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Molecular Diversity of *Bacillus anthracis* in Italy

Antonio Fasanella,[1]* Matthew Van Ert,[2] Settimia A. Altamura,[1] Giuliano Garofolo,[1] Canio Buonavoglia,[3] Guido Leori,[4] Lynn Huynh,[2] Shaylan Zanecki,[2] and Paul Keim[2]

Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata—Anthrax Reference Institute of Italy, Foggia,[1] Dipartimento di Sanità, Patologia, Farmacotossicologia e Benessere degli Animali, Facoltà di Medicina Veterinaria, Bari,[3] and Istituto Zooprofilattico Sperimentale della Sardegna, Sassari,[4] Italy, and Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona 86011-5649[2]

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We used multiple-locus variable-number tandem repeat analysis (MLVA) to type 64 *Bacillus anthracis* isolates from outbreaks that have occurred during the past 40 years in Italy. MLVA of the 64 isolates revealed 10 unique genotypes; 9 of these genotypes and the majority of isolates (63/64) belonged to the previously described genetic cluster A1.a. Within the A1.a isolates, two previously described genotypes (G1 and G3), which differ by a single mutation in the pX01 locus, account for the majority of isolates in the country (53/63). The low diversity of *B. anthracis* genotypes in Italy suggests a single, dominant historical introduction, followed by limited localized differentiation.

Bacillus anthracis, the causative agent of anthrax, is a genetically homogeneous and recently emerged pathogen, which complicates efforts to subtype it (7, 8, 11). In 1995, Henderson et al. detected the first molecular difference among *B. anthracis* strains, and in the following year Andersen et al. demonstrated that the variation was due to a 12-nucleotide variable-number tandem repeat (VNTR), named *vrrA* (1, 4). The molecular basis for the variation was due to differences in the number of repeated units, and five different allelic forms could be detected (6). An expansion on this strategy that further advanced the subtyping of *B. anthracis* involved the analysis of multiple VNTR loci (8). Multiple-locus VNTR analysis (MLVA) typing systems use PCR amplification and fragment sizing to detect length polymorphisms in several VNTR regions (8, 10). MLVA has proven to be a powerful tool for distinguishing among *B. anthracis* isolates and has been applied to examination of bioterrorism events as well as naturally occurring anthrax outbreaks (5, 8, 9, 10, 13, 14). Keim et al. in 2000 used MLVA to examine a worldwide collection of *B. anthracis* isolates and described 89 unique genotypes (8). Further studies have used MLVA to examine regional patterns of anthrax diversity; these include studies of the genetic diversity of *B. anthracis* isolates in France and Poland (2, 3, 13, 14).

In Italy, anthrax is generally a sporadic disease that occurs, with a few exceptions, in the central and southern regions and in the major islands, where it almost exclusively affects animals at pasture. Since the late 1950s, the Italian anthrax epidemic situation has improved as a result of vaccination campaigns with an attenuated live vaccine, composed of spores in 1% saponin (Carbosap). This nationwide control system also includes disease outbreak surveillance, and over the past 5 years, the Anthrax Reference Institute of Italy has been recovering *B. anthracis* isolates from national outbreaks, including archived specimens from historical outbreaks. As a result of this collection effort, we have recovered 64 isolates of *B. anthracis* that are archived as spores at the maximum security laboratory of the Anthrax Reference Institute of Italy. However, the genetic composition of *B. anthracis* in Italy has not been adequately studied. Analysis of the genetic similarity or differences would lead to an understanding of the disease origins and transmission patterns. In this study we used MLVA to genotype the *B. anthracis* isolates from Italian outbreaks in order to examine relationships among these outbreaks and to understand the diversity of *B. anthracis* isolates in Italy as it relates on a regional and a global scale.

* Corresponding author. Mailing address: Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata—Anthrax Reference Institute of Italy, Foggia 71100, Italy. Phone: 39.0881.786330. Fax: 39.0881.786362. E-mail: a.fasanella@izsfg.it.

