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Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins

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BACKGROUND AND HISTORY OF PESTICIDAL CRYSTAL PROTEIN NOMENCLATURE

Since the first cloning of an insecticidal crystal protein gene from *Bacillus thuringiensis* (91), many other such genes have been isolated. Initially, each newly characterized gene or protein received an arbitrary designation from its discoverers: *icp* (64); *cry* (21, 121); *kurhd1* (31); Bta (88); bt1, bt2, etc. (40); type B and type C (43); and 4.5 kb, 5.3 kb, and 6.6 kb (55). The first systematic attempt to organize the genetic nomenclature relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes (44). The *cryI* genes encoded proteins toxic to lepidopterans; *cryII* genes encoded proteins toxic to both lepidopterans and dipterans; *cryIII* genes encoded proteins toxic to coleopterans; and *cryIV* genes encoded proteins toxic to dipterans alone.

This system provided a useful framework for classifying the ever-expanding set of known genes. Inconsistencies existed in the original scheme, however, due to attempts to accommodate genes that were highly homologous to known genes but did not encode a toxin with a similar insecticidal spectrum. The *cryIIB* gene, for example, received a place in the lepidopteran-dipteran class with *cryIIA*, even though toxicity against dipterans could not be demonstrated for the toxin designated CryIIB. Other anomalies arose after the nomenclature was established. The protein named CryIC, for example, was reported to be toxic to both dipterans and lepidopterans (103), while the protein designated CryIB was reported to be toxic to both lepidopterans and coleopterans (8). Because the nomenclature system provided no central committee or database to maintain standardization, new genes encoding a diverse set of proteins without a common insecticidal activity each received the name *cryV*, based on the next available Roman numeral (32, 46, 67, 100, 102, 108).

PROPOSED NOMENCLATURE

We propose in this review a revised nomenclature for the *cry* and *cyt* genes. To organize the wealth of data produced by genomic sequencing efforts, a new nomenclatural paradigm is emerging, exemplified by the internationally recognized cytochrome P-450 superfamily nomenclature system (68a, 122a). Our proposal conforms closely to this model both in conceptual basis and in nomenclature format. The underlying basis of this type of system is to assign names to members of gene superfamilies according to their degree of evolutionary divergence as estimated by phylogenetic tree algorithms. The nomenclature format in such a system is designed to convey rich informational content about these relationships by appending to the mnemonic root a series of numerals and letters assigned in a hierarchical fashion to indicate degrees of phylogenetic divergence. This change from a function-based to a sequence-based nomenclature allows closely related toxins to be ranked together and removes the necessity for researchers to bioassay each new protein against a growing series of organisms before assigning it a name.

In our proposed revision, Roman numerals have been exchanged for Arabic numerals in the primary rank (e.g., Cry1Aa) to better accommodate the large number of expected new proteins. The mnemonic Cyt to designate crystal proteins showing a general cytolytic activity in vitro has been retained because of its historical precedent and entrenchment in the research literature. Our definition of a Cry protein is rather broad: a parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein. Similarly, Cyt denotes a parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein. By these criteria, the nontoxic 40-kDa crystal protein from *B. thuringiensis* subsp. *thompsoni*, for example, has been excluded from our list, but the lepidopteran-active 34-kDa protein (now Cry15A) encoded by an adjacent gene has been included (11).

The freely available software applications CLUSTAL W (110) and PHYLIP (27) define the sequence relationships among the toxins to form the framework of the new nomenclature. In the first step, CLUSTAL W aligns the deduced amino acid sequences of the full-length toxins and produces a distance matrix, quantitating the sequence similarities among the set of toxins. CLUSTAL W default settings are employed, except that the "delay divergent sequences" setting in the multiple-alignment parameter menu is reduced from 40 to 0%. The NEIGHBOR application within the PHYLIP package then constructs a phylogenetic tree from the distance matrix by an unweighted pair-group method using arithmetic averages

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(UPGMA) algorithm. The TREEVIEW application (73), with the "phylogenetic tree" and "ladderize left" options selected, produces a graphic presentation of the resulting tree.

