

Characterization of photochromogenic *Mycobacterium* spp. from Chesapeake Bay striped bass *Morone saxatilis*

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ABSTRACT: A large diversity of *Mycobacterium* spp. has been isolated from striped bass *Morone saxatilis* in Chesapeake Bay, USA. The new species *M. shottsii* and *M. pseudoshottsii* are the dominant isolates, while the classical fish pathogen *M. marinum* is found much less frequently. *M. fortuitum* and *M. chelonae*, other *Mycobacterium* spp. known to commonly infect fishes, have not yet been aseptically isolated from striped bass within Chesapeake Bay. While *M. pseudoshottsii* and *M. shottsii* have been phenotypically and genotypically characterized, other less common mycobacterial isolates have not. In the present study, we describe 17 photochromogenic isolates from Chesapeake Bay striped bass using phenotypic characterization and multilocus sequencing of 16S rRNA, *hsp65* and *rpoB* genes. Genetic characterization reveals that these isolates are related to widely divergent portions of the mycobacterial phylogeny; however, some interesting trends are observed, such as a majority of isolates (10/17) belonging to the *M. simiae*-related grouping. Five additional isolates were assigned to the slow-growing mycobacteria (including 2 identified as *M. marinum*), while 2 are clearly shown to belong genetically to the fast-growing mycobacteria.

KEY WORDS: *Mycobacterium* spp. · Photochromogenic · Striped bass · *Morone saxatilis* · Chesapeake Bay · Multilocus sequencing

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INTRODUCTION

The genus *Mycobacterium* currently contains 148 recognized species, of which 61 attained named status within the last decade (Euzéby 1997). The previously unrecognized diversity of this group is further attested to by the large and growing number of sequences on public databases (e.g. GenBank) that group, phylogenetically, with the mycobacteria, but which differ from recognized species and strains. Many newly described species are associated with human infections and clinical outbreaks (e.g. Schinsky et al. 2004, Cloud et al. 2006), while others are derived from a range of sources, including environmental samples (e.g. Torkko et al. 2000, 2002, Hennessee et al. 2009) and non-human vertebrates (e.g. Shojaei et al. 2000, Cousins et

al. 2003). Aquatic poikilotherms in particular have been a productive source of novel *Mycobacterium* spp., including *M. montefiorensis* from moray eels *Gymnothorax funebris* (Levi et al. 2003), *M. shottsii* and *M. pseudoshottsii* from striped bass *Morone saxatilis* (Rhodes et al. 2003, 2005), *M. stomatepieae* from the cichlid *Stomatepia mariae* (Pourahmad et al. 2008), and *M. salmoniphilum* from salmonids, a revived species name which was recently differentiated from *M. chelonae* (Whipps et al. 2007).

Striped bass in Chesapeake Bay, USA, have been experiencing a high prevalence of mycobacteriosis since the initial description of this disease in these fish in 1997 (see Gauthier & Rhodes 2009). The striped bass is a commercially and recreationally important species as well as a keystone predator in the Chesapeake Bay

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ecosystem, so that this disease creates concern for the continued health of the population. The 3 species of *Mycobacterium* traditionally associated with infections in fishes are *M. marinum*, *M. chelonae* and *M. fortuitum* (Gauthier & Rhodes 2009), and reports continue to implicate these species in fish disease worldwide. It was therefore surprising when bacteriological surveys of Chesapeake Bay striped bass conducted from 1997 to 2001 indicated that *M. marinum* comprised only about 3% of Chesapeake Bay striped bass isolates, and neither *M. chelonae* nor *M. fortuitum* was detected (Rhodes et al. 2004). Since this first report, *M. chelonae* has been isolated from only a single striped bass in Delaware Bay (Ottinger et al. 2007), and *M. fortuitum* has not yet been isolated from internal tissues of this fish. Instead, a suite of biochemically diverse, primarily slow-growing mycobacteria has been isolated, including *M. shottsii* and *M. pseudoshottsii* (Rhodes et al. 2001, 2003, 2004, 2005), which are related to *M. marinum* and *M. ulcerans*. *M. pseudoshottsii* and *M. shottsii* appear to be the dominant isolates, although a phenotypic grouping of non-pigmented, slow-growing mycobacteria similar to *M. triplex* and *M. interjectum* are also numerically relevant (Rhodes et al. 2004). More recent work using fatty acid methyl ester (FAME) analysis and DNA sequence data on mycobacterial isolates recovered from Chesapeake Bay striped bass and other fishes has further demonstrated the large diversity of *Mycobacterium* species and/or strains capable of infecting fishes in this ecosystem (Stine et al. 2010). It therefore appears that infections with *Mycobacterium* spp. in Chesapeake Bay striped bass represent a situation unique to those previously reported for wild fishes elsewhere, with a large diversity of newly described and undescribed slow-growing species involved in infections, and potentially, disease.

