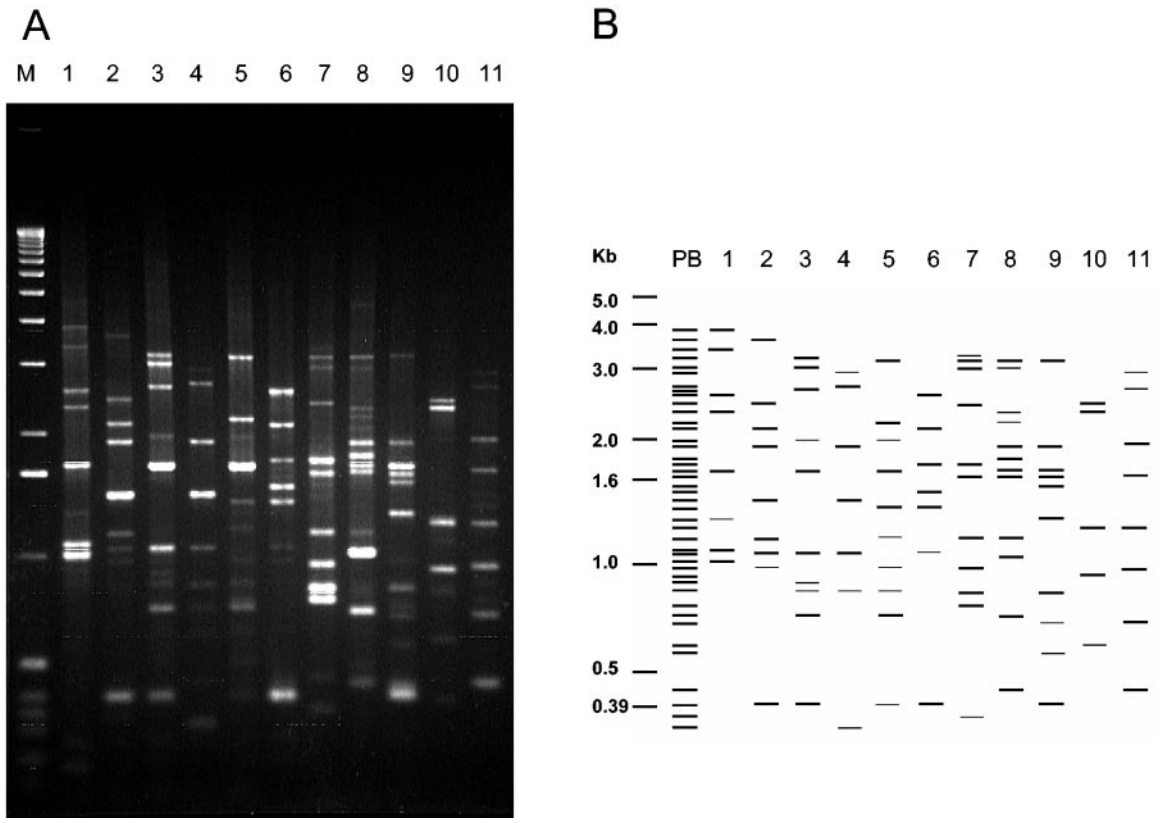

FIG. 2. Bc-Rep-PCR fingerprinting (A) and schematic representation (B) of 11 *B. thuringiensis* type strains. Lane 1, serovar amagiensis; lane 2, serovar israelensis; lane 3, serovar jinghongiensis; lane 4, serovar sumoyoshiensis; lane 5, serovar coreanensis; lane 6, serovar pakistani; lane 7, serovar konkukian; lane 8, serovar guiyangiensis; lane 9, serovar kurstaki; lane 10, serovar vazensis; lane 11, serovar brasiliensis; lane PB, polymorphic banding; lane M, molecular weight marker (1-kb DNA ladder; Invitrogen).

nonserotypable strain (LBIT-196) (Fig. 7). Also, the β-exotoxin-producing strains, which belong to the serovar thuringiensis (H1) (except for the self-agglutinated LBIT-398 strain), showed Bc-Rep-PCR patterns identical to that of the type strain T01 001 (serovar thuringiensis; H1) (Fig. 7).

