Chromobacterium vaccinii sp. nov., isolated from native and cultivated cranberry (Vaccinium macrocarpon Ait.) bogs and irrigation ponds

Scott D. Soby,1 Sudhindra R. Gadagkar,1 Cristina Contreras2 and Frank L. Caruso3

1Department of Biomedical Sciences, Midwestern University, 19555 N 59th Avenue, Glendale, AZ 85308, USA
2Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721, USA
3Cranberry Station, University of Massachusetts, PO Box 569, East Wareham, MA 02538, USA

A large number of Gram-negative, motile, mesophilic, violacein-producing bacteria were isolated from the soils and roots of Vaccinium macrocarpon Ait. and Kalmia angustifolia L. plants and from irrigation ponds associated with wild and cultivated cranberry bogs in Massachusetts, USA. Phylogenetic analyses of 16S rRNA gene sequences placed these isolates in a clade with Chromobacterium species, but the specialized environment from which they were isolated, their low genonomic DNA relatedness with Chromobacterium violaceum ATCC 12472T and C. subsugae PRAA4-1T, significant differences in fatty acid composition and colony morphology indicate that the cranberry and Kalmia isolates comprise a separate species of Chromobacterium, for which the name Chromobacterium vaccinii sp. nov. is proposed. Strain MWU205T (=ATCC BAA-2314T=DSM 25150T) is proposed as the type strain for the novel species. Phenotypic analysis of 26 independent isolates of C. vaccinii sp. nov. indicates that, despite close geographical and biological proximity, there is considerable metabolic diversity among individuals within the population.

Chromobacterium is a genus of saprophytic, Gram-negative bacteria within the Neisseriaceae (Betaproteobacteria) which has generally been isolated from soil and freshwater. Both tropical and subtropical isolates of Chromobacterium have occasionally been associated with rapid and lethal infections of humans (Ke et al., 2012; Yang & Li, 2011; Teoh et al., 2006; de Siquera et al., 2005) and other mammals (Ajitdoss et al., 2009; Baldi et al., 2010). Although non-pigmented isolates can often be isolated in culture or found in nature (Han et al., 2008; Yang, 2011), the fundamental property of the genus is the production of the intense purple pigments violacein and deoxyviolacein (Rettori & Duran, 1998), the biosynthesis of which is controlled by quorum sensing (Morohoshi et al., 2010). These pigments have been widely investigated for antibiological activity, and have been associated with inhibition of the growth of chytrids (Becker et al., 2009), filamentous fungi (Barreto et al., 2008), bacteria (Durán & Menck, 2001), protozoa (Matz et al., 2004), viruses (Durán et al., 2007) and human tumour cells (Kodach et al., 2006; Durán & Menck, 2001).

The genus Chromobacterium has undergone numerous revisions, expansions and contractions since it was first described a century ago (see, for example Gilman, 1953; Leifson, 1956; Moss & Ryall, 1981). The current edition of Bergey’s Manual of Systematic Bacteriology lists only the type species, Chromobacterium violaceum (Gillis & Logan, 2005), but five additional species have been proposed since 2007, based on the recognition of significant genetic, metabolic and ecological differences: Chromobacterium subsugae (Martin et al., 2007), C. aquaticum (Young et al., 2008), C. haemolyticum (Han et al., 2008), C. piscinae (Kämpfer et al., 2009) and C. pseudoviolaceum (Kämpfer et al., 2009). Detailed analyses of C. violaceum collected from geographically diverse tropical regions indicate that there is considerable genetic variability within Chromobacterium (Ponnusamy et al., 2011; Dall’Agnol et al., 2008; Lima-Bittencourt et al., 2007, 2011; Barreto et al., 2008), a finding which tends to support the recognition of additional species within Chromobacterium, particularly if these differences can be tied to variations in vertebrate pathogenicity or significant differences in ecological adaptation (Vinatzer & Bull, 2009).

Correspondence
Scott D. Soby
ssobyx@midwestern.edu

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MWU300, MWU205T and MWU328 are JN117594, JN120869 and JN120870, respectively.