We have applied this procedure to the set of holotype sequences given in Table 1 to produce the phylogenetic tree presented in Fig. 1. Vertical lines drawn through the tree show the boundaries used to define the various nomenclatural ranks. The name given to any particular toxin depends on the location of the node where the toxin enters the tree relative to these boundaries. A new toxin that joins the tree to the left of the leftmost boundary will be assigned a new primary rank (an Arabic number). A toxin that enters the tree between the left and central boundaries will be assigned a new secondary rank (an uppercase letter). It will have the same primary rank as the other toxins within that cluster. A toxin that enters the tree between the central and right boundaries will be assigned a new tertiary rank (a lowercase letter). Finally, a toxin that joins the tree to the right of the rightmost boundary will be assigned a new quaternary rank (another Arabic number). Toxins with identical sequences but isolated independently will receive separate quaternary ranks.

By this method each toxin will be assigned a unique name incorporating all four ranks. A completely novel toxin would currently be assigned the name Cry23Aa1. For the sake of convenience, however, we propose that the inclusion of the tertiary rank a and quaternary rank 1 be optional, their use dictated only by a need for clarity. This new toxin could therefore simply be referred to as Cry23A.

In choosing locations for rank boundaries, we attempted to construct a nomenclature reflecting significant evolutionary relationships while at the same time minimizing changes from the gene names assigned under the old system. In the resulting system, proteins with a common primary rank are similar enough that the percent identity can be defined with some confidence. Proteins with the same primary rank often affect the same order of insect; those with different secondary and tertiary ranks may have altered potency and targeting within an order. At the tertiary rank, differences can be due to the accumulation of dispersed point mutations, but often they appear to have resulted from ancestral recombination events between genes differing at a lower rank level (9). The quaternary rank was established to group "alleles" of genes coding for known toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing. To avoid confusion, however, the reader should bear in mind the differences between the quaternary rank number and the classical concept of the allele. Any *cry* gene specified with a quaternary rank is a natural isolate. No assumption about functionality is implied by the presence of this rank number in the gene name. In contrast, an allele number would be assumed, unless parenthetical or subscripted information indicated otherwise, to denote a nonfunctional mutant form of a wild-type gene found at a discrete genetic locus. Because of the somewhat modular nature of the Cry proteins and the effect that various segmental relationships could have on the clustering algorithm, it is likely that these boundaries will move slightly or even bend as the addition of new sequences changes the topology of the phylogenetic tree. Currently the boundaries represent approximately 95, 78, and 45% sequence identity.

A *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee, consisting of the authors of this paper, will remain as a standing committee of the *Bacillus* Genetic Stock Center (BGSC) to assist workers in the field of *B. thuringiensis* genetics in assigning names to new Cry and Cyt toxins. The corresponding gene or protein sequences must first be deposited into a publicly accessible database (GenBank, EMBL, or PIR) and released by the repository for electronic publication in the database so that the scientific community may conduct an independent analysis. Researchers should submit new sequences directly to the BGSC director (D. R. Zeigler), either by electronic mail (zeigler.1@osu.edu) or on computer diskette. The director will analyze the amino acid sequence as described above and suggest the appropriate name, subject to the approval of the committee. The committee will periodically review the literature of the Cry and Cyt toxins and publish a comprehensive list. This list, alongside other relevant information, will also be available via the Internet at the following URL: http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/.

The current list of *cry* and *cyt* genes (including quaternary ranks) is given in Table 1. New gene names are listed with their previous names, their GenBank accession numbers, and published references. The quaternary ranks were assigned in the order that the gene sequences were discovered in the literature or submitted to the committee. Genes assigned the quaternary rank 1 represent holotype sequences.

The boundaries shown in Fig. 1 allow most *cry* genes to retain the names they received under the system of Höfte and Whiteley (44), after a substitution of Arabic for Roman numerals. There are a few notable exceptions: *cryIG* becomes *cry9A*, *cryIIIC* becomes *cry7Aa*, *cryIIID* becomes *cry3C*, *cryIVC* becomes *cry10A*, *cryIVD* becomes *cry11A*, *cytA* becomes *cyt1A*, and *cytB* becomes *cyt2A* (Table 1). Under the revised system, the known Cry and Cyt proteins fall into 24 sets at the primary rank—Cyt1, Cyt2, and Cry1 through Cry22.