DISCUSSION

Serotyping is the best-known technique for identifying and characterizing *B. thuringiensis* strains. So far, 84 serotypes and two biovars are known (29, 32); however, serotyping shows some constraints, such as its inability to process the so-called self-agglutinated and immobile (nonflagellar) strains. Also, this technique is unable to differentiate between *B. thuringiensis* and some *B. cereus* strains; it cannot show a phylogenetic relationship between the serotypes (32). Although serotyping is a reliable and straightforward technique, it is performed only in a few laboratories around the world, in particular, the Pasteur Institute in France, where the *B. thuringiensis* type collection is held. Therefore, alternative techniques (47), especially molecular techniques, are being developed to try to overcome those constrains.

Rep-PCR has been widely used on a variety of bacterial (and nonbacterial) species (5, 9, 15, 17, 38, 54) to characterize and identify strains. It has also been used for strains within the genus *Bacillus* (including *B. thuringiensis)*, but based on the use of REPs found in other unrelated bacteria, such as the streptococcal BOX (10), the enterobacterial REP (35, 46), and the enterobacterial ERIC (35). In fact, a quick search for all these sequences in the six *B. cereus* group genomes reported showed no significant matches, indicating that actual BOX, REP, or ERIC analyses of these strains may be uncertain and should be reviewed. However, partial homology of enterobacterial REPs was found in *Bacillus sporothermodurans*, which allowed a real Rep-PCR analysis of the strains (20).

The presence of REP sequences in prokaryotes is common (36) and has been used for the design of species- or group-specific primers. That is the case of a 26-bp REP found in *Neisseria* spp., which allowed the design of specific primers for the analysis of *N. gonorrhoeae* and *N. meningitides* strains (57). Based on this approach, we looked for and found a 26-base REP common in the six *B. cereus* group reported genomes, which also include *B. anthracis* and *B. thuringiensis*. This REP (Bc-REP) allowed the design of two specific primers for the *B. cereus* group and proved their applicability by amplifying discrete and reproducible patterns in *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides* strains. Their specificity was corroborated when a *B. subtilis* strain showed only faint bands and an undefined pattern, which may be caused to a partial homology with the Bc-REP.

The strong relationship between these species has been corroborated before, either by DNA hybridization (53), ribotyping

