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Genetic structure of environmental *Bacillus cereus sensu lato* strains isolated from Northeastern Poland

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I dedicate this dissertation to my husband Mariusz for his remarkable patience and unwavering love and support over the course of my research.

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Contents

Streszczenie

Frederick Cohan w 2002 roku zaproponował specjację ekologiczną jako główny proces różnicowania bakterii. Zgodnie z teorią Cohan'a każdy gatunek bakterii zawiera zmienną liczbę ekotypów. Ekotyp bakteryjny jest definiowany jako homogeniczna grupa o wysokim podobieństwie ekologicznym. Poszczególne ekotypy danego gatunku charakteryzują się specyficznymi adaptacjami, pozwalającymi na efektywne wykorzystywanie składników odżywczych lub innych warunków środowiska. Teoria specjacji ekologicznej wśród bakterii została potwierdzona w populacjach *Bacillus subtilis-Bacillus licheniformis* pochodzących z Parku Narodowego Doliny Śmierci, sinic z Parku Narodowego Yellowstone oraz *Bacillus simplex* z Izraela. W ramach rozprawy doktorskiej badałam czy naturalne populacje gramdodatnich laseczek *Bacillus cereus sensu lato* wykazują ekotypowy charakter.

Przedstawiciele *B. cereus s.l.* występują powszechnie w środowisku naturalnym i wywierają ogromny wpływ na zdrowie człowieka, przemysł spożywczy oraz rolnictwo i leśnictwo. Te tlenowe, sporulujące laseczki z jednej strony produkują toksyny szkodliwe dla ludzi, zwierząt roślinożernych oraz bezkręgowców, ale też są znane jako producenci enzymów i wtórnych metabolitów, degradujących niebezpieczne związki chemiczne i/lub wspomagających wzrost roślin. Znane są też szczepy *B. cereus s.l.* stosowane jako probiotyki w paszy dla zwierząt. Powyższe właściwości były i są intensywnie badane, jednakże jedynie w odniesieniu do szczepów o szczególnym znaczeniu gospodarczym i medycznym, jak *B. cereus sensu stricto, B. thuringiensis* oraz *B. anthracis.* Tymczasem pokrewieństwo filogenetyczne tych laseczek wciąż budzi wiele kontrowersji. Również podłoże ekologicznej dywersyfikacji w grupie *B. cereus s.l.* nie jest dostatecznie poznane, przede wszystkim z powodu braku informacji na temat szczepów izolowanych z gleby, głównego rezerwuaru tych bakterii.

W ramach rozprawy doktorskiej analizowałam strukturę genetyczną oraz zidentyfikowałam potencjalne ekotypy wśród 297 szczepów *B. cereus s.l.* wyizolowanych z prób gleby pobranych w (i) Narwiańskim Parku Narodowym (Załącznik 1), jak również w (ii) Białowieskim Parku Narodowym, (iii) Biebrzańskim Parku Narodowym oraz (iv) w gospodarstwie rolnym w Jasienówce (Załącznik 2 i 3). Ekologiczna specjacja w grupie *B. cereus* wydaje się być silniejsza, aniżeli zdolność tych bakterii do horyzontalnego transferu genów. W związku z tym założyłam, iż wśród badanych populacji występują ekotypy, jak ekotyp termiczny, cytotoksyczny i melaninowy. W pierwszej kolejności wykazałam, iż termiczne ekotypy (izolaty zdolne do wzrostu w niskich temperaturach) występują wśród naturalnych populacji *B. cereus s.l.* z północno-wschodniej Polski (Załącznik 1, Załącznik 2). Początkowo wykazałam również, iż potencjał cytotoksyczny jest znacząco związany z dwoma liniami filogenetycznymi *B. thuringiensis* (Załącznik 1). Dalsze analizy ujawniły, iż szczepy potencjalnie cytotoksyczne występują przede wszystkim w środowisku, w którym działalność człowieka jest znacząca (gleba uprawna). Ponadto cytotoksyczność nie jest cechą gatunkową i występuje wśród szczepów należących do różnych grup filogenetycznych, co potwierdza oportunistyczny model patogenności grupy *B. cereus s.l.* (Załącznik 2). Następnie zidentyfikowałam potencjalny ekotyp melaninowy, utworzony przez szczepy *B. weihenstephanensis* pochodzące z Białowieskiego Parku Narodowego oraz pola uprawnego w Jasienówce, zdolne do produkcji rozpuszczalnego w wodzie pigmentu (Załącznik 3). Właściwości fizyko-chemiczne tego pigmentu jednoznacznie wskazują, iż jest to melanina. Synteza melaniny może znacząco wpływać na funkcjonowanie mikroorganizmów w niekorzystnych warunkach środowiskowych. Z tego względu zdolność do syntezy melaniny przez glebowe izolaty *B. weihenstephanensis,* a także ich psychrotroficzny charakter, wydają się być doskonałym dostosowaniem do miejscowych warunków środowiska. Przeprowadzenie analiz w odniesieniu do stosunkowo wysokiej liczby szczepów *B. cereus s.l.* pochodzących z ekologicznie zróżnicowanych środowisk, pozwoliło testować hipotezę stanowiącą, że specyficzne genotypy tych bakterii występują w poszczególnych naturalnych środowiskach (Załącznik 2). Analizy MLST (*ang.* Multi-Locus Sequence Typing) wykazały, iż aż 92% typów sekwencyjnych (ST) jest charakterystyczna dla poszczególnych środowisk. Ponadto 78% badanych izolatów posiadało niezidentyfikowane do tej pory STs. Powyższe wyniki wskazują na istnienie

specyficznych genotypów wśród naturalnych populacji *B. cereus s.l.* Obecność ekologicznie odrębnych genotypów wynika raczej z adaptacji tych bakterii do specyficznych środowisk, aniżeli z przypadkowej kolonizacji.

Taksonomia grupy *B. cereus s.l.* budzi wiele kontrowersji. Na podstawie literatury odnoszącej się głównie do szczepów związanych z działalnością człowieka, założyłam, iż szczepy środowiskowe *B. cereus s.l.* są genetycznie blisko spokrewnione i powinny być sklasyfikowane do jednego gatunku (Załącznik 2). Wprawdzie wykazałam istnienie czterech głównych grup filogenetycznych, obejmujących zmienną liczbę *B. cereus/B. weihenstephanensis, B. thuringiensis* i *B. mycoides/B. pseudomycoides*, jednakże tylko niewielka liczba typów sekwencyjnych (ST) zawierała szczepy należące do różnych gatunków. Ponadto, izolaty sklasyfikowane do tego samego gatunku, ale pochodzące z różnych środowisk, wykazywały tendencję do grupowania się w kompleksy klonalne. Powyższe wyniki dały podstawę do wyciągnięcia wniosku, iż środowiskowe izolaty *B. cereus s.l.* nie reprezentują jednego gatunku.

Szczegółowe analizy genetyczne, fenotypowe oraz biochemiczne przeprowadzone w odniesieniu do wysokiej liczby środowiskowych szczepów *B. cereus s.l.*, rzuciły nowe światło na ewolucję oraz ekologiczną adaptację tych bakterii. Ponadto, luki w wiedzy na temat biologii tych tlenowych laseczek, zostały znacząco uzupełnione poprzez uwzględnienie przeze mnie po raz pierwszy *B. mycoides* w badaniach struktury genetycznej *B. cereus s.l.*

Abstract

In 2002, Cohan proposed ecological speciation as the major way of bacterial diversification. He suggested that each bacterial species contains a variable number of ecotypes, defined as homogenous groups of bacteria that are ecologically similar and share genetic adaptation(s) to certain habitats, resources, and/or conditions. Now, it is generally accepted, that ecological speciation is likely to occur frequently in the bacterial world and that new ecotypes appear to originate in complex communities at a fast rate, in both natural and laboratory conditions. Specific ecotypes have been already observed within *Bacillus subtilis-Bacillus licheniformis* from Death Valley National Park, cyanobacteria from Mushroom Spring, Yellowstone National Park, and *Bacillus simplex* from Israel. In my doctoral thesis I tested whether natural populations of Gram-positive bacilli of *Bacillus cereus sensu lato* have an ecotypic character.

B. cereus s.l. are widespread in natural environments and have a significant impact on human health, food industry, and agriculture. On one hand, members of this group synthetize various toxins harmful to humans, herbivores and invertebrates. On the other hand, they are also known as producers of various valuable enzymes and secondary metabolites whereby they degrade pollutants and promote the growth of plants and animals. These aspects have been intensively studied especially with regard to *B. cereus*, *B. thuringiensis*, and *B. anthracis*, the *B. cereus* group members with the highest impact on human health and economy. Meanwhile, the phylogenetic relationships between these bacilli are still under controversial discussion. Likewise, the basis of ecological diversification of *B. cereus s.l.* remains largely undescribed, especially in relation to strains isolated from soil being their primary environment.

Within the doctoral thesis I investigated the genetic structure and identified potential ecotypes among 297 soil *B. cereus s. l.* isolates from diverse habitats in

Northeastern Poland, such as (i) the Narew National Park (Appendix 1), as well as (ii) the Białowieża National Park, (iii) Biebrza National Park, and (iv) agricultural land in Jasienowka (Appendixes 2 and 3). Since ecological speciation among *B. cereus s.l.* is stronger than the potential of these bacteria for horizontal gene transfer, I assumed that particular *B. cereus s.l.* genetic lineages contain isolates possessing the same specific ecological properties (thermal ecotype, cytotoxin K pathotype, and melanotype). First of all, I confirmed the presence of a thermal ecotype (isolates adapted to low temperature) among natural populations of *B. cereus s.l.* (Appendixes 1 and 2). However, while the first studies showed the presence of two lineages of cytotoxic *B. thuringiensis* strains (Appendix 1), upon further examination I found the *cytK* gene to be mainly associated with strains from farm samplings, representing all studied species of the *B. cereus* group, and to be intermixed among the clades on the phylogenetic tree. This observation supports the opportunistic pathogenicity model of *B. cereus s.l.* (Appendix 2). Next, for the first time, I identified a melanotype within *B. weihenstephanensis* strains from Białowieża National Park and farmland in Jasienowka, which were able to synthesize a water-soluble melanin-like pigment (Appendix 3). The pigment may give a survival advantage in the environment for many microorganisms. The ability to synthesize melanin by soil isolates and their psychrotrophic character seem to be a local adaptation to a specific niche. A relatively large number of samples of *B. cereus s.l.* from ecologically different environments allowed testing the hypothesis that specific genotypes of these bacilli occur in particular natural habitats (Appendix 2). The multi-locus sequence typing (MLST) revealed 92% of sequence types (STs) in bacilli originated from one habitat only. Together with the fact that there were new STs described for 78% of the isolates, it strongly indicates the existence of specific genotypes within the natural *B. cereus s.l.* populations. Presumably, the presence of ecologically distinct genotypes resulted from adaptations to specific habitats. The taxonomy of the *B. cereus s.l.* is complex and rises a lot of controversy. I assumed that environmental *B. cereus s.l.* are highly genetically related and should be classified as one species (Appendix 2). Indeed, phylogenetic analyses revealed three major clades, in which *B. cereus/B. weihenstephanensis*, *B. thuringiensis* and *B. mycoides/B. pseudomycoides* were intermixed. However, only few STs contain bacilli classified into different species. Moreover, the isolates originated from different habitats and classified to the same species

were grouped into particular clonal complexes. Thus, certainly environmental *B. cereus s.l.* do not represent one species.

Detailed genetic, phenotypic and biochemical analyses of the environmental *B. cereus s.l.* strains shed new light on the evolution and ecological adaptation of these bacteria to specific soil habitats differing in scope of human activity. In addition, the gaps in the knowledge on this group's biology are further complemented by taking into account the *B. mycoides* species which are often omitted by researchers when studying the *B. cereus s.l.* group.

Introduction

In 2002, Cohan proposed ecological speciation as the major way of bacterial diversification, and suggested that each bacterial species contains a variable number of ecotypes [Cohan, 2002]. In Cohan's framework, an ecotype is defined as a homogenous group of bacteria, that are ecologically similar and share genetic adaptation(s) to certain habitats, resources, and/or conditions. Genetic diversity within an ecotype is limited by cohesive forces, such as periodic selection and/or genetic drift. Thus, genetic lineages within one ecotype are ecologically too homogeneous to allow their indefinite co-existence [Cohan, 2002; Cohan & Perry, 2007; Cohan & Koeppel, 2008; Koeppel *et al.*, 2013]. Such ecotypes are usually monophyletic groups, because they are founded by a single individual. In some cases, if niche-determining plasmids are acquired or lost in horizontal gene transfer, an ecotype may never appear as a separate cluster [Cohan, 2007]. Now, it is generally accepted, that ecological speciation is likely to occur frequently in the bacterial world and that new ecotypes appear to originate in complex communities at a fast rate, in both natural and laboratory conditions [Dolittle & Zhaxybayeva, 2009; Vos *et al.*, 2013]. Several aspects of bacterial population dynamics, such as (i) the rarity of recombination, (ii) the promiscuity of genetic exchange, (iii) the small size of recombining segments, and (iv) the large population sizes, can entail the rapid evolution of bacteria [Koeppel *et al.*, 2013]. Especially the low rate of genetic exchange, being insufficient to impede adaptive divergence, may split a population into ecologically distinct populations which coexist without developing sexual and geographic isolation.

Data reported over the last decade on ecotypes within (i) *Bacillus subtilis-Bacillus licheniformis* from Death Valley National Park [Connor *et al.*, 2010], (ii) cyanobacteria from Mushroom Spring, Yellowstone National Park [Ward *et al.*, 2006], and (iii) in *Bacillus simplex* from 'Evolution Canyons' in Israel [Sikorski & Nevo, 2007], has allowed

to conclude that closely related populations within these bacteria are present along environmental gradients. Especially soil, due to its three-dimensional spatial network, and heterogeneity in nutrients, particle size, pH, humidity, and microbiota [McArthur, 2006; Vos *et al.,* 2013], creates favorable conditions for bacterial evolution [Connor *et al.,* 2010; Horner-Devine *et al.,* 2004; Materna *et al.,* 2012; Ward *et al.,* 2006]. Although soil is the primary niche also for *Bacillus cereus sensu lato (s.l.)* [Ceuppens *et al.,* 2013; Von Stetten *et al.,* 1999], the ecotypic character of these bacilli in their natural environment has not been thoroughly studied yet.

B. cereus s.l. comprises Gram-positive endospore-forming bacilli persisting ubiquitously in different natural habitats, food products, and in hospital environments, where they display a wide variety of different lifestyles [Ceuppens *et al.,* 2013]. From ecological and economical points of view, the most known members of this group are (i) *Bacillus cereus sensu stricto* (*B. cereus s.s.*), an opportunistic pathogen occasionally involved in foodborne illnesses [Logan, 2012], (ii) *Bacillus thuringiensis*, an entomopathogen used worldwide as a biopesticide [Crickmore, 2006], and (iii) *Bacillus anthracis,* an etiologic agent of anthrax, which primarily causes the disease in ruminants and secondarily in humans or other animals [Mock & Fouet, 2001]. *B. cereus s.l.* also includes (iv) *Bacillus mycoides* and (v) *Bacillus pseudomycoides* which form characteristic rhizoidal colonies on solid media and are distinguished by DNA relatedness and fatty acid composition [Nakamura, 1998], (vi) psychrotolerant *Bacillus weihenstephanensis* [Lechner *et al.,* 1998], as well as recently described (vii) thermotolerant *Bacillus cytotoxicus* [Guinebretière *et al.,* 2013], and (viii) *Bacillus toyonensis,* a probiotic organisms used in animal feed [Jiménez *et al.,* 2013].

The classical "Biological Species Concept" (BSC) [Mayr, 1944] defines a species as “groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups”. Thus, sexual isolation, arising mostly from the appearance of prezygotic barriers or geographical separation, is essential for species divergence. In this context, bacteria which reproduce clonally, exchange genes even with distant relatives through horizontal gene transfer (HGT), and often co-occur in nature without geographical boundaries, cannot be considered in the framework of Mayr's BSC

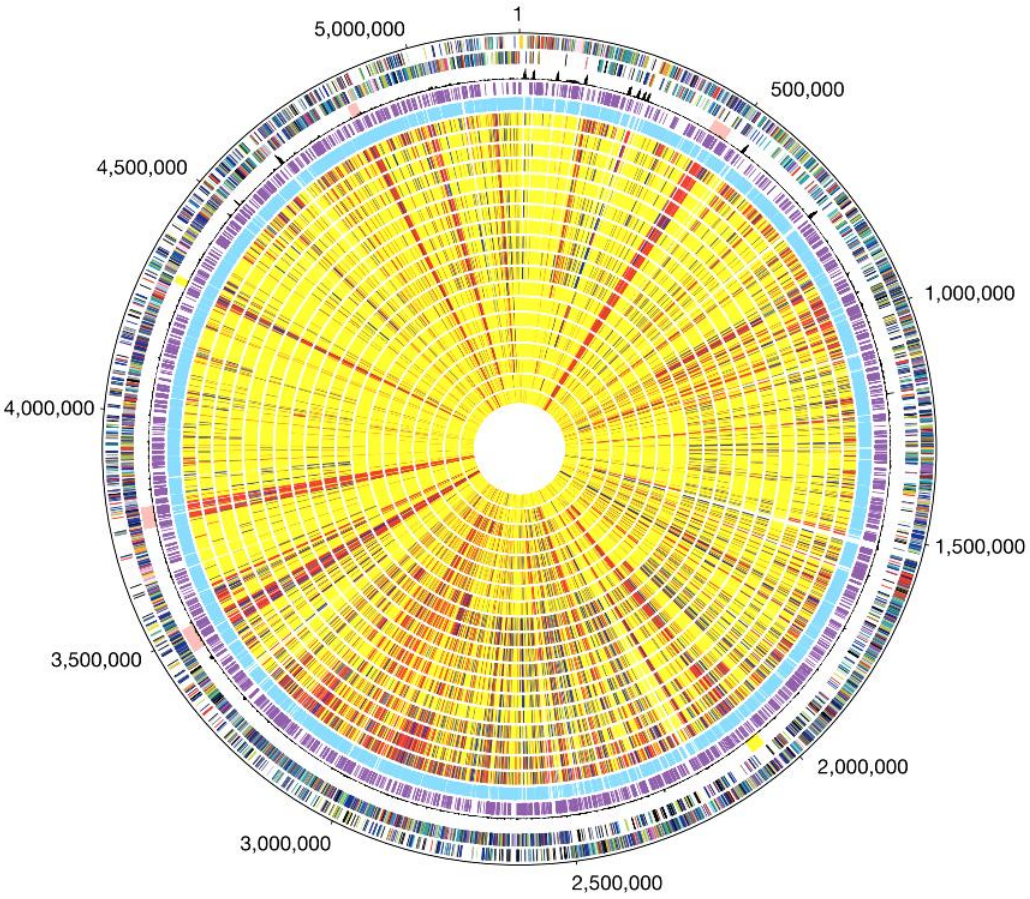

Figure 1. Genome similarity of the *B. cereus s.l.* strains in comparison to the *B. anthracis* Ames genome.

The outer circle 1 and 2, predicted coding regions on the plus strand, and minus strand, respectively; circle 3, atypical nucleotide composition curve; circle 4, genes not represented on the array; circle 5, genes present on the array. Genes were classified into: (i) genes present in the query strain (yellow), (ii) genes absent in the query strain (red), and (iii) diverged genes (blue). Missing data are in grey. The B. cereus group strains other than B. anthracis Ames *are displayed as circles 6-24 [Read et al., 2003].*

[Cohan, 2002]. Although, the development of molecular approaches in the last thirty years has made the identification of bacterial species much more accessible, the *B. cereus s.l.* taxonomy is so complex that it rises a lot of controversy. Early attempts to delineate bacterial species by DNA-DNA hybridization, in which bacterial species were defined as those isolates sharing at least 70% hybridization under standardized conditions, indicated that members of *B. cereus s.l.* should be considered as a different species [Nakamura & Jackson, 1995]. Afterwards, it became clear that DNA-DNA hybridization was not appropriate due to the level of variability over the lifetime of a species, especially in connection with *B. cereus s.l.* adaptations to various habitats [Riley & Lizotte-Waniewski, 2009]. More recently, the comparison of highly conserved sequences of the 16S rRNA gene of *B. anthracis, B. cereus* and *B. thuringiensis* showed >99% similarity, indicating that these bacilli belong to a single species [Sacchi *et al.,* 2002]. This view is supported by the fact that *B. cereus s.l.* share a large core set of conserved genes and their chromosomes display high level of synteny (Figure 1) [Read *et al.,* 2003*;* Rasko *et al.,* 2005].

Nevertheless, despite genetic similarity, the *B. cereus s.l.* members are mainly distinguished based on phenotypic features, which often arise from the presence of plasmids. However, these extra-chromosomal molecules of DNA could be naturally lost or gained in horizontal gene transfer (HGT), changing dramatically phenotype of the given isolate (Table 1) [Ceuppens *et al.,* 2013; Mock & Fouet, 2001; Hoton *et al.,* 2009]. From ecological perspective, the taxonomy of *B. cereus s.l.* is even more complicated when considering variations in their symbiotic associations and species-specific virulence [Swiecicka, 2008; Mock & Fouet, 2001; Bullied *et al.,* 2002; Swiecicka & Mahillon, 2006]. For example, cereulide, typically associated with the emetic strains of *B. cereus s.s.*, is also produced by psychrotolerant *B. weihenstephanensis* [Thorsen *et al.*, 2006]. On the other hand, *B. cereus s.s.* may cause clinical symptoms similar to those of inhalation anthrax [Hoffmaster *et al*, 2004]. Also other properties attributed to a particular species may not be intrinsically unique. For example, some *B. thuringiensis* strains have been reported as a potential source of human diarrheal infections similar to those caused by *B. cereus s.s.* [Hansen & Hendriksen, 2001], while some were able to grow at low temperature [Bartoszewicz *et al.,* 2009; Soufiane & Côtè, 2010], being a primary feature of *B. weihenstephanensis* [Lechner *et al.,* 1998].

Table 1. Diagnostic phenotypic features used for the species-level classification of *B. cereus s.l.* isolates.

Diagnostic feature	***B. cereus s.s***	***B. thuringiensis***	***B. anthracis***	***B. mycoides***	***B. pseudomycoides***	***B. weihenstephanensis***	***B. cytotoxicus***	***B. toyonensis****
Sheep blood hemolysis	+	+		+	+	+	+	+
Rhizoidal growth				+	+			
Growth at 7^{o}C		-/+		-/+		+		
Growth at 50^{o}C							+	
Plasmid-borne signatures:								
Parasporal crystal (Cry)		+						
Anthrax toxin(s)			+					
Emetic toxin	-/+					-/+		

-/+, variable; the lack of particular properties has been omitted for clarity.

**, B. toyonensis BCT-7112^{T} was primarily identified as B. cereus var toyoi, which could be distinguished from the closest relatives by the optimum growth temperature (35^{o}C), higher salinity tolerance (up to 5% NaCl), and optimal pH (6.5) [Jimenez et al., 2013].*

Due to the medical importance and economic significance, a vast number of investigations have focused on *B. cereus* and *B. anthracis* originated from clinical cases and food products, and *B. thuringiensis* as a potential bioinsecticidal [Priest *et al.,* 2004; Fagerlund *et al.,* 2007; Ehling-Schulz *et al.,* 2005; Sorokin *et al.,* 2006; Hoffmaster *et al.,* 2006; Vassileva *et al.,* 2007; Marston *et al.,* 2006; Hoffmaster *et al.,* 2008; Cardazzo *et al.,* 2008; Didelot *et al.,* 2009; Raymond *et al.,* 2010; Zahner *et al.,* 2013, Blackburn *et al.,*

2013]. Whereas, some global analysis have been concentrated on independent strains from miscellaneous geographic locations, substrate origins, and date of isolation [Guinebretiere *et al.,* 2008, Tourasse & Kolsto, 2007; Tourasse *et al.,* 2010], which makes it difficult to infer about ecological speciation of *B. cereus s.l.* General patterns emerging from these studies are the following: (i) particular species are phylogenetically intermixed, (ii) most of the clinical *B. cereus s.l.* isolates group together with *B. anthracis* strains, and (iii) other clusters contain mostly isolates from soil and dairy. In addition, Guinebretière *et al.* [2008] identified seven genetic *B. cereus s.l.* groups (I-VII) with the ecotypic structure associated with particular thermal niches. Furthermore, they showed that cytotoxic activity and toxin distributions varied according to the phylogenetic group I-VII rather than species affiliation [Guinebretiere *et al.,* 2010]. Despite these studies, still little is known about genetic structure and ecological speciation among natural populations of *B. cereus s.l.* originated from soil.

Interestingly, *B. cereus* group members are versatile producers of secondary metabolites, such as antimicrobial substances [Abriouel *et al.,* 2011], extracellular enzymes [Liang *et al.,* 2013], and fluorescent pigments [Benerjee *et al.,* 2013]. Nevertheless, these phenotypic features are very often omitted when the genetic structure of *B. cereus s.l.* is studied. From evolutionary and ecologic points of view, especially the production of melanin is highly significant for their producers as the pigment absorb a broad spectrum of electromagnetic radiation [Moeller *et al.,* 2005], protects from reactive oxygen species [Tada *et al.,* 2010], prevents from heavy metals toxicity [Garcia-Rivera & Casadevall, 2001], and from extreme temperatures [Rosas & Casadevall, 1997]. It is worth nothing, that melanin is potentially also involved in the development of symbiosis between soil bacteria and plants [Pinero *et al.,* 2007]. Yet, so far only two melanin-positive wild strains, *B. thuringiensis* subsp. *dendrolimus* L-7601 [Chen *et al.,* 2004] and *B. thuringiensis* subsp. *kurstaki* CCTCC AB90010 [Liu *et al.,* 2004], have been described. Nevertheless, several studies have managed to obtain the production of the blackish-brown pigment in *B. cereus s.l.* through chemical mutagenesis [Aghajanyan *et al.,* 2005] or genetic modification [Ruan *et al.,* 2002].

Considering the above issues, I decided to verify whether soil *B. cereus s.l.* populations have "the ecotype character". Altogether, I studied 297 *B. cereus s.l.* isolates, 24 *B. thuringiensis* from soil sampled in the Narew National Park (Appendix 1) and 273 *B. cereus/B. weihenstephanensis, B. thuringiensis*, and *B. mycoides/B. pseudomycoides* originated from soil samples from three highly geographically and ecologically diverse habitats: (i) Białowieża National Park (Białowieża NP), the last European natural forest with the primeval character and limited human activity (a World Heritage site and a biosphere reserve), (ii) Biebrza National Park (Biebrza NP), the largest Europe's marshes with limited human activity, located in the Biebrza River basin, and (iii) the agricultural soil in Jasienowka, a small village on the south of Podlasie province (Appendixes 2 and 3).

The first hypothesis is: **particular *B. cereus s.l.* genetic lineages cover isolates possessing the same specific ecological properties encoded by chromosomal genes,** such as (i) the ability to grow at low temperature (thermal ecotype) (Appendixes 1 and 2), (ii) cytotoxicity due to the presence and expression of the *cytK* gene (CytK pathotype) (Appendixes 1 and 2), and (iii) the ability to produce the melanin pigment (melanotype) (Appendix 3). The study on bacteria from environments differing significantly in human activity or type of soil, allowed me to investigate the impact of natural environment on the genetic structure of *B. cereus s.l.*, and to verify the second hypothesis, which is: ***B. cereus s.l.* revealing distinct genotypes (ecotypes) occur in particular habitats** (Appendix 2). In addition, taking into account genetic properties of *B. cereus/B. weihenstephanensis, B. thuringiensis* and *B. mycoides/B. pseudomycoides*, I was able to determine whether bacteria belonging to *B. cereus s.l.* should be classified as ecotypes within a single species or as separate species. I postulated that **environmental *B. cereus s.l.* are highly genetically related and should be classified as one species** (Hypothesis 3; Appendix 2).

Whereas the banding-based subtyping methods, such as multilocus enzyme electrophoresis (MLEE), amplified fragment length polymorphism (AFLP), or pulsed-field gel electrophoresis (PFGE), are not specific enough for studying the genetic structure of bacterial populations, multilocus sequence typing (MLST), based on a detailed analysis of the nucleotide sequence of seven housekeeping genes, has become the "gold standard" in the investigation of the population structure, evolution of bacteria, and identification of

epidemic clones [Maiden *et al.*, 1998; Priest *et al.*, 2004; Helgason *et al.*, 2004; Barker *et al.*, 2005; Hoffmaster *et al.*, 2008, Bolt *et al.*, 2010]. Most importantly, MLST allows classifying bacteria into "clonal complexes" (CCs) containing isolates that are identical at six or more loci, which correspond remarkably to ecologically distinct groups; therefore, they should effectively correspond to bacterial ecotypes [Cohan, 2000]. Taking all of these into account, I chose this approach as the primary method in my studies on the eco-genetic structure of *B. cereus s.l.* populations from Northeastern Poland.

Appendix 1

Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates

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My contribution: setup of experimental details, laboratory work (detection of δ-endotoxin and the *cytK* gene, the psychrotolerance testing of Polish isolates), data analysis, contribution in the manuscript preparation.