To date, *Mycobacterium pseudoshottsii* and *M. shottsii* have been the only mycobacteria infecting striped bass to have had significant genotypic characterization in addition to phenotypic description. The goal of the present study was to elucidate the molecular phylogeny of an additional 17 *Mycobacterium* spp. isolated from Chesapeake Bay striped bass, and to place them in context with other deposited sequences from mycobacteria from the same host and region. We used multilocus sequencing to improve the robustness of phylogenetic positioning for the 17 isolates described in the present study, and 16S rRNA gene data for other comparisons. We also provide phenotypic characterization of the 17 isolates, as well as basic data on the fish from which they were isolated.

The majority of isolates (16/17) in the present study are classified as Type I according to the scheme of Runyon (1959), in that they produce pigment upon exposure to light and take >7 d to form visible colonies

on agar media. One isolate (324-016) would be classified as Runyon Type IV based on its growth characteristics (visible colonies in <7 d), but is photochromogenic, unlike most mycobacteria in this group. The Runyon classification is entirely phenotypic and therefore produces artificial groupings of distantly-related mycobacteria, as well as splitting closely related bacteria between types. For example, the unpigmented *M. shottsii* (Type III), is grouped separately from the genetically closely related photochromogenic *M. pseudoshottsii* (Type I). However, this scheme is still in use today, and it provides a convenient descriptive grouping tool to divide the large panel of isolates that have been recovered from striped bass in Chesapeake Bay. Multilocus sequencing of slow-growing scotopigmented (Type II) and non-pigmented (Type III) *Mycobacterium* spp. isolated from striped bass is ongoing and will be dealt with in a future manuscript.

As with other bacterial genera, the 16S rRNA gene is useful for phylogenetic identification of the mycobacteria (Rogall et al. 1990); however, the high degree of conservation of this gene within *Mycobacterium* (>94%) presents limitations for differentiation of species which may be markedly different in phenotype. For example, *M. gastri* and *M. kansasii* are 100% similar in 16S rRNA gene sequence, and *M. marinum* strain M and *M. ulcerans* strain Agy99 differ by only 5 bp over the full 1542 bp length of the gene. Due to the occasionally low discriminatory power of the 16S rRNA gene for this genus, sequence data from other housekeeping genes have been used for phylogenetic and diagnostic purposes, including *erp* (de Mendonca-Lima et al. 2001), *gyrB* (Kasai et al. 2000), *hsp65* (Telenti et al. 1993), internal transcribed spacer (ITS) (Roth et al. 1998), *recA* (Blackwood et al. 2000), *rpoB* (Kim et al. 1999, 2004), and *sod* (Zolg & Philippi-Schulz 1994, Devulder et al. 2005). The utility of multilocus sequencing (MLS) for robust phylogenetic differentiation of the mycobacteria has been demonstrated (Devulder et al. 2005), and alternative loci (e.g. *crtB*, *fbpA*, *adk*, *ppk*, *aroE* and *glcB*) have been successfully used for generating phylogenies within closely related complexes, such as the *M. marinum* grouping (Stinear et al. 2000, Yip et al. 2007).