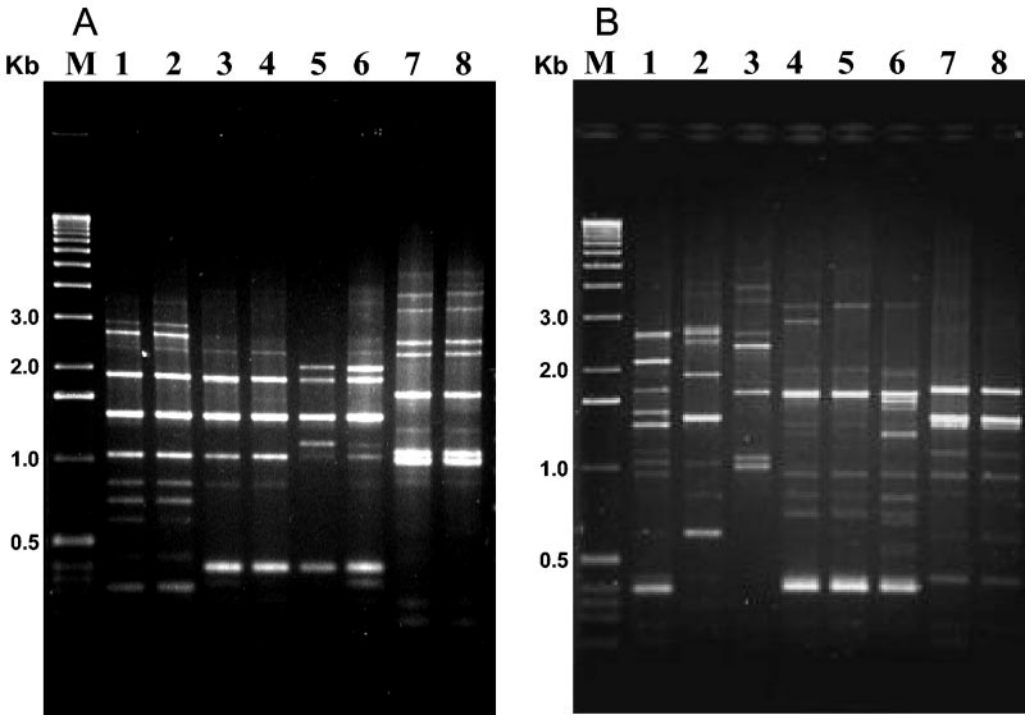

FIG. 3. Bc-Rep-PCR fingerprinting of 17 different type strains of *B. thuringiensis*. (A) Lane 1, serovar sumiyoshiensis; lane 2, serovar fukuokaensis; lane 3, serovar dakota; lane 4, serovar sotto; lane 5, biovar dendrolimus; lane 6, serovar leesis; lane 7, serovar amagiensis; lane 8, serovar seoulensis. (B) Lane 1, serovar pakistani; lane 2, serovar alesti; lane 3, serovar kyushuensis; lane 4, serovar galleriae; lane 5, serovar aizawai; lane 6, serovar kurstaki; lane 7, serovar entomocidus; lane 8, biovar subtoxicus. Lanes M, molecular weight marker (1-kb DNA ladder; Invitrogen).

(48), AFLP (21, 49, 55), or BOX-PCR (10) analyses of a number of strains. In all cases, strains from all these species intermingle within the same dendrogram, with, in general, the *B. anthracis* strains being the most homogeneous, the *B. cereus* and *B. thuringiensis* strains being the most diverse, and the *B. mycoides* strains being the least related to the rest. In our study, only three *B. cereus*, one *B. anthracis*, and one *B. mycoides* strains were analyzed; although all five strains intermingled in the same dendrogram with the *B. thuringiensis* strains, more strains from the other species were required to corroborate the same trend when Bc-REP-PCR analysis was used.

The main purpose of this report is the Rep-PCR characterization of the *B. thuringiensis* type strains, using specific primers for the *B. cereus* group. All the type strains were included, except for the most recent serotype described last year (29). Bc-REP-PCR fingerprinting of the type strains showed that practically all the serotypes displayed a distinct pattern. It also shows the putative phylogenetic relationship between the 83 serotypes and two biovars included in the analysis. Only a few strains showed identical patterns, such as serovars entomocidus and its biovar subtoxicus, both isolated by Heimpel (26) in Canada. Similar results were obtained by Phucharoen et al. (46) and Brousseau et al. (7), but they differ from the results obtained by Priest et al. (48) and Joung and Côté (27). On the other hand, Bc-REP-PCR patterns from the serovar sotto and its biovar dendrolimus slightly differ from each other, with the serovar sotto pattern identical to that of serovar dakota and the serovar leesis pattern the closest to that of biovar dendrolimus. Interestingly, ribotyping of these strains (27) indicated that while biovar dendrolimus and serovars leesis and dakota were phylogenetically related, serovar sotto was located in a separate group. Other serovars that shared the same Bc-REP-PCR pattern were serovars amagiensis and seoulensis. This is in agreement with a previous random amplified polymorphic DNA analysis of these strains (16); ribotyping also connects both strains in the same group (27).

Other serovars highly related by their Bc-REP-PCR pattern were serovars galleriae, aizawai, and kurstaki, which agrees with previously reported DNA hybridization and RFLP analyses (40, 48). These results may indicate that these associated and highly common serovars form a tight phylogenetically related group, whose segregation should be reviewed. Likewise, such segregation should be reviewed for the highly related serovars sumiyoshiensis and fukuokaensis, indiana and thompsoni; amagiensis, seoulensis, and kyushuensis; thuringiensis and sooncheon; azorensis and vazensis; and monterrey and oswaldocruzi. All these serovars appear closely related among each other in the phylogenetic dendrogram generated by the Bc-REP-PCR fingerprinting and by previously reported ribotyping (27). Other serovars highly related by their Bc-REP-PCR pattern, such as serovars silo and ostriniae, palmanyolensis and darmstadiensis, japonensis and kenyae, and colmeri and mexicanensis show less of a relationship by ribotyping analysis (27).