RESEARCH ARTICLE

Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates

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Abstract

Ecological diversification of *Bacillus thuringiensis* soil isolates was examined to determine whether bacteria adapted to grow at low temperature and/or potentially pathogenic correspond to genetically distinct lineages. Altogether, nine phylogenetic lineages were found among bacilli originating from North-Eastern Poland ($n = 24$) and Lithuania ($n = 25$) using multi-locus sequence typing. This clustering was chiefly confirmed by pulsed-field gel electrophoresis. One third of the bacilli were found to be psychrotolerant, which strongly supports the hypothesis of the existence of thermal ecotypes among *B. thuringiensis*. PCR screening was also performed to detect potential enterotoxin genes and *Bacillus anthracis* pXO1- and pXO2-like replicons. The *cytK*-positive isolates (22%) were significantly associated with two phylogenetic lineages (potential CytK pathotypes), whereas there was no correlation between phylogenetic grouping and the presence of the potential tripartite enterotoxin pathotypes (86% of strains). A statistically significant association between phylogenetic lineages and ecologic properties was found with regard to the *cry1*-positive Lithuanian isolates, while the *cry* genes in Polish isolates and the pXO1- and pXO2 replicon-like elements showed scattered distribution across phylogenetic lineages. Our results support the hypothesis that *B. thuringiensis* comprises strains belonging to different phylogenetic lineages, which exhibit specific ecological properties.

Introduction

Bacillus thuringiensis, an entomopathogen lethal to many insects due to the synthesis of δ-endotoxins, also called Cry toxins (Ben-Dov *et al.*, 1997; de Maagd *et al.*, 2001), is present in a wide range of environments and exhibits a high level of phenotypic and genotypic diversity (Gaviria Rivera & Priest, 2003; Swiecicka & Mahillon, 2005). Yet, the unique ecological role(s) played by *B. thuringiensis* in their environmental niches is not fully understood (Swiecicka, 2008). One possible way to tackle this issue is to investigate the ecological changes that distinguish the *B. thuringiensis* isolates into ecotypes, defined as groups of closely related, but ecologically different, genetic variants (Connor *et al.*, 2010). Indeed, bacterial isolates of particular ecotype occupy similar ecological niches and possess specific properties that allow them to exploit and adapt to their immediate environment (Cohan & Perry, 2007; Stefanic *et al.*, 2012). *Bacillus thuringiensis* belongs to the *Bacillus cereus sensu lato* group, which also includes *B. cereus sensu stricto* implicated in food poisoning (Granum & Lund, 1997), *Bacillus mycoides* and *Bacillus pseudomycoides*, characterized by their rhizoid growth on solid agar plates (Nakamura, 1998), *Bacillus anthracis*, the etiologic agent of anthrax (Mock & Fouet, 2001), and the psychrotolerant *Bacillus weihenstephanensis* (Lechner *et al.*, 1998). Although these bacteria are genetically closely related (Pannucci *et al.*, 2002a; Helgason *et al.*, 2004), they display distinct virulence spectra whose genetic determinants are mostly plasmid-borne. For instance, the production of entomotoxins by *B. thuringiensis*, the cereulide toxin by emetic *B. cereus* pathotypes,

and the anthrax toxins by *B. anthracis* all depend on plasmid-borne genes (Mock & Fouet, 2001; Hoton *et al.*, 2005, 2009; Reyes-Ramírez & Ibarra, 2008).

Despite its entomopathogenic properties, *B. thuringiensis* has occasionally been reported as a potential source of human infections similar to those caused by *B. cereus* (Jackson *et al.*, 1995). In fact, most *B. thuringiensis* and *B. mycoides/B. pseudomycoides* isolates harbour the genetic determinants of potential enterotoxins thought to be involved in diarrhoeal gastrointestinal infections (Hansen & Hendriksen, 2001; Swiecicka *et al.*, 2006). Whether these potential pathogenic ecotypes of *B. thuringiensis* are authentic pathotypes remains an open issue. The temperature adaptation is another *B. cereus s.l.* property of significant importance from both an environmental and a food industry point of view. Bartoszewicz *et al.* (2009) and Soufiane & Côté (2010) have pointed out that some *B. thuringiensis* were able to grow at low temperature and displayed specific motifs in their *cspA* gene (coding the major cold shock protein CspA), two features originally described as characteristic of the psychrotolerant *B. weihenstephanensis* (Francis *et al.*, 1998; Lechner *et al.*, 1998). These observations, together with an earlier report by Sorokin *et al.* (2006), indicated that psychrotolerance is a common feature among *B. cereus s.l.* isolates rather than a specific attribute of *B. weihenstephanensis*. Moreover, all the isolates demonstrating the ability to grow at low temperature can be regarded as thermal ecotypes of *B. cereus s.l.* (Guinebretière *et al.*, 2008).

It is generally accepted that closely related but ecologically distinct ecotypes may represent distinct evolutionary lineages within a particular species (Connor *et al.*, 2010). Accordingly, the primary hypothesis is that particular *B. thuringiensis* genetic lineages consist of isolates possessing the same specific ecological properties encoded by chromosomal genes, such as the ability to grow at low temperature (thermal ecotype) or the presence of the same set of enterotoxin genes (pathotypes). Conversely, the isolates displaying properties encoded by plasmid-borne genes may belong to different lineages. The multilocus sequence typing (MLST) approach extensively used in the phylogenetic typing of the *B. cereus* group members (Helgason *et al.*, 2004; Ko *et al.*, 2004; Priest *et al.*, 2004; Guinebretière *et al.*, 2008; Hu *et al.*, 2009), and pulsed-field gel electrophoresis (PFGE) applied to estimate the population structure of these bacilli (Gaviria Rivera & Priest, 2003; Swiecicka & Mahillon, 2005), were used in the present study to assess the genetic structure and phylogenetic lineages of 49 *B. thuringiensis* environmental isolates, including their relationship with the other members of the *B. cereus* complex. Furthermore, the following parameters were analysed in relation to the MLST lineages: (1) the ability to grow at low temperature, a cardinal feature of psychrotrophy as exemplified by strains of *B. weihenstephanensis*; (2) the presence of putative enterotoxin genes; and (3) the distribution of the pXO1- and pXO2-like replicons. To get a broader view of the genetic diversity and population structure, bacteria originating from two geographically distinct locations, North-Eastern Poland and Lithuania (about 400 km apart) were analysed.

Materials and methods

Bacterial strains, growth conditions, and bacterial identification

A total of 49 *B. thuringiensis* isolates from forest soil rich in organic material were tested (Fig. 1, Supporting Information Table S1). Among them, 25 strains were isolated from soil taken in the Narew National Park (North-Eastern Poland), and eight and 17 isolates were obtained from samples taken in central Lithuania (Vilnius) and North Lithuania (Pakruojis district), located 100–400 km apart (Fig. 1). Only one *B. thuringiensis* strain was analysed from each soil sample. To isolate *B. thuringiensis*, a 10% soil solution (w/v) in 0.85% NaCl was shaken for 1 h (200–250 rpm) and then preheated in a water bath

Fig. 1. Geographic map of Poland and Lithuania with the location of the sample sites in Lithuania (✦, Vilnius; isolates: DK-1.8, DK-1.18, DK-1.2, DK-1.6; DK-2.6, DK-2.9, DK-2.4, DK-3.3; ⅄, Pakruojis district; isolates: DK-3AB, DK-4AB, DK-1AC, DK-2AC, DK-4AD, DK-2AG, DK-3AG, DK-4AG, DK-C32, DK-2E, DK-8E, DK-6F, DK-11F, DK-13F, DK-15F, DK-2H, DK-3H), and in Poland (⌑, Narew National Park; all isolates).

for 5 min at 72 °C to eliminate vegetative cells and to select the spores. For each serial dilution (10^{-1} to 10^{-4}), 100 μL were plated on MYP agar (Oxoid, Basingstoke, UK), a selective medium for isolating members of the *B. cereus* group, and incubated at 30 °C for 48 h. Those bacteria forming rough and dry colonies with a violet pink background surrounded by egg yolk precipitation on the MYP agar and with parasporal crystals observed under phase-contrast microscopy, were identified as *B. thuringiensis*. The selected isolates were screened for haemolytic activity on Columbia Blood Agar (Oxoid) at 30 °C. *Bacillus thuringiensis* HD1, HD2, HD3, HD12, HD73, and HD146 strains (BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Ohio, USA) were used as references. *Bacillus weihenstephanensis* DSMZ 11821 (German Collection of Microorganisms and Cell Cultures), *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 (American Type Culture Collection) were also included as reference strains for psychrotolerant properties and the presence of the potential enterotoxin genes, respectively.

DNA extraction

Genomic DNA was extracted from overnight cultures grown in Luria–Bertani (LB) broth using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The quantity and the purity of the extracted DNA were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

MLST-based phylogenetic analysis

In total, seven housekeeping genes – *glpF* (glycerol uptake facilitator protein), *gmk* (putative guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (phosphoribosylaminoimidazolecarboxamide), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase) – were amplified with the primers described at the *B. cereus* PubMLST database (http://pubmlst.org/bcereus/info/primers.shtml) and given in Table S2. The PCR programs were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 56 °C (*gmk, pta, pur*), 57 °C (*pycA*), 58 °C (*ilvD, tpi*) or 59 °C (*glpF*) for 30 s, and 72 °C for 1 min; 72 °C for 10 min. PCRs were performed with the Veriti 96-Well thermal cycler (Applied Biosystems, Foster City, CA) in the final volume of 25 μL containing 0.65 U *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 250 ng DNA, 0.5 mM of each of the four dNTP, 2.0 mM $MgCl_2$, and 0.5 μM of each of the primers. The presence of PCR products was tested with the capillary electrophoresis QIAxcel system (Qiagen).

Amplification products were obtained from all 49 isolates and then purified using the QiaAmp PCR purification kit (Qiagen). Sequencing reactions were performed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems) and the primers described at the *B. cereus* PubMLST website. Products of cycle sequencing reactions were purified using the ExTerminator Kit (A&A Biotechnology, Gdynia, Poland) and sequenced with the ABI3130 automated sequencer (Applied Biosystems). The sequences were aligned and analysed with regard to their conservative sites and number of polymorphic nucleotide positions with the BIOEDIT SEQUENCE ALIGNMENT EDITOR version 7.0.1. program. The sequences of each of the seven housekeeping genes were assigned allele numbers based on the locus queries at http://pubmlst.org/bcereus/, and sequence types (ST) based on the combination of seven alleles. To assess the relationship of the *B. thuringiensis* isolates under this study, a phylogenetic tree was generated from their allelic profiles using the neighbour-joining (NJ) algorithm with the aid of START2 software (Jolley *et al.*, 2004). Genetic distances, based on the nucleotide polymorphism in housekeeping alleles, were calculated using the neighbour-joining method and Kimura 2-parameter mathematical model. The correctness of the results was evaluated using a 1000-step bootstrap test.

Psychrotolerance

To determine the potential 'psychrotolerance' genetic profile, all the *B. thuringiensis* isolates were screened by PCR for *cspA*, using a pair of primers cspA_f and cspA_r (Table S2), and the presence of specific ^{4}ACAGTT^{9} motif in the gene sequence, as reported by Bartoszewicz *et al.* (2009). The purified amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using the ABI3130 automated sequencer. Alignments of the sequences were performed as described above. To determine their potential psychrotrophic character, the isolates were screened for their ability to grow at 7 °C in nutrient broth with vigorous agitation for 7–10 days, as well as at 43 °C on nutrient agar plates for 48 h.

Presence of potential enterotoxin genes

All the *B. thuringiensis* isolates were tested for the presence of the *hblA* and *nheA* genes with the pairs of primers (Table S2) designed by Hansen & Hendriksen (2001), and for the *cytK* gene with the primer pair described by Swiecicka *et al.* (2006) (Table S2). PCR amplifications were carried out using the Veriti 96-Well thermal cycler for 30 reaction cycles in a final volume of 15 μL containing 0.35 U *Taq* DNA polymerase (MBI Fermentas),

150 ng DNA, 0.5 mM of each of the four dNTP, 1.5 mM $MgCl_2$, and 0.5 μM of each of the primers. The conditions for PCRs were as follows: an initial denaturation step for 3 min at 95 °C, denaturation of DNA template at 95 °C for 1 min, annealing templates and oligonucleotide primers at 50 °C (*cytK, hblA*) and 55 °C (*nheA*) for 1 min, and extension of PCR products at 72 °C for 1 min. An extra extension step was performed at 72 °C for 10 min. The PCR products were analysed in the capillary electrophoresis system QIAxcel.

Detection of δ-endotoxin genes, and pXO1- and pXO2-like replicons

To assess the potential entomopathogenic properties, the presence of *cry1*, *cry2*, *cry3*, *cry4*, *cry7*, *cry8*, and *cry9* genes was tested as described by Ben-Dov *et al.* (1997, 1999) with the pairs of primers given in Table S2. The presence of the *repX* and *repA* genes, encoding the replication proteins of the pXO1 and pXO2 plasmids, respectively, was tested by PCR as described by Hu *et al.* (2009) with the pairs of primers provided in Table S2. The PCR products were analysed in the capillary electrophoresis system QIAxcel.

PFGE of genomic DNA

Genomic DNA in LMP agarose (Sigma Chemical Co., St. Louis, MO) plugs was prepared according to Gaviria Rivera & Priest (2003). The agarose-imbedded DNA was then digested with 30 U of NotI (MBI Fermentas) and electrophoresed in the CHEF-DR II System (Bio-Rad, Hercules, CA) followed by staining DNA with ethidium bromide solution (1 μg mL^{-1}) and then analysed as described previously (Swiecicka & Mahillon, 2005). Each DNA profile was compared with the other DNA profiles using the Dice similarity coefficient. To reflect these similarities, a dendrogram was performed by UPGMA algorithm with the NTSYS version 2.02 g program (Exeter Software, Setauket, NY).

Statistical analysis

The Fisher exact test, performed with the R version 2.15.2 program, was used to investigate whether the variable MLST group is pairwise independent of each of the following variables: *hblA*, *nheA*, *cytK*, the δ-endotoxin genes, *B. anthracis* replicon-like, and psychrotrophy. The null hypothesis of the test is that the two analysed variables are independent. The significance level was set at 0.05. The MLST group that consisted of only one isolate was omitted in the analysis.

Results

Environmental *B. thuringiensis* isolates display broad diversity

The sequences of seven housekeeping genes of the 49 soil *B. thuringiensis* isolates were compared with allelic sequences available at PubMLST database (http://pubmlst.org/bcereus). Among the environmental isolates, sequence variability for each fragment ranged from 4.6% (*tpi*) to 21.4% (*ilv*) of polymorphic sites, with the number of alleles per locus varying from 9 (*gmk*) to 18 (*ilv*). With the exception of *gmk*, the number of alleles of each locus among Polish isolates was slightly higher than isolates from Lithuania (Table 1). When the nucleotide sequences of all the genes were compared with the PubMLST database, 43 STs could be distinguished, including 11 and 21 new STs among the Lithuanian and Polish isolates, respectively (Table S1). The remaining 11 known STs were shared by 17 isolates. The vast majority of the STs were present only once in the tested bacilli. Only ST305

Table 1. Genetic diversity at seven loci of the environmental *B. thuringiensis* isolates.

				Origin of *B. thuringiensis*			
		B. thuringiensis (*n* = 49)		Lithuania (*n* = 25)		Poland (*n* = 24)	
Locus	Fragment length (bp)	Polymorphic sites*	No. of alleles	Polymorphic sites*	No. of alleles	Polymorphic sites*	No. of alleles
glp	372	34 (9.1)	17	29 (7.8)	10	29 (7.8)	14
gmk	504	53 (10.5)	9	42 (8.3)	7	38 (7.5)	5
ilv	393	84 (21.4)	18	65 (16.5)	10	71 (18.1)	13
pta	414	37 (8.9)	15	28 (6.8)	8	30 (7.2)	11
pur	384	58 (15.1)	17	51 (13.3)	9	52 (13.5)	13
pyc	363	66 (18.2)	13	58 (16.0)	7	57 (15.7)	10
tpi	435	20 (4.6)	13	12 (2.8)	6	16 (3.7)	9

*No. of polymorphic sites and percentage (in parentheses) calculated as the number of polymorphic sites divided by the length of the sequenced fragment.

and ST312, consisting of two and four isolates, were represented by bacteria from both locations (Table S1).

To evaluate the genetic relationships among the environmental *B. thuringiensis*, the sequences of the seven housekeeping genes were compared using the NJ clustering method. The resulting dendrogram based on the concatenated nucleotide sequences revealed nine major groups (A–I) with the number of strains varying from one to 16 (Fig. 2). Groups B and D comprised Lithuanian isolates only, whereas Polish isolates were predominant in groups E and I. All the *B. thuringiensis* reference strains (except HD3), the *B. cereus* ATCC14579 type strain, and five Polish environmental isolates formed Cluster C. Quite interestingly, all the psychrotolerant isolates were grouped in a single cluster (F) together with the reference strain of cold-adapted *B. weihenstephanensis* DSMZ11821 (see also below).

PFGE macrorestriction profiling confirmed the *B. thuringiensis* genetic diversity

PFGE genotyping of the 48 *B. thuringiensis* (one strain could not be pulsotyped) revealed 46 distinct profiles (Fig. 3). Only two pairs of strains, IS5004 and IS5005, and DK-1.2 and DK-2.9, showed indistinguishable patterns, whereas the rest of the DNA profiles differed by at least one band. NotI cleaved the *B. thuringiensis* genome into five to eleven DNA fragments, ranging from 80 to 2540 kb. Although most strains exhibited a high number of common bands, differences were observed throughout all the DNA patterns. A numerical analysis of this diversity grouped the 46 profiles into six clusters (Fig. 3). Four clusters (I, III, IV, and VI) contained both Polish and Lithuanian bacilli, whereas groups II and V comprised exclusively Lithuanian isolates.

All the isolates pertaining to PFGE clusters I, II, and V were classified into groups F, D, and B, respectively, in the MLST analysis (Figs 2 and 3). The strains assigned to the other PFGE groups belonged to distinct MLST clusters (Fig. 3, Table S1). In most cases, though, the detailed branching (among close neighbours) observed between MLST and PFGE clusterings were congruent.

Psychrotolerant *B. thuringiensis* isolates belong to a separate phylogenetic lineage

The low temperature adaptation of the soil *B. thuringiensis* isolates was evaluated by the assessment of their growth at 7 and 43 °C. Interestingly, 15 bacteria (five Lithuanian isolates from the Northern part of Lithuania and 10 Polish isolates) grew well at the low cardinal temperature and showed no growth at 43 °C. As indicated above, these bacilli, along with the psychrotolerant *B. weihenstephanensis* DSMZ 11821 reference strain, formed a single MLST group defined as cluster F (Fig. 2). In the PFGE analysis, these isolates fell into groups I (12) and IV (3) (Fig. 3). Furthermore, it was confirmed that all these bacilli had the ^{4}ACAGTT^{9} signature in their *cspA* gene sequence. In the statistical analysis the null hypothesis was rejected due a *P*-value of < 0.001. This means that there is a statistically significant association between the psychrotrophic strains and the MLST group F.

Potential tripartite enterotoxin genes, but not *cytK*, are scattered among environmental *B. thuringiensis*

The *B. thuringiensis* isolates were screened by PCR to determine the occurrence of the *hblA*, *nheA*, and *cytK* genes, encoding the subunit A of the haemolytic enterotoxin (HBL), the subunit A of the non-haemolytic enterotoxin (NHE), and cytotoxin K (CytK), respectively. Of 49 soil bacilli, 44 (90%) showed positive results for the presence of *nheA*. Similarly, *hblA* was also found in a high proportion (86%) of the isolates. Similar results have been observed among the reference strains. In contrast, the *cytK* gene was observed in only 11 (22%) of the *B. thuringiensis* isolates. Bacilli with both enterotoxin genes, *nheA* and *hblA*, exhibited scattered distribution over all of the tree clusters (Fig. 2) and belonged to different PFGE patterns (Fig. 3). In contrast, the *cytK*-positive isolates constituted two separate clusters (A and E) in the MLST and made up part of the MLST clusters C and G (as defined in Fig. 2). The *cytK*-positive *B. thuringiensis* reference strains tested in this study pertained to the MLST cluster C (five of six). In PFGE analysis, the 11 *cytK*-positive isolates exhibited different DNA fingerprints, placed in groups III (2), IV (5), and VI (4).

All the isolates harboured at least one potential enterotoxin gene, but the numbers of those genes differed significantly among STs (Fig. 2, Table S1). The combination of both *hblA* and *nhe* was spread among (1) 22 different STs found for 28 soil isolates belonging to all phylogenetic groups except cluster E and (2) three STs defined for the reference strains HD3, HD12, and HD146 from the cluster G and C, respectively. The triad *hblA*, *nheA*, and *cytK* was only observed in 13 STs (nine among soil isolates and four references), all from clusters A and C. With the exception of ST312, strains representing the same ST type harboured the same enterotoxin genes. In fact, all four ST312 isolates possessed *hblA*, while the *nheA* gene was absent from DK-1.2. In Fisher's exact test the null hypothesis was not rejected with regard to the *hblA* and *nheA* genes, as the *P*-value was 0.066 and 1, respectively. Conversely, in the case of the *cytK* gene, the null hypothesis was rejected ($P < 0.001$). Thus, the

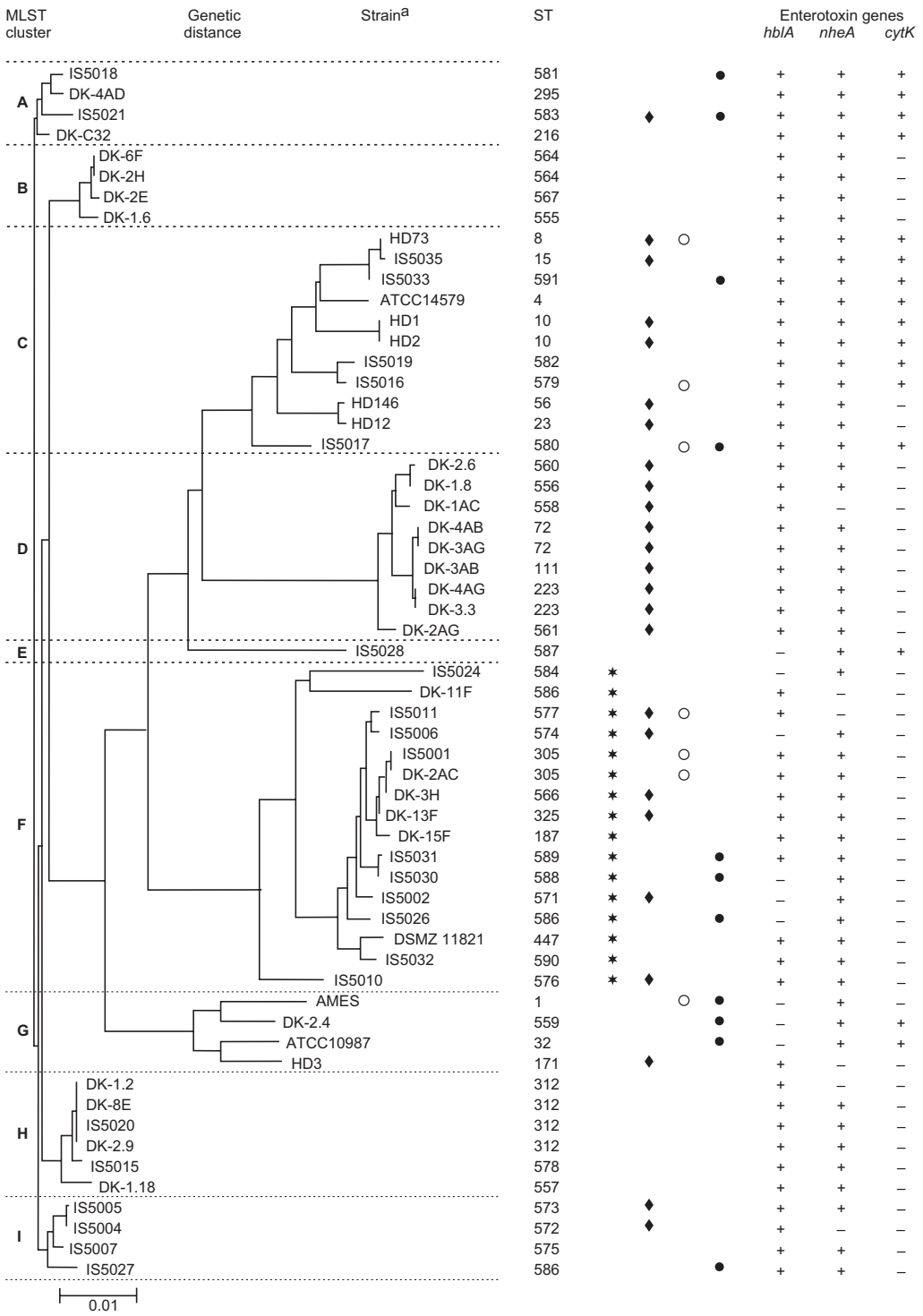

Fig. 2. NJ dendrogram of MLST analysis of Polish (IS) and Lithuanian (DK) soil *Bacillus thuringiensis* isolates and the references strains of *B. thuringiensis* HD1, *B. thuringiensis* HD2, *B. thuringiensis* HD3, *B. thuringiensis* HD12, *B. thuringiensis* HD73, and *B. thuringiensis* HD146, *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. weihenstephanensis* DSMZ 11821, and *B. anthracis* Ames, based on the DNA sequences of fragments of seven housekeeping genes (see Materials and methods). The scale bar indicates the scale of genetic distance measured as an average number of nucleotide differences per site. Symbols are as follows: stars, psychrotrophic strains; rhombus, strains containing *cry1*, *cry4* (only the DK-3H isolate) or *cry9* (only the IS3035 isolate) genes; hollow circles, bacilli containing pXO2-like *repA*; solid circles, isolates with pXO1-like *repX*.

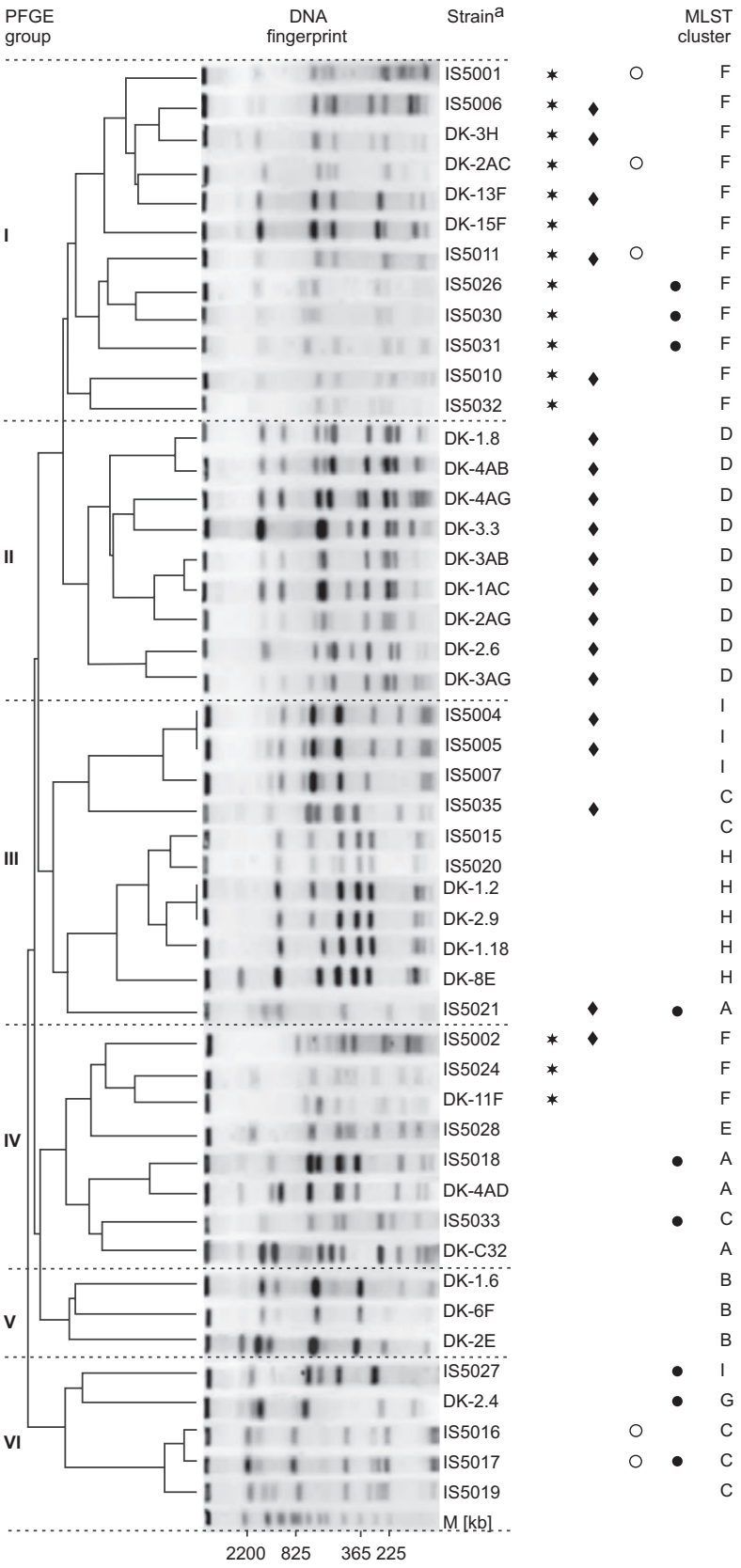

Fig. 3. PFGE patterns of Polish (IS) and Lithuanian (DK) *Bacillus thuringiensis* isolates and the dendrogram of similarity based on PFGE patterns of NotI-digested genomic DNA. M: Yeast chromosome PFGE marker (Sigma Chemical Co., St. Louis, MO). See Fig. 2 for description of symbols.

statistical results indicate (1) a lack of evidence of association between the *hblA*- and *nheA*- positive strains and the MLST groups, and (2) a strong association between the *cytK*-positive strains and the MLST groups A and C.

pXO1- and pXO2-related replicons and *cry* genes of Polish isolates are also randomly distributed

The *B. thuringiensis* isolates were also PCR-screened for the presence of the *B. anthracis* pXO1- and pXO2 replicon-like sequences, resulting in nine and five positive bacilli, respectively (Figs 2 and 3). Only one isolate IS5017 (ST580) gave a positive signal for both replicons. Interestingly, the majority of the *B. anthracis* plasmid-like replicons were observed in the Polish *B. thuringiensis* population (12 isolates), whereas among Lithuanian strains, only two bacilli displayed pXO1- (isolate DK-2.4; ST559) or pXO2-like replicons (isolate DK-2AC; ST305). *B. thuringiensis* containing pXO1-like replicon were classified into MLST groups A, C, F, G, and I, whereas isolates harbouring pXO2-like replicons were found in clusters C and F (Fig. 2). There is no statistical evidence that the strains with the *B. anthracis*-like replicons and the MLST clusters are dependent ($P = 0.462$ and 0.175 for *repA*- and *repX*-positive strains, respectively).