Our choice of loci for MLS in the present study was primarily based on availability of sequence in public databases for comparative purposes, as well as demonstrated phylogenetic discriminatory power of loci by previous authors. We used a portion of the 16S rRNA gene, including variable regions A and B, to which considerable attention has been paid in previous studies of mycobacterial phylogeny (e.g. Rogall et al. 1990, Tortoli 2003). Region A includes helices 8 to 11 of the folded 16S rRNA molecule, and a cytosine insertion in this region is characteristic of thermotolerant fast-

growing mycobacteria (Tortoli 2003). Region B includes helix 18 of the 16S rRNA molecule, and insertions within this region are, with exceptions, characteristic of slow-growing mycobacteria, while the presumably ancestral short helix is characteristic of fast growers (Tortoli 2003). The second locus used in the present study is the so-called 'Telenti fragment' of the mycobacterial *hsp65* gene which has been used extensively in previous studies for both sequencing and restriction fragment polymorphism analysis (Telenti et al. 1993). The third and final locus used in the present study is a fragment of the *rpoB* gene shown previously by Kim et al. (1999) to have discriminatory power among the mycobacteria. For reference, the first 2 loci in the present study are consistent in terms of sequence position with those reported by Devulder et al. (2005). The *rpoB* fragment here is consistent with that of Kim et al. (1999) and includes 301 bp found at the 5' end of the fragment reported by Devulder et al. (2005). In order to improve phylogenetic placement of photochromogenic isolates from Chesapeake Bay striped bass within known *Mycobacterium* spp., we compared artificial concatemers of the 3 sequenced fragments described above and compared them with homologous concatemers generated from a deposited sequence.

MATERIALS AND METHODS

Fish collections and bacterial isolation. Striped bass *Morone saxatilis* from the Rappahannock River, Virginia, were collected via pound net as part of ongoing tag-recapture studies to examine the prevalence of, and mortality related to, mycobacteriosis. Striped bass were collected from the mainstem of Chesapeake Bay via otter trawl using the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAAP; see Gauthier et al. 2008a). Fish were placed on ice immediately and necropsy was performed within 24 h after collection. Bacterial isolation and phenotypic characterization were as described in previous work (Rhodes et al. 2004, Gauthier et al. 2008b). Briefly, 0.1 to 0.5 g of spleen was homogenized using a Tenbroek tissue grinder, and dilutions were made in Butterfield's buffer + 0.05% Tween-80. Dilutions were plated (0.2 ml per plate) on Middlebrook 7H10 agar with oleic albumin dextrose catalase (OADC) enrichment (MDA), and plates were incubated at room temperature ($23 \pm 1^\circ\text{C}$) for up to 3 mo. All isolations were made from aseptically collected spleen samples. Individual colonies were picked from initial isolation plates and grown in Middlebrook 7H9 broth with OADC enrichment and 0.05% Tween-80 (MDB) at room temperature until turbid. Broth cultures were restreaked on

MDA to ensure purity, regrown in MDB, and DNA was extracted from cell pellets after centrifugation. Phenotypic characterization of study isolates, including growth at room temperature, 30°C and 37°C , is presented in Table 1.

Histology. Preparation of spleen samples for histological examination and enumeration of granulomas was performed as previously described (Gauthier et al. 2008a). Splenic area measurements were taken by photographing sections at $6.3\times$ magnification on an Olympus SZX-ZB9 dissecting microscope equipped with a Nikon DXM1200 digital camera. Splenic area (mm^2) was calibrated and measured with MetaMorph software (Universal Imaging). Area measurements were taken on all sections examined for granulomas, excluding fish with no granulomas. The severity index (SI) was calculated as $\log_{10}(\text{granulomas } \text{mm}^{-2} + 1)$, and disease severity was assigned as follows: negative (0): $\text{SI} = 0$; minor (1): $0 < \text{SI} \leq 0.1$; moderate (2): $0.1 < \text{SI} \leq 0.5$; severe (3): $\text{SI} > 0.5$.