ROBUSTNESS OF THE NOMENCLATURE

The robustness of the current naming process was assessed by a number of additional analyses. The choice of clustering algorithm (unweighted pair-group method using arithmetic averages) was driven largely by the consistent location of a root and constant branch lengths, resulting in a common vertical alignment of sequence names and essentially allowing a "ruler across the tree" approach to naming. It has the drawback of imposing a common evolutionary clock on the clustering process, an assumption that cannot be assured. The distance metric related to percent identity (essentially 1 minus the fraction of identical residues of the total compared without gaps) is the one most commonly found as the output of sequence comparison programs, including CLUSTAL W. For phylogenetic analysis, a more usual distance metric relates to the number of substitutions per site to convert one sequence to the other (e.g., Dayhoff's point accepted mutation [PAM]) and accounts for the possibility of multiple substitutions per site as the sequences are more divergent. The latter method has the drawback of being more computationally intensive, and, for very divergent sequences, requiring too large a value, resulting in numeric computation failures. They also differ in the way sequences of unequal length are handled, with the percent identity method typically ignoring excess sequence and the other methods assigning a penalty. This is particularly important for crystal proteins, since a number of them lack the C-terminal protoxin segments yet are quite related to some longer toxins in the N-terminal toxin segment; we feel that the stronger association of such relationships found by the percent identity method is preferred.

To assess the effect of using the neighbor-joining method to generate an unrooted tree, CLUSTAL W routines were used to generate such a tree with 1,000 bootstraps of the sequence alignment we used for Fig. 1. When an appropriate outgroup was chosen, the resulting tree (not shown) resembled our Fig. 1. The bootstrap values indicated that the tree thus generated

TABLE 1. Known *cry* and *cyt* gene sequences with revised nomenclature assignments