The highest proportion of the *cry*-positive isolates harboured *cry* genes encoding toxins against Lepidoptera. Altogether, 19 bacilli (eight Polish and 11 Lithuanian) yielded PCR products with the *cry1* primers (Fig. 2, Table S1), and one strain from Poland, IS5035, possessed the *cry9* gene. IS5035 was classified into ST15, the MLST type displayed by *B. thuringiensis* serovar *aizawai*. Only one bacterium, DK-3H (ST566; cluster F), was found to be positive for the *cry4* gene, encoding toxin potentially active against Diptera. Interestingly, nine Lithuanian bacilli, all positive for the *cry1* gene, formed a single cluster D in the MLST study and the PFGE group II. In Fisher's exact test the null hypothesis, that the *cry1*-positive isolates and MLST clusters are independent, was rejected ($P < 0.0001$). All the isolates turned out to be negative for the genes encoding δ-endotoxins against Coleoptera (Cry3, Cry7, and Cry8).

Discussion

In this study, ecological diversification among *B. thuringiensis* soil isolates was examined to determine whether the bacteria adapted to grow at low temperature and/or potentially pathogenic correspond to genetically distinct lineages. Based on the MLST scheme, nine lineages (clusters) were found among 49 *B. thuringiensis* isolates originating from North-Eastern Poland ($n = 24$) and Lithuania ($n = 25$). The genetic diversity of the isolates that pertained to particular MLST lineages was high (Fig. 2, Table 1). The *B. thuringiensis* polymorphism was also confirmed by PFGE, in which only two pairs of strains were found to be apparently clonal (Fig. 3). A high degree of genetic diversity among *B. thuringiensis* soil isolates can be explained by the existence of ecologically distinct ecotypes (Cohan, 2002), which recently have been considered the fundamental unit of bacterial diversity (Cohan & Perry, 2007; Koeppel *et al.*, 2008). The presence of specific ecotypes in *Bacillus subtilis-Bacillus licheniformis* from Death Valley National Park (Connor *et al.*, 2010), in cyanobacteria from Mushroom Spring, Yellowstone National Park (Ward *et al.*, 2006), or in *B. subtilis* from the sandy bank of the Sava River in Slovenia (Stefanic *et al.*, 2012), confirms the importance of ecotypes in the assessment of bacterial ecology.

Temperature is one of the most important factors which bacteria have to respond to in their environment. Guinebretière *et al.* (2008) have found temperature diversity in *B. cereus s.l.* and have proposed an 'ecotypic' structure of populations in the group. The existence of psychrotolerance variants among *B. thuringiensis* has been demonstrated by Bartoszewicz *et al.* (2009) and Soufiane & Côté (2010). In the present study, one-third of *B. thuringiensis* soil isolates were found to be psychrotolerant. Quite remarkably, all these bacilli formed the separate MLST cluster F, which also included the reference strain of *B. weihenstephanensis* (Fig. 2). The genetic similarity of these bacteria was confirmed by PFGE (Fig. 3). These observations strongly support the hypothesis of the existence of a thermal ecotype among *B. thuringiensis* soil isolates. Furthermore, the present data provide additional evidence to that of earlier observations (Bartoszewicz *et al.*, 2009; Soufiane & Côté, 2010) that the ability to grow at low temperature, first described for *B. weihenstephanensis* (Lechner *et al.*, 1998), is also shared by other species of the *B. cereus* group. Contrary to the results reported by Guinebretière *et al.* (2008), where the psychrotolerance *B. cereus s.l.* strains were classified into two phylogenetic groups, *B. thuringiensis* isolates growing at low temperature were assigned, in the present work, to a single phylogenetic lineage. In the work of Guinebretière *et al.* this discrepancy may be due to the incorporation of strains from a wider range of sources (natural environment, such as plants, water, soil, air, dairy products and other foods), whereas the bacteria tested in our study originated from soil only. Similar to our results, Sorokin *et al.* (2006) classified the psychrotolerant *B. thuringiensis* and *B. cereus* soil strains into one MLST phylogenetic cluster. Nevertheless, the existence of a higher number of psychrotolerant ecotypes among the *B. cereus* group originating from different ecological niches cannot be excluded.

Bacillus thuringiensis isolates displayed a high diversity with respect to potential toxicity (Hansen & Hendriksen, 2001; Swiecicka *et al.*, 2006; Oh *et al.*, 2012). To assess the existence of potential pathotypes among the *B. thuringiensis* soil isolates and their association with particular lineages, the presence of potential enterotoxin genes was tested by PCR, notwithstanding the fact that the mere presence of a gene is not necessary correlated with the actual enterotoxicity of the host strain. Contrary to the *hblA* and *nheA* genetic determinants, which were found in more than 86% of the strains, *cytK*-positive reactions were only observed in *c.* 22% of the isolates (MLST cluster A and within cluster B; Fig. 2). Due to the fact that the significant association of the *cytK*- positive strains with the lineages A and B was statistically confirmed, the isolates pertaining to these groups can be regarded as potential *cytK* pathotypes, although they also bear the *hblA* and *nheA* genes. Conversely, the potential pathotypes of the tripartite enterotoxins (HBL and NHE) are not associated with any particular phylogenetic lineage. Interestingly, whereas the *nheA* and *hblA* genes were found in proportions similar to what was previously reported for *B. thuringiensis* isolates recovered from the intestines of small free-living mammals from North-Eastern Poland (Swiecicka *et al.*, 2006), the percentage of the *cytK*-positive soil isolates was significantly lower than those reported in previous experiments (e.g. Gaviria Rivera *et al.*, 2000; Hansen & Hendriksen, 2001), which indicates specific properties of *B. thuringiensis* isolates under study.

Although δ-endotoxin crystals were observed under phase contrast microscope in all the isolates, only 19/49 (39%) gave positive PCR reactions to the *cry* genes (*cry1-4* and *cry7-9*). The bacilli which did not react with the primers used in this study could carry a potential pool for new *cry* genes not yet described or may harbour other δ-endotoxin genes not tested in our study. It is worth emphasizing that in previous investigations, the *cry* genes were found in similar proportions in populations of *B. thuringiensis* isolated from small mammals (Swiecicka & Mahillon, 2005). Interestingly, whereas the majority of Lithuanian strains with *cry1* created one separated cluster D and the PFGE group II, Polish *cry*-positives belonged to different clusters (Figs 2 and 3). The strong association of the Lithuanian *cry1*-positive isolates with the MLST cluster D may suggest that those bacilli are specific pathotypes against Lepidopteran insects. However, this assumption needs to be further characterized by bioassays. Moreover, only five Polish isolates were placed in the same cluster as *B. thuringiensis* HD1 (ST10), HD2 (ST10), HD12 (ST23), HD73 (ST8), and HD146 (ST56) and the *B. cereus* type strain ATCC 14579 (ST4). Similarly, Priest *et al.* (2004) classified these reference strains into one clade.

In the *B. cereus* group, plasmids are very common and individual strains differed from each other in plasmid number, size, and profile (Hoton *et al.*, 2005; Reyes-Ramírez & Ibarra, 2008). Although the *B. cereus s.l.* phylogeny is based on chromosomal markers, the main features of *B. thuringiensis* and *B. anthracis* are plasmid-borne. Thus, *B. cereus s.l.* plasmids are essential not only for defining the phenotypic traits associated with pathogenesis but also for enabling them to occupy different environmental niches. In this regard, special concern is given to the anthrax toxin genes of *B. anthracis*. Our study indicated that the genetic elements related to the *B. anthracis* pXO1- and pXO2-like replicons are present in distinct MLST clusters and PFGE groups. Similarly, Pannucci *et al.* (2002a,b) did not found a correlation between the presence of *B. anthracis*-like extrachromosomal elements and genomic relatedness of the *B. cereus* group members.

It is worth noting that, contrary to previous observations on the presence of clonal *B. thuringiensis* in the alimentary track of free-living animals from North-Eastern Poland (Swiecicka & Mahillon, 2005), there is no indication of clonal groups among soil isolates obtained from the same area (Figs 2 and 3). It is therefore plausible that among *B. thuringiensis* are lineages or ecotypes adapted to specific animals. Moreover, we cannot exclude the presence of highly diverse *B. thuringiensis* ecotypes in soil and specific ecotypes in the animal hosts, as similar relationships have been found for its relative *B. anthracis* (Harrell *et al.*, 1995; Mock & Fouet, 2001) and virulent *B. cereus* (Helgason *et al.*, 2000). To further address this issue, it will be necessary to analyse the genetic structure of *B. thuringiensis* from soil and animals obtained at the same time and from the same area. Soils are extremely heterogeneous matrices that vary even in a very small scale with regard to the size of particles, amount of organic matter, pH, nutrient concentration, type of vegetation, and root mass (McArthur, 2006). These factors interact with each other, as well as with temperature, and may have an impact on soil bacteria adaptation and evolution. The *B. thuringiensis* isolates under study were obtained from soil rich in organic material, a condition that may support or even stimulate horizontal gene transfers (Bizzarri & Bishop, 2007; Hu *et al.*, 2009).

To sum up, the present study showed a high degree of genetic polymorphism among soil *B. thuringiensis* strains, and provided new insights into the population structure. Our results strongly support the hypothesis that *B. thuringiensis* comprises strains belonging to different phylogenetic lineages and exhibiting specific ecological properties.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characterisation of the soil *B. thuringiensis* isolates from Northeaster Poland and central Lithuania.
Table S2. Primers used in this study.

Table S1. Characterisation of the soil *B. thuringiensis* isolates from Northeaster Poland and central Lithuania.

				Genes presence						Psychrotolerance		
				Putative enterotoxins			δ-endo-toxins[c]	*B. anthracis* plasmid-like replicons			Growth at	
Strain[a]	ST[b]	MLST group	PFGE group	*hblA*	*nheA*	*cytK*		*repA*	*repX*	*cspA*[d]	7°C	43°C
IS5021	583[N]	A	III	+	+	+	+	-	+	M	-	+
IS5018	581[N]	A	IV	+	+	+	-	-	+	M	-	+
DK-4AD	295	A	IV	+	+	+	-	-	-	M	-	+
DK-C32	216	A	IV	+	+	+	-	-	-	M	-	+
DK-2H	564[N]	B	N.D.	+	+	-	-	-	-	M	-	+
DK-6F	564[N]	B	V	+	+	-	-	-	-	M	-	+
DK-2E	567[N]	B	V	+	+	-	-	-	-	M	-	+

DK-1.6	555[N]	B	V	+	+	-	-	-	-	M	-	+
IS5033	591[N]	C	IV	+	+	+	-	-	+	M	-	+
IS5035	15	C	III	+	+	+	+[9]	-	-	M	-	+
IS5016	579[N]	C	VI	+	+	+	-	+	-	M	-	+
IS5017	580[N]	C	VI	+	+	+	-	+	+	M	-	+
IS5019	582[N]	C	VI	+	+	+	-	-	-	M	-	+
DK-2.6	560[N]	D	II	+	+	-	+	-	-	M	-	+
DK-1.8	556[N]	D	II	+	+	-	+	-	-	M	-	+
DK-1AC	558[N]	D	II	+	-	-	+	-	-	M	-	+
DK-4AB	72	D	II	+	+	-	+	-	-	M	-	+
DK-3AG	72	D	II	+	+	-	+	-	-	M	-	+
DK-3AB	111	D	II	+	+	-	+	-	-	M	-	+
DK-4AG	223	D	II	+	+	-	+	-	-	M	-	+
DK-3.3	223	D	II	+	+	-	+	-	-	M	-	+
DK-2AG	561[N]	D	II	+	+	-	+	-	-	M	-	+
IS5028	587[N]	E	IV	-	+	+	-	-	-	M	-	+
IS5024	584[N]	F	IV	-	+	-	-	-	-	P	+	-
DK-11F	568[N]	F	IV	+	-	-	-	-	-	P	+	-

IS5002	571[N]	F	IV	-	+	-	+	-	-	P	+	-
IS5011	577[N]	F	I	+	-	-	+	+	-	P	+	-
IS5006	574[N]	F	I	-	+	-	+	-	-	P	+	-
IS5001	305	F	I	+	+	-	-	+	-	P	+	-
DK-2AC	305	F	I	+	+	-	-	+	-	P	+	-
DK-3H	566[N]	F	I	+	+	-	+[4]	-	-	P	+	-
DK-13F	325	F	I	+	+	-	+	-	-	P	+	-
DK-15F	187	F	I	+	+	-	-	-	-	P	+	-
IS5031	589[N]	F	I	+	+	-	-	-	+	P	+	-
IS5030	588[N]	F	I	-	+	-	-	-	+	P	+	-
IS5026	585[N]	F	I	-	+	-	-	-	+	P	+	-
IS5032	590[N]	F	I	+	+	-	-	-	-	P	+	-
IS5010	576[N]	F	I	+	+	-	+	-	-	P	+	-
DK-2.4	559[N]	G	V	-	+	+	-	-	+	M	-	+
DK-1.2	312	H	III	+	-	-	-	-	-	M	-	+
DK-8E	312	H	III	+	+	-	-	-	-	M	-	+
IS5020	312	H	III	+	+	-	-	-	-	M	-	+
DK-2.9	312	H	III	+	+	-	-	-	-	M	-	+

IS5015	578[N]	H	III	+	+	-	-	-	-	M	-	+
DK-1.18	557[N]	H	III	+	+	-	-	-	-	M	-	+
IS5004	572[N]	I	III	+	-	-	+	-	-	M	-	+
IS5005	573[N]	I	III	+	+	-	+	-	-	M	-	+
IS5007	575[N]	I	III	+	+	-	-	-	-	M	-	+
IS5027	586[N]	I	VI	+	+	-	-	-	+	M	-	+
DSMZ 11821	115	F	N.D.	+	+	-	-	-	-	P	+	-
HD1	10	C	N.D.	+	+	+	+	-	-	M	-	+
HD2	10	C	N.D.	+	+	+	+	-	-	M	-	+
HD12	23	C	N.D.	+	+	-	+	-	-	M	-	+
HD73	8	C	N.D.	+	+	+	+	+	-	M	-	+
HD146	56	C	N.D.	+	+	-	+	-	-	M	-	+
HD3	171	G	N.D.	+	-	-	+	-	-	M	-	+
ATCC 10987	32	G	N.D.	-	+	+	-	-	+	M	-	+
ATCC 14579	4	C	N.D.	+	+	+	-	-	-	M	-	+
Ames	1	G	N.D.	-	+	-	-	+	+	M	N.D.	N.D.

[a] IS, *B. thuringiensis* isolates from Poland (the Narew National Park); DK, *B. thuringiensis* isolates from Lithuania (Vilnius district: DK-1.8, DK-1.18, DK-1.2, DK-1.6; DK-2.6, DK-2.9, DK-2.4, DK-3.3; Pakruojis district: DK-3AB, DK-4AB, DK-1AC, DK-2AC, DK-4AD, DK-2AG, DK-

3AG, DK-4AG, DK-C32, DK-2E, DK-8E, DK-6F, DK-11F, DK-13F, DK-15F, DK-2H, DK-3H; DSMZ 11821, *B. weihenstephanensis* type strain; HD1, *B. thuringiensis* HD1; HD2, *B. thuringiensis* HD2; HD3, *B. thuringiensis* HD3; HD12, *B. thuringiensis* HD12; HD73, *B. thuringiensis* HD73; HD146, *B. thuringiensis* HD146; ATCC 10987, *B. cereus* ATCC 10987; ATCC 14579, *B. cereus* ATCC 14579; Ames, *B. anthracis* Ames.

[b] New STs are indicated with a N index.

[c] +, *cry1*; $+^{4}$, *cry*^{4}; $+^{9}$, *cry9*;

[d] M, mesotrophic isolates with the signature ^{4}GCAGTA^{9} in the *cspA* gene; P, psychrotrophic isolates with the signature ^{4}ACAGTT^{9} in the *cspA* gene.

Table S2. Primers used in this study.

Target gene	Primer name	Nucleotide (5'-3')	PCR product size (bp)	Reference
Primers for MLST analysis				
glpF	glpF_F	5'-GCGTTTGTGCTGGTGTAAGT-3'	548	www.pubmlst.org/bcereus/info/primers.shtml
	glpF_R	5'-CTGCAATCGGAAGGAAGAAG-3'		
gmk	gmk_F	5'-ATTTAAGTGAGGAAGGGTAGG-3'	599	www.pubmlst.org/bcereus/info/primers.shtml
	gmk_R	5'-GCAATGTTCACCAACCACAA-3'		
ilvD	ilvD_F	5'-CGGGGCAAACATTAAGAGAA-3'	553	www.pubmlst.org/bcereus/info/primers.shtml
	ilvD_R	5'-GGTTCTGGTCGTTTCCATTC-3'		
	ilvD4_F	5'-GCAGAGATTAAAGATAAGGA-3'	568	www.pubmlst.org/bcereus/info/primers.shtml
	ilvD2_R	5'-GTT ACC ATT TGT GCA TAA CGC-3'		
pta	pta_F	5'-GCAGAGCGTTTAGCAAAAGAA-3'	575	www.pubmlst.org/bcereus/info/primers.shtml
	pta_R	5'-TGCAATGCGAGTTGCTTCTA-3'		
pur	pur_F	5'-CTGCTGCGAAAAATCACAAA-3'	534	www.pubmlst.org/bcereus/info/primers.shtml
	pur_R	5'-CTCACGATTCGCTGCAATAA-5'		

pycA	pycA_F	5’-GCGTTAGGTGGAAACGAAAG-3’	549	www.pubmlst.org/bcereus/info/primers.shtml
	pycA_R	5’-CGCGTCCAAGTTTATGGAAT-3’		
tpi	tpi_F	5’-GCCCAGTAGCACTTAGCGAC-3’	557	www.pubmlst.org/bcereus/info/primers.shtml
	tpi_R	5’-CCGAAACCGTCAAGAATGAT-3’		
Primers for *cspA* signature				
cspA	cspA_f	5’-TGCTGAAAACGAACGAGAAA-3’	332	Bartoszewicz *et al.*, 2009
	cspA_r	5’-TTATAGTTTGATTACGTTTTA-3’		
Primers for enterotoxin genes				
hblA	HBLA1	5’-GTGCAGATGTTGATGCCGAT	301	Hansen and Hendriksen, 2001
	HBLA2	5’-ATGCCACTGCGTGGACATAT-3’		
nheA	nheA 344 S	5’-TACGCTAAGGAGGGGCA-3’	478	Hansen and Hendriksen, 2001
	nheA 843 A	5’-GTTTTTATTGCTTCATCGGCT-3’		
cytK	CytKF	5’-GATAATATGACAATGTCTTTAAA-3’	617	Swiecicka *et al*., 2006
	CytKR	5’-GGAGAGAAACCGCTATTTGT-3’		
Primers for δ-endotoxin genes				
cry1	Un1(d)	5’-CATGATTCATGCGGCAGATAAAC-3’	277	Ben-Dov *et al.*, 1997
	Un1(r)	5’-TTGTGACACTTCTGCTTCCCATT-3’		
cry2	Un2(d)	5’-GTTATTCTTAATGCAGATGAATGGG-3’	701	Ben-Dov *et al.*, 1997
	Un2(r)	5’-CGGATAAAATAATCTGGGAAATAGT-3’		

cry3	Un3(d)	5’-CGTTATCGCAGAGAGATGACATTAAC-3’	589	Ben-Dov *et al.*, 1997
	Un3(r)	5’-CATCTGTTGTTTCTGGAGGCAAT-3’		
cry4	Un4(d)	5’-GCATATGATGTAGCGAAACAAGCC-3’	439	Ben-Dov *et al.*, 1997
	Un4(r)	5’-GCGTGACATACCCATTTCCAGGTCC-3’		
cry7,8	Un7,8(d)	5’-AAGCAGTGAATGCCTTGTTTAC-3’	420	Ben-Dov *et al.*, 1997
	Un7,8(r)	5’-CTTCTAAACCTTGACTACTT-3’		
cry9	Un9(d)	5’-CGGTGTTACTATTAGCGAGGGCGG-3’	354	Ben-Dov *et al.*, 1999
	Un9(r)	5’-GTTTGAGCCGCTTCACAGCAATCC-3’		
Primers for pXO1 and pXO2 replicon-like sequences				
repX	repX_F1	5’- CCATATCGTGCGATTCTTG-3’	1,163	Hu *et al.*, 2009
	repX_R2	5’- GATAATTTCTTCTTTGYTGGTTGTGA-3’		
repX	repX_F1	5’-CCATATCGTGCGATTCTTG-3’	583	Hu *et al.*, 2009
	repX_R1	5’-GAGCAAATTCACTCGCATCA-3’		
repA	repA_F1	5’- TAAATCTAAAAAYTCRAAAGCTG-3’	1,183	Hu *et al.*, 2009
	repA _R 2	5’- GTGTAAAGTATAGCACAGGT-3’		
repA	repA_F2	5’- GGACARGCTTCTCACTGGAC-3’	386	Hu *et al.*, 2009
	repA_R3	5’- GGCATTCTGAAGAAVCCAAA-3’		

W=A or T; Y=C or T; R=A or G; V=A or G or C; H=A or C or T; D=A or G or T; S=G or C; K=G or C; N=A or G or C or T; B=G or C or T.

Appendix 2

Eco-genetic structure of *Bacillus cereus sensu lato* populations from different environments in Northeastern Poland

Drewnowska JM, Swiecicka I (2013) Eco-genetic structure of Bacillus cereus sensu lato populations from different environments in northeastern Poland. PLoS ONE 8(12), e80175.

My contribution: setup of experimental details, laboratory work (isolation and identification of *B. cereus s.l.* isolates, psychrotolerance and termotolerance testing, DNA extraction, detection of the δ-endotoxins genes and *cytK* gene, real-time PCR of the *cytK* gene, multi-locus sequence typing analysis), data analysis including bioinformatics (ClonalFrame v1.2, PHYLOViZ v1.0, DnaSP v5, START v2, R v2.15.2, MEGA4), contribution in the manuscript preparation.

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Eco-Genetic Structure of *Bacillus cereus sensu lato* Populations from Different Environments in Northeastern Poland

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Abstract

The *Bacillus cereus* group, which includes entomopathogens and etiologic agents of foodborne illness or anthrax, persists in various environments. The basis of their ecological diversification remains largely undescribed. Here we present the genetic structure and phylogeny of 273 soil *B. cereus s.l.* isolates from diverse habitats in northeastern Poland, with samplings acquired from the last European natural forest (Białowieża National Park), the largest marshes in Europe (Biebrza National Park), and a farm. In multi-locus sequence typing (MLST), despite negative selection in seven housekeeping loci, the isolates exhibited high genetic diversity (325 alleles), mostly resulting from mutation events, and represented 148 sequencing types (131 STs new and 17 STs already described) grouped into 19 complexes corresponding with bacterial clones, and 80 singletons. Phylogenetic analyses showed that 74% of the isolates clustered with *B. cereus s.l.* environmental references (clade III), while only 11 and 15%, respectively, grouped with isolates of clinical origin (clade I), and *B. cereus* ATCC 14579 and reference *B. thuringiensis* (clade II). Predominantly within clade III, we found lineages adapted to low temperature (thermal ecotypes), while putative toxigenic isolates (*cytK*-positive) were scattered in all clades of the marsh and farm samplings. The occurrence of 92% of STs in bacilli originating from one habitat, and the description of new STs for 78% of the isolates, strongly indicate the existence of specific genotypes within the natural *B. cereus s.l.* populations. In contrast to the human-associated *B. cereus s.l.* that exhibit a significant level of similarity, the environmental isolates appear more complex. Thus we propose dividing *B. cereus s.l.* into two groups, the first including environmental isolates, and the second covering those that are of clinical relevance.

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Introduction

Bacillus cereus sensu lato (*s.l.*) are Gram-positive endospore-forming bacilli that persist in different ecological environments [1,2]. This group of bacteria includes various strains of *Bacillus thuringiensis*, an enthomopathogen successfully exploited in biocontrol worldwide [3], *Bacillus cereus sensu stricto (B. cereus s.s.)* which is associated with foodborne illness [4], and *Bacillus anthracis*, the etiologic agent of anthrax [5]. *Bacillus cereus s.l.* also include psychrotolerant *Bacillus weihenstephanensis* [6], thermotolerant *Bacillus cytotoxicus* [7], as well as *Bacillus mycoides* and *Bacillus pseudomycoides* that form characteristic rhizoidal colonies on solid media [8]. The species-specific properties of these bacteria are generally based on plasmid-borne signatures [5], entomopathogenicity [9], and the ability to synthesize cereulide [10]. In contrast to extra-chromosomal profiles, the chromosomes of these bacilli exhibit a high level of synteny [11], thus giving rise to controversy regarding their taxonomy. Based on genetic evidence, *B. thuringiensis*, *B. cereus s.s.* and *B. anthracis* have been designated as one species [12] or are contained in one genetic cluster [13], although others have reported that at least *B. anthracis* represents an independent taxon [14]. From an ecologic perspective, the taxonomy of *B. cereus s.l.* is even more complicated when variations in their symbiotic associations, including species-specific virulence, are considered [1,5,15,16]. For example, cereulide, typically associated with the emetic strains of *B. cereus s.s.*, is also produced by *B. weihenstephanensis* [17], while *B. cereus s.s.* may cause clinical symptoms similar to those of inhalation anthrax [18]. Other properties attributed to a particular species may not be intrinsically unique, for example, psychrotolerance, a primary feature of *B. weihenstephanensis* [6], has recently been reported among *B. thuringiensis* isolates [19,20].

Data reported over the last decade have revealed the importance of the environment to bacterial adaptation, diversification, and evolution [21,22,23,24,25]. Soil, which is the primary niche for *B. cereus s.l.* [2,26], creates favorable conditions for these processes to occur due to its heterogeneity in nutrients, particle size, pH, humidity and microbiota [22,27,28]. Thus, so called ecotypes, defined as a cohesive group of bacteria that are ecologically similar to one another and are closely related at the molecular level, evolve and proliferate as a result of acquired genetic determinants or favorable mutations that confer selective advantages conducive to a particular niche or ecosystem [29]. From this point of view, ecotypes represent distinct evolutionary lineages within a particular species [21].

The mechanisms involved in ecological diversification of *B. cereus s.l.* are poorly understood. Considering the group's biological and ecologic properties we postulate that (1) in particular habitats

distinct genotypes (ecotypes) of *B. cereus s.l.* occur, and (2) environmental *B. cereus s.l.* are highly related genetically and should be classified as one species. To test these hypotheses, we assessed the genetic structure and phylogenetic relationship of soil isolates of *B. cereus s.l.* using the multi-locus sequence typing (MLST) approach based on seven housekeeping genes. These isolates were collected from geographically and ecologically distinct locations in northeastern Poland, namely, (i) Białowieża National Park (Białowieża NP), the last European natural forest with a primeval character and limited human activity (a World Heritage site and a biosphere reserve), (ii) Biebrza National Park (Biebrza NP), the largest of Europe's marshes and also with limited human activity, located in the Biebrza River basin, and (iii) the agricultural soil in Jasienowka, a small village south of Podlasie province. The results of our study provide new insights into population structure of environmental *B. cereus s.l.* and address key issues regarding their ecology and phylogeny.