Serovars israelensis and malaysiensis also share the same Bc-REP-PCR pattern, which is in agreement with their high relationship (mosquitocidal specificity, *cry* gene content, and crystal morphology) (50); however, ribotyping is unable to rec-

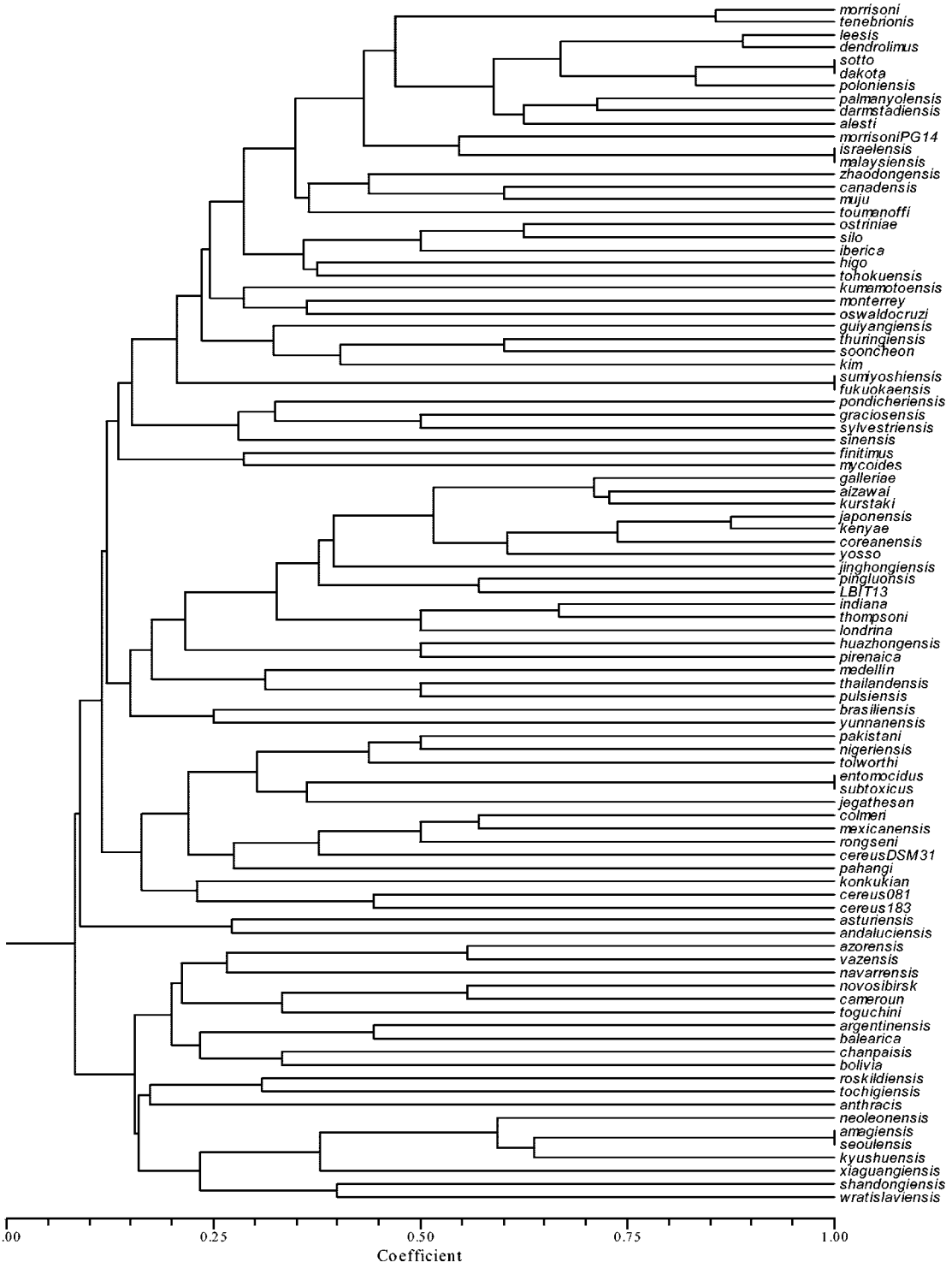

FIG. 4. Dendrogram estimated from the Bc-Rep-PCR patterns obtained from 83 type serovars, two biovars, three isolates of *B. thuringiensis*, and five *B. cereus* group strains, using the Jaccard coefficient and UPGMA.

ognize such a relationship and locate both strains in separate groups (27). Interestingly, other mosquitocidal strains with attributes practically identical to those of serovar israelensis such as K6 (AAT028), B51 (AATO21), canadensis 11S2.1 (T05A030), and serovar thompsoni B175 (T12007) (50) also display Bc-REP-PCR patterns identical to those of serovars israelensis and malaysiensis. On the other hand, the mosquitocidal serovar medellin, which has attributes different from those of serovar israelensis (43, 50), also shows a very different Bc-REP-PCR pattern. These results may indicate that the