Three supplementary tables are available with the online version of this paper.
Cranberry (*Vaccinium macrocarpon* Ait.; Ericaceae) is a plant native to North America that is grown commercially in close proximity to its native origin, and is one of the few domesticated crop species that is nearly genetically identical to its wild progenitors. This regionally important speciality crop is susceptible to a number of serious diseases, including *Phytophthora* root rot, fairy ring and field and storage rots caused by a complex of ascomycete fungi (Caruso & Ramsdell, 1995). Nematodes, insects, weeds and parasitic plants have also re-emerged as pest challenges due to the withdrawal of chemical control agents over the past decade. Despite its importance, little is known about the microbiota associated with this crop species. In order to understand the effects of the cranberry microbiota on plant growth and development, as well as disease incidence and severity, we have begun to isolate bacteria from the phyllospheres and rhizospheres of cultivated and wild cranberry, as well as associated flora. Among these bacteria are violacein-producing *Chromobacterium* isolates that are physiologically and genetically distinct from previously described species. Here, we propose a novel species of *Chromobacterium* isolated from cranberry rhizospheres, bog soils and irrigation ponds in south-east Massachusetts, USA.

Plant roots and attached soil were sampled from the dune swales of coastal heathland containing wild cranberry plants, sheep laurel (*Kalmia angustifolia* L.; Ericaceae) and bearberry (*Arctostaphylos uva-ursi* L. Spreng.; Ericaceae) in the Cape Cod National Seashore in Truro and Provincetown, Massachusetts, and from commercial bogs in East Wareham and Carver, Massachusetts, during the growing seasons of 2010, 2011 and 2012. Commercial cranberry irrigation ponds were sampled in July 2011. Water samples were pre-filtered through Whatman Millipore filters, and the filters were placed directly on *KMB* to assure clonal purity.

Isolates were obtained directly for *C. vaccinii* sp. nov., *C. piscinae* ATCC 12472†, *C. aquatilum* CC-SEYA-1T (data from Young et al., 2008); 6, *C. haemolyticum* CC-SEYA-1T (Kämpfer et al., 2009); 7, *C. aquaticum* CC-MDA0585T (Han et al., 2009); 1). Motility plates were inoculated in the centre from individual colonies with a toothpick and incubated at 26 °C for 24 h. Cells were rod-shaped, with occasional curved cells. Colonies were initially cream-coloured, turning deep purple medial to distal in the colony, with the whole colony becoming deeply pigmented by the third day. Pigments were produced in colonies but not secreted when cells were grown on agar media, whereas planktonic growth in KMB broth resulted in secretion of violacein and deoxyviolacein into the medium prior to intracellular accumulation (Table S2). Colonies were smooth, regular and raised on both KMB and LB (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar l⁻¹), with a deep-brown coastal heathland, nor were there any detectable *Chromobacterium* isolates in woodland soils, leaf litter or tidal wetlands adjacent to cranberry bogs. Isolate MWU205† was selected as the type strain of the proposed novel species. An isolate from wild cranberry, MWU205†, and two isolates from different commercial cranberry bogs, MWU300 and MWU328, were assayed for pigment production and for confirmation that the purple pigments were violacein and deoxyviolacein. Membership of the genus *Chromobacterium* was determined by a combination of verification of the production of violacein pigments as well as phenotypic and genetic tests (Table 1 and Tables S1 and S2, available in IJSEM Online).

All of the *Chromobacterium* isolates were Gram-negative and motile in soft-agar plates (1.0 g yeast extract, 0.75 g K₂HPO₄, 1.6 g MgSO₄. H₂O and 3.0 g agar l⁻¹). Motility plates were inoculated in the centre from individual colonies with a toothpick and incubated at 26 °C for 24 h. Cells were rod-shaped, with occasional curved cells. Colonies were initially cream-coloured, turning deep purple medial to distal in the colony, with the whole colony becoming deeply pigmented by the third day. Pigments were produced in colonies but not secreted when cells were grown on agar media, whereas planktonic growth in KMB broth resulted in secretion of violacein and deoxyviolacein into the medium prior to intracellular accumulation (Table S2). Colonies were smooth, regular and raised on both KMB and LB (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar l⁻¹), with a deep-brown coastal heathland, nor were there any detectable *Chromobacterium* isolates in woodland soils, leaf litter or tidal wetlands adjacent to cranberry bogs. Isolate MWU205† was selected as the type strain of the proposed novel species. An isolate from wild cranberry, MWU205†, and two isolates from different commercial cranberry bogs, MWU300 and MWU328, were assayed for pigment production and for confirmation that the purple pigments were violacein and deoxyviolacein. Membership of the genus *Chromobacterium* was determined by a combination of verification of the production of violacein pigments as well as phenotypic and genetic tests (Table 1 and Tables S1 and S2, available in IJSEM Online).