MATERIALS AND METHODS

***B. anthracis* isolates.** We have analyzed a collection of 64 *B. anthracis* isolates that are representative samples from outbreaks occurring in Italy during the past 40 years. *B. anthracis* isolates were collected from the following regions: Puglia, Campania, Basilicata, Calabria, Tuscany, Sicily, Sardinia, Lazio, and Veneto.

DNA preparation. Each *B. anthracis* strain was streaked onto tryptose agar plates and then incubated at +37°C for 24 h. The single colonies were suspended in 1 ml of TE (Tris-HCl [pH 8], 1.0 mM EDTA) and incubated at +98°C for 20 min. After centrifugation at 15,000 × *g* for 1 min, the supernatant was collected and filtered using 0.22-μm filters.

MLVA PCR. For the MLVA test, we utilized fluorescently labeled primers that flank the eight VNTR regions (*vrrA*, *vrrB*$_1$, *vrrB*$_2$, *vrrC*$_1$, *vrrC*$_2$, CG3, pXO1, pXO2) as described by Keim et al. in 2000 (8). Three different dyes are used for the reaction primers (6-carboxyfluorescein, VIC, and NED), while one is reserved for molecular weight size standards (ROX). Primers amplifying VNTRs that exhibit overlapping allele size ranges are labeled with different fluorophores to ensure that the VNTRs can be examined in a single electrophoretic injection. The final volume (50 μl) of reaction mixture contained 200 μM deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Piscataway, N.J.), 0.2 μM primers (Applied Biosystems, Foster City, Calif.), 10 mM Tris-HCl, pH 8.3 (Perkin-Elmer, Wellesley, Mass.), 50 mM KCl (Perkin-Elmer), 1.5 mM $MgCl_2$ (Perkin-Elmer), 1.25 U of *Taq* DNA polymerase gold (Perkin-Elmer), and 5.0 μl of extracted DNA. The PCR samples were amplified in a Mastercycler Personal (Eppendorf AG, Hamburg, Germany) as follows: 95°C for 10 min, followed by 30 cycles of 92°C for 30 s, annealing for 30 s (57°C for VrrA, VrrB$_1$, VrrB$_2$, and VrrC$_1$; 60°C for VrrC$_2$; 55°C for CG3, pX01, and pX02), and extension at 72°C for 30 s. To ensure that the loci were amplified, 20.0 μl of each amplified sample was subjected to electrophoresis in a 2.0% agarose gel with 0.5 μg/ml of ethidium

TABLE 1. Characteristics of *Bacillus anthracis* isolates in this study

Region	Province	No. of isolates	G_I[a]	G_K[b]	Genetic cluster[c]	MLVA genotype score[d]								No. of isolates from the following source:						
						vrrA	$vrrB_1$	$vrrB_2$	$vrrC_1$	$vrrC_2$	CG3	pXO1	pXO2	Sheep	Goats	Cattle	Horses	Donkeys	Humans	Unknown
Puglia	Foggia	18	A	1	A1.a	313	229	162	613	604	153	123	137	14	1	3				
		5	F	3	A1.a	313	229	162	613	604	153	126	137	1	1	3				
		2	I	(–)	A1.a	313	229	153	613	604	153	126	137		1		1			
		1	D	(–)	A1.a	313	229	162	613	604	153	123	143	1						
	Bari	1	A	1	A1.a	313	229	162	613	604	153	123	137			1				
		3	F	3	A1.a	313	229	162	613	604	153	126	137	2		1				
		1	C	(–)	A1.a	313	229	162	613	604	153	123	139	1						
	Brindisi	2	A	1	A1.a	313	229	162	613	604	153	123	137	1		1				
		1	F	3	A1.a	313	229	162	613	604	153	126	137	1						
	Taranto	1	A	1	A1.a	313	229	162	613	604	153	123	137	1						
		1	F	3	A1.a	313	229	162	613	604	153	126	137			1				
Campania	Avellino	2	F	3	A1.a	313	229	162	613	604	153	126	137			2				
	Naples	1	A	1	A1.a	313	229	162	613	604	153	123	137			1				
Basilicata	Potenza	3	A	1	A1.a	313	229	162	613	604	153	123	137	1		1				1
	Matera	1	A	1	A1.a	313	229	162	613	604	153	123	137			1				
Calabria	Cosenza	1	A	1	A1.a	313	229	162	613	604	153	123	137		1					
Tuscany		1	B	2	A1.a	313	229	162	613	604	153	123	135			1				
Sicily		3	F	3	A1.a	313	229	162	613	604	153	126	137	2				1		
		3	E	19	A1.a	289	229	162	613	604	153	123	137	2		1				
Sardinia		1	F	3	A1.a	313	229	162	613	604	153	126	137			1				
		1	H	6, 7[e]	A1.a	301	229	162	613	604	153	126								1
	Nuoro	6	F	3	A1.a	313	229	162	613	604	153	126	137		3					3
		1	J	57, 58, 59, 61, 63, 65[f]	A3.b	313	229	162	583	532	158				1					
	Cagliari	1	F	3	A1.a	313	229	162	613	604	153	126	137			1				
Lazio		2	F	3	A1.a	313	229	162	613	604	153	126	137	1					1	
Veneto	Verona	1	G	(–)	A1.a	325	229	162	613	604	153	126	137			1				