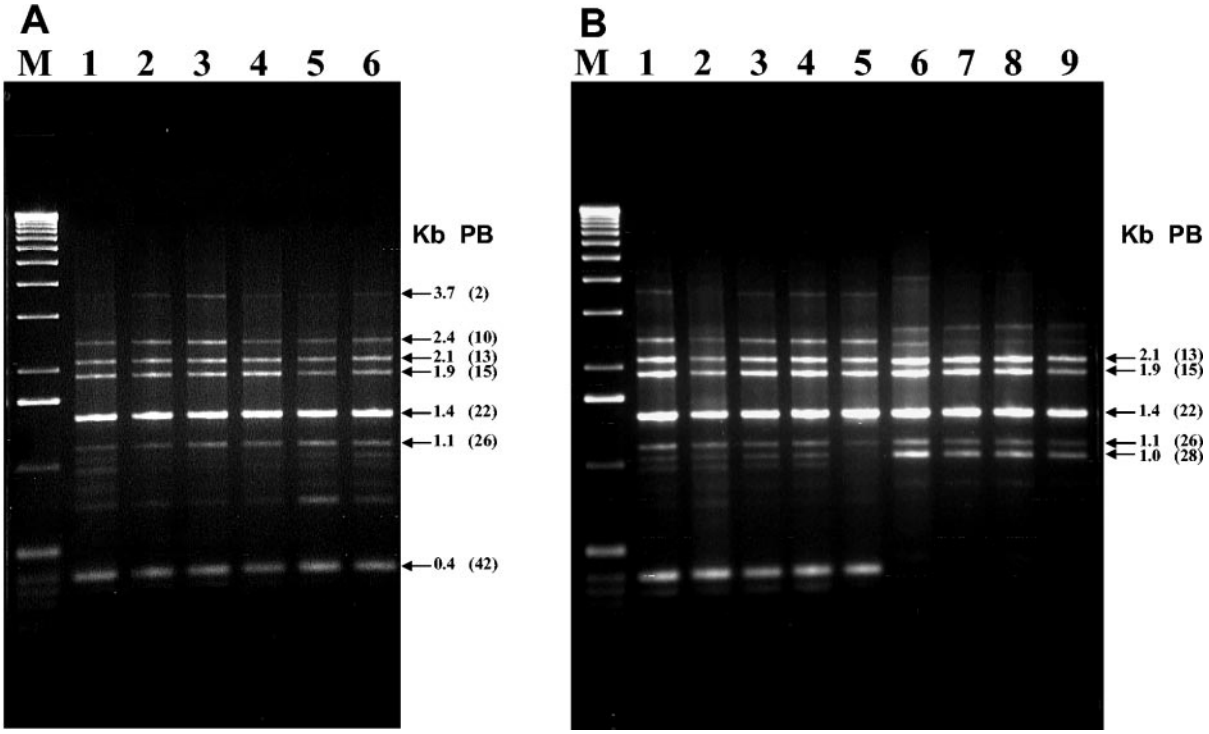

FIG. 5. Bc-Rep-PCR fingerprint patterns of native mosquitocidal *B. thuringiensis* isolates. (A) Lane 1, serovar israelensis IPS-82; lane 2, LBIT-52 (H4a,4b); lane 3 LBIT-58 (H6); lane 4, LBIT-153 (H14); lane 5, LBIT-163 (not serotyped); lane 6, LBIT-201 (not serotyped). (B) Lane 1, LBIT-62 (not serotyped); lane 2, LBIT-388 (self agglutinated); lane 3, LBIT-393 (H 14); lane 4, LBIT-396 (self agglutinated); lane 5, LBIT-432 (not serotyped); lane 6, morrisoni PG14 (H8a,8b); lane 7, LBIT-93 (H8a,8b); lane 8, LBIT-94 (H6), lane 9, LBIT-426 (not serotyped). Lanes M, molecular weight marker (1-kb DNA ladder; Invitrogen); Kb, band size; PB, polymorphic band.

genomic relationship between the *B. thuringiensis* strains is not necessarily defined only by their toxic specificity, but by a series of attributes, such as *cry* gene content, crystal morphology, and plasmid pattern. This was also corroborated when native mosquitocidal isolates, highly related to serovar israelensis, showed identical Bc-REP-PCR patterns, even when some of these isolates were self agglutinated or belonged to a serotype different from that of israelensis. These results not only corroborate the reported genomic homogeneity of the serovar israelensis strains (2) but also imply that the same homogeneity occurs in other non-israelensis strains, as long as they share other attributes.