### Table 1. Metabolic phenotypes of the type strains of *Chromobacterium* species

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reduction</td>
<td>88</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose assimilation</td>
<td>26</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adipic acid assimilation</td>
<td>93</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate assimilation</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Strains: 1, 26 isolates of *C. vaccinii* sp. nov.; 2, *C. violaceum* ATCC 12472†; 3, *C. subtsgae* PRAA4-1T; 4, *C. aquaticum* CC-SEYA-1T (data from Young et al., 2008); 5, *C. haemolyticum* MDA0585T (Han et al., 2008); 6, *C. piscinae* LMG 3947T (Kämpfer et al., 2009); 7, *C. pseudoviolaceum* LMG 3953T (Kämpfer et al., 2009). Results were obtained directly for *C. vaccinii* sp. nov. strains, *C. subtsgae* PRAA4-1T and *C. violaceum* ATCC 12472† at 48 h post-inoculation. Percentages of the 26 *C. vaccinii* sp. nov. isolates that tested positive for the metabolic test are shown. All strains were positive for oxidase, catalase, motility, arginine dihydrolase and assimilation of D-glucose, N-acetylglucosamine, gluconate, capric acid and malic acid and negative for indole production, glucose fermentation, urease, β-glucosidase, β-galactosidase and assimilation of L-arabinose, D-mannitol, maltose and phenylacetic acid. Na, No data available in published species description.
pigment diffusing in the agar by the fourth day after plating, with isolates varying in the timing and amount of brown pigment produced. Violacein and deoxyviolacein were not produced at temperatures above 37 °C. Some isolates frequently produced white to beige violacein-deficient colonies, particularly when grown on NaCl concentrations greater than 1%. Violacein-deficient colonies, which are readily apparent because they never produce the characteristic purple pigment, grew slightly faster than the wild-type, and purified violacein-deficient colonies continued to make the diffusible brown pigment. Isolates MWU205T, MWU300 and MWU328 did not grow on KMB adjusted to pH 6.5, but grew up to pH 8.5, though, at this alkalinity, the colonies appeared less glossy (data in columns 1 and 2 from Martin et al., 2007a). Ten colonies, which are readily apparent because they never produce the characteristic purple pigment, grew slightly faster than the wild-type, and purified violacein-deficient colonies continued to make the diffusible brown pigment. Isolates MWU205T, MWU300 and MWU328 did not grow on KMB adjusted to pH 6.5, but grew up to pH 8.5, though, at this alkalinity, the colonies appeared less glossy (data in columns 1 and 2 from Martin et al., 2007a). Table 2. Fatty acid methyl ester analysis of Chromobacterium strains

