Environmental mycobacteria, known as nontuberculous mycobacteria (NTM), comprise the majority of the genus *Mycobacterium* (Falkinham, 2002; Pontiroli et al., 2013). NTM have been found in various water and soil environments, and many are the causative agents of opportunistic infections in immunocompromised individuals (Chilima et al., 2006; Falkinham, 2002, 2009; Kazda, 2000; Primm et al., 2004). NTM have been found in numerous different environments including tap water, hospital water systems, acidic peatland, natural water systems and contaminated soils (Falkinham, 2002; Nichols et al., 2004; Wang et al., 2006). Despite the wide range of habitats that mycobacteria and humans share, these bacteria are still poorly understood in terms of their pathogenicity, natural reservoirs and evolutionary histories (Tortoli, 2012). Novel species from different environmental sources continue to be discovered, making it essential to determine the actual scope of NTM habitats in order to deter potential opportunistic mycobacterial infections.

In 2013, a novel species, *Mycobacterium minnesotense* JCM 17932T, was identified in the sphagnum moss of Minnesota peat bogs (Hannigan et al., 2013). In an effort to further characterize the ecology of mycobacteria in regional bogs, we continued our surveys focusing on a specific plant growing in the bogs, *Sarracenia purpurea*, commonly called the northern pitcher plant, is a carnivorous plant that is typically found in low-nutrient wetlands of North America such as bogs and fens (Juniper et al., 1989). These herbaceous perennials produce pitcher-shaped leaves that open up and fill with water after 2–3 weeks of growth (Fish & Hall, 1978; Wolfe, 1981). Prior to opening, the interior of the pitcher is sterile, only acquiring a microbial community after opening and exposure to rain water. The purpose of captured water is to entrap and digest insects that provide nitrogen to the plant growing in low-nutrient environments. Unlike other members of this

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**Mycobacterium sarraceniae** sp. nov. and *Mycobacterium helvum* sp. nov., isolated from the pitcher plant *Sarracenia purpurea*

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Several fast- to intermediate-growing, acid-fast, scotochromogenic bacteria were isolated from *Sarracenia purpurea* pitcher waters in Minnesota sphagnum peat bogs. Two strains (DL734T and DL739T) were among these isolates. On the basis of 16S rRNA gene sequences, the phylogenetic positions of both strains is in the genus *Mycobacterium* with no obvious relation to any characterized type strains of mycobacteria. Phenotypic characterization revealed that neither strain was similar to the type strains of known species of the genus *Mycobacterium* in the collective properties of growth, pigmentation or fatty acid composition. Strain DL734T grew at temperatures between 28 and 32 °C, was positive for 3-day arylsulfatase production, and was negative for Tween 80 hydrolysis, urease and nitrate reduction. Strain DL739T grew at temperatures between 28 and 37 °C, and was positive for Tween 80 hydrolysis, urea, nitrate reduction and 3-day arylsulfatase production. Both strains were catalase-negative while only DL739T grew with 5 % NaCl. Fatty acid methyl ester profiles were unique for each strain. DL739T showed an ability to survive at 8 °C with little to no cellular replication and is thus considered to be psychrotolerant. Therefore, strains DL734T and DL739T represent two novel species of the genus *Mycobacterium* with the proposed names *Mycobacterium sarraceniae* sp. nov. and *Mycobacterium helvum* sp. nov., respectively. The type strains are DL734T (=JCM 30395T=NCCB 100519T) and DL739T (=JCM 30396T=NCCB 100520T), respectively.

†These authors contributed equally to this work.

**Abbreviations**: NTM, nontuberculous mycobacteria; OADC, oleic, albumin, dextrose, catalase.
genus, S. purpurea does not produce its own digestive enzymes (Adams & Smith, 1977) but relies upon the microbial and invertebrate inquilines inside it to break down and digest captured prey (Heard, 1994). Field and laboratory studies show that the bacterial-protozoan-invertebrate food web of S. purpurea changes dynamically after the web is established (Cochran-Stafira & von Ende, 1998). Most notably, the pH of this detritus-based aquatic food web drops from pH 6.8 to as low as pH 3.5 within a month of being established.