Another mosquitocidal strain, serovar morrisoni PG14, is known to share only some of the serovar israelensis characteristics (toxic specificity, some *cry* gene content, and crystal morphology) (25, 50); accordingly, its Bc-REP-PCR pattern is similar but not identical to that of serovar israelensis. Interestingly, its pattern is significantly different from that of the serovar morrisoni type strain. This serovar also includes the coleopteran-active strains tenebrionis and san diego, previously reported to be the same strain (30); however, contrary to the PG14 strain, the identical Bc-Rep-PCR pattern of both strains is very similar to that of the serovar morrisoni type strain. Also, similar to the results obtained with the mosquitocidal strains, the native coleopteran-active strains share the same Bc-Rep-PCR pattern as the serovar tenebrionis strain, most of them serotyped as serovar morrisoni but also including two nonserotypable strains. Genomic homogeneity may also

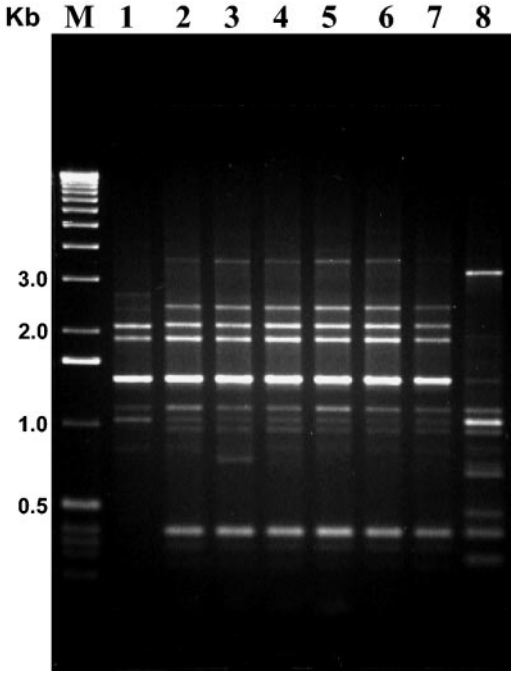

FIG. 6. Bc-Rep-PCR fingerprint patterns of mosquitocidal *B. thuringiensis* strains. Lane 1, serovar morrisoni PG14 (H8a,8b); lane 2, serovar israelensis (H14); lane 3, serovar malaysiensis (H 36); lane 4, serovar canadensis 11S2.1 (H5a,5c); lane 5, serovar thompsoni B175 (H12); lane 6, K6 (self agglutinated); lane 7, B51 (self agglutinated); lane 8, serovar medellin 163.131 (H30); lane M, molecular weight marker (1-kb DNA ladder; Invitrogen).