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10:0</td>
<td>–</td>
<td>–</td>
<td>0.21</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>C10:1 3-OH</td>
<td>5.15</td>
<td>4.33</td>
<td>3.20</td>
<td>3.37</td>
<td>2.8</td>
<td>3.0</td>
<td>5.1</td>
<td>4.6</td>
</tr>
<tr>
<td>C12:0</td>
<td>5.58</td>
<td>4.95</td>
<td>3.75</td>
<td>3.82</td>
<td>4.5</td>
<td>3.2</td>
<td>4.9</td>
<td>8.8</td>
</tr>
<tr>
<td>C12:0 2-OH</td>
<td>3.02</td>
<td>2.85</td>
<td>1.86</td>
<td>2.02</td>
<td>1.2</td>
<td>1.9</td>
<td>3.3</td>
<td>0.2</td>
</tr>
<tr>
<td>C12:0 3-OH</td>
<td>4.96</td>
<td>3.95</td>
<td>3.25</td>
<td>3.40</td>
<td>2.3</td>
<td>2.8</td>
<td>4.8</td>
<td>4.4</td>
</tr>
<tr>
<td>C14:0 10:0</td>
<td>1.74</td>
<td>1.37</td>
<td>2.32</td>
<td>2.11</td>
<td>1.6</td>
<td>3.1</td>
<td>3.5</td>
<td>2.6</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.62</td>
<td>1.25</td>
<td>–</td>
<td>–</td>
<td>0.7</td>
<td>2.3</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>C16:1 10:0</td>
<td>35.80</td>
<td>41.88</td>
<td>42.72</td>
<td>41.94</td>
<td>41.7</td>
<td>28.7</td>
<td>27.5</td>
<td>33.4</td>
</tr>
<tr>
<td>C16:1 9:0</td>
<td>0.30</td>
<td>0.33</td>
<td>0.31</td>
<td>1.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.93</td>
<td>24.95</td>
<td>28.40</td>
<td>29.56</td>
<td>27.6</td>
<td>32.0</td>
<td>26.6</td>
<td>25.8</td>
</tr>
<tr>
<td>C17:0 10:6</td>
<td>0.20</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>cyclo-C17:0</td>
<td>1.71</td>
<td>–</td>
<td>0.42</td>
<td>–</td>
<td>–</td>
<td>13.2</td>
<td>4.3</td>
<td>–</td>
</tr>
<tr>
<td>C18:1 9:0</td>
<td>15.02</td>
<td>10.62</td>
<td>13.11</td>
<td>12.63</td>
<td>14.9</td>
<td>8.7</td>
<td>14.8</td>
<td>18.8</td>
</tr>
<tr>
<td>C18:0</td>
<td>–</td>
<td>–</td>
<td>0.44</td>
<td>0.45</td>
<td>0.6</td>
<td>0.3</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Comparison under identical conditions. KMB and LB agar plates supplemented with NaCl (LB normally contains 1% NaCl) were streaked with 200 μl mid-exponential-phase culture and incubated at 4–45 °C for up to 2 weeks to test growth and pigment production at different temperatures and salinities. Cranberry and Kalmitia isolates were able to grow at up to 43 °C on KMB or LB, though there was no pigment production at 43 °C (Table S1). C. violaceum ATCC 12472T grew at up to 45 °C on KMB, but only to 37 °C on LB. C. subttsugae PRAA4-1T grew at 37 °C, but not at higher temperatures. A metabolic panel was performed on all of the isolates using API 20NE test strips according to the manufacturer’s instructions (bioMérieux). All strains were positive for oxidase, catalase, indole production, arginine dihydrolase, β-galactosidase and assimilation of D-glucose, N-acetylglucosamine, glucosate, capric acid and malic acid, but negative for glucose fermentation, urease and β-glucosidase. None of the isolates assimilated L-arabinose, D-mannitol, maltose or phenylacetic acid. There was some variation in nitrate reduction and protease production (gelatinase), as well as in mannose, adipic acid and citrate assimilation (Table 1). Single, polar flagella were visualized with a silver flagella stain (Presque Isle Cultures) using light microscopy (Olympus AHBS3) and measured electronically with a Zeiss AxioCam MRC5 and Axiovision software (Zeiss).

The production of violacein by isolates MWU205T, MWU300 and MWU328 was compared by growth in liquid KMB at 30 °C with aeration (200 r.p.m.) for 32 h. Samples were taken at the time of inoculation and at 8-h intervals for determining cell counts by dilution series and for extracting pigments. After a 100 μl sample was taken for dilution plating, 5 ml samples were centrifuged to pellet the cells. The broth medium was decanted into fresh tubes, extracted by vigorous shaking at room temperature with two volumes of 2-butanol and allowed to separate overnight. The final volume of the 2-butanol fraction and absorbance at 585 nm were measured and compared with a standard curve of violacein plus deoxyviolacein in water-saturated 2-butanone, and the production of pigment produced per cell was determined by dividing the amount of violacein plus deoxyviolacein by the live cell count. No violacein production was evident until 16 h post-inoculation. Though violacein is known as a non-mobile pigment based on its containment within the cell when Chromobacterium strains are grown on agar medium, during planktonic growth in KMB it is detected in the medium before it is evident in pelleted cells, indicating an active transport system for export of these compounds (Table S2).