Preparation of DNA and PCR. Turbid bacterial cultures were mechanically disrupted with 0.1 mm zirconium-silicon beads in a beadmill (FastPrep, Thermo Fisher). This was followed by column-based (QIAGEN DNeasy) or phenol-chloroform purification (Ausubel et al. 1999) of total genomic DNA. PCR was performed in a C1000 Thermal Cycler (Bio-Rad) in a 25 μl final volume containing 1 μl of extracted mycobacterial DNA template. Each reaction contained $1\times$ Q-solution (QIAGEN), 0.2 mM dNTPs, 1 μM each of forward and reverse primer, $1\times$ PCR buffer containing 1.5 mM MgCl_2 (QIAGEN), and 1.25 U *Taq* polymerase (TopTaq, QIAGEN). PCR was performed as follows: initial denaturation of 3 min at 94°C ; 36 cycles of denaturation at 94°C for 30 s, annealing at 61°C (16S rRNA gene), 55°C (*hsp65*), or 58°C (*rpoB*) for 45 s, and extension at 72°C for 60 s; and a 7 min final extension at 72°C . Primer pairs used for amplification of 16S, *hsp65* and *rpoB* were, respectively, M16seq-1F (5'-CTC AGG ACG AAC GCT GGC GG-3') and M16seq-1R (5'-ACT GCC CAG AGA CCC GCC TT-3'); Tb11 and Tb12 (Ringuet et al. 1999); and MF and MR (Kim et al. 1999). Expected product sizes for the 3 primer sets were 696 to 717 bp for 16S, 439 bp for *hsp65*, and 342 bp for *rpoB*. Amplified DNA was electrophoresed on a 2% agarose gel, stained with SybrSafe (Invitrogen), and imaged with 360 nm UV light.

Sequencing. PCR products were purified with ExoSAP-IT (USB Scientific) according to the manufacturer's directions. Direct sequencing was performed on PCR products bi-directionally using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers used for initial amplification. At least 2 separate PCR reactions were sequenced for each isolate.

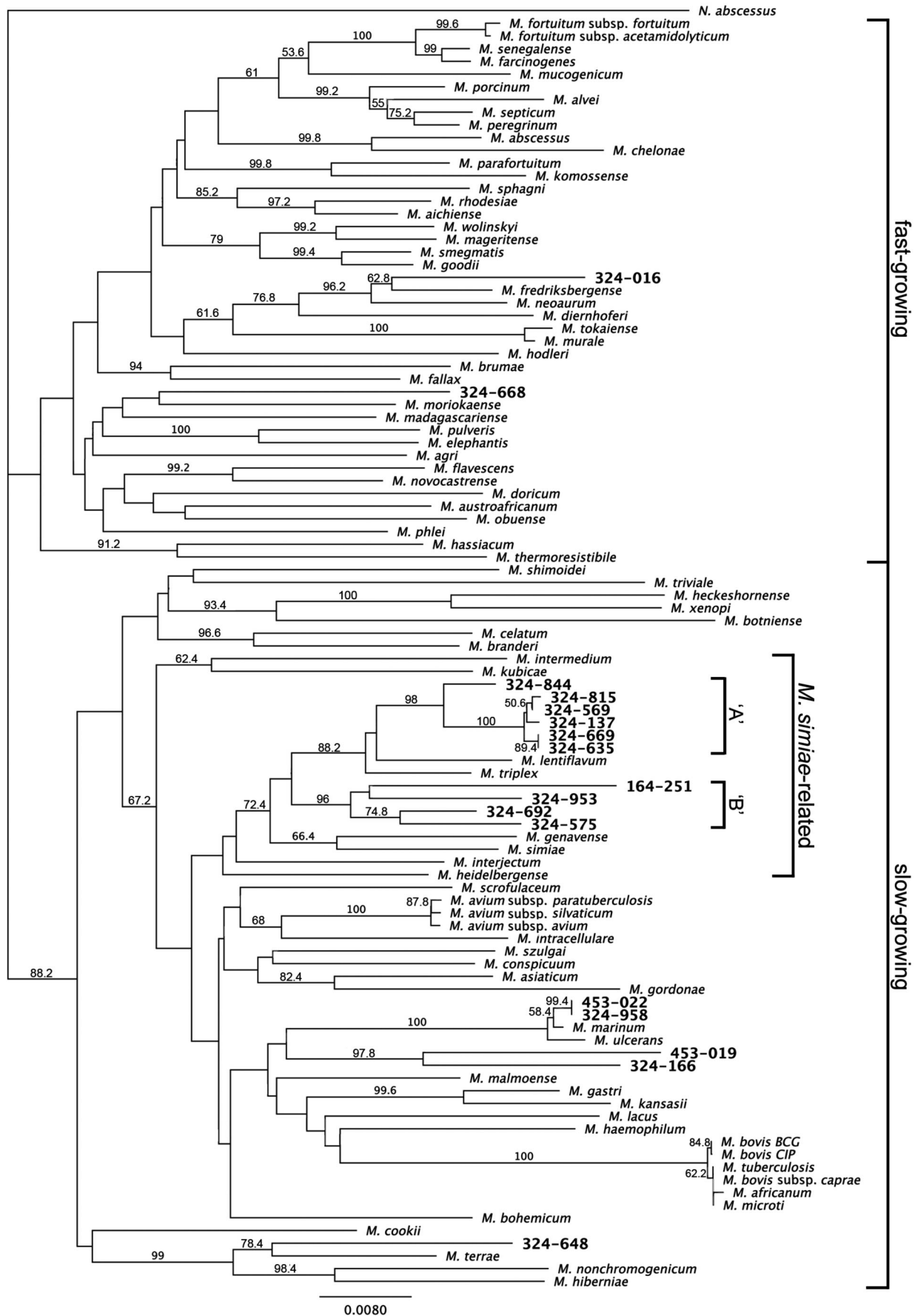
Table 1. Phenotypic characterization of photochromogenic *Mycobacterium* spp. isolated from Chesapeake Bay striped bass *Morone saxatilis*. Groupings are as discussed in the text. Relative growth at room temperature (RT), 30°C and 37°C (when growth was observed) is shown under 'Growth at temp. (°C)'. Arylsulfatase activity is that observed on Day 3 and Day 14. Catalase tests are for heat tolerance at 68°C and semiquantitative production (SQ). Pyrazinamidase activity is that observed on Day 4 and Day 7. ND: no data; ±: variable to weak reaction