Materials and Methods

Soil sampling, and chemical and physical analysis of soil

Soil samples were obtained from Białowieża NP (forest bacilli), Biebrza NP (marsh bacilli), and agricultural land in Jasienowka (farm bacilli), northeastern Poland. The marsh collections consisted of soil samples taken adjacent to water. All samples from the parks were collected with consent according to the Nature Conservation Act adopted on 16 April, 2004 by Polish Parliament (Parliament Diary 2004, No. 92: 880). The owner of the farm also permitted collection of soil samples for our study. The locations of the sampling areas are ~60–200 km apart (Figure 1). At each location 60 to 90 soil samples were collected from sites distributed over an area of about 50,000 m^2. Samples were analyzed by the Institute of Soil Science and Plant Cultivation in Puławy, Poland, for pH, organic content, humic substances, carbon, nitrogen, phosphorus, calcium, and sulfur.

Isolation and identification of *B. cereus sensu lato*

B. cereus s.l. were isolated as described previously [30]. Bacilli showing rhizoidal growth on the MYP agar (Oxoid, Basingstoke, UK), a selective medium for the *B. cereus* group, were classified as *B. mycoides/B. pseudomycoides* (referred hereafter as *B. mycoides*). Bacteria forming rough and dry colonies with a violet-pink background surrounded by egg yolk precipitation, were designated as *B. cereus* or *B. thuringiensis* if parasporal crystals were observed under phase-contrast microscopy (Olympus BX61). From each sample, only one isolate of *B. mycoides* and *B. cereus s.s.* or *B. thuringiensis* were selected for further study. Occasionally, two isolates of the same species or those classified as *B. cereus s.s.* or *B. thuringiensis*, but showing visible differences in colony appearance on MYP, were selected from one sample for further analysis. Each isolate was subsequently screened for hemolytic activity on Columbia Blood Agar (Oxoid) at 30°C and for psychrotolerance and thermotolerance by growth in Luria-Bertani (LB) broth at 7°C and 50°C, respectively. The diagnostic features used for species-level classification of the *B. cereus s.l.* isolates are provided in Table S1. Based on colony morphology and microscopic observation, ~30 isolates of *B. cereus s.s.*, *B. thuringiensis*, and *B. mycoides* from each location were selected randomly for further analysis.

DNA extraction

Genomic DNA was extracted from overnight LB broth culture using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) and the QIAcube automat (Qiagen). The quantity and purity of DNA were determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

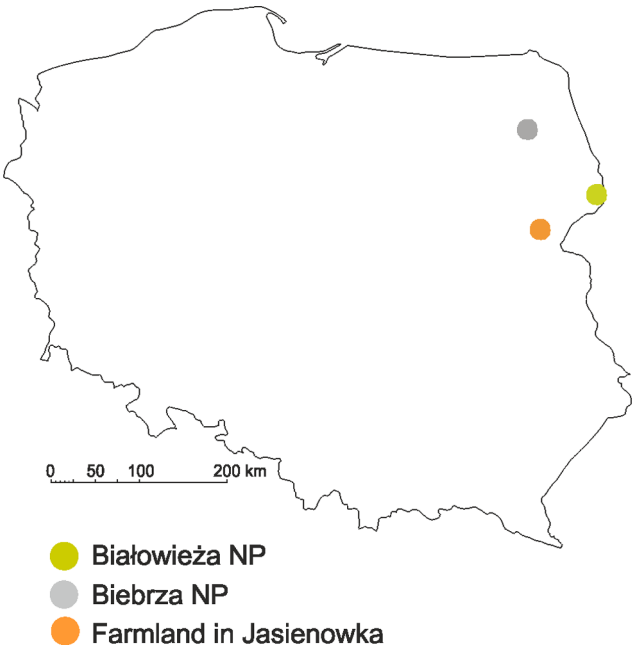

Figure 1. Geographic map of Poland with locations of soil sampling. Soil samples were collected in Białowieża National Park (Białowieża NP; N 52°42′, E 23°54′), the natural forest with no human activity, in marshes of Biebrza National Park (Biebrza NP; N 53°36′, E 22°56′) with limited human activity, and a farmland in Jasienowka (N 52°30′, E 22°58′) in northeastern Poland.
doi:10.1371/journal.pone.0080175.g001

Detection of the δ-endotoxins genes and the *cytK* gene

All isolates were screened for the presence of *cytK* encoding cytotoxin K in *B. cereus s.l.* [16] and the most frequently occurring genes encoding δ-endotoxins in *B. thuringiensis*: *cry1*, *cry2*, *cry4*, and *cry9* [31], as described by Ben-Dov et al. [32,33] (PCR primer sequences are provided in the Table S2). PCR (15 µl reactions containing 0.35 U *Taq* DNA polymerase (MBI Fermentas), 150 ng of DNA, 0.5 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 µM of each of the primers] was performed using the Veriti 96-Well thermal cycler (Applied Biosystems, Foster City, USA), and amplicons were analyzed in the capillary electrophoresis system QIAxcel (Qiagen). *B. thuringiensis* subsp. *kurstaki* HD1, *B. thuringiensis* subsp. *aizawai* HD133, and *B. thuringiensis* subsp. *israelensis* HD567 (BGSC, *Bacillus* Genetic Stock Centre, Ohio State University, Ohio, USA) were used as reference strains for the *cry* genes. *B. cereus* ATCC 14579 (ATCC, American Type Culture Collection) was used as the reference strain for *cytK*.

Real-Time PCR of *cytK*

RNA was isolated using the Total RNA Mini Plus Kit (A&A Biotechnology, Gdynia, Poland), and cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Reactions were performed in the Veriti 96 Well thermal cycler (Applied Biosystems) in a final volume of 20 µl, as follows: 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and 5 min at 4°C. The cDNA was analyzed using the Step One Plus Real-Time PCR System (Applied Biosystems). The reactions were performed using the Real-Time 2×PCR Master Mix SYBR A Kit (A&A Biotechnology) in 20 µl of the reaction mixture, containing 10 µl of RT

2×PCR Master Mix, 0.1–1 µM of each forward and reverse *cytK* primers (Table S2), 10 pg – 1 µg template cDNA and water added to a final volume of 20 µl. The thermal protocols for *cytK* and *udp* (uridine phosphorylase), genes used as a reference [34], were as follows: 95°C for 3 min (*cytK*) and 2 min (*udp*); 40 cycles of 95°C for 15 sec (*cytK*) and 30 sec (*udp*), 60°C (*cytK*) and 57°C (*udp*) for 30 sec, 72°C for 45 sec (*cytK*) and 1 min (*udp*). Threshold cycle (C_T) was normalized to the C_T of the *udp* gene amplified from the corresponding sample. The expression of *cytK* was calculated using the Pfaffl's method [35] with comparison to the reference *B. cereus* ATCC 14579.

Multi-locus sequence typing (MLST)

All isolates were characterized by the MLST scheme using seven housekeeping loci, *glpF* (glycerol uptake facilitator protein), *gmk* (putative guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (phosphoribosylamino-imidazolecarboxamide), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase). The 534–599 bp fragments originating from these loci were amplified as described previously [30] in the Veriti 96-Well thermal cycler (Applied Biosystems) with the pairs of primers recommended in the *B. cereus* PubMLST database (http://pubmlst.org/bcereus) (see Table S2). Amplicons were analyzed by the capillary electrophoresis QIAxcel system (Qiagen). The products were purified with the CleanUp Kit (A&A Biotechnology, Gdynia, Poland) and sequences were determined with the ABI3500 automated sequencer (Applied Biosystems) using Big Dye Terminator cycle sequencing Kit (Applied Biosystems).

The MLST scheme sequences (348–504 bp) were assembled with the BioEdit Sequence Alignment Editor version 7.0.1 software. Each unique sequence was assigned an arbitrary allele number by reference to the *B. cereus* group MLST database. The combination of allele numbers for all seven loci of a given isolate allowed assessing the specific sequence type (ST), regarded also as genotype. New allele sequences and STs were submitted to the *B. cereus* PubMLST database. The χ^2 test with Bonferroni correction, performed with the R version 2.15.2 software, was used for pairwise analysis of statistical differences in proportions of the new STs in the habitats. The null hypothesis of the test is that the proportions are even. The significance level was set at 0.05.

Diversity of the loci

In order to assess loci diversity and their significance in selection, allele frequencies of each loci and the number of polymorphic sites were calculated using the DnaSP version 5 software [36]. The same software was also used to calculate pairwise ratios of nonsynonymous (*dN*) substitutions to synonymous (*dS*) substitutions (*dN/dS*) according to Nei and Gojobori [37]. This statistic allows measuring the significance of selection, as follows: $dN/dS<1$, purifying selection; $dN/dS=1$, neutral selection; $dN/dS>1$, positive selection [38]. The relative impact of homologous recombination on the variation of the *B. cereus s.l.* populations was estimated by calculation of the r/m ratio using ClonalFrame software version 1.2 [39]. The analysis was performed on the complete dataset (N = 273) and with regard to each environment with four independent runs. The basis of 100,000 iterations including 50,000 burn in iterations was used in all runs of the algorithm.

Population genetic analysis

The sequence types (STs) were assigned to clonal complexes using PHYLOViZ v1.0 [40] with goeBURST algorithm and 1,000 bootstrap resampling, according to Feil et al. [41]. Clonal complexes (CCs) were defined as Single Locus Variants (SLV) of two or more independent isolates that shared identical alleles at six or seven loci. To assess the correlation between alleles in the populations and the clonal versus panmixia status [42], the standard index of association (I^S_A) was calculated using the START version 2 software [43]. This statistic allowed for estimating the degree of linkage disequilibrium among loci. $I^S_A \sim 0$ indicates equilibrium between recombination and mutation rates. $I^S_A > 1$ means low rate of recombination in relation to mutation. The χ^2 test with Bonferroni correction, performed with the R version 2.15.2 software, was used for pairwise analysis of the statistical differences in proportions of bacilli forming clonal complexes with regard to their species designation. The null hypothesis of the test is that the proportions are even. The significance level was set at 0.05.

Phylogenetic analysis

Phylogenetic trees based on the concatenated loci were constructed for each population and for all isolates jointly with the MEGA4 software using the Neighbor-Joining method (NJ). Branch quality was evaluated using 1,000 replicates bootstrap test [44]. Altogether 28 reference sequences from the *B. cereus* MLST database were used for comparative analysis.

Results

Occurrence of *B. cereus s.l.* in different types of soil

The estimated density of *B. cereus s.l.* isolates from the farm, and Białowieża and Biebrza national parks (NPs) were, respectively, $0.7\pm0.7\times10^5$, $1.4\pm1.7\times10^5$ and $1.5\pm2.2\times10^5$ (Table 1). At each location, the density differed significantly among samplings. Moreover, while *B. cereus s.s.* and *B. thuringiensis* were isolated from each sample, *B. mycoides* was present in all marsh and farm samples, but only in 77% of forest samples. Chemical analysis revealed significant differences in nutrients from different locations, for example, the amount of humic substances and organic matter were ~20 times higher in soil from the NPs than the farm soil, which allowed the classification of the soil in the parks as organic, and the farm as mineral (Table 1).

Genetic diversity of the environmentally different *B. cereus s.l.* populations

The 273 *B. cereus s.l.* isolates were analyzed with the MLST approach to assess their genetic relationship and ecological diversification. The characteristic of each locus is presented in Table 2 and Figure 2 (additional data are shown in Table S3). The polymorphic sites ranged from 307 to 392 among the forest and marsh isolates, and was much higher in *B. cereus s.s.* and *B. thuringiensis* than *B. mycoides* originating from the parks, that was similar for all three species isolated from the farm samplings. However, the number of polymorphic sites among the farm *B. mycoides* was influenced by the number of polymorphic sites in two strains further classified to a separate phylogenetic clade (see below). Altogether 325 alleles were identified. The ratio of nonsynonymous to synonymous mutations (*dN/dS*) was less than one for all loci in all isolates, indicating purifying selection among the genes. The relative frequency of recombination and mutation among all *B. cereus s.l.* isolates (N = 273; 95% credibility interval) varied from 0.9 to 1.5. When single environments were considered the recombination to mutation ratio was slightly higher among strains from Białowieża NP (1.2–2.7) than those from Biebrza NP (0.8–1.9) and farmland (0.8–1.8).

The isolates were classified into 148 STs, 131 new STs (N = 212) and 17 STs (N = 61) already described in the MLST database. Almost all STs (56/57) detected in bacilli originating from

Table 1. Soil types, chemical properties and density of *B. cereus sensu lato* in the samples.

Origin	Soil type[a]	pH	Content in air-dry soil [%] SOM[b]	HS[c]	C	N	P org.	Ca	S	Number of *B. cereus s.l.*[d]
Białowieża	O	4,75	24,1	21,4	14,8	0,753	0,041	0,39	0,06	$1.4\pm1.7\times10^5$
National Park										
Biebrza	O	7,15	17,3	18,9	13,1	1,005	0,126	1,87	0,24	$1.5\pm2.2\times10^5$
National Park										
Farmland in	M	5,25	1,68	0,98	0,66	0,062	0,037	0,127	0,01	$0.7\pm0.7\times10^5$
Jasienowka										

[a]M, mineral soil; O, organic soil;
[b]soil organic matter;
[c]humic substances;
[d]given as an average CFU per gram of soil.
doi:10.1371/journal.pone.0080175.t001

Białowieża NP were found to be new, whereas with the marsh and farm isolates the number of new STs was 43/55 and 40/49, respectively. According to the χ^2 test, the proportion of the new STs to all STs of bacilli from Białowieża NP (86/93) differed significantly from the corresponding proportions in samples from Biebrza NP (61/95) and Jasienowka farm (65/85), with p-value of 0.0076 and 0.0293, respectively. However, the proportions in the samples from the latter two habitats did not differ significantly (p-value = 1).

Altogether 105 STs were present only once, while the most numerous, ST222, ST625, and ST624, were detected 27, 19, and 13 times, respectively. With regard to the environment, 50, 47, and 40 STs were associated only with the forest, marsh, and farm isolates, respectively. Additionally, nine STs (N = 41) were found in two environment simultaneously: ST646 and ST665 in the forest and marsh isolates; ST624, ST650 and ST712 in bacteria isolated from the forest and the farm; ST196, ST218, ST410; and ST647 with the marsh and farm isolates. Only ST222 (N = 27) and ST625 (N = 19) contained bacteria from all three environments. Considering the species classification, 68, 54, and 19 STs were identified among *B. cereus s.s.*, *B. thuringiensis* and *B. mycoides*, respectively. *B. cereus* and *B. thuringiensis* originating from the same environment were found simultaneously in five STs (ST657, ST683, ST705, ST708, ST737), while *B. cereus* and *B. mycoides* were present in ST222 (two *B. cereus* plus 25 *B. mycoides*) and ST624 (one *B. cereus* and 12 *B. mycoides*). *B. thuringiensis* with *B. mycoides* or three species together did not occur in one ST.

Isolate-based analysis of population genetic structure

The goeBURST approach allowed assigning the isolates to 19 clonal complexes (CCs) containing 68 STs (N = 172), and 80 singletons (N = 101) (Figure 3). All CCs were named after putative founders of STs. The most numerous complex, CC650, comprised 20 STs (N = 89) originating from the three environments, including the most frequent STs ST222 (N = 27), ST625 (N = 19), and ST624 (N = 13). The second clonal complex, CC223, included seven STs (N = 16) also from the three habitats. The CC658–668 complex, the two putative founder, included 4

Table 2. Genetic diversity in the seven loci within three *B. cereus sensu lato* populations from northeastern Poland.

	Białowieża National Park			Biebrza National Park			Farmland		
	N = 93 (*B.c.*-25; *B.t.*-38; *B.m.*-30)[a]			N = 95 (*B.c.*-39; *B.t.*-26; *B.m.*-30)[a]			N = 85 (*B.c.*-38; *B.t.*-23; *B.m.*-24)[a]		
	ST = 57 (56)[b]			ST = 55 (43)[b]			ST = 49 (40)[b]		
Locus	Polymorphic sites	Alleles[c]	Index dN/dS[d]	Polymorphic sites	Alleles[c]	Index dN/dS[d]	Polymorphic sites	Alleles[c]	Index dN/dS[d]
glpF	34	22 (10)	0.046	41	24 (8)	0.101	34	21 (3)	0.125
gmk	41	11 (7)	0.023	57	16 (4)	0.018	86	20 (7)	0.017
ilvD	73	20 (13)	0.014	94	25 (14)	0.015	103	17 (4)	0.017
pta	39	24 (12)	0.020	44	24 (8)	0.019	54	21 (5)	0.024
pur	49	21 (12)	0.031	62	24 (9)	0.018	80	24 (8)	0.016
pycA	56	14 (6)	0.030	67	18 (5)	0.029	80	21 (6)	0.030
tpi	15	12 (5)	0.082	27	21 (8)	0.076	32	17 (4)	0.070
Total	307	124 (65)	0.030	392	152 (56)	0.032	389	141 (37)	0.033

[a]*B.c.*, *Bacillus cereus s.s.*; *B.t.*, *Bacillus thuringiensis*; *B.m.*, *Bacillus mycoides*.
[b]ST, sequencing type; number of new STs described in this study is given in the parentheses.
[c]Number of new alleles described in this study are given in the parenthesis.
[d]Ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions per nucleotide site where $dN/dS<1$ indicates that the loci is subjected to purifying selection.
doi:10.1371/journal.pone.0080175.t002

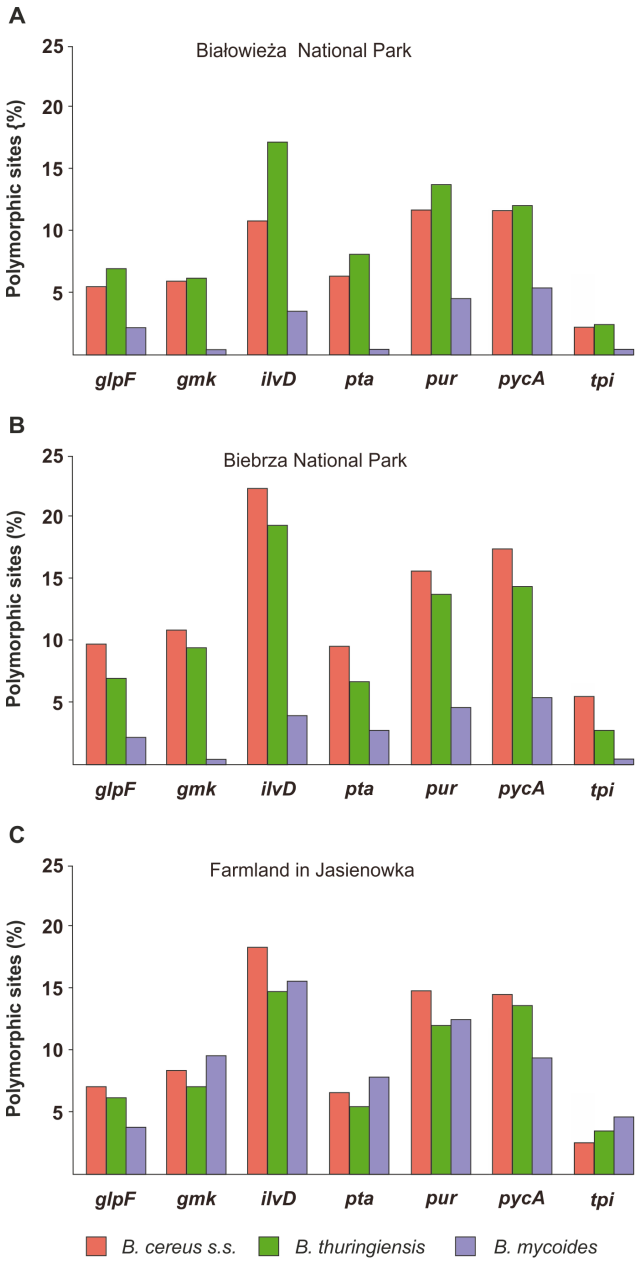

Figure 2. Genetic diversity in housekeeping genes within *B. cereus s.l.* originated from three locations in northeastern Poland. The percentage of the polymorphic sites was much higher in *B. cereus* and *B. thuringiensis* than in *B. mycoides* originating from Białowieża National Park (A) and Biebrza National Park (B). Contradictory for bacteria isolated from the farm soil (C), the percentage of the polymorphic sites was similar among bacilli classified to the three species.
doi:10.1371/journal.pone.0080175.g002

STs (N = 6). The other 16 CCs, comprised either three or two STs mostly originating from one environment.

In general, particular clonal complexes were composed of bacilli classified as the same species (Figure 4). Even in the most numerous complex, CC650 and CC223, a similar tendency was observed. In CC650, 11 STs consisting only of *B. cereus* (N = 20) composed one subcomplex around ST650, while nine STs with 65 *B. mycoides* and four *B. cereus* created the second subcomplex. The CC223 complex included 14 *B. thuringiensis* from all environments and only two *B. cereus* from Białowieża NP. The next two complexes, CC705–723 and CC656–657, comprised *B. cereus* and single *B. thuringiensis* of the same allelic profiles. CC732 and CC730–739 consisted of two STs with either *B. cereus* or *B. thuringiensis*.

Considering origins, eight CCs (N = 56) and 33 singletons (N = 37) were found among the forest isolates, nine CCs (N = 55) and 31 singletons (N = 40) in the marsh isolates, and four CCs (N = 48) and 25 singletons (N = 37) in the farm isolates (Figure S1). On the other hand, with regard to species classification, nine CCs (N = 52) and 44 singletons (N = 50) or six CCs (N = 34) and 40 singletons (N = 53), respectively, were observed among *B. cereus* and *B. thuringiensis*, while *B. mycoides* were classified only in four CCs (N = 78) and six singletons (N = 6) (Figure S2). It is worthy to note that ~77% of *B. mycoides* belonged to CC650. According to the χ^2 test, the proportion of the isolates forming clonal complexes within *B. mycoides* (78/84) differed significantly from the corresponding proportions within *B. cereus* (55/105) and *B. thuringiensis* (34/88); p-value of the test were <0.000 in both cases. However, the proportions in the latter two species did not differ significantly (p-value = 0.821).

The clonality of the isolates and the degree of association between alleles were assessed with the standardized index of association (I^S_A). The index for all 273 *B. cereus s.l.* isolates was calculated to be 0.477 (P<0.000). The separate calculation for each environment were $I^S_A = 0.364$ (P<0.000) for the forest isolates, $I^S_A = 0.651$ (P<0.000) for the marsh *B. cereus s.l.*, and $I^S_A = 0.476$ (P<0.000) for the farm isolates. Among particular species, the I^S_A indices were 0.452 (P = 0.000), 0.390 (P<0.000), and 0.520 (P = 0.000) for *B. cereus s.s.*, *B. thuringiensis*, and *B. mycoides*, respectively.

Phylogenetic analysis

The multiple alignment of 148 unique MLST concatenated sequences (2,829 bases long) of the environmental *B. cereus s.l.* and 28 reference sequences available at the MLST *B. cereus* database, revealed four major strain groups, defined according to Priest et al. (2004), as clade I, II, III and IV (Figure 5). Clade I contained approximately 11% of the environmental isolates (one *B. thuringiensis* and 28 *B. cereus*) mostly clustered with *B. anthracis* Ames (ST1), *B. cereus* ATCC 10987 (ST32), an atypical xylose-positive strain isolated from cheese, and *B. thuringiensis* HD868 (ST104). Based on the PubMLST dataset, this clade also contained strains isolated from clinical samples (ST123, ST133, ST141), food (ST123, ST152) and environment (ST309, ST152). Clade II containing approximately 15% of all environmental bacilli (28 *B. thuringiensis* and 11 *B. cereus*) clustered together with *B. cereus* type strain ATCC 14579 (ST4), *B. cereus* ATCC 11778 (ST34), and *B. thuringiensis* HD1 (ST10), HD12 (ST23), and HD73 (ST8), the lepidopteran-active strains. This clade also contained isolates from clinical samples (ST72, ST85), food (ST43) or natural environment (ST223, ST278, ST315). Clade III consisting of 74% of the bacteria isolated in this study grouped with *B. cereus s.l.* references designated as environmental isolates (ST286, ST305, ST332) in the MLST database, *B. weihenstephanensis* WSBC 10364 (ST42) and DSMZ 11821 (ST447), as well as *B. mycoides* ATCC 6462 (ST116). Clade IV contained only two *B. mycoides* from Jasienowka and *B. pseudomycoides* DSMZ 12442 (ST83).

Analysis of the sample collection sites and the phylogenetic tree showed a strong correlation between the clade designation and origin of isolates (Figure 6). Almost all *B. cereus s.l.* (97%) recovered from Białowieża NP clustered in clade III, while only one and two isolates from this habitat clustered with the references in clade II and III. Similarly, the majority (71%) of bacteria isolated from the

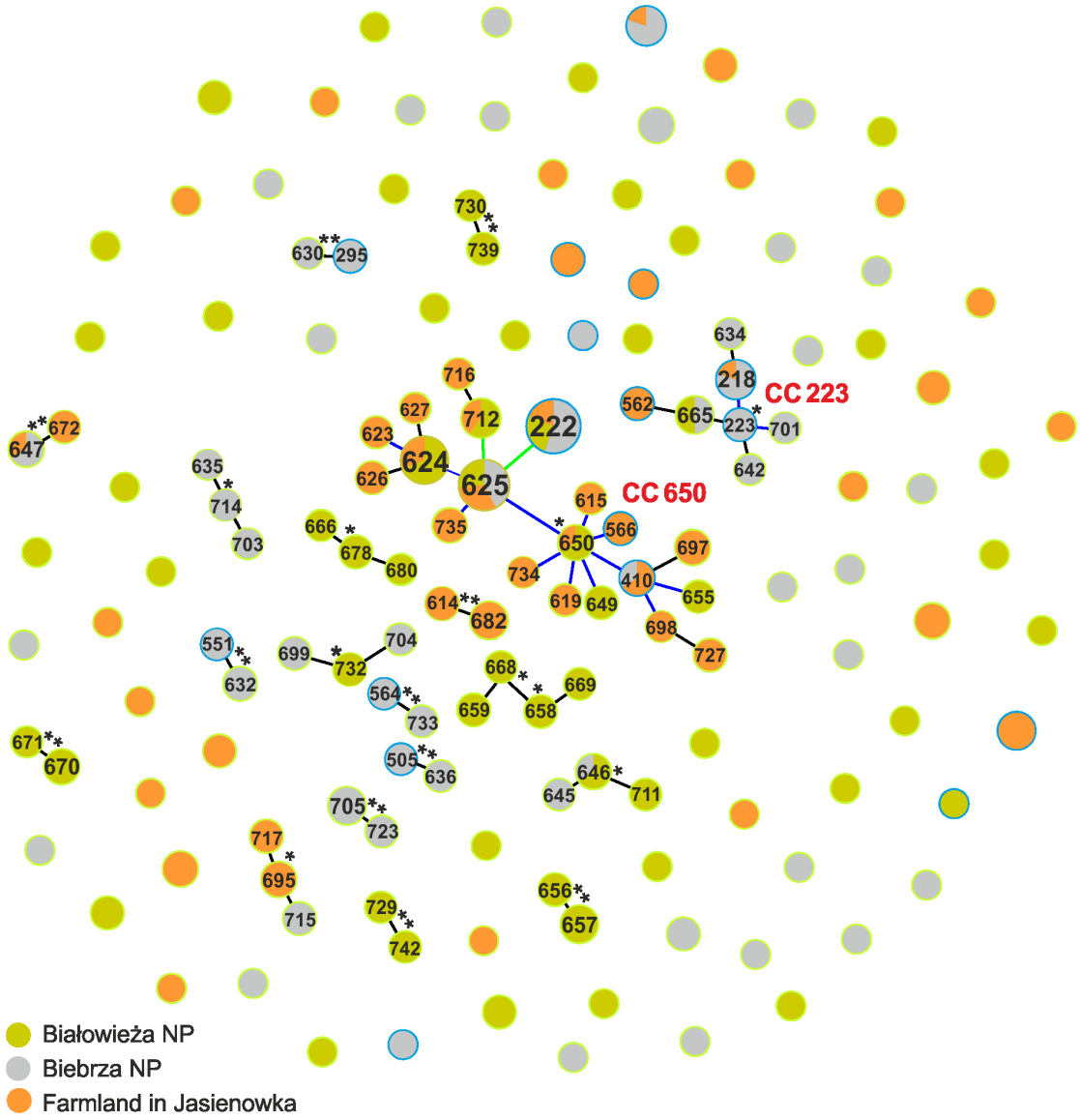

Figure 3. geoBURST analysis showing the clonal assignment of STs present in *B. cereus s.l.* Bacteria originated from Białowieża National Park, Biebrza National Park, and the Jasienowka farm in northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.
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farm clustered within clade III, and only 14 and 13% of isolates from this habitat were found in clade I and II. The bacilli originating from Biebrza NP clustered in clade I, II, and III in proportions of 17, 28, and 55%, respectively.

Detection of toxin genes

The *cytK* gene was present in four isolates from Biebrza NP (three *B. cereus*, and one *B. thuringiensis*) and in 29 from the farm (five *B. cereus*, five *B. thuringiensis*, and 19 *B. mycoides*) (Figure 6, Table S4). However, based on Real-Time PCR data, the expression of the *cytK* gene was established for eight isolates (one and seven from the Biebrza NP and the farm, respectively), identified as *B. cereus* (N = 4), *B. thuringiensis* (N = 2), and *B. mycoides* (N = 2) (Figure 6). The relative expression varied from 0.55 (*B. mycoides* 35/1 from the farmland, ST627) to 1.02 (*B. thuringiensis* 56/3 from Biebrza NP, ST707) (Table S4).