[a] Genotype designations of Italian isolates as defined in this study.
[b] Genotype designations as described by Keim et al. in 2000 (8). (–) indicates that the Italian isolate had a novel genotype; therefore, no equivalent genotype was described by Keim et al. in 2000.
[c] As defined by Keim et al. in 2000 (8).
[d] Allele sizes determined by electrophoresis on the ABI 310, with results rounded to the nearest whole number and calibrated to the sizes in the report of Keim et al. (8).
[e] As a result of missing data for the pXO2 locus, this isolate matches genotypes 6 and 7 across the seven remaining markers.
[f] As a result of missing data for the pXO1 and pXO2 loci, this isolate matches several genotypes in the A3.b cluster across the six markers analyzed.

bromide at 100 V for 120 min. The amplified DNA bands were visualized upon UV light exposure (Eagle Eye II; Stratagene, La Jolla, Calif.).

Automated genotype analysis. The amplified MLVA PCR products were subjected to capillary electrophoresis in polyacrylamide gels under denaturing conditions on an ABI Prism 310 automated DNA sequencer (Applied Biosystems) and were analyzed using Genescan and Genotyper software (Applied Biosystems).

Data analysis. UPGMA (unweighted-pair group method using arithmetic averages) cluster analysis was performed on genotype scores from a combined data set of the Italian isolates and a panel of 89 diverse genotypes as reported by Keim et al. in 2000 (8). To ensure comparability of genotype scores between the 89 diverse genotypes and Italian isolates, a subset of isolates was genotyped at both the Keim Genetics Laboratory (Northern Arizona University) and the Anthrax Reference Institute of Italy (Foggia, Italy), and raw VNTR sizes were compared and normalized.

RESULTS

Geographical distribution and sources of Italian isolates. While anthrax occurs in much of Italy, the majority of anthrax outbreaks occurred in central Italy, southern Italy, and major islands (Table 1; Fig. 1B). The samples from this study are representative of anthrax activity for the past 40 years.

Genetic diversity of *B. anthracis* in Italy. While MLVA of 64 *B. anthracis* isolates revealed 10 unique genotypes, these were very closely related genetically (Fig. 1A; Table 1). UPGMA cluster analysis grouped 63/64 isolates with the previously described A1.a cluster (8), with only a single isolate associated with the A3.b cluster (Table 1). Four of the genotypes were exact matches to previously described genotypes, including the two dominant Italian genotypes (G_I) A ($n = 28$) and F ($n = 25$), which match the Keim et al. 2000 genotypes (G_K) 1 and 3, respectively (Fig. 1; Table 1). Not all of the Italian isolates matched previously described genotypes (G_K); those that did not were assigned only Italian genotype designations (G_I). For two samples, assigning genotypes was difficult due to missing data. One A1.a isolate is missing the pXO2 plasmid marker (G_I H), whereas the single A3.b representative (G_I J) is missing both the pXO2 and pXO1 markers. Therefore, these samples could match several previously defined genotypes in these groups or could represent novel genotypes.