In order to ascertain that the purple pigments were authentic violacein and deoxyviolacein, large-volume (1 l) extractions of pigments violacein and deoxyviolacein were made by inoculating with 1 ml overnight culture of MWU205T for each litre of liquid culture in KMB. Cells were grown at 26 °C for 72 h with aeration (200 r.p.m.). Culture medium and cells were harvested and separated by
centrifugation at 6000 r.p.m. in a GSA rotor (Sorvall) for 20 min. Cell pellets were washed with 600 ml water and then extracted directly with 50 ml ethanol and shaken overnight at room temperature. Cell debris was removed by filtration through Whatman #1 filter paper and washed with 50 ml ethanol and the extracts were combined. Ethanol was allowed to evaporate, and the precipitate was triturated with CHCl₃ and back-extracted with one volume of water. The CHCl₃ phase was then evaporated to dryness and the product was subjected to electrospray ionization mass spectrometry (ESI-MS) or redissolved in DMSO-d₆ for proton NMR spectroscopy. The culture medium was extracted twice with equal volumes of 2-butanol, extracts were pooled and back-extracted with an equal volume of water and the 2-butanol phase was allowed to evaporate to dryness. This was then redissolved in ethanol for ESI-MS or in DMSO-d₆ for proton NMR spectroscopy. Proton NMR spectra were obtained at 500 or 600 MHz on a Bruker spectrometer in DMSO-d₆. ESI-MS spectra were obtained on a Bruker 9.4 Tesla Apex Qh spectrometer. Violacein and deoxyviolacein were identified by their proton NMR spectra in DMSO-d₆ (Rettori & Duran, 1998; Wille & Steglich, 2001). Their relative amounts in mixtures were determined by integration of these spectra (data not shown). ESI-MS mass spectra showed peaks for the monoanions of these pigments, but were not used for quantitative analysis.

Fatty acids were analysed with the Sherlock Microbial Identification system (Microbial ID, Inc.), MWU205T and MWU300 were grown in KMB to exponential phase at 25 °C, and the analysis was repeated three times. Fatty acids were identified by GLC using the standard TSBA40 method (MIDI, 2002) and confirmed by GC-MS (Agilent 5890 GC and 5970 mass spectrometer). The predominant fatty acid of MWU205T and MWU300 was C₁₆:₀ and C₁₆:₁ω7c, accounting for 41.9–42.7 % of total peak area (Tables 2 and S3). Fatty acids C₁₆:₀ and C₁₆:₁ω7c are major components of all other Chromobacterium species, but the contents differed from both C. violaceum ATCC 12472T and C. subtusguae PRAA4-1T in the novel strains, as did those of a number of the minor constituents.

The Sherlock similarity index (SI) identified strains MWU205T and MWU300 as Pseudomonas syringae pv. tomato (SI=0.699) with a ‘good’ species match, and as C. violaceum with a much lower SI (0.540). The same MIDI algorithm had placed C. subtusguae PRAA4-1T with Pseudomonas syringae pv. coronafaciens (Martin et al., 2007). From these and other fatty acid data collected from Chromobacterium strains, it is apparent that fatty analysis is less useful in this taxon than it has been in others for differentiating within the genus, and even between subphyla, unless much more sensitive algorithms become available.