The entomopathogenic properties of *B. thuringiensis* were assessed based on the presence of genes encoding δ-endotoxins. The *cry1* gene was found among 38, 24 and 22 bacilli from Białowieża NP, Biebrza NP and the farm, respectively, and four isolates (BPN 40/2, ST710; BPN 43/2, ST680; BPN 51/1, ST667; BPN 54/2, ST668) from Białowieża NP were also *cry2*-positive. Only *B. thuringiensis* BB 48/1 (ST721) and BB 56/3 (ST707) isolated from Biebrza NP, and *B. thuringiensis* JAS 10/2 (ST694) from the farmland harbored *cry4*. Interestingly, based on the phylogenetic trees (Figure 6) the BB 56/3 isolate (ST707) appeared to be closely related to *B. thuringiensis* HD12 (ST23), and JAS 10/2 (ST694) to HD868 (ST104).

Temperature adaptation

Altogether, 189 environmental *B. cereus s.l.* isolates (69.2%) were able to grow at low temperature (Table S4). The majority of these

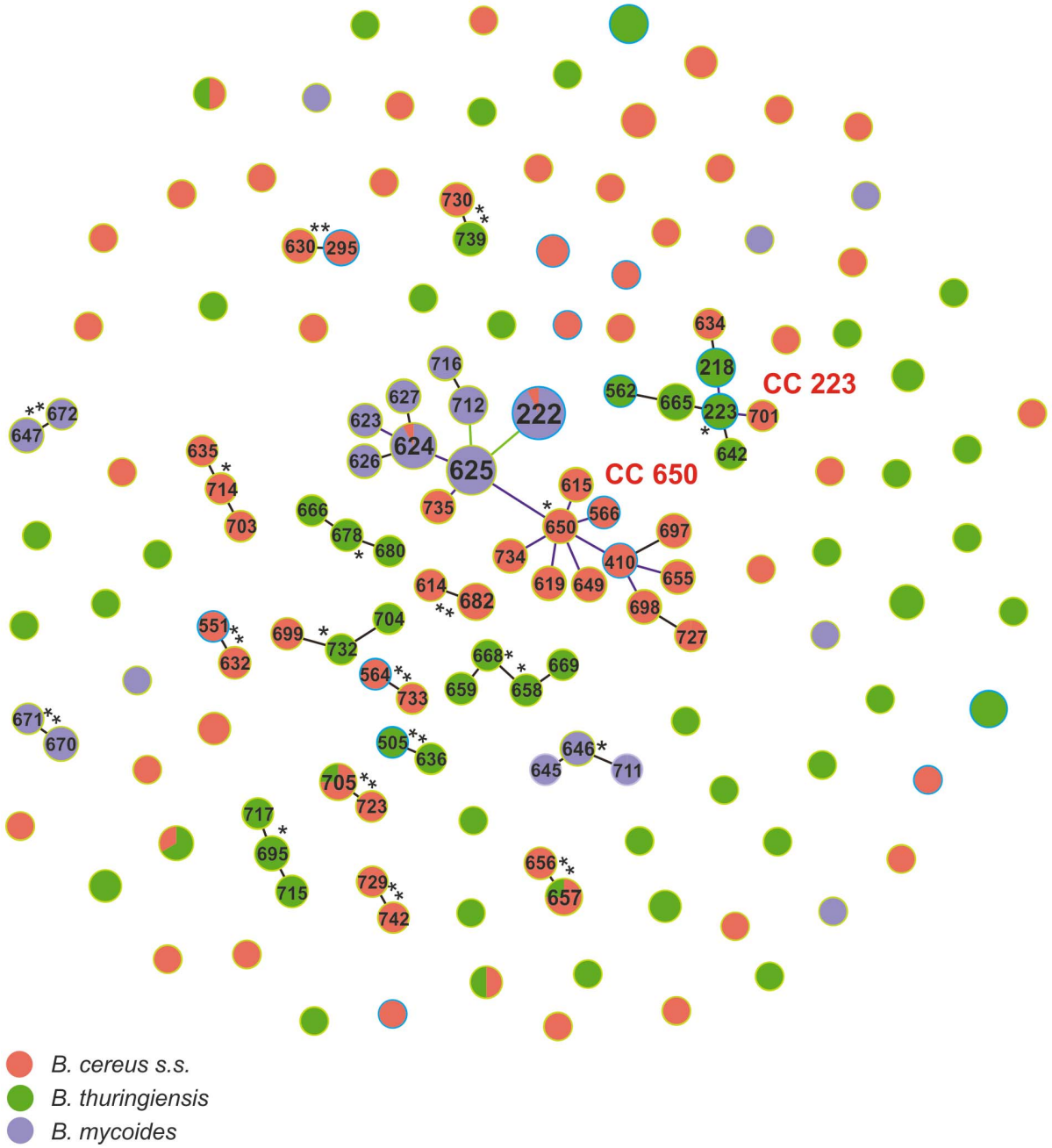

Figure 4. geoBURST analysis showing the clonal assignment of the STs present in soil isolates of *B. cereus*, *B. thuringiensis*, and *B. mycoides* originating from northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.
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isolates (95.8%) belonged to clade III (Figure 6). The largest number of isolates with psychrotrophic properties was found among bacilli isolated from Białowieża NP (95.7%), while growth at 7°C was observed only among 43.2 and 69.4% isolates from Biebrza NP and farmland, respectively. None of the isolates grew at 50°C.

Discussion

In this report we present various aspects of the genetic structure and phylogeny of *B. cereus s.l.* isolates originating from three highly diverse habitats in northeastern Poland. In general, the density of these bacilli in "organic soil" was approximately twice as high when compared to "mineral soil". Evidently these differences correlated with the concentration of nutrients and biogenic elements as suggested by Broughton et al. [45]. *B. cereus s.l.* isolated in this study demonstrated a high genetic diversity, but similar in all populations, as measured in MLST by the number of polymorphic sites, alleles, and sequence types. Heterogeneous habitats, such as soil, even with low concentration of nutrients as noted in the farm sampling, facilitates bacterial genetic divergence [46]. Zwick et al. [47]

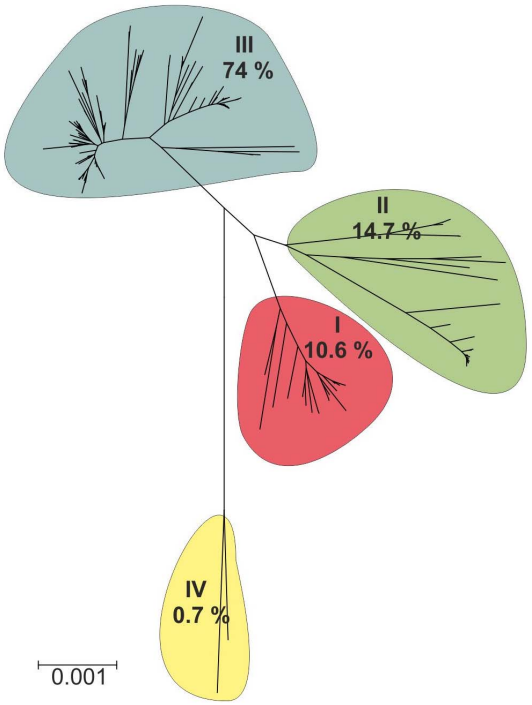

Figure 5. Phylogenetic tree of 273 *B. cereus s.l.* environmental isolates from northeastern Poland. The tree was constructed based on seven concatenated housekeeping loci (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*) representing a total of 2,829 nucleotides, using the Neighbor-Joining (NJ) method implemented in MEGA4 software. Branch quality was evaluated using 1,000 replicates bootstraps [44]. The *B. cereus s.l.* isolates originated from northeastern Poland were separated into four major clades containing 10.6% (Clade I), 14.7% (Clade II), 74.0% (Clade III), and 0.7% (Clade IV) of environmental isolates. The pathogenic *B. cereus s.l.* references were clustered mostly with the minority of the environmental isolates of the clades I and II, whereas the environmental references were grouped together with the bacilli isolated in this study classified as the clade III.
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suggested that genes associated with metabolic processes under selection offer ecological specialization and may result from adaptation to specific deficiencies in the environment. Based on the *r/m* ratio, the diversity observed within the studied populations resulted mostly from mutation events, rather than from recombination events. Yet, some differences were noted at the level of recombination when forest bacilli (*r/m*: 1.2–2.7) were compared with marsh (*r/m*: 0.8–1.9) and farm (*r/m*: 0.8–1.8) populations. Thus, it is more likely that environmental conditions may influence recombination rates. *B. cereus s.l.* isolated in northeastern Poland undergo recombination slower than those tested by Didelot and Falush [39] (*r/m*: 1.3–2.8) or Didelot et al. [48] (*r/m*: 1.08–1.57). A comprehensive study by Vos and Didelot [49] comparing homologous recombination rates in bacteria and archaea showed significant variation from species to species, with the *r/m* ratio from 0.02 for *Leptospira interrogans* to even 63.3 for *Flavobacterium psychrophillum*. Apparently, recombination rates are not only influenced by bacterial lifestyle (pathogenic, commensal or mutualistic), but also by environmental conditions. Nevertheless, it is necessary to emphasize the impact of horizontal gene transfer (HGT) on bacterial diversity, particularly mediated by conjugation, which occurs especially in soil rich in organic material [50,51].

The relatively large samples of *B. cereus s.l.* from three different ecologic habitats, allowed testing the hypothesis that specific genotypes of these bacilli occur in a particular natural habitat. This assumption was supported by the presence of 137 (92%) STs in bacilli originating from one specific habitat. Moreover, the description of new STs for 77% of the isolates provided additional evidence for the existence of specific genotypes within natural *B. cereus s.l.* populations. Owing to high dispersal ability of *Bacillus* spp. [21], the presence of ecologically distinct genotypes within environmental *B. cereus s.l.* populations must be based on adaptations to habitats, not on accidental colonization. In this regard, of particular importance is the statistically significant abundance of new genotypes within *B. cereus s.l.* originated from Białowieża NP when compared with those from Biebrza NP and the farm. The protection of Białowieża NP [52] appears to have an impact on the heterogeneity of ecological "microniches", which affects the diversification of *B. cereus s.l.* into genetically different lineages. Nevertheless, it cannot be excluded that the forest environment itself may select for distinct ecotypes. Yet, the incidence of 11 STs (8%) simultaneously in more than one habitat is, to a minor extent, in contradiction to the hypothesis about association between bacterial genotypes and ecological niches. The latter STs probably represent polyphyletic genotypes persisting in a variety of environmental habitats, similarly as noted for *B. thuringiensis* HD73 (ST8) [28]. Thus, some genotypes within environmental *B. cereus s.l.* populations do not seem to be niche specialists. This observation probably also applies to other microorganisms in natural population. For instance Hunt et al. [53] noted remarkable narrow preferences within some *Vibrio* spp. genotypes, whereas others were broadly distributed in marine ecosystem.

In contrast to the diversity, the I^S_A index, significantly different from zero, inferred a clonal population structure. It is generally accepted, that in clonal bacterial populations the genetic diversity is purged by selective sweeps [54]. Recombination in *Bacillus* spp. is unlikely to be frequent enough to prevent periodic selection events from occurring [54]. Indeed, the selective pressure on the alleles among the bacilli studied, as measured by *dN/dS* ratio, indicates a negative selection. Nevertheless, our data highlight the ecological diversity among very close relatives (e.g. within group III), which indicates that a selective sweep would not be expected to purge the diversity within an entire species. For instance, goeBURST grouped 172 bacilli isolated in this study into 19 complexes corresponding to bacterial clones, and 101 bacteria into 80 singletons. Thus, although the *dN/dS* and I^S_A indices suggested clonal population structures, dynamic genetic diversification exists in natural populations of *B. cereus s.l.*, presumably allowing these bacteria to adapt to different ecological niches, and thereby increasing the number of ecologically distinct subpopulations, so called ecotypes [55]. In some cases ecotypes are identified as DNA sequence lineages, but more often an ecotype can encompass distinct evolutionary lineages [54]. In this study we found lineages of isolates, mostly within clade III, capable of growth at low temperature (thermal ecotypes), as previously observed among *B. cereus s.l.* and *B. thuringiensis* [30,56]. However, in contrast to the previous report [30], in which we found association between the cytotoxic potential with some *B. thuringiensis* ecotypes, in this study the presence of *cytK* was mainly associated with isolates from farm samplings where human activity is extensive, while the gene was absent in isolates originating from Białowieża NP. Moreover, the *cytK* presence was intermixed among the lineages and species. This supports the opportunistic pathogenicity model of *B. cereus s.l.*, where the potential or ability to cause various diseases have no association with specific pathotypes [57]. The presence of *cytK* among *B. cereus s.s.*, *B. thuringiensis* and *B. mycoides*, indicate that food poisoning potential is not associated with species affiliation as suggested by Guinebretière et al. [57]. From an ecological point of

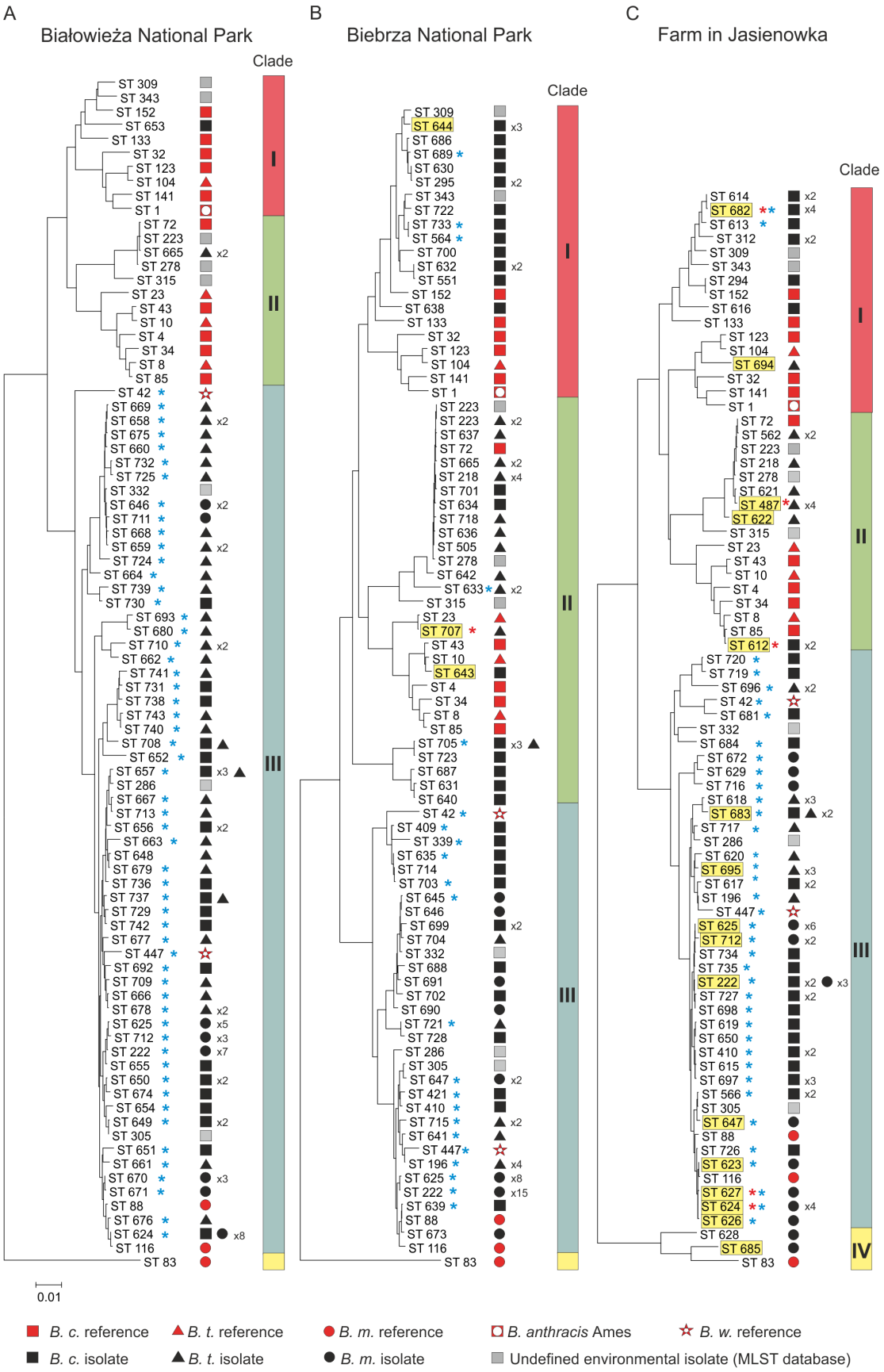

Figure 6. NJ phylogenic trees of the *B. cereus s.l.* environmental isolates originated from three locations in northeastern Poland and

28 reference strains. The trees prepared separately for each population were constructed as given in Figure 5. The isolates originated from Białowieża and Biebrza National Parks pertained to three clades, while those isolated from the farm samples clustered to four clades, with only two isolates in clade IV containing *B. pseudomycoides* reference strains. Detailed information on the isolates and the reference strains used in phylogenetic trees are given in Table S3. Yellow box indicates the *cytK* positive isolates. Red asterisk indicates the isolates that express the *cytK* gene in Real-Time PCR analysis. Blue asterisk indicates isolates able to grow at 7°C. *B.c., B. cereus s.s.*; *B.t., B. thuringiensis*; *B.m., B. mycoides, B.a., B. anthracis*, *B.w., B. weihenstephanensis*.
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view, it could be considered that toxin production is considerably "expensive" for the bacterial host, and presumably toxigenic genes would not be maintained if harboring them were disadvantageous [2]. Nevertheless, the problem of *B. cereus s.l.* pathogenicity and its relation to ecology of the group seems to be more complex and needs further investigation.

In phylogenetic analyses, only a small number of isolates from the marsh and farm samples clustered in clade I, a clade composed primarily of pathogenic bacilli [13]. These bacilli formed singletons and low-numbered complexes, which may suggest they are "atypical" environmental isolates. The part of STs forming clade III, created the CC650 complex (N = 89/202) and were classified as *B. cereus s.s.* and *B. mycoides*, whereas, clade II STs forming CC223 (N = 16/40) were mostly *B. thuringiensis* and *B. cereus s.s.* These two clonal complexes seem to be representative for these clades, and indicate their adaptation to specific niches, i.e., CC650 - soil matrices, and CC223 - insect larvae. Some taxonomists, based on studies of mostly human-associated isolates, disagree with the separation of *B. cereus s.l.* into distinct species [12,13]. Hence, we hypothesized that the environmental *B. cereus s.l.* are also genetically highly related and should be classified to one species. However, the above hypothesis was not confirmed in our study as (i) only seven STs included bacilli classified into different species, (ii) the isolates classified to the same species but originated from different habitats exhibited a tendency of clustering to particular complexes, and (iii) *B. mycoides* isolates showed higher genetic similarity than *B. cereus s.s.* and *B. thuringiensis* confirmed statistically by significantly lower number of clonal complexes with high number of isolates (4 CCs; N = 78/84). The previous reports largely focused on *B. cereus s.s.*, *B. anthracis*, and/or *B. thuringiensis* [12,28,30]. Here we present for the first time the genetic structure for *B. mycoides*. Indeed this bacterium has no proven medical and/or economic significance, but it could alter bacterial populations dynamics, for instance by aiding other bacteria to adapt to specific environments or by facilitating horizontal gene transfer, as has been described for *B. cereus* and *B. thuringiensis* [28]. Although the idea of classification of the *B. cereus s.l.* isolates into one species has many proponents [12,13,18], this issue is still unresolved, especially as it relates to environmental isolates of the group. It seems that in contrast to the human-associated *B. cereus s.l.* exhibiting high levels of similarities [12], the environmental isolates are more intricate. We propose dividing *B. cereus s.l.* into two groups, the first which contains environmental isolates, and the second composed of those that may be clinically significant.

In summary, although high genetic diversity measured with MLST was observed among natural *B. cereus s.l.* isolates from three varied habitats in northeastern Poland, the populations appear to be clonal in nature. Nevertheless, the bacilli undergo dynamic genetic diversification, mostly resulting from mutation events. Further, a significantly high number of genotypes found within *B. cereus s.l.* populations are habitat-specific. In phylogenetic analyses described in this report, only a small number of isolates, mostly from environments associated with the anthropogenic factors (the Jasienowka farm and the Biebrza NP) clustered in clade I and were associated with pathogenic *B. cereus s.l.*, while the majority of isolates clustered in clade III comprising environmental bacilli from the MLST database. Based on genetic properties of the isolates we did not find strong arguments for merging of the particular species into one taxon. It seems that in contrast to human-associated *B. cereus s.l.*, which exhibit significant similarity, the environmental isolates are more complex.

Supporting Information

Table S1 The diagnostic feature used for species-level classification of the *B. cereus s.l.* isolates.
(DOCX)

Table S2 Primers used in this study.
(DOCX)

Table S3 Genetic diversity in the seven housekeeping loci within *B. cereus s.s.*, *B. thuringiensis*, and *B. mycoides* originated from northeastern Poland.
(DOCX)

Table S4 Characterization of the *B. cereus s.l.* isolates originated from Białowieża National Park (BPN), Biebrza National Park (BB), and the Jasienowka farm (JAS), and the 12 reference strains used in the phylogenetic analysis.
(DOCX)

Figure S1 geoBURST analysis. The figures are showing the clonal assignment of the STs present in *B. cereus s.l.* originating from Białowieża National Park (A), Biebrza National Park (B), and the Jasienowka farm (C) in northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.
(TIF)

Figure S2 geoBURST analysis. The figures are showing the clonal assignment of the STs present in soil isolates of *B. cereus s.s.* (A), *B. thuringiensis* (B), and *B. mycoides* (C) originating from northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.
(TIF)

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Author Contributions

Conceived and designed the experiments: JMD IS. Performed the experiments: JMD. Analyzed the data: JMD IS. Contributed reagents/materials/analysis tools: IS. Wrote the paper: JMD IS.

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Table S1. The diagnostic feature used for species-level classification of the *B. cereus s.l.* isolates.

Diagnostic feature	***B. cereus***	***B. thuringiensis***	***B. anthracis***	***B. mycoides/ B. pseudomycoides***	***B. weihen-stephanensis***	***B. cytotoxicus***
Parasporal crystal synthesis	no	yes	no	no	no	no
Rhizoidal growth	no	no	no	yes	no	no
Sheep blood hemolysis	yes	yes	no	yes	yes	yes
Growth at 7 ^{o}C	no	variable	no	no	yes	no
Growth at 50 ^{o}C	no	no	no	no	no	yes

Table S2. Primers used in this study.

Target gene	Primer name	Nucleotide	Product size (bp)	Reference
Primers for the δ-endotoxin genes				
cry1	Un1(d)	5'-CATGATTCATGCGGCAGATAAAC-3'	277	Ben-Dov et al., 1997
	Un1(r)	5'-TTGTGACACTTCTGCTTCCCATT-3'		
cry2	Un2(d)	5'-GTTATTCTTAATGCAGATGAATGGG-3'	701	Ben-Dov et al., 1997
	Un2(r)	5'-CGGATAAAATAATCTGGGAAATAGT-3'		
cry3	Un3(d)	5'-CGTTATCGCAGAGAGATGACATTAAC-3'	589	Ben-Dov et al., 1997
	Un3(r)	5'-CATCTGTTGTTTCTGGAGGCAAT-3'		
cry4	Un4(d)	5'-GCATATGATGTAGCGAAACAAGCC-3'	439	Ben-Dov et al., 1997
	Un4(r)	5'-GCGTGACATACCCATTTCCAGGTCC-3'		
cry7,8	Un7,8(d)	5'-AAGCAGTGAATGCCTTGTTTAC-3'	420	Ben-Dov et al., 1997
	Un7,8(r)	5'-CTTCTAAACCTTGACTACTT-3'		
cry9	Un9(d)	5'-CGGTGTTACTATTAGCGAGGGCGG-3'	354	Ben-Dov et al., 1999
	Un9(r)	5'-GTTTGAGCCGCTTCACAGCAATCC-3'		
Primers for the cytK gene				

cytK (PCR)	CytKF CytKR	5’-GATAATATGACAATGTCTTTAAA-3’ 5’-GGAGAGAAACCGCTATTTGT-3’	1,011	Swiecicka and Mahillon, 2006
cytK (RealTime PCR)	CytK(RT)-F CytK(RT)-R	5’-GGCCATTAGGCGTTACAGAA-3’ 5’-CTGGCGCTAGTGCAACATTA-3’	180	M. Bartoszewicz (personal com.), 2013
udp	udpF udpR	5′-ACTAGAGAAACTTGGAAATGATCG-3′ 5′-GACGCTTAATTGCACGGAAC-3′	101	Reiter et al., 2011
Primers used in MLST analysis				
glpF	glpF_F glpF_R	5’-GCGTTTGTGCTGGTGTAAGT-3’ 5’-CTGCAATCGGAAGGAAGAAG-3’	548	www.pubmlst.org/bcereus/info/primers.shtml
gmk	gmk_F gmk_R	5’-ATTTAAGTGAGGAAGGGTAGG-3’ 5’-GCAATGTTCACCAACCACAA-3’	599	www.pubmlst.org/bcereus/info/primers.shtml
ilvD	ilvD_F ilvD_R ilvD4_F ilvD2_R	5’-CGGGGCAAACATTAAGAGAA-3’ 5’-GGTTCTGGTCGTTTCCATTC-3’ 5’-GCAGAGATTAAAGATAAGGA-3’ 5’-GTTACCATTTGTGCATAACGC-3’	553 568	www.pubmlst.org/bcereus/info/primers.shtml www.pubmlst.org/bcereus/info/primers.shtml
pta	pta_F pta_R	5’-GCAGAGCGTTTAGCAAAAGAA-3’ 5’-TGCAATGCGAGTTGCTTCTA-3’	575	www.pubmlst.org/bcereus/info/primers.shtml

pur	pur_F	5’-CTGCTGCGAAAAATCACAAA-3’	534	www.pubmlst.org/bcereus/info/primers.shtml
	pur_R	5’-CTCACGATTCGCTGCAATAA-5’		
pycA	pycA_F	5’-GCGTTAGGTGGAAACGAAAG-3’	549	www.pubmlst.org/bcereus/info/primers.shtml
	pycA_R	5’-CGCGTCCAAGTTTATGGAAT-3’		
tpi	tpi_F	5’-GCCCAGTAGCACTTAGCGAC-3’	557	www.pubmlst.org/bcereus/info/primers.shtml
	tpi_R	5’-CCGAAACCGTCAAGAATGAT-3’		

Table S3. Genetic diversity in the seven housekeeping loci within *B. cereus*, *B. thuringiensis*, and *B. mycoides* originated from northeastern Poland.

Białowieża National Park

	B. cereus (N = 25; ST = 20)			*B. thuringiensis* (N = 38; ST = 33)			*B. mycoides* (N = 30; ST = 8)		
Locus	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**
glpF	21 (5.6)	11 (5)	0.030	26 (7.0)	15 (7)	0.045	8 (2.2)	5 (1)	0.040
gmk	31 (6.2)	6 (4)	0.030	32 (6.3)	9 (7)	0.037	3 (0.6)	2 (0)	0.145
ilvD	43 (10.9)	11 (6)	0.016	68 (17.3)	14 (9)	0.016	14 (3.6)	4 (1)	0.023
pta	27 (6.5)	13 (7)	0.024	34 (8.2)	18 (9)	0.016	3 (0.7)	2 (0)	0.000
pur	41 (11.8)	10 (4)	0.014	48 (13.8)	17 (11)	0.028	16 (4.6)	5 (1)	0.062
pycA	43 (11.8)	9 (3)	0.047	44 (12.1)	9 (4)	0.019	20 (5.5)	3 (0)	0.041
tpi	10 (2.3)	8 (3)	0.095	11 (2.5)	7 (2)	0.060	2 (0.5)	2 (0)	0.000

Biebrza National Park

	B. cereus (N = 39; ST = 32)			*B. thuringiensis* (N = 26; ST = 16)			*B. mycoides* (N = 30; ST = 8)		
Locus	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**
glpF	37 (9.9)	18 (6)	0.094	26 (7.0)	9 (3)	0.118	7 (1.9)	5 (1)	0.052
gmk	57 (11.3)	14 (4)	0.015	49 (9.7)	8 (1)	0.017	3 (0.6)	2 (0)	0.145

ilvD	91 (23.2)	18 (10)	0.018	79 (20.1)	8 (3)	0.018	16 (4.1)	5 (2)	0.016
pta	41 (9.9)	19 (5)	0.015	28 (6.8)	7 (2)	0.020	11 (2.7)	4 (2)	0.029
pur	57 (16.4)	16 (6)	0.013	50 (14.4)	8 (3)	0.013	16 (4.6)	7 (2)	0.056
pycA	66 (18.2)	14 (4)	0.031	54 (14.9)	8 (3)	0.023	20 (5.5)	3 (0)	0.041
tpi	25 (5.7)	18 (8)	0.086	12 (2.8)	8 (0)	0.042	2 (0.5)	2 (0)	0.000

Farmland in Jasienowka

	B. cereus (N = 38; ST = 25)			*B. thuringiensis* (N = 23; ST = 13)			*B. mycoides* (N = 24; ST = 13)		
Locus	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**	**Polymorphic sites[a]**	**Number of alleles[b]**	**Index *d*N/*d*S[c]**
glpF	28 (7.5)	15 (2)	0.124	24 (6.5)	9 (0)	0.121	15 (4.0)	5 (1)	0.122
gmk	45 (8.9)	14 (5)	0.013	38 (7.5)	6 (2)	0.021	53 (10.5)	3 (0)	0.027
ilvD	77 (19.6)	11 (3)	0.014	62 (15.8)	8 (2)	0.019	66 (16.8)	4 (1)	0.018
pta	29 (7.0)	10 (2)	0.021	25 (6.0)	10 (2)	0.039	35 (8.5)	5 (1)	0.018
pur	55 (15.8)	13 (3)	0.028	45 (12.9)	8 (4)	0.016	47 (13.5)	6 (1)	0.014
pycA	56 (15.4)	13 (4)	0.029	53 (14.6)	7 (2)	0.027	38 (10.5)	4 (0)	0.026
tpi	12 (2.8)	9 (1)	0.098	17 (3.9)	6 (1)	0.080	22 (5.1)	4 (2)	0.052

[a] Number of polymorphic sites and percentage (in parentheses) calculated as the number of polymorphic sites divided by the length of the sequenced fragment.