A mutational-step model attempts to describe genetic relationships based on the minimum number of mutations needed to create the observed diversity in the Italian *B. anthracis* genotypes (Fig. 1A). The A3.b G_I genotype J, which is multiple mutational steps different from the A1.a genotypes and which lacks data for the plasmid markers, is not included in this

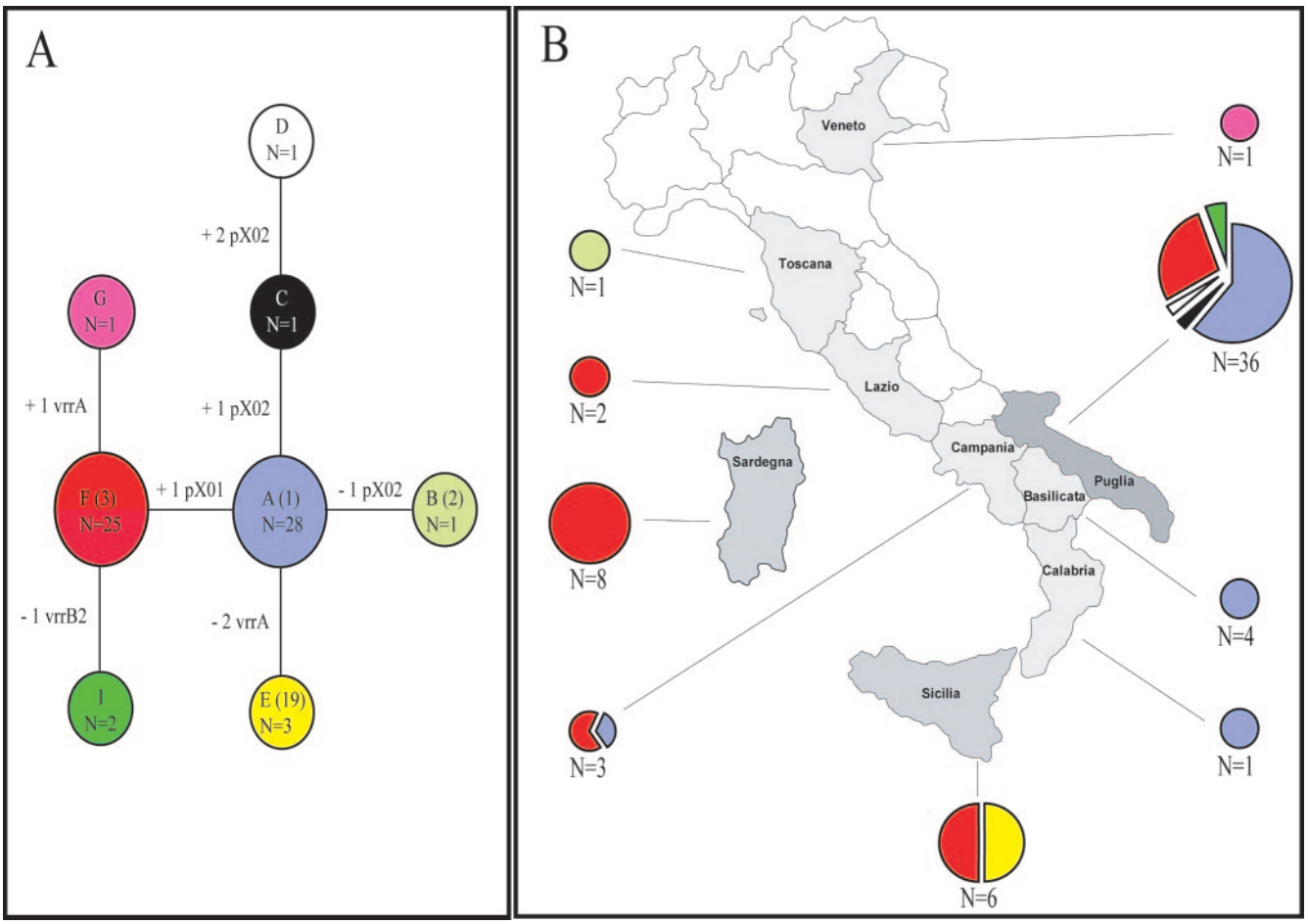

FIG. 1. Genetic and geographic relationships among Italian *B. anthracis* isolates. (A) Mutational-step model for isolate genetic relationships. Shown is a network of genotypes with the minimum mutational changes needed to convert one genotype into another. Note that genotype designations are consistent with those in Table 1. The G_I designation is given first, and G_K designation is given in parentheses following G_I if an equivalent genotype was described by Keim et al. in 2000 (8). Connecting lines are labeled with the genetic locus and the number of repeat units that differ between individual genotypes. (B) Geographic distribution of *B. anthracis* isolates. Genotype distribution and frequency are indicated by colors corresponding to designations in panel A. Pie chart sizes are scaled categorically according to the number of isolates (in three categories: 1 to 5, 6 to 10, and >10 isolates). Note that the two isolates with missing data (plasmid markers) were not included in either panel.

model. The most common genotypes (G_I genotypes A and F, G_K genotypes 1 and 3) differ by only a single mutation in the pXO1 marker locus. With the exception of G_I genotypes D and E, there is only a single mutational step separating the rarer genotypes from either of the two dominant genotypes. In this study, with one exception (G_I J), the *B. anthracis* isolates are closely genetically related within the A1.a cluster.

Different Italian regions have distinctive genotype compositions and population structures (Fig. 1B). The A genotype (G_K 1) dominates the peninsular provinces of Puglia, Basilicata, and Calabria. This genotype is not found in Sardinia or Sicily, where the F genotype (G_K 3) is dominant. The close genetic relationship between these genotypes argues for a historical relationship, but the spatial differences indicate that recent anthrax outbreaks are now independent between the regions.