Revised gene name	Original gene or protein name	Accession no.	Coding region[a]	Reference
cry1Aa1	*cryIA*(*a*)	M11250	527–4054	92
cry1Aa2	*cryIA*(*a*)	M10917	153–>2955	98
cry1Aa3	*cryIA*(*a*)	D00348	73–3600	99
cry1Aa4	*cryIA*(*a*)	X13535	1–3528	62
cry1Aa5	*cryIA*(*a*)	D17518	81–3608	113
cry1Aa6	*cryIA*(*a*)	U43605	1–>1860	63
cry1Ab1	*cryIA*(*b*)	M13898	142–3606	119
cry1Ab2	*cryIA*(*b*)	M12661	155–3622	111
cry1Ab3	*cryIA*(*b*)	M15271	156–3620	31
cry1Ab4	*cryIA*(*b*)	D00117	163–3627	50
cry1Ab5	*cryIA*(*b*)	X04698	141–3605	40
cry1Ab6	*cryIA*(*b*)	M37263	73–3537	37
cry1Ab7	*cryIA*(*b*)	X13233	1–3465	36
cry1Ab8	*cryIA*(*b*)	M16463	157–3621	69
cry1Ab9	*cryIA*(*b*)	X54939	73–3537	13
cry1Ab10	*cryIA*(*b*)	A29125	—[b]	28
cry1Ac1	*cryIA*(*c*)	M11068	388–3921	3
cry1Ac2	*cryIA*(*c*)	M35524	239–3769	117
cry1Ac3	*cryIA*(*c*)	X54159	339–>2192	18
cry1Ac4	*cryIA*(*c*)	M73249	1–3534	84
cry1Ac5	*cryIA*(*c*)	M73248	1–3531	83
cry1Ac6	*cryIA*(*c*)	U43606	1–>1821	63
cry1Ac7	*cryIA*(*c*)	U87793	976–4509	38
cry1Ac8	*cryIA*(*c*)	U87397	153–3686	71
cry1Ac9	*cryIA*(*c*)	U89872	388–3921	33
cry1Ac10		AJ002514	388–3921	107
cry1Ad1	*cryIA*(*c*)	M73250	1–3537	79
cry1Ae1	*cryIA*(*e*)	M65252	81–3623	60
cry1Af1	*icp*	U82003	172–>2905	49
cry1Ba1	*cryIB*	X06711	1–3684	10
cry1Ba2		X95704	186–3869	105
cry1Bb1	ET5	L32020	67–3753	25
cry1Bc1	*cryIB*(*c*)	Z46442	141–3839	6
cry1Bd1	*cryE1*	U70726		12
cry1Ca1	*cryIC*	X07518	47–3613	45
cry1Ca2	*cryIC*	X13620	241–>2711	88
cry1Ca3	*cryIC*	M73251	1–3570	79
cry1Ca4	*cryIC*	A27642	234–3800	114
cry1Ca5	*cryIC*	X96682	1–>2268	106
cry1Ca6	*cryIC*	X96683	1–>2268	106
cry1Ca7	*cryIC*	X96684	1–>2268	106
cry1Cb1	*cryIC*(*b*)	M97880	296–3823	48
cry1Da1	*cryID*	X54160	264–3758	42
cry1Db1	*prtB*	Z22511	241–3720	56
cry1Ea1	*cryIE*	X53985	130–3642	115
cry1Ea2	*cryIE*	X56144	1–3513	7
cry1Ea3	*cryIE*	M73252	1–3513	82
cry1Ea4		U94323	388–3900	47
cry1Eb1	*cryIE*(*b*)	M73253	1–3522	81
cry1Fa1	*cryIF*	M63897	478–3999	14
cry1Fa2	*cryIF*	M73254	1–3525	80
cry1Fb1	*prtD*	Z22512	483–4004	56
cry1Ga1	*prtA*	Z22510	67–3564	56
cry1Ga2	*cryIM*	Y09326	692–4210	96
cry1Gb1	*cryH2*	U70725		12
cry1Ha1	*prtC*	Z22513	530–4045	56
cry1Hb1		U35780	728–4195	53
cry1Ia1	*cryV*	X62821	355–2511	108
cry1Ia2	*cryV*	M98544	1–2157	34
cry1Ia3	*cryV*	L36338	279–2435	100
cry1Ia4	*cryV*	L49391	61–2217	54
cry1Ia5	*cryV159*	Y08920	524–2680	94
cry1Ib1	*cryV465*	U07642	237–2393	100
cry1Ja1	ET4	L32019	99–3519	25