[b] Number of new alleles described in this study is given parentheses.

[c] Ratio of nonsynonymous(d_N) to synonymous (d_S) substitutions per nucleotide site. dN/dS < 1 indicates that the loci is subjected to purifying selection.

Table S4. Characterization of the *B. cereus s.l.* isolates originated from three habitats in northeastern Poland, and 12 reference strains used in the phylogenetic analysis.

Isolate[a]	**Origin**[b]	**Growth at 7°C**[c]	***cry***[d]	***cytK***[e]	**ST**[f]	**Clade**[g]	**CC**[h]
B.c. 33/4	BPN				653[N]	I	S
B.t. 44/2	BPN		+		665[N]	II	CC223
B.t. 45/4	BPN		+		665[N]	II	CC223
B.t. 55/2	BPN	+	+		669[N]	III	CC658-668
B.t. 05/4	BPN	+	+		658[N]	III	CC658-668
B.t. 60/3	BPN	+	+		658[N]	III	CC658-668
B.t. 05/2	BPN	+	+		675[N]	III	S
B.t. 10/2	BPN	+	+		660[N]	III	S
B.t. 59/2	BPN	+	+		732[N]	III	CC732
B.t. 50/1	BPN	+	+		725[N]	III	S
B.m. 06/1	BPN	+			646[N]	III	CC646
B.m. 42/1	BPN	+			646[N]	III	CC646
B.m. 03/1	BPN	+			711[N]	III	CC646
B.t. 54/2	BPN	+	+, +[2]		668[N]	III	CC658-668
B.t. 07/3	BPN	+	+		659[N]	III	CC658-668
B.t. 56/1	BPN	+	+		659[N]	III	CC658-668
B.t. 05/3	BPN	+	+		724[N]	III	S
B.t. 42/2	BPN	+	+		664[N]	III	S
B.t. 57/2	BPN	+	+		739[N]	III	CC730-739
B.c. 42/1	BPN	+			730[N]	III	CC730-739
B.t. 53/3	BPN	+	+		693[N]	III	S
B.t. 43/2	BPN	+	+, +[2]		680[N]	III	CC678
B.t. 36/4	BPN	+	+		710[N]	III	S
B.t. 40/2	BPN	+	+, +[2]		710[N]	III	S
B.t. 37/2	BPN	+	+		662[N]	III	S
B.t. 41/1	BPN	+	+		741[N]	III	S
B.c. 54/1	BPN	+			731[N]	III	S
B.c. 57/3	BPN	+			738[N]	III	S
B.t. 38/4	BPN	+	+		743[N]	III	S
B.t. 57/4	BPN	+	+		740[N]	III	S
B.c. 58/4	BPN	+			708[N]	III	S

B.t. 03/1	BPN	+	+	708^{N}	III	S
B.c. 32/3	BPN	+		652^{N}	III	S
B.c. 50/3	BPN	+		657^{N}	III	CC656-657
B.c. 52/2	BPN	+		657^{N}	III	CC656-657
B.c. 56/3	BPN	+		657^{N}	III	CC656-657
B.t. 30/3	BPN	+	+	657^{N}	III	CC656-657
B.t. 51/1	BPN	+	+, $+^{2}$	667^{N}	III	S
B.t. 30/4	BPN	+	+	713^{N}	III	S
B.c. 43/4	BPN	+		656^{N}	III	CC656-657
B.c. 55/4	BPN	+		656^{N}	III	CC656-657
B.t. 40/1	BPN	+	+	663^{N}	III	S
B.t. 06/1	BPN		+	648^{N}	III	S
B.t. 35/2	BPN	+	+	679^{N}	III	S
B.c. 30/1	BPN	+		736^{N}	III	S
B.c. 34/4	BPN	+		737^{N}	III	S
B.t. 12/1	BPN	+	+	737^{N}	III	S
B.c. 21/1	BPN	+		729^{N}	III	CC729-742
B.c. 36/3	BPN	+		742^{N}	III	CC729-742
B.t. 51/2	BPN	+	+	677^{N}	III	S
B.c. 53/1	BPN	+		692^{N}	III	S
B.t. 02/2	BPN	+	+	709^{N}	III	S
B.t. 47/1	BPN	+	+	666^{N}	III	CC678
B.t. 29/1	BPN	+	+	678^{N}	III	CC678
B.t. 54/4	BPN	+	+	678^{N}	III	CC678
B.m. 08/1	BPN	+		625^{N}	III	CC650
B.m. 09/1	BPN	+		625^{N}	III	CC650
B.m. 37/1	BPN	+		625^{N}	III	CC650
B.m. 38/1	BPN	+		625^{N}	III	CC650
B.m. 48/1	BPN	+		625^{N}	III	CC650
B.m. 05/1	BPN	+		712^{N}	III	CC650
B.m. 15/1	BPN	+		712^{N}	III	CC650
B.m. 20/1	BPN	+		712^{N}	III	CC650
B.m. 13/1	BPN	+		222	III	CC650
B.m. 16/1	BPN	+		222	III	CC650
B.m. 29/1	BPN	+		222	III	CC650
B.m. 32/1	BPN	+		222	III	CC650

B.m. 52/1	BPN	+			222	III	CC650
B.m. 56/1	BPN	+			222	III	CC650
B.m. 57/1	BPN	+			222	III	CC650
B.c. 37/1	BPN	+			655^{N}	III	CC650
B.c. 13/1	BPN	+			650^{N}	III	CC650
B.c. 36/2	BPN	+			650^{N}	III	CC650
B.c. 38/2	BPN	+			674^{N}	III	S
B.c. 35/4	BPN	+			654^{N}	III	S
B.c. 08/1	BPN	+			649^{N}	III	CC650
B.c. 08/4	BPN	+			649^{N}	III	CC650
B.c. 28/4	BPN	+			651^{N}	III	S
B.t. 32/4	BPN	+	+		661^{N}	III	S
B.m. 01/1	BPN	+			670^{N}	III	CC670-671
B.m. 04/1	BPN	+			670^{N}	III	CC670-671
B.m. 28/1	BPN	+			670^{N}	III	CC670-671
B.m. 53/1	BPN	+			671^{N}	III	CC670-671
B.t. 23/2	BPN	+	+		676^{N}	III	S
B.c. 60/1	BPN	+			624^{N}	III	CC650
B.m. 12/1	BPN	+			624^{N}	III	CC650
B.m. 26/1	BPN	+			624^{N}	III	CC650
B.m. 31/1	BPN	+			624^{N}	III	CC650
B.m. 35/1	BPN	+			624^{N}	III	CC650
B.m. 36/1	BPN	+			624^{N}	III	CC650
B.m. 39/1	BPN	+			624^{N}	III	CC650
B.m. 40/1	BPN	+			624^{N}	III	CC650
B.m. 45/1	BPN	+			624^{N}	III	CC650
B.c. 04/1	BB				644^{N}	I	S
B.c. 51/4	BB			+	644^{N}	I	S
B.c. 54/4	BB			+	644^{N}	I	S
B.c. 20/4	BB				686^{N}	I	S
B.c. 25/4	BB	+			689^{N}	I	S
B.c. 08/1	BB				630^{N}	I	CC295-630
B.c. 01/3	BB				295	I	CC295-630
B.c. 54/3	BB				295	I	CC295-630
B.c. 01/1	BB				722^{N}	I	S
B.c. 18/1	BB	+			733^{N}	I	CC564-733

B.c. 26/4	BB	+			564	I	CC564-733
B.c. 18/3	BB				700[N]	I	S
B.c. 19/4	BB				632[N]	I	CC551-632
B.c. 50/5	BB				632[N]	I	CC551-632
B.c. 25/1	BB				551	I	CC551-632
B.c. 16/1	BB				638[N]	I	S
B.t. 51/3	BB		+		223	II	CC223
B.t. 56/2	BB		+		223	II	CC223
B.t. 15/2	BB		+		637[N]	II	S
B.t. 52/5	BB		+		665[N]	II	CC223
B.t. 58/1	BB		+		665[N]	II	CC223
B.t. 17/4	BB		+		218	II	CC223
B.t. 21/3	BB		+		218	II	CC223
B.t. 44/5	BB		+		218	II	CC223
B.t. 47/4	BB		+		218	II	CC223
B.c. 23/4	BB				701[N]	II	CC223
B.c. 28/4	BB				634[N]	II	CC223
B.t. 48/5	BB		+		718[N]	II	S
B.t. 10/5	BB		+		636[N]	II	CC505-636
B.t. 42/3	BB		+		505	II	CC505-636
B.t. 43/5	BB		+		642[N]	II	CC223
*B.t.*06/1	BB	+	+		633[N]	II	S
B.t. 27/1	BB		+		633[N]	II	S
B.t. 56/3	BB		+[4]	+ (1.02)	707[N]	II	S
B.c. 45/2	BB			+	643[N]	II	S
B.c. 01/2	BB				705[N]	II	CC705-723
B.c. 25/5	BB	+			705[N]	II	CC705-723
B.c. 34/5	BB	+			705[N]	II	CC705-723
B.t. 32/5	BB		+		705[N]	II	CC705-723
B.c. 16/2	BB				723[N]	II	CC705-723
B.c. 02/1	BB				687[N]	II	S
B.c. 17/5	BB				631[N]	II	S
B.c. 31/5	BB				640[N]	II	S
B.c. 33/2	BB	+			409	III	S
B.c. 22/2	BB	+			339	III	S
B.c. 29/2	BB	+			635[N]	III	CC714

B.c. 02/3	BB			714[N]	III	CC714
B.c. 14/1	BB	+		703[N]	III	CC714
B.m. 10/1	BB	+		645[N]	III	CC646
*B.m.*57/1	BB			646[N]	III	CC646
B.c. 11/4	BB			699[N]	III	CC732
B.c. 14/5	BB			699[N]	III	CC732
B.t. 30/5	BB		+	704[N]	III	CC732
B.c. 05/3	BB			688[N]	III	S
B.m. 48/1	BB			691[N]	III	S
B.c. 12/1	BB			702[N]	III	S
B.m. 46/1	BB			690[N]	III	S
B.t. 48/1	BB	+	+[4]	721[N]	III	S
B.c. 53/3	BB			728[N]	III	S
B.m. 33/1	BB	+		647[N]	III	CC647-672
B.m. 58/1	BB	+		647[N]	III	CC647-672
B.c. 52/3	BB	+		421	III	S
B.c. 20/1	BB	+		410	III	CC650
B.t. 27/4	BB	+	+	715[N]	III	CC695
B.t. 46/2	BB	+	+	715[N]	III	CC695
B.t. 40/3	BB	+	+	641[N]	III	S
B.t. 07/2	BB		+	196	III	S
B.t. 15/1	BB	+	+	196	III	S
B.t. 24/2	BB	+	+	196	III	S
B.t. 58/3	BB	+	+	196	III	S
B.m. 01/1	BB	+		625[N]	III	CC650
B.m. 02/1	BB			625[N]	III	CC650
B.m. 08/1	BB			625[N]	III	CC650
B.m. 14/1	BB	+		625[N]	III	CC650
B.m. 40/1	BB	+		625[N]	III	CC650
B.m. 51/1	BB	+		625[N]	III	CC650
B.m. 54/1	BB	+		625[N]	III	CC650
B.m. 55/1	BB			625[N]	III	CC650
B.m. 04/1	BB	+		222	III	CC650
B.m. 05/1	BB	+		222	III	CC650
B.m. 07/1	BB			222	III	CC650
B.m. 16/1	BB	+		222	III	CC650

B.m. 21/1	BB	+			222	III	CC650
B.m. 22/1	BB				222	III	CC650
B.m. 24/1	BB	+			222	III	CC650
B.m. 26/1	BB	+			222	III	CC650
B.m. 30/1	BB	+			222	III	CC650
B.m. 31/1	BB	+			222	III	CC650
B.m. 37/1	BB	+			222	III	CC650
B.m. 41/1	BB	+			222	III	CC650
B.m. 45/1	BB	+			222	III	CC650
B.m. 49/1	BB	+			222	III	CC650
B.m. 50/1	BB	+			222	III	CC650
B.c. 23/3	BB	+			639[N]	III	S
B.m. 34/1	BB				673[N]	III	S
B.c. 31/2	JAS				614[N]	I	CC614-682
B.c. 67/5	JAS				614[N]	I	CC614-682
B.c. 08/1	JAS			+ (0.90)	682[N]	I	CC614-682
B.c. 11/1	JAS				682[N]	I	CC614-682
B.c. 23/4	JAS			+ (0,70)	682[N]	I	CC614-682
B.c. 36/3	JAS	+			682[N]	I	CC614-682
B.c. 28/2	JAS	+			613[N]	I	S
B.c. 05/4	JAS				312	I	S
B.c. 07/5	JAS				312	I	S
B.c. 90/2	JAS				294	I	S
B.c. 92/2	JAS				616[N]	I	S
B.t. 10/2	JAS		+[4]	+	694[N]	I	S
B.t. 03/3	JAS		+		562	II	CC223
B.t. 100/3	JAS		+		562	II	CC223
B.t. 22/1	JAS		+		218	II	CC223
B.t. 42/3	JAS		+		621[N]	II	S
B.t. 04/4	JAS		+		487	II	S
B.t. 09/5	JAS		+		487	II	S
B.t. 13/1	JAS		+		487	II	S
B.t. 32/4	JAS		+	+ (0,71)	487	II	S
B.t. 63/5	JAS		+	+	622[N]	II	S
B.c. 24/2	JAS			+ (1.01)	612[N]	II	S
B.c. 35/2	JAS			+ (0.86)	612[N]	II	S

B.c. 74/1	JAS	+			720^{N}	III	S
B.c. 82/3	JAS	+			719^{N}	III	S
B.t. 30/2	JAS	+	+		696^{N}	III	S
B.t. 32/2	JAS	+	+		696^{N}	III	S
B.c. 01/4	JAS	+			681^{N}	III	S
B.c. 84/1	JAS	+			684^{N}	III	S
B.m. 100/1	JAS	+			672^{N}	III	CC647-672
B.m. 91/1	JAS	+			629^{N}	III	S
B.m. 76/1	JAS	+			716^{N}	III	CC650
B.t. 12/5	JAS	+	+		618^{N}	III	S
B.t. 58/5	JAS	+	+		618^{N}	III	S
B.t. 62/4	JAS		+		618^{N}	III	S
B.c. 80/5	JAS	+			683^{N}	III	S
B.t. 60/4	JAS	+	+		683^{N}	III	S
B.t. 83/2	JAS	+	+	+	683^{N}	III	S
B.t. 100/4	JAS	+	+		717^{N}	III	CC695
B.t. 41/1	JAS	+	+		620^{N}	III	S
B.t. 05/5	JAS	+	+		695^{N}	III	CC695
B.t. 21/4	JAS	+	+		695^{N}	III	CC695
B.t. 78/1	JAS	+	+	+	695^{N}	III	CC695
B.c. 06/3	JAS	+			617^{N}	III	S
B.c. 74/3	JAS	+			617^{N}	III	S
B.t. 98/4	JAS	+	+		196	III	S
B.m. 20/1	JAS	+		+	625^{N}	III	CC650
B.m. 23/1	JAS	+		+	625^{N}	III	CC650
B.m. 27/1	JAS	+		+	625^{N}	III	CC650
B.m. 39/1	JAS	+		+	625^{N}	III	CC650
B.m. 58/1	JAS	+		+	625^{N}	III	CC650
B.m. 81/1	JAS	+		+	625^{N}	III	CC650
B.m. 07/1	JAS	+			712^{N}	III	CC650
B.m. 30/1	JAS	+		+	712^{N}	III	CC650
B.c. 78/2	JAS	+			734^{N}	III	CC650
B.c. 30/3	JAS	+			735^{N}	III	CC650
B.c. 06/1	JAS	+		+	222	III	CC650
B.c. 15/2	JAS	+			222	III	CC650
B.m. 17/1	JAS	+		+	222	III	CC650

B.m. 48/1	JAS	+		+	222	III	CC650
B.m. 62/1	JAS	+		+	222	III	CC650
B.c. 23/1	JAS	+			727^{N}	III	CC650
B.c. 68/2	JAS	+			727^{N}	III	CC650
B.c. 76/3	JAS	+			698^{N}	III	CC650
B.c. 26/2	JAS	+			619^{N}	III	CC650
B.c. 84/4	JAS	+			650^{N}	III	CC650
B.c. 21/5	JAS	+			410	III	CC650
B.c. 85/1	JAS	+			410	III	CC650
B.c. 83/3	JAS				615^{N}	III	CC650
B.c. 39/1	JAS				697^{N}	III	CC650
B.c. 81/4	JAS	+			697^{N}	III	CC650
B.c. 86/1	JAS	+			697^{N}	III	CC650
B.c. 19/1	JAS	+			566	III	CC650
B.c. 21/1	JAS	+			566	III	CC650
B.m. 45/1	JAS	+		+	647^{N}	III	CC647-672
B.c. 94/5	JAS	+			726^{N}	III	S
B.m. 09/1	JAS	+		+	623^{N}	III	CC650
B.m. 35/1	JAS	+		+ (0.55)	627^{N}	III	CC650
B.m. 15/1	JAS	+		+ (0.76)	624^{N}	III	CC650
B.m. 41/1	JAS	+		+	624^{N}	III	CC650
B.m. 60/1	JAS	+		+	624^{N}	III	CC650
B.m. 82/1	JAS	+		+	624^{N}	III	CC650
B.m. 21/1	JAS	+		+	626^{N}	III	CC650
B.m. 72/1	JAS				628^{N}	IV	S
B.m. 51/1	JAS			+	685^{N}	IV	S
DSMZ 11821	DSMZ	+			447	III	N.D.
DSMZ 12442	DSMZ				83	IV	N.D.
WSBC 10364	WSBC	+			42	III	N.D.
HD1	BGSC		+	+ (N.D.)	10	II	N.D.
HD12	BGSC		+		23	II	N.D.
HD73	BGSC		+	+ (N.D.)	8	II	N.D.
HD868	BGSC	N.D.	N.D.	N.D.	104	I	N.D.
ATCC 10987	ATCC			+ (N.D.)	32	I	N.D.
ATCC 14579	ATCC			+ (ref.)	4	II	N.D.
ATCC 11778	ATCC	N.D.		N.D	34	II	N.D.

ATCC 6462	ATCC	+	N.D.	116	III	N.D.
Ames	-	N.D.		1	I	N.D.

[a] *B.c.*, *B. cereus*; *B.t.*, *B. thuringiensis*; *B.m.*, *B. mycoides*; *B.a.*, *B. anthracis*; *B.w.*, *B. weihenstephanensis*; DSMZ 11821, *B. weihenstephanensis* type strain; DSMZ 12442, *B. pseudomycoides* DSMZ 12442; WSBC 10364, *B. weihenstephanensis* WSBC 10364; HD1, *B. thuringiensis* HD1; HD12, *B. thuringiensis* HD12; HD73, *B. thuringiensis* HD73; HD868, *B. thuringiensis* HD868; ATCC 10987, *B. cereus* ATCC 10987; ATCC 14579, *B. cereus* ATCC 14579; ATCC 11778, *B. cereus* ATCC 11778; ATCC 6462, *B. mycoides* ATCC 6462; Ames, *B. anthracis* Ames.

[b] BNP, Białowieża National Park; BB, Biebrza National Park; JAS, the Jasienowka farm; DSMZ, German Collection of Microorganisms and Cell Cultures; WSBC, Weihenstephan *Bacillus* collection; BGSC, *Bacillus* Genetic Stock Center; ATCC, American Type Culture Collection.

[c] +, the growth at 7 ^{o}C was observed. The negative results were omitted.

[d] +, *cry1*; $+^{2}$, *cry2*; $+^{4}$, *cry4*. The negative results were omitted.

[e] +, the presence of the *cytK* gene tested in PCR. In parenthesis the relative expression calculated according to the method of Pfaffl [Pfaffl 2001]. The negative results were omitted.

[f] ST, sequencing type. New STs are indicated with a N index.

[g] The clades designation corresponds with Figure 5, and Figure 6.

[h] CC, clonal complexes; S, singleton. The CCs designation corresponds with Figure 3, Figure 4, Figure S1, and Figure S2.

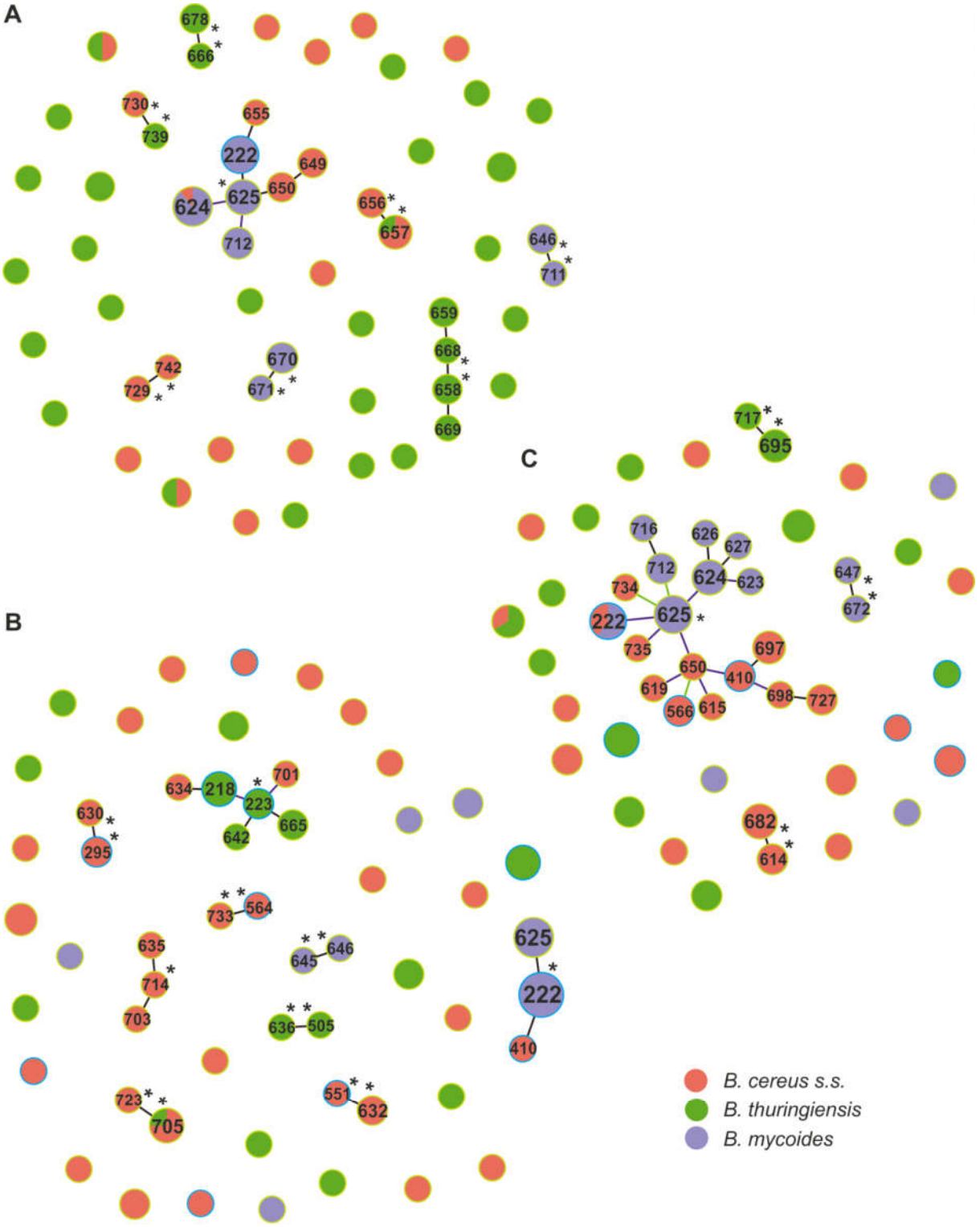

Figure S1. geoBURST analysis. The figures are showing the clonal assignment of the STs present in *B. cereus s.l.* originating from Białowieża National Park (A), Biebrza National Park (B), and the Jasienowka farm (C) in Northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.

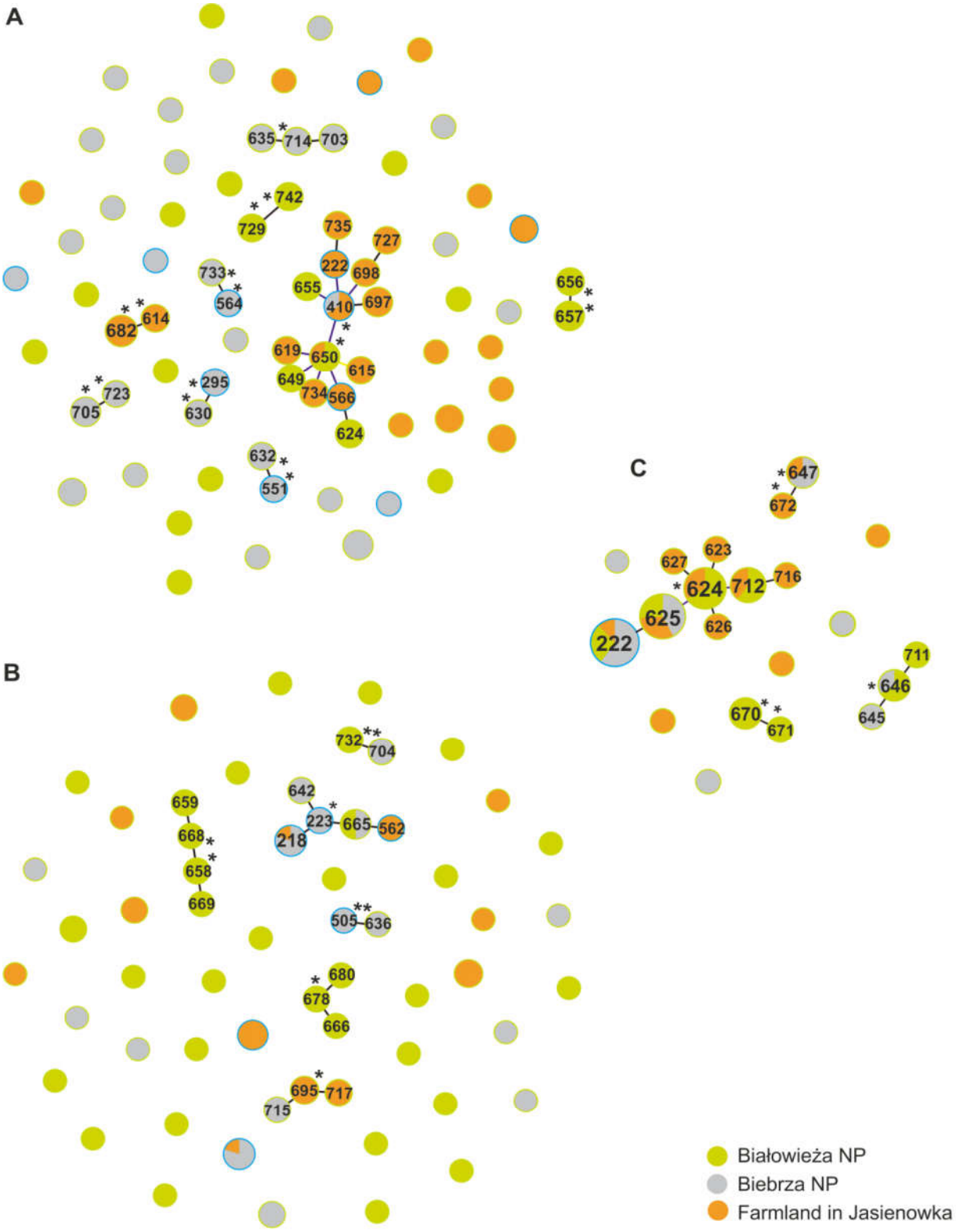

Figure S2. geoBURST analysis. The figures are showing the clonal assignment of the STs present in soil isolates of *B. cereus s.s.* (A), *B. thuringiensis* (B), and *B. mycoides* (C) originating from Northeastern Poland. The CCs are named based on the ST assigned as a founder genotype (marked with a star) of the complex. The relative size of the circles indicates their prevalence among the *B. cereus s.l.* isolates. New STs characterized in this study are accentuated by a green halo, while STs present in the MLST database are accentuated by a blue halo.