We repeatedly cultured bacteria from S. purpurea, obtaining a total of 11 isolates belonging to the genus Mycobacterium as described by 16S rRNA gene sequencing. These isolates were characterized using a polyphasic approach. Enough differences in genetic and morphological features were found to suggest that the isolates are members of two novel species of the genus Mycobacterium.

S. purpurea water samples were collected from two Minnesota glacial peat bogs: Repose Lake north of Ely, MN, USA, in August 2012 (15 plants sampled), and the Laurentian Environmental Center, MN (47.57 N, 92.57 W), in July 2013 (23 plants sampled). The contents of pitcher plants were siphoned out in the field and transported to the laboratory, as described by Fish & Hall (1978). Mycobacteria were isolated from the samples using modified protocols from Chilima et al. (2006) and Wang et al. (2006). Brieﬂy, aliquots collected directly from the pitcher structures of S. purpurea were allowed to incubate in nutrient broth (Difco) at 32°C for 2–4 h to facilitate bacterial and fungal spore germination. Cells were then pelleted (4000 g for 15 min) and resuspended in 11 ml mycobacterial selection buffer (3.6 mg NaOH ml⁻¹, 45 µg cycloheximide ml⁻¹ and 0.115 µg malachite green ml⁻¹ in distilled H₂O, pH 12). After incubating at room temperature for 20 min, cells were pelleted (4000 g for 15 min), resuspended in 11 ml acid buffer (0.2 M HCl and 0.2 M KCl in distilled H₂O, pH 2) and incubated at room temperature for 20 min. After adding 10 ml phosphate buffer (pH 6.9), cells were pelleted (4000 g for 15 min) and plated onto Middlebrook 7H11 agar plates supplemented with 17.5 µg cycloheximide ml⁻¹, 250 µg nalidixic acid ml⁻¹ and oleic albumin dextrose catalase (OADC; Becton Dickinson). Plates were incubated at either 28 or 32°C and examined for growth at 3, 5 and 7 days and then every 2 weeks thereafter. Ziehl-Neelsen acid-fast staining was conducted (Beveridge et al., 2007), and 27 acid-fast isolates were discovered that were designated strains DL725-DL752. All isolates formed colonies within 7 days and were considered ‘fast-growing’.