The 16S rRNA genes of six cranberry-associated isolates were cloned and sequenced from multiple PCR clones to assure sequence fidelity. Genomic DNA was amplified by PCR using universal 16S primers 27f (5′-AGAGTTTGATCMTGGCCTCAG-3′) and 1525r (5′-AAGAGGTTGWTCCARCC-3′). Amplicons were cloned into the TOPO2.1 vector (Invitrogen) and sequenced with a redundancy of 4- to 5-fold. The 16S rRNA gene sequence of MWU205T was used to search GenBank using BLAST (Altschul et al., 1990) for other bacterial 16S rRNA gene sequences, and 845 sequences were obtained. These sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 5 (Tamura et al., 2011) with default parameter values, after adding MWU300 and MWU328. Phylogenetic analysis of the entire alignment revealed that the novel isolates clearly clustered with the genus Chromobacterium (not shown). The novel isolates were then compared with only the Chromobacterium sequences, with the 16S rRNA gene sequences of two other betaproteobacteria (Thiobacillus denitrificans ATCC 25259T and Azorarcus sp. BH72) included as an outgroup (Fig. 1). This group of 37 sequences was realigned as before. The model selection tool in MEGA 5 (Tamura et al., 2011) indicated that Tamura’s 3-parameter model (Tamura, 1992) with a discrete gamma distribution to model evolutionary rate differences among sites [five categories (+G; parameter=0.1752)] was the most appropriate nucleotide substitution model for the alignment. Phylogenetic inference was done using the maximum-likelihood method based on this model. The tree with the lowest Bayesian information criterion score (9616.526) is shown. The numbers at nodes show bootstrap support (500 replications). The maximum-likelihood method was used as implemented in MEGA 5 (Tamura et al., 2011). The initial tree(s) for a heuristic search was obtained automatically as follows: when the number of common sites was <100 or less than a quarter of the total number of sites, the maximum-parsimony method was used; otherwise, the BIONJ method (Gascuel, 1997) with maximum composite likelihood distance matrix was used. All positions containing gaps and missing data were eliminated, leaving a total of 1371 shared positions in the final dataset. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. A major node (labelled ‘X’) of the Chromobacterium 16S rRNA gene sequence phylogenetic tree contains most of the strains identified as C. violaceum. The branch emanating from this node divides into three groups. Group A is composed primarily of isolates from the Brazilian rainforest, group B contains C. violaceum ATCC 12472T (South Asia) and a few closely related strains and group C contains isolates MWU205T, MWU300 and MWU328, among others. Thus, the novel isolates are neither monophyletic, nor do they group with any other clade (specifically A or B). Rather, they cluster in group C along with strains CV24 (Lima-Bittencourt et al., 2011) and MBIC3901 at the root of the node marked ‘X’. Interestingly, isolate CV24 was obtained from a river in South America, more than 9000 km from Massachusetts.

For DNA–DNA hybridizations, cells were disrupted using a Constant Systems TS 0.75 kW (IUL Instruments) and the
DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was performed as described by De Ley et al. (1970), as modified by Huss et al. (1983), using a model Cary 100 Bio UV/Vis-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian). DNA–DNA hybridization in 2 × SSC with 10% formamide at 69 °C resulted in a relatedness with *Chromobacterium violaceum* ATCC 12472T of 44.2% (n=2) and with *C. subttsuga* PRA4-1T of 28.0% (n=2). Thus, the cranberry *Chromobacterium* isolates do not belong to the same species as either *C. violaceum* ATCC 12472T or *C. subttsuga* PRA4-1T according to the 70% criterion established by Wayne et al. (1987).

The cranberry isolates from both wild and cultivated bogs cluster in the 16S rRNA gene phylogenetic tree with strains CV24 and MBIC3901 as part of a broader clade containing many *Chromobacterium* 16S rRNA gene sequences. It is notable that these isolates segregate from both the type strain of the type species, *C. violaceum* ATCC 12472T, and the newly established species of *Chromobacterium* (*C. piscinae*, *C. pseudoviolaceum*, *C. subttsuga*, *C. aquaticum* and *C. haemolyticum*), indicating that, although these isolates belong in the genus *Chromobacterium*, they are genetically distinct. The colony morphology of *C. violaceum* ATCC 12472T is clearly different from that of the cranberry and *Kalmia* isolates, in that *C. violaceum* ATCC 12472T colonies appear much less glossy, particularly as the culture ages, and they produce violacein and deoxyviolacein later in culture. However, the production of violacein, a close similarity of metabolic capabilities and fatty acid composition with other *Chromobacterium* species, and placement in the *Chromobacterium* clade in the 16S rRNA gene tree indicate clearly that the cranberry isolates are correctly placed in the genus *Chromobacterium*.