Isolate name	Growth at temp. (°C)	Aryl-sulfatase Day 3/14	Catalase 68°C/SQ	Nitrate reductase	Pyrazin-amidase Day 4/7	5% NaCl tolerance	Tween hydrolysis	Urease
<i>M. simiae</i>-related Group A								
324-844	30 > RT	-/+	+/+	-	-/-	-	±	+
324-569	30 ≥ RT	-/+	+/+	-	-/ND	ND	±	+
324-137	30 = RT	-/-	+/+	+	-/-	-	-	+
324-815	30 = RT	-/+	+/+	-	-/-	-	-	+
324-635	30 = RT	-/+	+/+	-	-/-	-	-	±
324-669	30 > RT	-/+	ND/ND	-	-/-	ND	-	+
<i>M. simiae</i>-related Group B								
164-251	30 = RT	-/+	±/±	-	-/-	-	-	-
324-575	30 = RT	-/-	+/+	+	-/-	-	-	+
324-692	30 > RT	-/-	+/+	+	-/±	ND	-	+
324-953	30 = RT	+/+	ND/+	-	-/-	-	-	+
Fast-growing								
324-016	30 = RT	+/+	±/±	-	+/+	+	+	+
324-668	30 > RT > 37	-/-	-/-	+	-/-	-	-	±
<i>M. marinum</i>								
453-022	30 > RT	+/+	-/-	-	-/-	-	±	+
324-958	30 = RT > 37	-/+	ND/-	-	+/+	±	+	+
Misc. slow-growing								
324-166	30 > RT	+/+	-/-	-	-/-	-	-	+
453-019	30 > RT	+/+	±/-	-	-/ND	-	-	-
324-648	30 = RT	±/+	+/+	+	-/-	-	-	+

Sequence analysis. Sequence editing and alignment, as well as BLAST searches and construction of phylogenetic trees, was performed with Geneious software (Biomatters; Drummond et al. 2010). Consensus sequences were generated from at least 6 total forward and reverse sequences, and including sequences from least 2 PCR reactions. 16S rRNA, *hsp65* and *rpoB* gene sequences from Chesapeake Bay striped bass isolates were aligned and trimmed for primer sequences. Additionally, for consistency with previous literature, 16S rRNA gene sequences were trimmed to match position coverage with sequences deposited by Devulder et al. (2005). Sequences were then concatenated in the order 16S rRNA-*hsp65*-*rpoB* and aligned with homologous concatemers generated from *Mycobacterium* spp. sequences from GenBank. In order to maintain