Appendix 3

Melanin-like pigment synthesis by soil *Bacillus weihenstephanensis* isolates from northeastern Poland

Drewnowska JM, Zambrzycka M, Kalska-Szostko B, Fiedoruk K, Swiecicka I (2015) Melanin like pigment synthesis by soil Bacillus weihenstephanensis isolates from northeastern Poland. PLoS ONE 10(4), e0125428.

My contribution: general concept of the work, experimental setup, laboratory work (selection of melanin-like pigment producers, biochemical and genetic characterization of melanin-positive strains, PFGE analysis, mechanisms of pigment production, next generation whole genome sequencing), data analysis, reagents contribution, manuscript preparation, proposal preparation and receiving of funding from National Science Centre (Preludium grant No UMO-2013/09/N/NZ8/03209).

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RESEARCH ARTICLE

Melanin-Like Pigment Synthesis by Soil *Bacillus weihenstephanensis* Isolates from Northeastern Poland

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Abstract

Although melanin is known for protecting living organisms from harmful physical and chemical factors, its synthesis is rarely observed among endospore-forming *Bacillus cereus sensu lato*. Here, for the first time, we reported that psychrotolerant *Bacillus weihenstephanensis* from Northeastern Poland can produce melanin-like pigment. We assessed physicochemical properties of the pigment and the mechanism of its synthesis in relation to *B. weihenstephanensis* genotypic and phenotypic characteristics. Electron paramagnetic resonance (EPR) spectroscopy displayed a stable free radical signal of the pigment from environmental isolates which are consistent with the commercial melanin. Fourier transform infrared spectroscopy (FT-IR) and physicochemical tests indicated the phenolic character of the pigment. Several biochemical tests showed that melanin-like pigment synthesis by *B. weihenstephanensis* was associated with laccase activity. The presence of the gene encoding laccase was confirmed by the next generation whole genome sequencing of one *B. weihenstephanensis* strain. Biochemical (API 20E and 50CHB tests) and genetic (Multi-locus Sequence Typing, 16S rRNA sequencing, and Pulsed-Field Gel Electrophoresis) characterization of the isolates revealed their close relation to the psychrotrophic *B. weihenstephanensis* DSMZ 11821 reference strain. The ability to synthesize melanin-like pigment by soil *B. weihenstephanensis* isolates and their psychrotrophic character seemed to be a local adaptation to a specific niche. Detailed genetic and biochemical analyses of melanin-positive environmental *B. weihenstephanensis* strains shed some light on the evolution and ecological adaptation of these bacteria. Moreover, our study raised new biotechnological possibilities for the use of water-soluble melanin-like pigment naturally produced by *B. weihenstephanensis* as an alternative to commercial non-soluble pigment.

collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Melanin is a heterogenous and polymeric pigment found in many Prokaryote and Eukaryote organisms. Melanin production has been considered to be of a great significance, especially with regard to microorganisms in which it has been often associated with virulence in the host and survival advantage in the environment [1]. For instance, melanin synthesized by free-living microorganisms absorbs a broad spectrum of electromagnetic radiation, from visible light to ionizing radiation [2] protects their producers from reactive oxygen forms [3], heavy metals toxicity [4], and extreme temperatures [5]. These properties of melanin make it an attractive biomaterial used as an ingredient in sunscreens [6], wool fabric dyes [7] and *Bacillus thuringiensis*-based biopesticides for UV protection [8]. Moreover, melanin protects pathogens against immune responses of a microbe's host [9], and appears to play an important role in the development of symbiosis between soil bacteria and plants [10]. In most organisms, melanin production starts with an enzymatic reaction of L-tyrosine via L-DOPA (L-3,4-dihydroxyphenylalanine) to DOPA-quinone, which involves enzymes such as tyrosinase or tyrosine hydroxylase [11, 12]. In some fungi, such as *Cryptococcus neoformans*, the conversion of L-DOPA to DOPA-quinone occurs with the participation of laccase [13]. Subsequently, a series of non-enzymatic reactions leads to the formation of black or brown eumelanin or orange-yellow pheomelanin [11–13]. The alternative pathway, involving degradation of L-tyrosine to homogentisic acid (HGA) which can polymerize to brown pyomelanin, also has been observed in some fungi and bacteria, such as *Aspergillus fumigatus*, *Legionella pneumophila*, *Pseudomonas aeruginosa* or *Vibrio cholerae* [14–16]. Moreover, dark green pigment synthetized by DHN- or HPQ-melanin pathway was characterized in fungi [17] and bacteria [18], respectively.

Bacillus weihenstephanensis is a psychrotrophic Gram-positive aerobic or facultative anaerobic bacterium [19], commonly present in food matrices [20] and soil [21]. This bacterium belongs to the *Bacillus cereus* group (*B. cereus sensu lato*) which includes seven other species commonly occurring in different environments and being extremely important for medical and economic reasons [21, 22]. The most extensively studied are (i) *Bacillus anthracis*, etiologic agent of anthrax [23], (ii) *Bacillus cereus*, an opportunistic pathogen occasionally involved in foodborne illnesses [24], and (iii) *B. thuringiensis*, entomopathogen universally used as a biopesticide [25]. Much less is known about other members of the group, *Bacillus mycoides* and *Bacillus pseudomycoides*, characterized by rhizoidal growth on solid media, antifungal activity and stimulation of plants' growth [26]. *B. cereus s.l.* also contains recently described thermotolerant *Bacillus cytotoxicus* [27] and *Bacillus toyonensis*, a probiotic organisms used in animal feed [28]. Although it has been established that the *B. cereus* group members are versatile producers of secondary metabolites, such as antimicrobial substances [29], extracellular enzymes [30] or fluorescent pigments [31], little is known about strains synthesizing melanin. Only two melanin-positive wild strains, *B. thuringiensis* subsp. *dendrolimus* L-7601 [32] and *B. thuringiensis* subsp. *kurstaki* CCTCC AB90010 [33], have been described. However, melanin producers among *B. weihenstephanensis* have not been reported so far. In several studies blackish-brown pigment production in *B. cereus s.l.* has been obtained through chemical mutagenesis [34] or genetic modification [35]. Nevertheless, the mechanism of melanin production among these species is poorly understood.

In this work, for the first time, we report the natural production of water-soluble melanin-like pigment by soil *B. weihenstephanensis* isolates from Northeastern Poland. We also assessed physicochemical properties of melanin-like pigment synthesized by these bacilli and proposed the mechanism of its synthesis. The unique properties of environmental strains prompted us to

conduct phenotypic, genotypic and phylogenetic analysis, which gave insight into their evolution.

Materials and Methods

Bacterial strains

A collection of about 950 *B. cereus s.l.* isolates from soil, arthropods, and foodstuffs. Soil samples were obtained from Bialowieza National Park (N 52° 72', E 23° 84'), Biebrza National Park (N 53° 36', E 22° 56'), and agricultural land in Jasienowka (N 52° 30', E 22° 58), Northeastern Poland. All samples from the parks were collected with consent according to Nature Conservation Act adopted on 16 April, 2004 by Polish Parliament (Parliament Diary 2004, No. 92: 880). The owner of the farm also permitted collection of soil samples for our study. The strains were isolated during previous studies [20, 36, 37]. The field studies did not involve endangered or protected species.

The isolates were screened by culturing on different media as Luria-Bertani (LB) agar, LB broth, nutrient agar, nutrient broth, and sporulation medium T3 (0.3% tryptone, 0.2% tryptose, 0.15% yeast extract, 0.05 M sodium phosphate pH 6.8, 0.0005% $MgCl_2$) in order to select producers of a blackish-brown pigment diffusing into the medium.The collection included bacilli identified in previous studies as *B. cereus*, *B. thuringiensis*, *B. mycoides/B. pseudomycoides*, and *B. weihenstephanensis*. *B. cereus* ATCC 10987 and ATCC 14579 (American Type Culture Collection), *B. thuringiensis* HD1, HD73, HD567, and HD867 (*Bacillus* Genetic Stock Center, Ohio State University), and *B. weihenstephanensis* DSMZ 11821 (German Collection of Microorganisms and Cell Cultures), were used as references during phenotypic and genotypic tests of the melanin-positive isolates tested in this study.

Psychrotolerance of pigment producers

The blackish-brown pigment-positive isolates were tested for the ability to grow at 7°C in LB broth and on nutrient agar plates. Then their psychrotrophic potential was confirmed in PCR by investigating the presence of specific signatures of *cspA* and 16S rDNA genes [19] tested with primers designed by Bartoszewicz et al. [20] and Lechner et al. [19], respectively. PCRs were carried out in the Veriti 96-Well thermal cycler (Applied Biosystems, Foster City, USA), followed by the analysis of amplicons in the QIAxcel capillary electrophoresis system (Qiagen GmbH, Hilden, Germany). Genes were sequenced using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and automated sequencer ABI3500 (Applied Biosystems). A phylogenetic tree of 16S rDNA partial homologs (1463 bp) was constructed with MEGA6 program using the Neighbor-Joining (NJ) method with the branch quality evaluated including 1,000 replicates bootstrap test [38].

Biochemical characterization

Biochemical properties of isolates and references were verified with API 50CHB and API 20E system (bio-Mérieux S.A., Mercy l'Etoile, France), according to the manufacturer's procedure and as recommended by Swiecicka and de Vos [39]. Biochemical similarity was calculated using the simple matching coefficient (SMC) and clustered with unweighted pair-group average linkage algorithm (UPGMA) performed with the NTSys ver. 2.02 g program (Exeter Software, E. Setauket, NY, USA) as described previously [39].

Phylogeny based on multi-locus sequence typing (MLST)

Nucleotide sequences of seven housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, *tpi*) deposited in the MLST database (http://pubmlst.org/bcereus/) during previous studies [37] and corresponding sequences of reference strains also available in the database, were used to construct a phylogenetic tree with MEGA6 program using the Neighbor-Joining (NJ) method. The branch quality was evaluated using 1,000 replicates bootstrap test [38].

Pulsed-field gel electrophoresis of genomic DNA

Overnight cultures of strains were grown in LB and centrifuged at 4°C and 2,500 x g for 15 min and resuspended in SE buffer (10 mM NaCl, 30 mM EDTA, pH 7.5) to an OD_{590} of approximately 2.0. Genomic DNA plugs were prepared by mixing bacterial suspension with 2% LMP agarose (Sigma-Aldrich, Poznan, Poland) at 1:1 ratio and placed into slots of a plug mold (Bio-Rad, Hercules, CA, USA). After solidification, plugs were treated according to Gaviria Rivera and Priest [40], DNA was digested with 30 U of NotI restrictase (MBI Fermentas, Vilnius, Lithuania) and electrophoresed in the CHEF-MAPPER System (Bio-Rad) following the protocol of Swiecicka et al. [41]. PFG Lambda Ladders and PFG Yeast chromosomes from New England BioLabs were used as markers. Gels were stained with ethidium bromide solution (1 μg ml^{-1}) and visualized in ChemiDOC XRS System (Bio-Rad).

Extraction, purification, and chemical characteristics of melanin-like pigment

The pigment was isolated from the four most productive strains: JAS 39/1, JAS 81/4, JAS 83/3, and JAS 86/1. Isolates were inoculated into 200 ml of nutrient broth and incubated at 30°C on rotary shaker at 180 rpm for 96 h until the liquid medium became dark-brown. When the cell mass was removed, the supernatant was acidified by lowering a pH to 2.0 using 1N HCl and incubated at room temperature for one week, followed by boiling of the suspension for 1 h and centrifuging at 14,000 x g for 15 min. The pigment pellet was washed with ethanol as described by Sajjan et al. [42]. The modified method of Fava et al. [43] was used to perform chemical analyses of the extracted melanin-like pigment. The pigment solubility was checked in deionized water, 1 N NaOH, 1 N HCl, acetone, benzene, chloroform, ethanol, and phenol. In addition, the reactions of melanin with 30% hydrogen peroxide (H_2O_2), 1% iron (III) chloride ($FeCl_{3)}$, and 5% sodium hydrosulfite ($Na_2S_2O_4$), were tested. Synthetic melanin (Sigma-Aldrich, Cat. No M8631) was used as a reference.

Electron paramagnetic resonance (EPR) measurement

The electron paramagnetic resonance measurement for the tested melanin-like pigments and synthetic melanin were performed with the X-band (9,3 GHz) EPR spectrometer (Radiopan, Poznan, Poland) and the Rapid Scan Unit (Jagmar, Krakow, Poland). Each sample was placed in a thin-walled glass tube free of paramagnetic impurities. Microwave frequency was obtained by the MCM101 Recorder (Eprad, Poznan, Poland) at magnetic modulation of 100 kHz. The total microwave power of the klystron was 70 mW. The numerical acquisitions of the first-derivative EPR spectra were done at low microwave power of 11 mW. The spectroscopic programs SWAMP (Jagmar) and LabVIEW 8.5 (National Instruments) were used.

Fourier transform infrared (FT-IR) spectroscopy

To quantify the extracted melanin-like pigment, IR spectra were recorded by Nicolet 6700 infrared spectrometer (Thermo Fisher Scientific, Warsaw, Poland). A small amount of pigment

was placed on a diamond window of the spectrometer, and a measurement was done in reflection mode, at a room temperature, by summary of 32 scans with a resolution of 4 cm^{-1}. The available spectra range was 400–4000 cm^{-1}. Synthetic melanin (Sigma-Aldrich, Cat. No M8631) was used as a standard.

Mechanisms of pigment production

LB was inoculated with melanin-positive *B. cereus s.l.* isolates and incubated at 30°C on a rotary shaker at 180 rpm for 24 h. After the preincubation, arginine [44] or kojic acid [45] as tyrosinase inhibitors were added to a final concentration of 0.01–0.5 mM and 10–100μg/ml, respectively. As a laccase inhibitor, sodium azide was added to a final concentration of 0.01–0.2 mM [46]. In addition, the sulcotrione [16] and tricyclazole [45] were used for 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) and hydroxynaphthalene reductase inhibition, respectively, to confirm/exclude the HGA or DHN pathways of melanin synthesis. Then, cultures were incubated at 30°C on the rotary shaker at 180 rpm for 96 h till the dark color occurred in the control culture.

Next generation whole genome sequencing

Genomic DNA of *B. cereus* strain JAS 83/3, one of the most melanin-productive isolate, was extracted in the QIAcube automat (Qiagen) using the DNeasy Blood and Tissue Kit (Qiagen) with a protocol for Gram-positive bacteria. The draft genome sequence of the strain was determined using an Ion Torrent PGM sequencer (Applied Biosystems) using the Ion 316 chip with 200-bp shotgun sequencing, according to the manufacturer's instructions. The annotation was performed using best-placed reference protein set GeneMarkS+ provided by the National Center for Biotechnology Information (NCBI).

GenBank accession numbers

The whole sequence of *B. weihenstephanensis* JAS 83/3 obtained by shotgun sequencing is under Accession Number: JNLY00000000 (BioProject: *Genome sequencing of melanin-positive Bacillus weihenstephanensis strain* JAS 83/3; NCBI; PRJNA246580). We also deposited three 16S rRNA homologs of *B. weihenstephanensis* JAS 83/3 (KP006648), *B. thuringiensis* HD567 (KP006649), and *B. thuringiensis* HD867 (KP006650). Remaining sequences of 16S rRNA were available under accession number: NR_024697 (*B. weihenstephanensis* DSMZ 11821), BTK_r29390 (*B. thuringiensis* HD1), HD73_r36 (*B. thuringiensis* HD73), BC0007 (*B. cereus* ATCC 14579), and BCE5759 (*B. cereus* ATCC 10987).

Results

Melanin-like pigment synthesis among *B. cereus s.l.* from Northeastern Poland is very rare and is restricted to *B. weihenstephanensis*

In order to study the production of melanin-like pigment by *B. cereus s.l.*, a large collection of about 950 strains identified as *B. cereus*, *B. thuringiensis*, *B. mycoides/B. pseudomycoides*, and *B. weihenstephanensis*, was screened by culturing on different growth media (LB, nutrient agar, T3 agar, LB and nutrient broths) and observed for the blackish-brown pigment diffused in the medium. Although the origins of the strains were diverse (soil, arthropods, mammals, and foodstuffs), the pigment-positive bacilli were found only among six *B. weihenstephanensis* isolates from soil samples obtained from Jasienowka farm (JAS 39/1, JAS 81/4, JAS 83/3, JAS 86/1) and Bialowieza National Park (BPN 08/1, BPN 08/4) in Northeastern Poland. However, the production of pigment by isolates from the park was low and observed only on NA plates. In

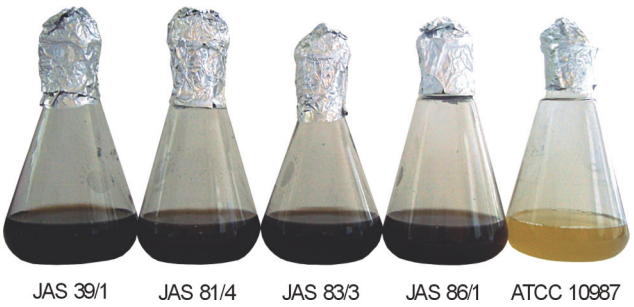

Fig 1. Melanin-like pigment production in Luria-Bertoni broth by *B. weihenstephanensis*. *B. cereus* ATCC 10987 was used as the negative control.

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contrast, the farm isolates synthesized the pigment at high quantity both on NA plates and in LB and nutrient broths (Fig 1). These bacilli grew well at 7°C. Their psychrotrophic adaptation was confirmed by the presence of unique nucleotide motifs: (i) ^{4}ACAGTT^{9} in the *cspA* gene encoding the major cold shock protein, and (ii) ^{1002}TCTAGAGATAGA^{1013} in the 16S rDNA (S1 Table).

Biochemical properties of pigment-positive and reference strains were tested using 60 different tests available with the API system (see summary in S1 Table). In short, all environmental isolates produced acetoin, liquefied gelatin and fermented 11 of 49 carbohydrates. Variable results were received for acid production from cellobiose and sucrose as well as for arginine dihydrolysis. Biochemical profiles of reference strains used in the study were similar to these found for the isolates (S1 Table). A dendrogram based on biochemical properties and calculated using the simple matching coefficient and UPGMA algorithm (Fig 2A), showed high similarity between soil isolates and reference *B. weihenstephanensis* DSMZ 11821. *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. thuringiensis* HD1, *B. thuringiensis* HD73, and *B. thuringiensis* HD867 reference strains grouped in a second cluster. *B. thuringiensis* HD567, known for anti-mosquito properties [47], differed biochemically from other strains and made cluster III.

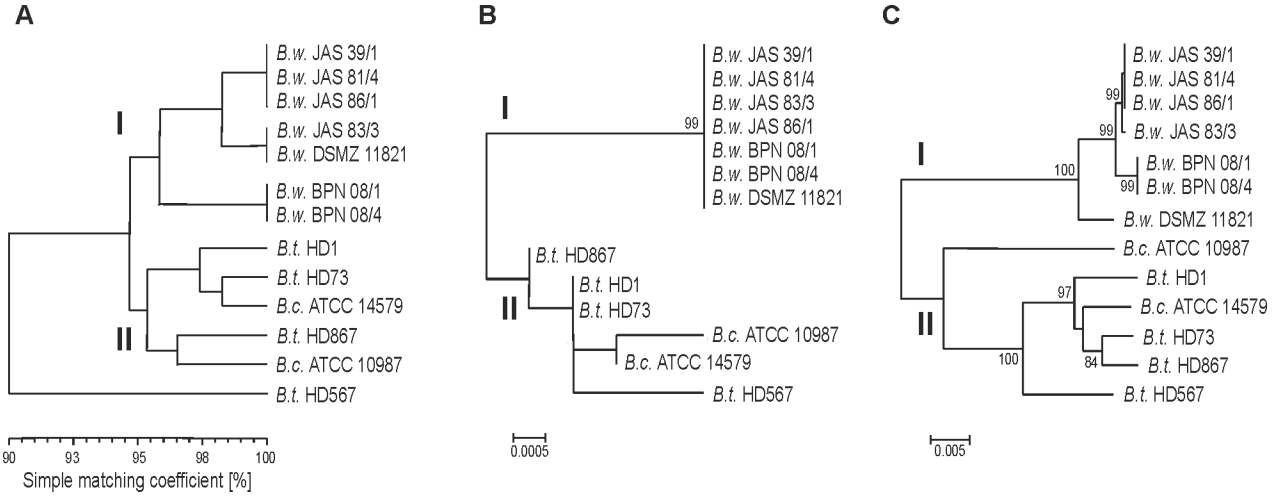

Fig 2. The phenotypic similarity and phylogeny among *B. weihenstephanensis* producing melanin-like pigment and reference strains. Comparisons between strains based on biochemical API 50CH and API 20E tests were made using simple matching coefficient and clustered with the UPGMA algorithm (A). Phylogenetic trees were constructed based on 16S rDNA gene (B) and seven concatenated housekeeping loci (MLST) (C) using the Neighbor-Joining (NJ) method implemented in MEGA6 software, where branch quality was evaluated using 1,000 replicates bootstraps.

doi:10.1371/journal.pone.0125428.g002

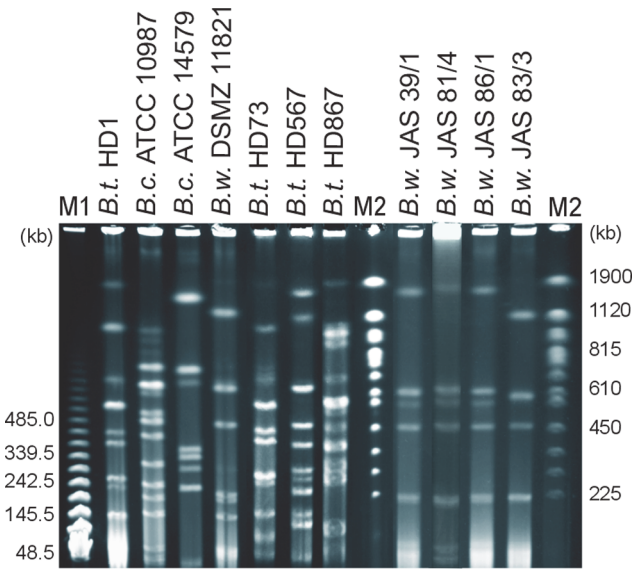

Fig 3. PFGE fingerprints of melanin-positive *B. weihenstephanensis* isolates and reference strains. Genomic DNA was digested using NotI. M1, PFG Lambda Ladders; M2, PFG Yeast chromosomes. The values on the left and right are molecular weight marker in kb.

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Comparative analysis of 16S rRNA confirmed close relation between melanin-positive isolates and *B. weihenstephanensis* DSMZ 11821 (Fig 2B). On the phylogenetic tree, isolates grouped together with DSMZ 11821, while other references gathered in the second cluster. Phylogeny based on multi-locus sequence typing also revealed two genetic clusters in the Neighbour-Joining dendrogram (Fig 2C). Soil isolates grouped in cluster I with DSMZ 11821, whereas other references were classified into cluster II. Pulsed-field gel electrophoresis (PFGE) confirmed the clonality of *B. weihenstephanensis* JAS 39/1, JAS 81/4 and JAS 86/1 (Fig 3), while *B. weihenstephanensis* BPN 08/1 and BPN 08/4 were not typeable by PFGE (profiles has not been achieved). Pulsed-field fingerprints of isolates do not overlap with PFGE profiles of reference strains.

Melanin-like pigment synthesized by soil *B. weihenstephanensis* isolates had phenolic character

EPR spectra of natural pigment produced by environmental strains of *B. weihenstephanensis* from the farm (isolates from Bialowieza National Park did not produce enough pigment to analyse) were similar to the EPR signal of the synthetic melanin and all revealed broad curves around 335 mT (Fig 4A). In addition, FT-IR spectroscopy of dark particles demonstrated similar spectra to synthetic one (Fig 4B). A broad band centered around 3268–3278 cm^{-1} for each studied pigment was observed, which is associated with-OH stretching. Furthermore, all samples had absorbance peaks in the 1511–1729 cm^{-1} area, due to the bonding vibration of C = C and C = O aromatic ring stretching, and double bonds in COOH. In contrast to standard melanin, *in vitro* synthesized melanin had signals in 2926–2970 cm^{-1} area indicating the presence of saturated carbon, as well as around 1045 cm^{-1} and 1220 cm^{-1}, what corresponds to carbonyl, alcoholic or phenolic groups, respectively. Detailed information on functional groups found in melanin-like pigment synthesized by environmental isolates and synthetic melanin is given in Table 1.

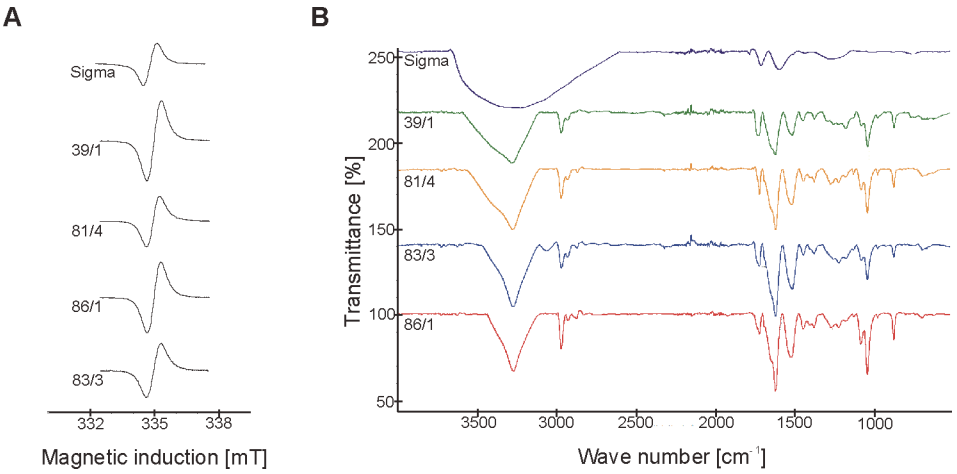

Fig 4. EPR (A) and FT-IR (B) spectra of commercial melanin and pigment obtained from *B. weihenstephanensis* isolates.

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Chemical properties of melanin-like pigment produced by soil *B. weihenstephanensis* isolates and of synthetic melanin were also similar (S2 Table). Pigments were soluble in alkaline solution (1N NaOH) and phenol, but were insoluble in ethanol, acetone, chloroform, and benzene. Moreover, dissolved pigments and synthetic melanin precipitated in hydrochloric acid (1N HCl) and ferric chloride (1% $FeCl_3$), and were decolorized by hydrogen peroxide (30% H_2O_2), as well as by sodium hydrosulfite (5% $Na_2S_2O_4$). The only feature which differed

Table 1. Functional groups of melanin-like pigment produced by soil *B. weihenstephanensis* isolates and commercial melanin obtained from Fourier Transform Infrared spectroscopy.

Assignment	Wave number [cm^{-1}]					Reference
	Bw JAS 39/1	*Bw* JAS 81/4	*Bw* JAS 83/3	*Bw* JAS 86/1	Commercial melanin	
-OH, stretching	3274	3278	3268	3271		[48]
Aliphatic-CH, stretching	2967	2970	3069	2964		[49]
			2964	2929		
			2926			
-COOH	1729	1723	1726	1723	1707	[49]
Aromatic ring C = C and C = O, stretching	1622	1615	1622	1619	1603	[49]
-COOH	1511	1517	1511	1524		[49]
C-H, bonding	1448	1451	1448	1448	1438	[48]
-COO, symmetric stretching	1375	1378	1375	1375		[50]
C-H, deformation	1264	1277		1277	1283	[51]
C = O	1178	1220	1220			[49]
C-O, close to aromatic ring	1042	1045	1045	1045		[51]
N-H, O-H, bending	874	874	874	871	757	[48]

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natural pigment and synthetic melanin was the solubility in water, observed only for pigments from *B. weihenstephanensis*.

Next generation whole genome sequencing and a set of biochemical tests indicate that melanin-like pigment synthesis by *B. weihenstephanensis* could be associated with the laccase activity

Shotgun sequencing of *B. weihenstephanensis* strain JAS 83/3 was performed to identify genes associated with melanin-like pigment synthesis. We found genes encoding enzymes potentially involved in the synthesis of melanin such as: laccase, phenylalanine 4-monooxygenase, pterin-4-alpha-carbinolamine dehydratase, aromatic amino acid aminotransferase, and 4-hydroxyphenylpyruvate dioxygenase. It is worth adding that JAS 83/3 genome consisted of 5,902,407 bp and contained 5,362 coding sequences (CDSs), 347 pseudogenes, and 99 RNA operons (seven rRNA, 91 tRNA and one ncRNA).

In a set of biochemical tests, no inhibitory effect on the melanin-like pigment synthesis by environmental *B. weihenstephanensis* isolates was observed after supplementation of bacterial culture with arginine and kojic acid (tyrosinase inhibitors), sulcotrione (an inhibitor of 4-hydroxyphenylpyruvate dioxygenase in the HGA pathway) and tricyclazole (an inhibitor of hydroxynaphthalene reductase in the DHN pathway). On the other hand, the addition of 0.1 mM sodium azide, which inhibits laccase, resulted in the inhibition of melanin-like pigment production, indicating that laccase is a key enzyme in the pigment synthesis among *B. weihenstephanensis* strains. The addition of higher concentrations of sodium azide resulted in inhibition of bacterial growth. The effects of tested chemicals on melanin-like pigment production in isolates are presented in Fig 5.