16S rRNA gene sequences were amplified for the 27 isolates as previously described, and nucleotide BLAST searches were performed to give comparisons and calculate homologies to known mycobacterial species (Skrnytek, 2010). Of the 27 acid-fast-positive isolates, 11 were confirmed as belonging to the genus Mycobacterium. Two of the 11 pitcher plant mycobacteria would be designated as type strains and these are DL734ᵀ and DL739ᵀ. Phylogenetic analysis was conducted on the 16S rRNA gene sequences of the isolates and closely related species. Genbank accession numbers for all sequences used are listed in Table S1 (available in the online Supplementary Material). Multiple alignments with CLUSTALX were performed on the sequences, and phylogenetic analyses were performed using MEGA6 software (Tamura et al., 2013). Evolutionary history was inferred by the maximum-likelihood and neighbour-joining methods. Distances were computed using the Kimura 2-parameter distance correction model with bootstrapping based on 1000 replications. Trees based on the 16S rRNA gene sequence indicated that DL739ᵀ most recently diverged from Mycobacterium rhodesiae DSM 44223ᵀ and DL734ᵀ diverged from a clade with Mycobacterium murale CCUG 39728ᵀ and Mycobacterium tokaiense ATCC 27282ᵀ (Fig. 1). The strains with the highest 16S rRNA gene sequence similarity values to strain DL734ᵀ were Mycobacterium aichiense JS618 with 98.36 % similarity, M. murale CCUG 39728ᵀ with 98.29 % similarity and M. tokaiense ATCC 27282ᵀ with 98.27 % similarity. The strains with the highest 16S rRNA gene sequence similarity values to strain DL739ᵀ were M. rhodesiae DSM 44223ᵀ with 99.24 %, M. aichiense JS618 with 99.04 % and Mycobacterium paraafricanum DSM 43528ᵀ with 99.04 % similarity. Because 16S rRNA gene sequences alone may provide low resolution in characterizing mycobacteria at the species level, four other conserved genes were also sequenced (Yamada-Noda et al., 2007). The genes rpoB (Adékambi et al., 2003), hsp65 (Ringuet et al., 1999) and dnaJ (Yamada-Noda et al., 2007) were amplified by PCR from the 11 isolates. Multilocus sequence typing was conducted for DL739ᵀ and its most closely related mycobacteria species listed above. The concatamer construct 16S rRNA–rpoB–dnaJ–hsp65 was used for comparison with M. rhodesiae DSM 44223ᵀ and M. paraafricanum DSM 43528ᵀ, while dnaJ was omitted from the comparison with M. aichiense JS618. Strain DL739ᵀ was 83.4 % similar to M. rhodesiae DSM 44223ᵀ, 90.3 % similar to M. aichiense JS618 and 87.9 % similar to M. paraafricanum DSM 43528ᵀ. Phylogenetic analyses based on the concatenator sequences of rpoB, dnaJ, hsp65 were conducted in the same method as described above (Fig. S1). The trees revealed that the pitcher isolates are most closely related to Mycobacterium flavescens CIP 104533ᵀ, Mycobacterium vaccae ATCC 13483ᵀ and Mycobacterium hiberniae ATCC 49874ᵀ, all of which have previously been found in sphagnum bogs (Kazda, 2000). The pitcher plant isolates formed a distinct clade branching away from the rest of the genus Mycobacterium. Within this clade, the isolates form two different sister clades with unresolved branching. The low similarity values and distinct branching of the pitcher plant isolates suggests the identification of two uncharacterized mycobacteria species. Strains DL734ᵀ and DL739ᵀ were chosen to represent each separate clade.

Upon determination of the most closely related species, cultural characteristics were determined for DL734ᵀ and DL739ᵀ and compared with those of the related species. All
pitcher plant isolates produced colonies that were smooth, shiny and scotochromogenic, producing orange to yellow colonies with increased pigmentation intensity when incubated in light conditions. Growth at temperatures of 28, 32, 37, 42 and 52 °C was tested. Optimal growth temperatures for strain DL734^T were between 28 and 32 °C. This makes it distinctly separate from *M. murale* CCUG 39728^T and *M. tokaiense* ATCC 27282^T, which are capable of forming colonies at 37 °C (Tsukamura et al., 1981). Optimal growth temperatures for strain DL739^T were between 28 and 37 °C. Neither strain grew at 42 or 52 °C. While both DL734^T and DL739^T are most closely related to *M. flavescens* CIP 104533^T and *M. vaccae* ATCC 15483^T by DNA analysis (Fig. S1), they showed very different growth properties at 28 and 32 °C from these two known species (Fig. S2). The morphologies of DL734^T and DL739^T on solid media were similar to that of *M. vaccae* ATCC 15483^T but noticeably different from that of *M. flavescens* CIP 104533^T (data not shown). High-resolution scanning electron microscopy images of cells of DL734^T and DL739^T showed that these two strains have different cell shapes from one another (Fig. S3).

14470<sup>T</sup> by saponification, methylation and extraction at Microbial ID (Newark, DE, USA). FAME profiles were then determined by gas chromatography and analysed by the Sherlock Microbial Identification System (MIDI). M. gordonae ATCC 14470<sup>T</sup> was correctly identified to the species level while the 11 pitcher plant isolates could only be identified to the genus *Mycobacterium* with no match to FAME profiles of known species. The majority of lipids produced in the pitcher isolates were the same as those produced by *M. gordonae* ATCC 14470<sup>T</sup>, but quantities of these lipids varied (Table S2). This FAME analysis indicated that strains DL734<sup>T</sup> and DL739<sup>T</sup> are more closely related to each other than to *M. gordonae* ATCC 14470<sup>T</sup>.