continuity with previous work, all reference sequences (i.e. those not generated from *Mycobacterium* spp. isolates from Chesapeake Bay fishes) were those deposited by Devulder et al. (2005), with exceptions noted in Fig. 1. A final 1277 bp concatemer alignment was generated and used for phylogenetic placement of photochromogenic isolates from Chesapeake Bay striped bass (Fig. 1). Phylogenies based on individual genes were also constructed (not shown). The optimal model for construction of phylogenetic trees was determined for individual alignments with jModelTest (Posada 2008) under default settings and calculating log likelihoods for all available models. Model fit was compared with the Δ AIC method, and the best-fit model was used to construct the phylogenetic tree by maximum likelihood using the PHYML plugin for

Fig. 1. *Mycobacterium* spp. Maximum likelihood analysis of global phylogeny (GTR+I+G model, 4 substitution rates) including concatenated fragments of 16S rRNA, *hsp65* and *rpoB* genes (1277 bp). Photochromogenic isolates from Chesapeake Bay striped bass *Morone saxatilis* are indicated in boldface. Other sequences are as described in Devulder et al. (2005), with the following exceptions: 16S rRNA gene sequences for *M. agri* (AY438067), *M. hiberniae* (AY438069), *M. kommosense* (AY438077) and *M. lacus* (AY438078); *hsp65* sequences for *M. agri* (AY438080), *M. bovis* BCG (AY438082), *M. hiberniae* (AY438083), *M. kommosense* (AY438649) and *M. lacus* (AY438090); *rpoB* sequence for *M. genavense* (AF057467; Kim et al. 2004). Groupings (i.e. fast-growing, slow-growing, *M. simiae*-related) are consistent with previous work (Rogall et al. 1990, Tortoli 2003). Bootstrap (n = 500) proportions > 50% are presented at nodes. The scale bar is substitutions per site



Geneious (Guindon & Gascuel 2003). All phylogenies were run under the General Time-Reversible model estimating invariable sites and 4 substitution rate categories (GTR+I+G). Five hundred bootstrap replicates were performed for all trees.

Sequences at multiple loci (i.e. 16S rRNA, *hsp65*, *rpoB*) from several mycobacteria isolated from Chesapeake Bay fishes other than *Mycobacterium pseudoshottsii* and *M. shottsii* have been deposited in GenBank (nos. EU619837 to EU619935), but, to our knowledge, an analysis of these sequences has not yet been published. Additional 16S rRNA gene sequences from isolates from Chesapeake Bay striped bass have also been deposited (nos. EU541619 to EU541622) and discussed briefly by Gauthier & Rhodes (2009). Using the procedures detailed above, we generated an alignment and phylogenetic tree based on a 458 bp fragment of the 16S rRNA gene common to photochromogenic isolates described in this report as well as to deposited sequences and *M. pseudoshottsii* and *M. shottsii* (Fig. 2). The Tamura–Nei model estimating invariable sites and rate variation (TN+I+G) had the best likelihood score for this analysis as estimated by jModelTest; however, the GTR+I+G model also had support ($\Delta\text{AIC} = 6.8$) and was used for consistency. Trees from both models had nearly identical topologies. Five hundred bootstrap replicates were performed for both analyses.

Sequences from 16S rRNA, *hsp65* and *rpoB* genes have been deposited in GenBank under accession nos. HQ446163 to HQ446213.

RESULTS

Biochemical characteristics of photochromogens recovered from striped bass demonstrated heterogeneity between isolates and within groupings (Table 1). All isolates grew at room temperature and at 30°C, and only 2 (324-688 and 324-016) were capable of growth at 37°C. One isolate in the study, 324-016, was characterized phenotypically as a fast-growing mycobacterium (Runyon Type IV), forming visible colonies on agar in less than 7 d.