Discussion

Although many microorganisms have been known to synthesize melanin [11–15], *B. cereus s.l.* species able to produce this pigment are extremely rare in nature [32, 33]. In this study we found only six *B. weihenstephanensis* melanin-positive isolates within a *B. cereus s.l.* collection of about 950 strains of different origin. However, only four strains originated from Jasienowka farm were able to produce melanin-like pigment in/on different media steadily when the experiments were conducted. Chemicals used in agriculture, such as mineral fertilizers or plant protection products, change bacterial communities [52] and may induce mutation(s) altering biochemical pathways associated with melanin synthesis in microorganisms [34, 53]. Melanin producers from Northeastern Poland showed phenotypic and genotypic similarity with psychrotolerant *B. weihenstephanensis* DSMZ 11821 reference strain [19]. Within *B. cereus* group, the adaptation to low temperatures was regarded to be a characteristic of *B. weihenstephanensis* [19]. However, recent studies have indicated that psychrotolerance is also present among other members of the *B. cereus* group isolated from Northeastern Poland, one of the coldest region in Poland (Institute of Meteorology and Water Management in Poland, http://www.imgw.pl/klimat/), and revealed the existence of thermal ecotype among soil isolates [37, 41]. *B. weihenstephanensis* strains able to grow at low temperatures seem to be perfectly adapted to the habitat they occupy in Northeastern Poland.

The cluster analysis based on biochemical properties, 16S rRNA and housekeeping genes sequences indicated a close relationship among melanin-like pigment producers. A previous study showed that the community of soil *B. cereus s.l.* strains from Northeastern Poland was genetically diverse, and this diversity mostly resulted from mutation events [37]. Belotte et al. [54] noted that environmental heterogeneity, selectable genetic variation and divergent selection were prerequisites for the emergence of local adaptation. Thus, high similarity of melanin-

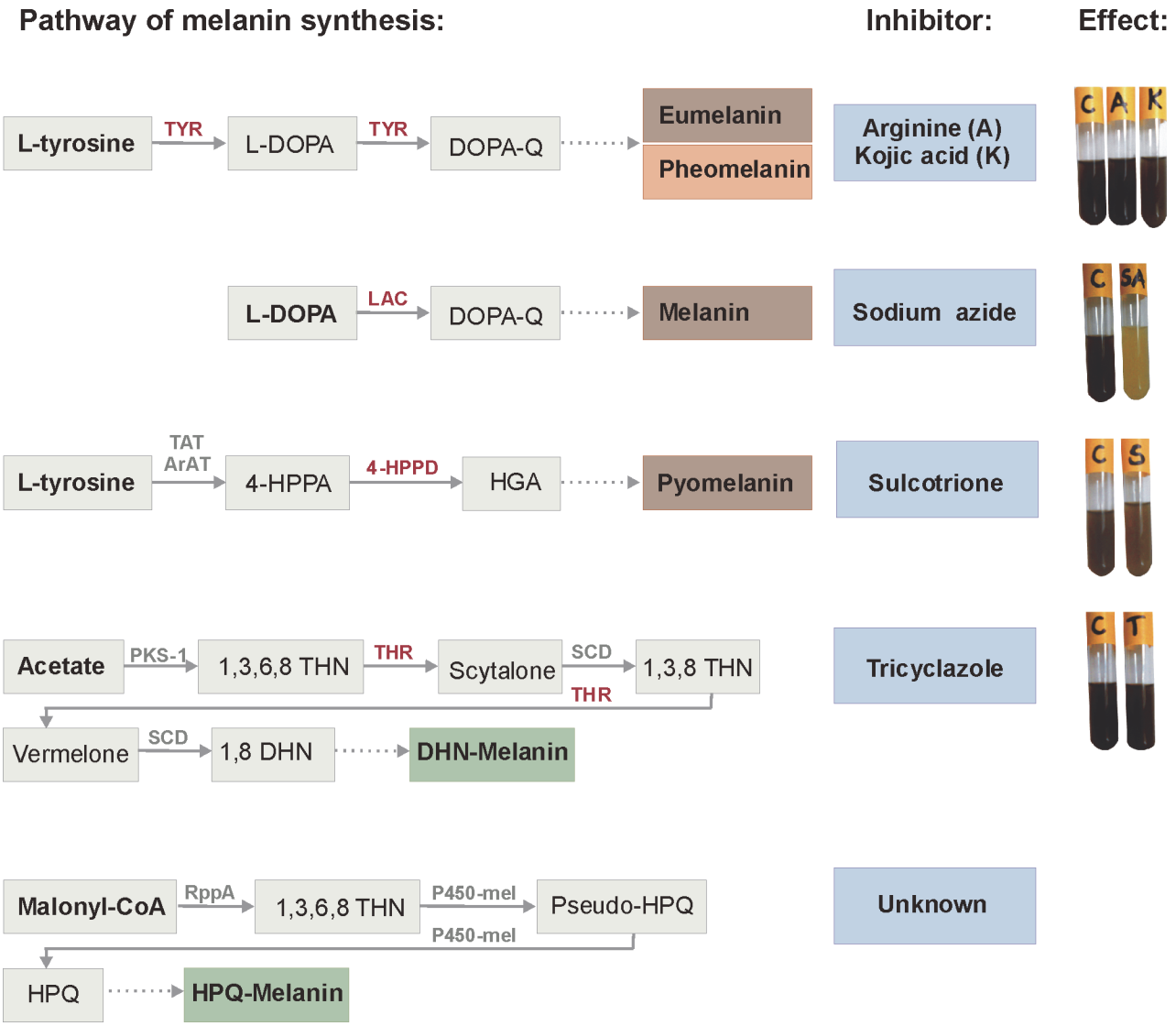

Fig 5. Putative pathways of melanin synthesis and the inhibition tests for pigment production by *B. weihenstephanensis* isolates. TYR, tyrosinase (EC 1.14.18.1); L-DOPA, L-3,4-dihydroxyphenylalanine; DOPA-Q, Dopa-quinone; LAC, laccase (EC 1.10.3.2); TAT, tyrosine aminotransferase (EC 2.6.1.5); ArAT, aromatic amino acid aminotransferase (EC 2.6.1.57); 4-HPPA, 4-hydroxyphenylpyruvic acid; 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27); HGA, homogentisic acid; PKS-1, polyketide synthase type I; 1,3,6,8 THN, 1,3,6,8-tetrahydroxynaphthalene; THR, Hydroxynaphthalene reductase; SCD, scytalone dehydratase (EC 4.2.1.94); 1,3,8 THN, 1,3,8-trihydroxynaphthalene; 1,8 DHN, 1,8-dihydroxynaphthalene; DHN-Melanin, dihydroxynaphthalene melanin; RppA, polyketide synthase type III; P450-mel, cytochrome P-450 enzyme; HPQ, 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone; HPQ-Melanin, hexahydroxyperylenequinone melanin; C, control. The dotted line represents the non-enzymatic reactions (oxidation and/or polymerization). Abbreviations of inhibited enzymes are marked in red color.

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positive strains derived from genetically polymorphic population indicated that melanogenesis among soil *B. weihenstephanensis* might be a local adaptation to specific environmental niches which protects these bacteria from adverse environmental factors, such as UV light [2] extreme temperatures [5] or heavy metals [55]. Similarly, Wang et al. [53] also observed high clonality among natural melanin-producing *Vibrio cholerae* strains and suggested an environmental protective function of melanin in the *V. cholerae* community. This raised a question, why bacteria able to produce melanin are very rare in natural environments? We assumed that

melanin-like pigment synthesis among *B. weihenstephanensis* entails a fitness cost due to the energy expensive pathway involving laccase and/or polymerization of the pigment. Yet, the benefit of extracellular melanin-like pigment production could be shared with co-occurring bacteria which do not synthesize the pigment. Such a cooperation of bacteria to reduce production costs were observed for entomopathogenic toxin synthesis by *B. thuringiensis* [56], a close relative of *B. weihenstephanensis* [19, 21].

The presence of stable free radicals in all melanin pigments allowed the identification of the pigment as melanin using EPR spectroscopy. The similar shapes of EPR spectra of natural dark particles and the synthetic melanin confirmed the production of melanin pigment by environmental *B. weihenstephanensis* isolates. It was shown that o-Semiquinone free radicals (S = 1/2) with unpaired electrons localized on oxygen atoms were responsible for these melanin spectra [57]. Moreover, the typical broadening of melanin spectral curves was caused by dipolar interactions of free radicals in these polymers [58]. FT-IR spectroscopy, which gives precise information on main functional groups of an organic compound, also revealed similar spectra for "environmental" melanin-like pigment produced by soil *B. weihenstephanensis* isolates and synthetic melanin, indicating their phenolic character. Some differences in FT-IR spectra of environmental pigment and commercial melanin could result from the procedure of pigment extraction and purification, e.g. the usage of ethanol. According to the actual definition, melanin is a dark in color substance, insoluble in aqueous or organic solvents, resistant to concentrated acid and susceptible to bleaching by oxidizing agents [1]. Physicochemical properties of *B. weihenstephanensis* melanin-like pigment and the synthetic one are comparable. It is worth emphasizing that the precipitation with ferric chloride, decolorization by hydrogen peroxide and infrared spectra, indicated the phenolic nature of melanin-like pigments synthesized by environmental *B. weihenstephanensis*. The solubility in water of "environmental" melanin was the only difference. Similar results, including also melanin-like pigment water-solubility, were observed by Aghajanyan et al. [34] for *B. thuringiensis* subsp. *galleriae* strain K1 obtained by chemical mutagenesis from industrial bioinsecticide *B. thuringiensis* 69–6. However, in general melanin produced by bacteria was water-insoluble [32, 59]. Certainly, water-soluble melanin-like pigment as this produced by our *B. weihenstephanensis* isolates might have broader biotechnological applications in comparison to the insoluble pigment.

In microbial melanogenesis the most important enzyme seems to be tyrosinase, a monooxygenase which binds two copper ions within the active site by three conserved histidines residues [12]. Indeed, Liu et al. [33] identified the heat-inducible tyrosinase responsible for the pigment production by wild *B. thuringiensis* CCTCC AB 90010; however, we did not identify the gene encoding tyrosinase in *B. weihenstephanensis* JAS 83/3 genome. Nevertheless, there are several other enzymes potentially involved in melanin production, such as laccase or polyketide synthases [12, 13, 17]. Also some species produce melanin in a way of homogentisic acid polymerization [14, 53], yet little is known about these mechanisms among *B. cereus s.l.* Here, for the first time, we pointed out that laccase might be involved in the production of melanin-like pigment among strains belonging to the *B. cereus* group. While laccase is widely distributed in fungi and plants, in which it can be involved in melanin formation, lignolysis or detoxification, the activity of this enzyme in bacteria has been rarely documented [60, 61]. Laccase enzyme possesses three conservative histidine residues and it has a similar function to tyrosinase, but at the same time, it is activated under different conditions in different species [12]. In fact, the metabolic pathway of melanin synthesis with the participation of laccase is poorly understood and requires further investigation.

Conclusions

Only a limited number of *B. weihenstephanensis* isolates naturally produce melanin-like pigment. This process is probably associated with laccase activity. *B. weihenstephanensis* pigment producers from Northeastern Poland demonstrated a high level of phenotypic and genotypic similarity. Our study shed new light on the evolution and adaptation of *B. weihenstephanensis* to specific habitats. Because of possible applications, water-soluble melanin-like pigment produced by *B. weihenstephanensis* might be an alternative to commercial pigment, thus, it is worth further investigation.

Supporting Information

S1 Table. Phenotypic and genotypic characteristic of melanin-positive *Bacillus weihenstephanensis* isolates and the reference strains.
(DOCX)

S2 Table. Chemical properties of melanin-like pigment synthetized by soil *B. weihenstephanensis* isolates in comparison to reference melanin.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JMD IS. Performed the experiments: JMD MZ BKS KF. Analyzed the data: JMD IS. Contributed reagents/materials/analysis tools: JMD IS. Wrote the paper: JMD IS.

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Supporting Information Table S1. Phenotypic and genotypic characteristic of melanin-positive *Bacillus weihenstephanensis* isolates and the reference strains.

Feature[a]	**Strain**[b]												
	B.w. JAS 39-1	*B.w.* JAS 81-4	*B.w.* JAS 83-3	*B.w.* JAS 86-1	*B.w.* BPN 08-1	*B.w.* BPN 08-4	*B.w.* DSM 11821	*B.t.* HD1	*B.t.* HD73	*B.t.* HD567	*B.t.* HD867	*B.c.* ATCC 10987	*B.c.* ATCC 14579
Growth at:													
7°C	+	+	+	+	+	+	+						
30°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Nucleotide motif in the *cspA* gene:													
Psychrophilic ^{4}ACAGTT^{9}	+	+	+	+	+	+	+						
Mesophilic ^{4}GCAGTA^{9}								+	+	+	+	+	+
Nucleotide motif in the 16S rDNA gene:													
Psychrophilic ^{1002}TCTAGAGATAGA^{1013}	+	+	+	+	+	+	+						
Mesophilic ^{1002}CCTAGAGATAGG^{1013}								+	+	+	+	+	+

Parasporal crystal presence								+	+	+	+		
Fermentation of[c]:													
Glycerol								+		+	+		
D-mannose										+	+	+	
Arbutin	+	+	+	+	+	+	+	+	+		+	+	+
Salicin	+	+	+	+	+	+	+	+	+		+	+	+
Cellobiose					+	+		+	+		+	+	+
Sucrose	+	+		+	+	+					+	+	+
Arginine dihydrolysis					+	+							
Liquefaction of gelatin	+	+	+	+	+	+	+					+	

[a] For clarity negative results are omitted.

[b] JAS, isolates from a farmland in Jasienowka in Northeastern Poland; BPN, isolates from Białowieża National Park; *B.w.*, *B. weihenstephanensis*; *B.t.*, *B. thuringiensis*; *B.c.*, *B. cereus*; *B.w.* DSMZ 11821, *B. weihenstephanensis* DSMZ 11821 type strain (German Collection of Microorganisms and Cell Cultures); *B.t.* HD1, *B. thuringiensis* HD1 (*Bacillus* Genetic Stock Center, Ohio State University, Columbus, USA, BGSC); *B.t.* HD73, *B. thuringiensis* HD73 (BGSC); *B.t.* HD567, *B. thuringiensis* HD567 (BGSC); *B.t.* HD867, *B. thuringiensis* HD867 (BGSC); *B.c.* ATCC 10987, *B. cereus* ATCC 10987 (American Type Culture Collection); ATCC 14579, *B. cereus* ATCC 14579 (American Type Culture Collection);

[c] Only variable results are given. All isolates and the reference strains fermented: ribose, D-glucose, D-fructose, N-acetyloglucosamine, aesculin, maltose, trehalose, glycogen, starch, and gave negative results in the VP test. All isolates and the reference strains were negative for fermentation of: erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl-D-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α methyl-D-mannoside, α methyl-D-glucoside, amygdalin, lactose, melibiose, inuline, melezitose, D-raffinose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate. In addition the bacilli under study were negative in the following tests: presence of ß-galactosidase (test ONPG), lysine decarboxylation, ornithine decarboxylation, citrate utilization, H_2S production, presence of urease, tryptophane deamination, and indole production.

Supporting Information Table S2. Chemical properties of melanin-like pigment synthetized by soil *Bacillus weihenstephanensis* isolates in comparison to reference melanin.

Test	**Melanin**	
	Produced by *Bacillus weihenstephanensis* isolates	**Comercial**
Color	Blackish-brown	Blackish-brown
Solubility in:		
Water	+	-
1 N NaOH	+	+
Ethanol	-	-
Acetone	-	-
Chloroform	-	-
Benzene	-	-
Phenol	+	+
Precipitation:		
1N HCl	+	+
1% $FeCl_3$	+	+
Decolorization in:		
30% H_2O_2	+	+
5% $Na_2S_2O_4$	+	+

Summary

In my doctoral thesis I have undertaken large-scale phylogenetic and genetic structure studies on *B. cereus s.l.* isolates in order to shed some light on the evolution and ecological speciation of this group of bacteria. As it has been proven that the diversity of habitats has a significant impact on the bacterial divergence [Zwick *et al.,* 2012], I tested 297 bacilli (*B. cereus/B. weihenstephanensis*, n=102; *B. thuringiensis*, n=111; *B. mycoides/B. pseudomycoides*, n=84), originated from four natural habitats in Northeastern Poland, which varied in the level of nutrients, physico-chemical properties, and the scope of human activity. It is generally accepted, that soil, which is heterogeneous in nutrients, particle size, pH, humidity and microbiota, creates favorable conditions for bacterial adaptation and diversification [Guinebretière *et al.,* 2008; McArthur, 2006; Raymond *et al.,* 2010]. Indeed, I demonstrated high genetic diversity in populations of *B. cereus s.l* and proved that this heterogeneity resulted mostly from mutation events (Appendix 2). I also observed that even soil with low concentrations of nutrients from farm sampling, facilitated bacterial genetic divergence.

Hypothesis 1:

Particular *B. cereus s.l.* genetic lineages cover isolates possessing the same specific ecological properties encoded by chromosomal genes.

Dynamic genetic diversification exists in natural populations of *B. cereus s.l.* allowing these bacteria to adapt to different ecological niches, and thereby splitting the population into ecologically distinct ecotypes, identified as DNA sequence lineages [Koeppel *et al.,* 2013]. More often an ecotype can encompass distinct evolutionary lineages [Cohan, 2006]. In my studies, I found 199 *B. cereus s.l.* isolates (67%) having the ability to grow at low temperature. The largest number of the psychrotrophic isolates was found

within the Białowieża National Park population. Based on the MLST methodology, lineages of psychrotolerant strains occurred predominantly within the clade III, what **strongly supports the hypothesis on the existence of thermal ecotypes within the *B. cereus* group** (Appendixes 1 and 2).

While the first report (Appendix 1) showed the cytotoxic pathotypes associated with particular lineages among 24 *B. thuringiensis* strains, further studies on 273 *B. cereus s.l.* isolates (Appendix 2), revealed that the *cytK* gene was mainly associated and expressed within the strains isolated from farm samplings. It is worth adding, that the *cytK*-positives were classified to all *B. cereus s.l.* species under study, and were intermixed among clades on the phylogenetic tree. **Although these results do not support the hypothesis that particular *B. cereus s.l.* genetic lineages contain isolates possessing cytotoxic potential (CytK pathotype),** it is in line with the opportunistic pathogenicity model of *B. cereus s.l.*, where the ability to cause various diseases has no association with specific pathotypes [Ceuppens *et al.*, 2013].

Interestingly, I showed that only a limited number of *B. cereus s.l.* isolates naturally produce the melanin pigment (Appendix 3). Altogether, six melanin-positive strains of *B. weihenstephanensis* from genetically polymorphic populations (the Białowieża National Park and farm in Jasienowka), demonstrated a high level of phenotypic and genotypic similarity. Certainly, melanogenesis among psychrotolerant *B. weihenstephanensis* strains might be a local adaptation to specific environmental niches. Moreover, these isolates did cluster together on the phylogenetic tree (Appendixes 2 and 3), what **strongly supports the hypothesis on the existence of a melanotype in the *B. cereus* group.** It is known that melanin protects microorganisms from adverse environmental factors, such as UV light [Moeller *et al.*, 2005], extreme temperatures [Rosas *et al.*, 1997] or heavy metals [Nair *et al.*, 1992]. If this is the case, then why *B. cereus s.l.* able to produce the melanin pigment are so rare in the nature? I assumed that melanin synthesis entails a fitness cost due to the energy expensive pathway involving laccase and/or the polymerization of the pigment. Another explanation is that, the benefit of extracellular melanin production could be shared with co-occurring bacteria which do not synthesize the pigment [Raymond *et al.*, 2012]. It is worth adding, that it has been the first report (Appendix 3) of the melanin synthesis by *B.*

weihenstephanensis. The application of the next generation whole genome sequencing and a set of biochemical tests, allowed me to conclude that melanin synthesis by *B. weihenstephanensis* is associated with laccase activity.

Hypothesis 2:

***B. cereus s.l.* revealing distinct genotypes (ecotypes) occur in particular habitats.**

A relatively large number of samples of *B. cereus s.l.* from ecologically different environments, allowed to test the hypothesis on the occurrence of distinct genotypes (ecotypes) of *B. cereus s.l.* in particular habitats (Appendix 2). The phylogenic studies showed a strong correlation between the clade designation and the origin of isolates. I demonstrated that almost all isolates originated from Białowieża NP clustered in clade III (97%). The bacilli isolated from farm clustered within clade I (14%), II (13%), III (71%), and IV (2%), while the bacteria from Biebrza NP clustered in proportions 17%, 28% and 55%, respectively. I identified new STs for 78% of the isolates (especially among strains acquired from Białowieża National Park) and the presence of a significantly high number of genotypes (92%) within *B. cereus s.l.* populations that are habitat-specific. In addition, geoBURST analyses grouped the strains into 19 clonal complexes and 80 singletons, which mostly comprised strains originating from single environments. The occurrence of the large number of specific genotypes within natural *B. cereus s.l.* populations, as well as the presence of cytotoxic strains mainly among farm samplings described above, **strongly support the hypothesis on the occurrence of distinct genotypes (ecotypes) of *B. cereus s.l.* in particular habitats.** On the other hand, 8 % of genotypes occurred simultaneously in more than one habitat and, to some extent, did seem to be niche specialists. The majority of these genotypes belong to the largest clonal complexes CC223 or CC650, which are representative for clades II and III, respectively. Such STs probably represent polyphyletic genotypes occupying different environmental habitats, as reported for *B. thuringiensis* HD73 [Raymond *et al.*, 2010].

Hypothesis 3:

Environmental *B. cereus s.l.* are highly genetically related and should be classified as one species.

The taxonomy of *B. cereus s.l.* is complex and some microbiologists disagree with the separation of *B. cereus s.l.* into distinct species [Helgason *et al.*, 2000; Priest *et al.*, 2004]. In the phylogenetic analyses I revealed four clades defined according to Priest *et al.* [2004], in which particular species were intermixed. I identified 68, 54, and 19 genotypes of *B. cereus s.s.*, *B. thuringiensis*, and *B. mycoides*, respectively. However, only seven STs contained isolates classified into two species, while three species together did not occur in one ST. Moreover, in geoBURST analyses the particular species tend to cluster into distinct clonal complexes. Especially *B. mycoides/B. pseudomycoides*, forming four CCs covering 93% strains, showed higher genetic similarity than *B. cereus/B. weihenstephanensis* or *B. thuringiensis.* **These results have not confirmed the hypothesis that environmental *B. cereus s.l.* should be classified to one species.** It seems that in contrast to the human-associated *B. cereus s.l.* exhibiting high levels of similarities, the environmental isolates are more intricate. I proposed dividing *B. cereus s.l.* into two groups, the first of which contains environmental isolates, and the second is composed of those that may be clinically significant.

Detailed genetic, phenotypic and biochemical analyses of the environmental *B. cereus s.l.* strains shed new light on the evolution and ecological adaptation of these bacteria to specific soil habitats differing in scope of human activity. In addition, the gaps in the knowledge on this group's biology are further complemented by taking into account the *B. mycoides* species which are often omitted by researchers when studying the *B. cereus s.l.* group.

Conclusions

1. The ability of growth at low temperatures (thermal ecotype) is a common adaptation among members of *B. cereus s.l.* from Northeastern Poland.

2. Cytotoxic pathotype within *B. cereus s.l.* is mainly associated with environments where human activity is extensive.

3. Melanotype among *B. cereus s.l.* appears occasionally and seems to be a local adaptation to specific niches.

4. Soil *B. cereus s.l* populations are clonal in nature and their genetic diversity results mostly from mutation events and purging effects of purifying selection.

5. Taxonomy of environmental *B. cereus s.l.* is more complex in contrast to the human-associated isolates of the group. *B. cereus s.l.* should be divided into environmental and clinically significant subgroups.

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Co-author's statements
indicating their individual contribution to the publications
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STATEMENT

I, undersigned Izabela ŚWIĘCICKA Ph.D., declare that my part:

in the paper: **Swiecicka I, Bartoszewicz M, Kasulyte-Creasey D, Drewnowska JM, Murawska E, Yernazarova A, Lukaszuk E, Mahillon J (2013) Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85:262-72,** included authorship of the general concept of the work, contribution of reagents, pulsed-field gel electrophoresis of genomic DNA, participation in data analysis and manuscript preparation, fulfilling the function of the corresponding author.

in the paper: **Drewnowska JM, Swiecicka I (2013) Eco-genetic structure of *Bacillus cereus sensu lato* population from different environments in northeastern Poland. PLoS ONE 8: e80175,** included authorship of the general concept of the work, contribution of reagents, participation in data analysis and manuscript preparation, fulfilling the function of the corresponding author.

in the paper: **Drewnowska JM, Zambrzycka M, Kalska-Szostko B, Fiedoruk K, Swiecicka I (2015) Melanin-like pigment synthesis by soil *Bacillus weihenstephanensis* isolates from northeastern Poland. PLoS ONE 10: e0125428,** included co-authorship of the work concept and participation in contribution of reagents, data analysis and editing the manuscript, fulfilling the function of the corresponding author.

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STATEMENT

I, undersigned PhD Marek BARTOSZEWICZ, declare that my part:

in the paper: **Swiecicka I, Bartoszewicz M, Kasulyte-Creasey D, Drewnowska JM, Murawska E, Yernazarova A, Lukaszuk E, Mahillon J (2013) Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85:262-72,** included multi locus sequence typing (MLST) of all isolates under study and identification of the *hblA* and *nheA* genes among Polish isolates.

Marek Bartoszewicz

London, March 11th, 2016

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To whom it may concern

STATEMENT

I, undersigned Daiva KASULYTE-CREASEY, declare that my part:

in the paper: **Święcicka I., Bartoszewicz M., Kasulyte-Creasey D., Drewnowska J.M., Murawska E., Yernazarova A., Łukaszuk E., Mahillon J., 2013. Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85: 262-72,** included (i) isolation and identification of *B. thuringiensis* strains from Lithuanian soil samples, (ii) DNA extraction, and (iii) detection of δ-endotoxin genes among Lithuanian isolates.

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I, undersigned Emilia MURAWSKA, declare that my part:

in the paper: **Swiecicka I., Bartoszewicz M., Kasulyte-Creasey D., Drewnowska J.M., Murawska E., Yernazarova A., Lukaszuk E., Mahillon J. (2013) Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85:262-72,** included detection of the *repX* and *repA* genes for all isolates and participation in manuscript preparation.

Emilia Murawska

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Almaty, March 11th, 2016

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I declare that my part:

in the paper: **Swiecicka I., Bartoszewicz M., Kasulyte-Creasey D., Drewnowska J.M., Murawska E., Yernazarova A., Lukaszuk E., Mahillon J., 2013. Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85: 262-72,**included PCR detection of potential enterotoxigenic isolates based on the presence of the *hblA, nheA,* and *cytK* genes within Lithuanian collection of *Bacillus thuringiensis*.

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Białystok, 19-03-2016

To whom it may concern

STATEMENT

I, undersigned Edyta ŁUKASZUK, declare that my part:

in the paper: **Swiecicka I., Bartoszewicz M., Kasulyte-Creasey D., Drewnowska J.M., Murawska E., Yernazarova A., Lukaszuk E., Mahillon J. (2013) Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85:262-272,** included isolation of *B. thuringiensis* strains from the Polish soil samples and DNA extraction from these isolates.

Edyta Łukaszuk

.....

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I, undersigned Pr Jacques MAHILLON, declare that my part:

in the paper: **Swiecicka I., Bartoszewicz M., Kasulyte-Creasey D., Drewnowska J.M., Murawska E., Yernazarova A., Łukaszuk E., Mahillon J., 2013. Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85: 262-72,** included co-authorship of the work concept and participation in editing the manuscript.

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I, undersigned Monika ZAMBRZYCKA, declare that my part:

in the paper: **Drewnowska J.M., Zambrzycka M., Kalska-Szostko B., Fiedoruk K., Swiecicka I. (2015) Melanin-like pigment synthesis by soil Bacillus weihenstephanensis isolates from northeastern Poland, PLoS ONE 10(4): e0125428,** included biochemical characteristic (API 50CHB and 20E tests) of melanin-positive strains, as well as pigment extraction and its purification.

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STATEMENT

I, undersigned Beata KALSKA-SZOSTKO PhD, declare that my part:

in the paper: **Drewnowska J.M., Zambrzycka M., Kalska-Szostko B., Fiedoruk K., Swiecicka I. (2015) Melanin-like pigment synthesis by soil *Bacillus weihenstephanensis* isolates from northeastern Poland, PLoS ONE 10(4): e0125428,** included characteristic of melanin pigment with Fourier transform infrared (FT-IR) spectroscopy.

.....

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STATEMENT

I, undersigned Krzysztof FIEDORUK PhD, declare that my part:

in the paper: **Drewnowska J.M., Zambrzycka M., Kalska-Szostko B., Fiedoruk K., Swiecicka I. (2015) Melanin-like pigment synthesis by soil Bacillus weihenstephanensis isolates from northeastern Poland, PLoS ONE 10(4): e0125428,** included cooperation in the performance of next generation whole genome sequencing.

Krzysztof Fiedoruk