Several pitcher plant mycobacteria strains, *M. gordonae* ATCC 14470<sup>T</sup> and *M. vaccae* ATCC 15483<sup>T</sup> were subjected to the following biochemical tests as described by Kent & Kubica (1985) and Leao et al. (2004): Positive for urea hydrolysis, Tween 80 hydrolysis, 3 and 14-day arylsulfatase production, and nitrate reduction. *M. helvum* is unable to reduce tellurite and does not produce heat-stable catalase (Tierno & Milstoc, 1981). Results are shown in Table 1. DL734<sup>T</sup> and associated strains lacked urease, which distinguished them from *M. murale* CCUG 39728<sup>T</sup> and *M. tokaïense* ATCC 27282<sup>T</sup> (Tsukamura et al., 1981; Vuorio et al., 1999). DL739<sup>T</sup> and associated strains did not produce catalase, did reduce nitrate and were only weakly positive for urease (Table 1). This suggests that strain DL739<sup>T</sup> is different from *M. rhodesiae* DSM 44223<sup>T</sup>, which produces urease and catalase, but does not reduce nitrate (Tsukamura et al., 1971).

Antibiotic susceptibilities were determined for *M. gordonae* ATCC 14470<sup>T</sup> and the 11 pitcher plant isolates on 7H11 plates containing antibiotic-impregnated discs. Because there are no standards to determine resistance or susceptibility for NTM, diameters of zones of inhibition (reported as mm±sd) were measured to compare relative sensitivity to the antibiotics. Antibiotics tested were (µg per disc) ampicillin (10), aztreonam (30), chloramphenicol (30), ciprofloxacin (5), neomycin (30), penicillin (10), streptomycin (10), tetracycline (30) and trimethoprim (30). *M. gordonae* ATCC 14470<sup>T</sup> was sensitive only to chloramphenicol and ciprofloxacin, with zones of inhibition of 15 mm and 30 mm, respectively. In contrast, all the pitcher plant isolates were susceptible to neomycin (32±3 mm) and tetracycline (50±7 mm). Strain DL734<sup>T</sup> was susceptible to ampicillin (55±20 mm), chloramphenicol (53±17 mm), ciprofloxacin (79±8 mm), streptomycin (58±10 mm) and penicillin (50±7 mm). Strain DL739<sup>T</sup> was susceptible to chloramphenicol (27±2 mm), ciprofloxacin (37±15 mm) and streptomycin (58±11 mm), and was completely resistant to penicillin.

Because Minnesota bogs undergo repeated rounds of freezing and thawing each year, the 11 isolates were assayed for their ability to tolerate near freezing temperatures. Five millilitre aliquots of 7H9 (Difco) were inoculated with fresh cells and equilibrated for starting optimal densities (OD<sub>600</sub> 0.5). The cultures were shaken at 8 °C for 2 weeks. No change in culture turbidity was observed over this period. Viable cell density, as measured in c.f.u. ml<sup>-1</sup>, did not change for DL739<sup>T</sup> but decreased for *M. gordonae* ATCC 14470<sup>T</sup>, *M. vaccae* ATCC 15483<sup>T</sup>, *M. flavescens* CIP 104533<sup>T</sup> and DL734<sup>T</sup> over this 2 week period. The DL734<sup>T</sup> culture was unable to produce any viable cells after this 2 week incubation at 8 °C. However, when the DL734<sup>T</sup> culture was allowed to warm to 28 °C and was shaken for an additional 7 days, c.f.u. were detected. Therefore, it appears that DL734<sup>T</sup> can temporarily enter a viable but non-culturable state at 8 °C. Strain DL739<sup>T</sup> appears to be more psychrotolerant at 8 °C since cells were still culturable immediately after incubation at this temperature.

By combining the phylogenetic, phenotypic, chemotaxonomic and growth analyses, it was determined that DL734<sup>T</sup> and DL739<sup>T</sup> represented two unique and previously unidentified species of the genus *Mycobacterium*. Therefore, the names *Mycobacterium sarraceniae* sp. nov. and *Mycobacterium helvum* sp. nov. are proposed to accommodate strains DL734<sup>T</sup> and DL739<sup>T</sup>, respectively.