DISCUSSION

Worldwide, *B. anthracis* exhibits a clonal population structure with ties among geographically distant regions (8, 10). A worldwide distribution is particularly true for the A1.a group, which has been highly successful. The major Italian genotypes are part of this very fit strain complex, which has succeeded in becoming ecologically established in many regions. This result is in contrast to that for adjacent southern France, where B2 genotypes have become established and are the most common subtype (2).

The close similarity of the *B. anthracis* isolates is consistent with a single introduction of the pathogen into Italy. Only a few postulated mutations are needed to explain most of the genotypes observed. Of the 10 genotypes observed in this study, the vast majority of samples (53/64) belonged to genotype A (G_K 1) or F (G_K 3), which differ only by a single 3-bp mutation in the pXO1 plasmid marker (Table 1; Fig. 1). Based on these data, it could be supposed that the *B. anthracis* strains responsible for anthrax outbreaks in Italy are the result of evolution from a recent, common ancestral strain. These genotyping markers are highly mutable loci, and the few mutational events that separate the isolates could have occurred in Italy after the

introduction of this strain. A single origin would be true for most of the recent anthrax outbreaks but not for genotype J, which likely was independently introduced into Sardinia, Italy. The center of diversity for Italian *B. anthracis* is in Puglia, where five different genotypes are observed. Other regions, notably Sardinia, are more homogeneous. It appears that the *B. anthracis* populations in Puglia are older and have had time to generate more diverse genotypes that were then dispersed to other sites. Dispersal can be associated with population bottlenecks that eliminate diversity due to founder effects in the new locations. Alternatively, the low sample sizes from other regions could result in underestimation of their diversity.