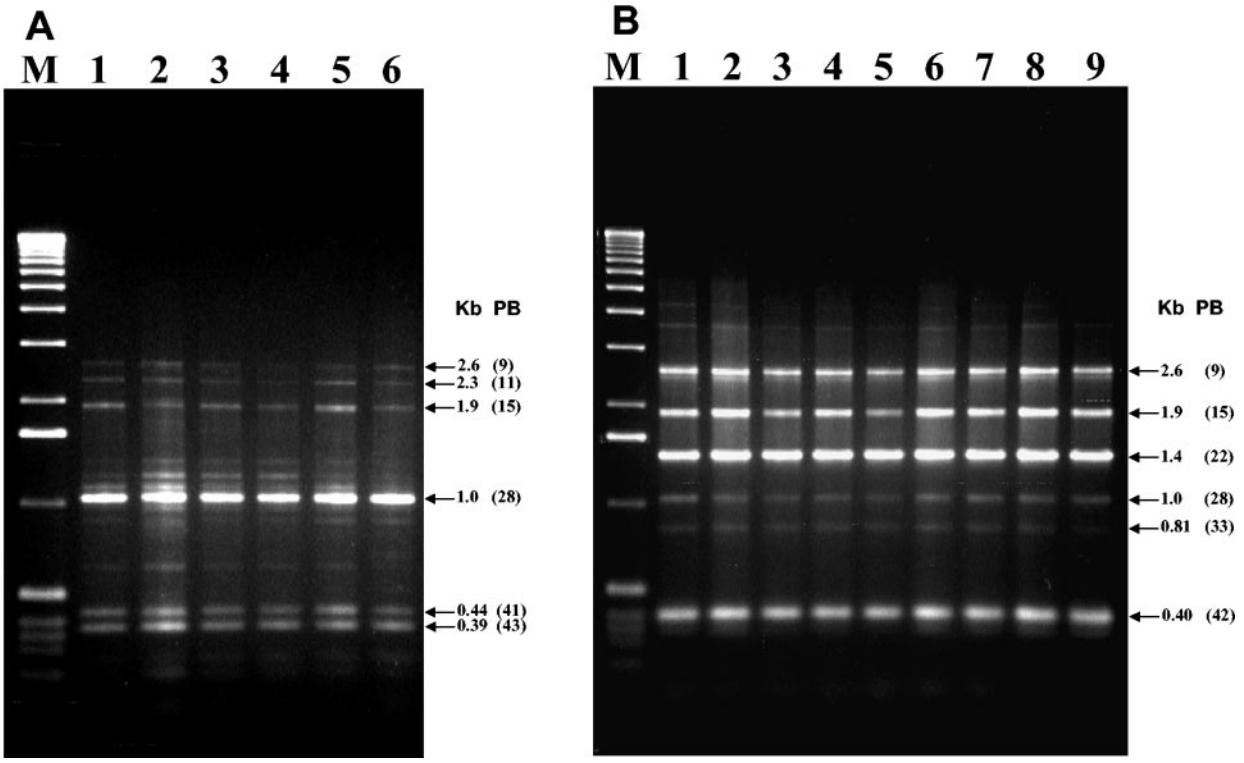

FIG. 7. Bc-Rep-PCR fingerprint patterns of β-exotoxin-producing (A) and coleopteran-active (B) *B. thuringiensis* isolates. (A) Lane 1, serovar thuringiensis (T01 001); lane 2, LBIT-63 (H1); lane 3, LBIT-279 (H1); lane 4, LBIT-299 (H1); lane 5, LBIT-301 (H); lane 6, LBIT-398 (self agglutinated). (B) Lane 1, strain tenebrionis (T08 017); lane 2, strain san diego (H8a,8b); lane 3 LBIT-18 (H8a,8b); lane 4, LBIT-24 (H a,8b); lane 5, LBIT-73 (H8a,8b); lane 6, LBIT-74 (self agglutinated); lane 7, LBIT-196 (nonserotypable); lane 8, LBIT-358 (H8a,8b); lane 9, LBIT-419 (H8a,8b). Lanes M, molecular weight marker (1-kb DNA ladder; Invitrogen); Kb, band size; PB, polymorphic band.

occur in this group, similar to that observed with the serovar israelensis group. Interestingly, the same homogeneity was found in the group of native strains that produce β-exotoxin and belong to serovar thuringiensis (H1) (except for the self-agglutinated LBIT-398 strain). These results may indicate that Bc-Rep-PCR fingerprinting of *B. thuringiensis* strains is useful not only to differentiate between serovars, but also to properly identify the nonserotypable strains and, most of all, to recognize more accurately the evolutionary relationship between strains, to whichever serovar they belong.

B. thuringiensis constitutes a genetically diverse species; the great number of strains known today may form distinctive groups, according to their phenetic and genetic traits. Serotyping has been a useful tool to try to discriminate those groups since its establishment in 1962 (12); however, as strains mounted up, shortcomings started to appear in the technique. Molecular tools have been developed in recent years, trying to offer a new typing alternative for *B. thuringiensis* strains and to recognize the actual phylogenetic relationships between subspecific groups. Bc-Rep-PCR offers a new tool to identify these groups, based on the use of specific primers designed from a REP sequence found in the *B. cereus* group. The potential of this technique was tested in this work and proved to be sensitive, specific, reproducible, and fast; it may become a standardized characterization procedure. It may also help in the establishment of a new subspecies-level classification of *B. thuringiensis*.

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