### Table 1. Phenotypic and biochemical properties of selected pitcher plant isolates and related of the genus *Mycobacterium*

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<td>Growth with 5% NaCl (w/v)</td>
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<td>Nitrate reduction</td>
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<td>3-Day arylsulfatase</td>
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</table>

Strains: 1, *M. gordonae* ATCC 14470<sup>T</sup>; 2, *M. vaccae* ATCC 15483<sup>T</sup>; 3, DL725; 4, DL726; 5, DL728; 6, DL729; 7, DL730; 8, DL731; 9, DL732; 10, DL734<sup>T</sup>; 11, DL742; 12, DL738; 13, DL739<sup>T</sup>. ++, Positive result; +, weakly positive result; +*, reaction occurred after 7 days; –, negative result. All strains tested were negative for tellurite reduction. *M. vaccae* ATCC 15483<sup>T</sup> was the only one positive for heat-stable catalase. All were positive for 14-day arylsulfatase.

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Description of Mycobacterium sarraceniae sp. nov.

Mycobacterium sarraceniae (sar. ra.ce’n.i. ae. N.L. fem. gen. n. sarraceniae of Sarracenia, for the pitcher plant from where the species was isolated).

Cells are acid-fast, slender and bent-rod-shaped. Growth on Middlebrook 7H11 with OADC reaches maturity in 7 days at 28 °C and 10 days at 32 °C. Growth is not observed at 37 °C. Cells exhibit polymorphism in shape, with some cells appearing as curved rods, and with a decrease in acid-fastness in older cells. Colonies are orange, smooth and shiny, exhibiting scotocromogenic and photochromogenic properties. Positive for 3- and 14-day arylsulfatase. M. sarraceniae does not produce catalase, reduce nitrate or tellurite, and does not hydrolyse Tween 80. There is minimal growth in the presence of 5 % NaCl. M. sarraceniae is susceptible to neomycin, tetacycline, ampicillin, chloramphenicol, ciprofloxacin, streptomycin and penicillin, but is resistant to aztreonam and trimethoprim. Demonstrates an ability to enter a reversible dormant state at 8 °C. 16S rRNA gene sequence data puts M. sarraceniae nearest to M. murale CCUG 39728T and M. tokaiense ATCC 27282T, but evaluation of the rpoB, dnak, secA and hsp65 sequence data places M. sarraceniae among the intermediate- to fast-growing species of the genus Mycobacterium, most closely related to strain DL739T.

The type strain, DL739T, was isolated from water samples collected from S. purpurea pitchers at Repose Lake in northern Minnesota. The type strain has been deposited in the Japan Collection of Microorganisms (JCM 30395T) and the Netherlands Culture Collection of Bacteria (NCCB 100519T) collection banks. Strains DL725, DL726, DL728, DL729, DL730, DL731, DL732 and DL742 also represent the species.

Description of Mycobacterium helvum sp. nov.

Mycobacterium helvum (hel’vum L. neut. adj. helvum pale yellow, intended to mean pale yellow-pigmented).

Cells are acid-fast and exhibit a coccolid-bacillus shape. Growth on Middlebrook 7H11 agar with OADC reaches maturity in 7 days at 28 °C and 5 days at 37 °C. Older cultures show a decrease in acid-fastness. Colonies are pale yellow, smooth with a slightly ridged margin and exhibit scotocromogenicity. Positive for 3- and 14-day arylsulfatase production, and nitrate reduction. M. helvum is unable to hydrolyse Tween 80, reduce tellurite or produce urease or catalase. Growth in the presence of 5 % NaCl is detected. Susceptible to chloramphenicol, ciprofloxacin, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin. Resistant to penicillin, aztreonam, and streptomycin.

The type strain, DL739T, was isolated from water samples collected from S. purpurea pitchers at the Laurentian Educational Center in northern Minnesota. The type strain has been deposited in the Japan Collection of Microorganisms (JCM 30396T) and the Netherlands Culture Collection of Bacteria (NCCB 100520T) collection banks. DL738 also represents the species.

Acknowledgements

We thank the Laurentian Educational Center, MN, for permission to collect samples. This research was supported by internal funds from the University of Minnesota.

References