cry1Jb1	ET1	U31527	177–3686	116
cry1Ka1		U28801	451–4098	52
cry2Aa1	*cryIIA*	M31738	156–2054	20
cry2Aa2	*cryIIA*	M23723	1840–3738	123
cry2Aa3		D86064	2007–3911	89
cry2Ab1	*cryIIB*	M23724	1–1899	123
cry2Ab2	*cryIIB*	X55416	874–2775	17
cry2Ac1	*cryIIC*	X57252	2125–3990	124
cry3Aa1	*cryIIIA*	M22472	25–1956	39
cry3Aa2	*cryIIIA*	J02978	241–2172	93
cry3Aa3	*cryIIIA*	Y00420	566–2497	41
cry3Aa4	*cryIIIA*	M30503	201–2132	65
cry3Aa5	*cryIIIA*	M37207	569–2500	22
cry3Aa6	*cryIIIA*	U10985	569–2500	1
cry3Ba1	*cryIIIB2*	X17123	25–>1977	101
cry3Ba2	*cryIIIB*	A07234	342–2297	85
cry3Bb1	*cryIIIBb*	M89794	202–2157	24
cry3Bb2	*cryIIIC*(*b*)	U31633	144–2099	23
cry3Ca1	*cryIIID*	X59797	232–2178	59
cry4Aa1	*cryIVA*	Y00423	1–3540	121
cry4Aa2	*cryIVA*	D00248	393–3935	95
cry4Ba1	*cryIVB*	X07423	157–3564	16
cry4Ba2	*cryIVB*	X07082	151–3558	112
cry4Ba3	*cryIVB*	M20242	526–3930	125
cry4Ba4	*cryIVB*	D00247	461–3865	95
cry5Aa1	*cryVA*(*a*)	L07025	1–>4155	102
cry5Ab1	*cryVA*(*b*)	L07026	1–>3867	67
cry5Ac1		I34543	1–>3660	76
cry5Ba1	PS86Q3	U19725	1–>3735	76
cry6Aa1	*cryVIA*	L07022	1–>1425	68
cry6Ba1	*cryVIB*	L07024	1–>1185	67
cry7Aa1	*cryIIIC*	M64478	184–3597	58
cry7Ab1	*cryIIIC*(*b*)	U04367	1–>3414	75
cry7Ab2	*cryIIIC*(*c*)	U04368	1–>3414	75
cry8Aa1	*cryIIIE*	U04364	1–>3471	29
cry8Ba1	*cryIIIG*	U04365	1–>3507	66
cry8Ca1	*cryIIIF*	U04366	1–3447	70
cry9Aa1	*cryIG*	X58120	5807–9274	104
cry9Aa2	*cryIG*	X58534	385–>3837	32
cry9Ba1	*cryX*	X75019	26–3488	97
cry9Ca1	*cryIH*	Z37527	2096–5569	57
cry9Da1	N141	D85560	47–3553	4
cry9Da2		AF042733	<1–>1937	122
cry10Aa1	*cryIVC*	M12662	941–2965	111
cry11Aa1	*cryIVD*	M31737	41–1969	21
cry11Aa2	*cryIVD*	M22860	<1–235	2
cry11Ba1	Jeg80	X86902	64–2238	19
cry11Bb1	94 kDa	AF017416		72
cry12Aa1	*cryVB*	L07027	1–>3771	67
cry13Aa1	*cryVC*	L07023	1–2409	90
cry14Aa1	*cryVD*	U13955	1–3558	77
cry15Aa1	34kDa	M76442	1036–2055	11
cry16Aa1	*cbm71*	X94146	158–1996	5
cry17Aa1	*cbm72*	X99478	12–1865	5
cry18Aa1	*cryBP1*	X99049	743–2860	126
cry19Aa1	Jeg65	Y07603	719–2662	86
cry19Ba1		D88381		87
cry20Aa1	86kDa	U82518	60–2318	61
cry21Aa1		I32932	1–3501	74
cry22Aa1		I34547	1–2169	76
cyt1Aa1	*cytA*	X03182	140–886	118
cyt1Aa2	*cytA*	X04338	509–1255	120
cyt1Aa3	*cytA*	Y00135	36–782	26
cyt1Aa4	*cytA*	M35968	67–813	30
cyt1Ab1	*cytM*	X98793	28–777	109
cyt1Ba1		U37196	1–795	78
cyt2Aa1	*cytB*	Z14147	270–1046	51
cyt2Ba1	*"cytB"*	U52043	287–655	35
cyt2Bb1		U82519	416–1204	15