This study establishes a genetic landscape for Italy that can be used for future epidemiological and forensic analyses. As was shown in the U.S. anthrax letter attacks, strain identity can differentiate between a natural outbreak and one associated with bioterrorism (5, 10, 12). We would expect to see genotypes A and F associated with future Italian anthrax outbreaks caused by current ecological foci. In contrast, association with an exotic *B. anthracis* strain would point to a new introduction via commodities or even the deliberate release of this pathogen. Strain identity is useful in these situations only if the natural population is characterized and its strain genotype composition known.

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REFERENCES

1. **Andersen, G. L., J. M. Simchock, and K. H. Wilson.** 1996. Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. **178:**377–384.
2. **Fouet, A., K. L. Smith, C. Keys, J. Vaissaire, C. Le Doujet, M. Lévy, M. Mock, and P. Keim.** 2002. Diversity among French *Bacillus anthracis* isolates. J. Clin. Microbiol. **40:**4732–4734.
3. **Gierczynski, R., S. Kaluzewski, A. Rakin, M. Jagielski, A. Zasada, A. Jakubczak, B. Borkowska-Opacka, and W. Rastawicki.** 2004. Intriguing diversity of *Bacillus anthracis* in eastern Poland—the molecular echoes of the past outbreaks. FEMS Microbiol. Lett. **239:**235–240.
4. **Henderson, I., D. Yu, and P. C. Turnbull.** 1995. Differentiation of *Bacillus anthracis* and other '*Bacillus cereus* group' bacteria using IS*231*-derived sequences. FEMS Microbiol. Lett. **128:**113–118.
5. **Hoffmaster, A. R., C. C. Fitzgerald, E. Ribot, L. W. Mayer, and T. Popovic.** 2002. Molecular subtyping of *Bacillus anthracis* and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg. Infect. Dis. **8:**1111–1116.
6. **Jackson, P. J., E. A. Walthers, A. S. Kalif, K. L. Richmond, D. M. Adair, K. K. Hill, C. R. Kuske, G. I. Andersen, K. H. Wilson, M. E. Hugh-Jones, and P. Keim.** 1997. Characterization of the variable-number tandem repeats in *vrrA* from different *Bacillus anthracis* isolates. Appl. Environ. Microbiol. **63:**1400–1405.
7. **Keim, P., A. Kalif, J. M. Schupp, K. K. Hill, S. E. Travis, K. L. Richmond, D. M. Adair, M. E. Hugh-Jones, C. R. Kuske, and P. Jackson.** 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. **179:**818–824.
8. **Keim, P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. Jackson, and M. E. Hugh-Jones.** 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. **182:**2928–2936.
9. **Keim, P., K. L. Smith, C. Keys, H. Takahashi, T. Kurata, and A. Kaufmann.** 2001. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. J. Clin. Microbiol. **39:**4566–4567.
10. **Keim, P., M. N. Van Ert, T. Pearson, A. J. Vogler, L. Y. Hyunh, and D. M. Wagner.** 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect. Genet. Evol. **4:**205–213.
11. **Pearson, T., J. D. Busch, J. Ravel, T. D. Read, S. D. Rhoton, J. M. U'Ren, T. S. Simonson, S. M. Kachur, R. R. Leadem, M. L. Cardon, M. N. Van Ert, L. Y. Huynh, C. M. Fraser, and P. Keim.** 2004. Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphism from whole-genome sequencing. Proc. Natl. Acad. Sci. USA **101:**13536–13541.
12. **Read, T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser.** 2002. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. Science **296:**2028–2033.
13. **Smith, K. L., V. De Vos, H. B. Bryden, M. E. Hugh-Jones, A. Klevytska, L. B. Price, P. Keim, and D. T. Scholl.** 1999. Meso-scale ecology of anthrax in southern Africa: a pilot study of diversity and clustering. J. Appl. Microbiol. **87:**204–207.
14. **Smith, K. L., V. DeVos, H. Bryden, L. B. Price, M. E. Hugh-Jones, and P. Keim.** 2000. *Bacillus anthracis* diversity in Kruger National Park. J. Clin. Microbiol. **38:**3780–3784.