The genus *Chromobacterium* was stable for a number of years, comprising only the type species, but it has become apparent that the amount of ecological, physiological and genetic variation in this taxon exceeds what may reasonably
be described in one or two species (see, for example, Lima-Bittencourt et al., 2007; Ponnusamy et al., 2011). The observation that phenotypic characteristics such as nitrate reduction (see also Table BXXII.β.85 in Gillis & Logan, 2005), indole production, gelatinase activity and the ability to assimilate mannose, adipic acid and citrate varies among the 26 _Chromobacterium_ isolates from cranberry and _Kalmia_ (Table 1) illustrates the limitations inherent in defining bacterial species by metabolic testing of a single isolate or small sample of isolates, even among closely related organisms that are likely to have co-evolved with the host plant and environment for long periods of time, grow in close proximity and presumably are subject to horizontal transfer of genes. It is interesting to note that (except for gelatin hydrolysis) the percentage of the 26 isolates that are positive for variable metabolic tests corresponds roughly with the number of species that were reported to be positive in published descriptions (Table 1), suggesting that what have been reported as phenotypic descriptions underlying species differentiation within the genus are actually a random sampling of isolate-to-isolate variation. Reliable definition of species within the genus may therefore rely on more robust genomic characterization (e.g. multilocus sequence analysis, rep-PCR) or upon quantitative analysis of multiple characteristics such as fatty acid composition and parameters that govern growth and virulence factor production (Table S1).

At least five _Chromobacterium_ species have been described in the past decade, a number that would be expected to increase as the microbiota of diverse terrestrial and limnic environments are sampled and analysed. Here, we propose a species of _Chromobacterium_ that inhabits the specialized habitat associated with the roots and soils of ericaceous plants and, not surprisingly, is found in irrigation ponds used for cranberry cultivation.

**Description of Chromobacterium vaccinii sp. nov.**

_Chromobacterium vaccinii_ [vacc.i’ni.i. L. n. vaccin ium a blueberry, whortleberry, and also a botanical generic name; N.L. gen. n. vaccin ium of Vaccinium, referring to the isolation of the type strain in association with wild and cultivated Vaccinium macrocarpon Ait. (cranberry) roots and soils].

Cells are Gram-negative, aerobic rods, 3.03 ± 0.555 by 1.99 ± 0.0198 μm (means ± st.dev) (range 2.01–3.91 by 0.96–1.78 μm). A single, relatively short (4.91 ± 0.730 μm; range 3.14–6.12 μm), polar flagellum is produced. Colonies grow well on KMB and LB, and are inherently resistant to penicillin and ampicillin at 50 μg ml⁻¹. Optimum growth occurs at 25–26 °C, growing up to 43 °C on KMB. Growth on solid agar produces round, smooth, glossy, convex colonies within 48 h, starting out as cream-coloured and rapidly turning deep purple starting from the centre of the colony. Violacein production is somewhat less at 37 °C and is absent at 43 °C. Some isolates produce violacein-deficient colonies at high frequencies. Cells grow freely in 2 % (w/v) NaCl and marginally in 3 % (w/v) NaCl. Major fatty acids are C₁₆:₀₁ω₇c, C₁₆:₀ and C₁₈:₁ω7c. Colonies do not fluoresce under either short- or long-wave UV irradiation, but produce large amounts of the pigments violacein and deoxyviolacein at up to 37 °C, eventually also producing a water-soluble brown pigment that diffuses freely in the medium. Known isolates are positive for catalase and oxidase, produce indole from tryptophan, produce arginine dihydrolase and ß-galactosidase and assimilate D-glucose, N-acetylglucosamine, gluconate, capric acid and malic acid, but are negative for glucose fermentation, urease and ß-galactosidase. Isolates do not assimilate L-arabinose, D-mannitol, maltose or phenylacetic acid. Some isolates have reduced nitrate reduction, and the ability to hydrolyse gelatin and to assimilate mannose, adipic acid and citrate varies by isolate, as does the production of an uncharacterized diffusible brown pigment.

The type strain, MWU205T (=ATCC BAA-2314T =DSM 25150T), was isolated from wild cranberry bog soil in Truro, MA, USA, in the Cape Cod National Seashore.

**Acknowledgements**

We thank Phyllis Martin and her colleagues at the USDA/ARS Invasive Insect Biocontrol and Behavior Laboratory for the kind gift of _C. subsuga_ PRAA4-1T. We would also like to thank Robert Bates, Douglas Barker and Agnes Pascual for their technical assistance, Pedro Chavez and Kim Cooper for helpful discussions and rotation students in the Midwestern University College of Health Sciences MBS program for their contributions to the project. This work was funded in part by a grant from the Cape Cod Cranberry Growers Association and internal support from the Office of Research and Sponsored Programs, Midwestern University.

**References**


environments of state of Pará, Brazilian Amazon. Mem Inst Oswaldo Cruz 103, 678–682.