[a] The symbols < and > indicate that the coding region extends up- or downstream, respectively, from the known sequence data.
[b] Only the polypeptide sequence has been reported.

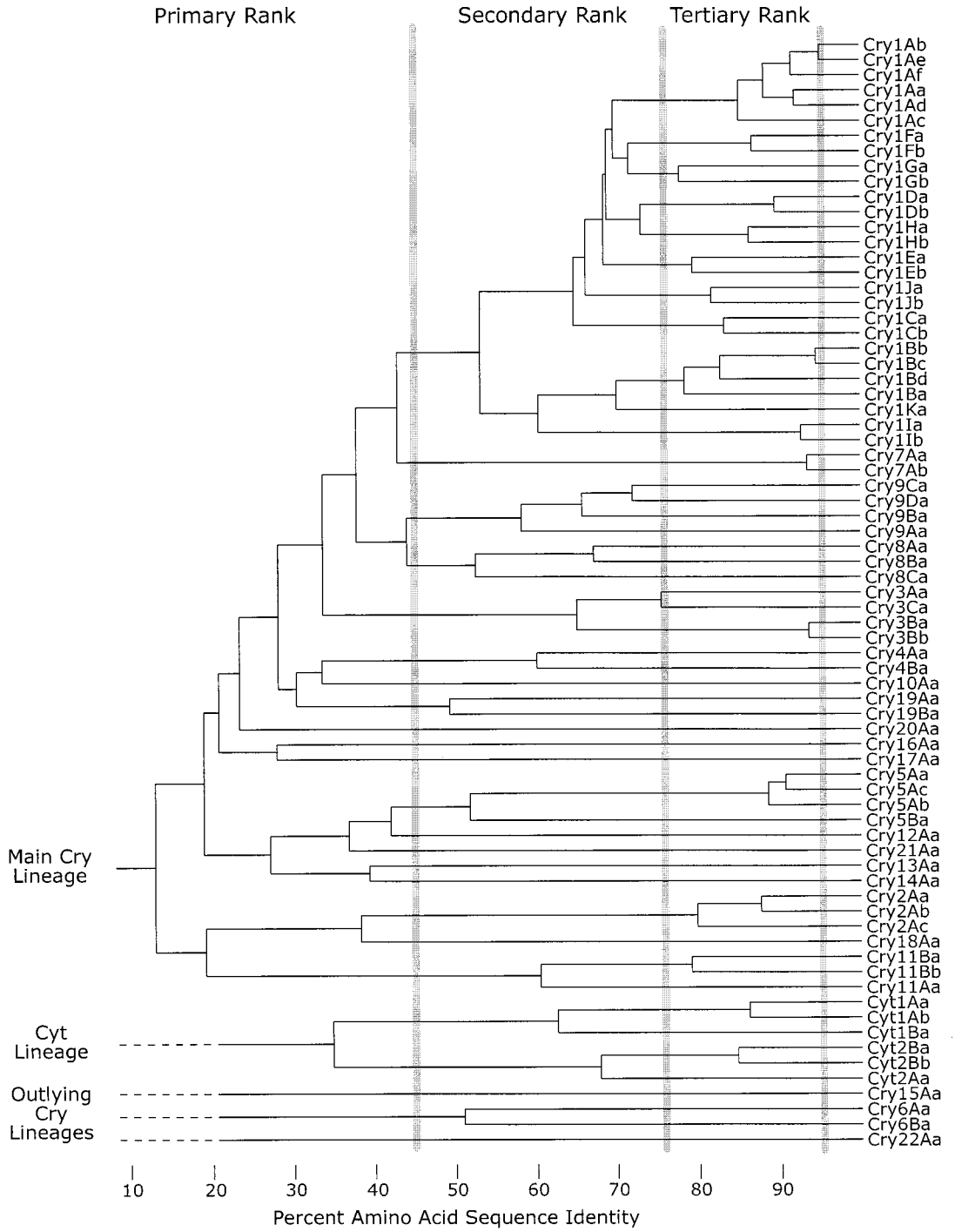

FIG. 1. Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBOR treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences, as described in the text. The gray vertical bars demarcate the four levels of nomenclature ranks. Based on the low percentage of identical residues and the absence of any conserved sequence blocks in multiple-sequence alignments, the lower four lineages are not treated as part of the main toxin family, and their nodes have been replaced with dashed horizontal lines in this figure.

had significant branch points deeper in the tree than the chosen primary rank in the nomenclature. This sort of analysis was rejected as unsuitable for the purposes of Cry nomenclature due to the generally ragged branch lengths it produced and the requirement for the careful choice of an outgroup.

An alternative method of clustering protein sequences, capable of handling sequences that are quite diverse, is parsimony analysis. A consensus tree generated from 100 bootstraps of such an analysis displaces the two incomplete Cry1 sequences (Cry1Bd and Cry1Af) and the two Cry1 sequences lacking the C-terminal protoxin segments (Cry1Ia and Cry1Ib) into a region of the tree populated with such shortened se-

quences (not shown). With the further exceptions of Cry12A being interjected into the Cry5 cluster and a number of sequences besides Cry6B clustering higher in the tree than Cry6A, the proposed nomenclature successfully reflects the grouping of sequences provided by this method of analysis as well.

As noted above, the usual distance metrics for phylogenetic analysis account for multiple substitutions per site; most commonly, the Dayhoff PAM metric is used. When this distance metric was applied to the alignment used to make Fig. 1, a large number of the sequence pairs were found to have infinite distance. Therefore, the main Cry lineage and the Cyt lineage were separately aligned, the distances were calculated, and the distance matrices were clustered by using the FITCH program (of the PHYLIP software package). This method of analysis revealed several strongly associated groups of sequences (>90% of trees) in the main Cry lineage that extend deeper into the tree than the primary rank assigned in the proposed nomenclature: Cry1; Cry3; Cry4; Cry7; the Cry5, Cry12-Cry13-Cry14-Cry21 group; the Cry8-Cry9 group; the Cry10-Cry19 group; the Cry16-Cry17 group; and the Cry2-Cry11-Cry18 group. Many of these groups, however, were separated by branch points that were either nonmajority or were found <60% of the time; thus, the arrangement of these groups would be likely to change with additional sequence additions. At the secondary rank, the only anomaly with respect to the proposed nomenclature was the interjection of the Cry1Ia and Cry1Ib sequences into the Cry1B group. This effect may be due to an artificially reduced distance between the Cry1I sequences and the incomplete Cry1Bd sequence caused by the particular distance metric used. The Cyt lineage sequences were separated into the expected two primary rank groups that separate into the expected secondary rank groupings. This more standard phylogenetic approach also suffers from an accentuated visual disorientation of uneven branch lengths and shortening of the more closely related branches, especially at the tertiary rank (lowercase letter), where a great deal of comparative work has been done among the Cry1 toxins.

In summary, the proposed nomenclature uses readily available software that can be easily interpreted by investigators in the field and meets their needs as well as, or better than, alternative methods of analysis and presentation. When the holotype toxins were analyzed by alternative phylogenetic methods, the hierarchy implied by the nomenclature was essentially consistent with the resulting phylogenetic clustering, and the few exceptions were largely explainable by known properties of the sequences in question.

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