The global phylogenetic tree for *Mycobacterium* spp., including data from photochromogens in the present study (Fig. 1), was consistent with that previously reported for a global phylogeny that also included the *sod* gene and a longer *rpoB* fragment (Devulder et al. 2005). Slow-growing and fast-growing mycobacteria were well separated in monophyletic clades; however, the previously reported grouping (Devulder et al. 2005) of the fast-growing *M. fallax* and *M. brumae* within the slow-growing clade was not observed in the present study. Greater than 50% bootstrap support was present for 56.4% of nodes, and bootstrap support for important groupings of mycobacteria, such as the slow growers, was similar to that previously reported (Devulder et al. 2005). The 16S rRNA gene-based phylogeny, including study isolates as well as deposited sequences from Chesapeake Bay fish isolates, was also consistent with previously published work (Rogall et al. 1990, Tortoli 2003, Devulder et al. 2005) in terms of both topology and bootstrap support.

Two of the 17 photochromogenic isolates analyzed in the present study, 324-016 and 324-668, grouped genetically with the fast-growing mycobacteria in the global and 16S phylogenies (Figs. 1 & 2). Isolate 324-016 was grouped with *M. frederiksbergense* and *M. neoaurum* in global and 16S rRNA gene trees, and additionally with *Mycobacterium* sp. strain FL04-58-70A (EU619851) in the 16S rRNA gene phylogeny including deposited sequences from Chesapeake Bay isolates (Fig. 2). Sequence identity with strain FL04-58-70A was 98.8%. Isolate 324-016 was recovered from a striped bass in moderately high density ($6.3 \times 10^4 \text{ g}^{-1}$), and in association with a non-pigmented *Mycobacterium* spp. Minor granulomatous inflammation was noted in the spleen of this fish, as were acid-fast bacteria (Table 2). Isolate 324-668 was grouped with *M. moriokaense* in the global tree, although with no (<50%) bootstrap support. The closest BLAST pairwise similarities to the 16S rRNA gene of isolate 324-668 were from *Mycobacterium* sp. A14N (GenBank no. AB251601) and 'Mycobacteriaceae bacterium MI-6.3 U19', (FN550143), at 99.4% and 99.1%, respectively. The closest pairwise similarity of the 16S rRNA gene with a named species was with *M. moriokaense* strain CIP 105393

Fig. 2. *Mycobacterium* spp. Maximum likelihood analysis of 16S rRNA gene fragment (458 bp) including variable sites A and B (Tortoli 2003). Sequences used are as for Fig. 1, with the addition of deposited 16S rRNA gene sequences obtained from *Mycobacterium* spp. derived from Chesapeake Bay fishes including *M. shottsii* (AY005147) and *M. pseudoshottsii* (AY570988). Bootstrap proportions and scale are as for Fig. 1. Certain clades are collapsed for clarity: *M. farcinogenes* group = *M. farcinogenes*, *M. senegalense*; *M. kommosense* group = *M. kommosense*, *M. aichiense*, *M. rhodesiae*; *M. murale* group = *M. murale*, *M. tokaiense*; *M. smegmatis* group = *M. smegmatis*, *M. goodii*, *M. madagascariense*, *M. elephantis*, *M. pulveris*, *M. moriokaense*, *M. thermoresistibile*, *M. flavescens*; *M. agri* group = *M. agri*, *M. doricum*, *M. austroafricanum*, *M. novocastrense*; *M. simiae*-like Group 'A' = study isolates as described in text; *M. xenopi* group = *M. xenopi*, *M. heckeshornense*, *M. botniense*; *M. avium* group = *M. avium* subsp. *avium*, *silvaticum* and *paratuberculosis*; *M. tuberculosis* group = *M. tuberculosis*, *M. bovis* BCG, *M. bovis* CIP, *M. bovis* subsp. *caprae*, *M. africanum*, *M. microti*; *M. cookii* group = *M. cookii*, *M. branderi*; *M. terrae* group = *M. terrae*, *M. hiberniae*, *M. nonchromogenicum*, *M. triviale*

Table 2. Details of isolation for photochromogenic *Mycobacterium* spp. in the present study. Density is given as \log_{10} bacteria g^{-1} spleen (see Rhodes et al. 2004). Acid-fast co-isolates from the same spleen sample are given as: Sc = scotochromogenic isolate, Mp = *M. pseudoshottsii*, Ms = *M. shottsii*, NP = non-pigmented isolate; *co-isolate numerically dominant in the sample. The collection location is given as Rappahannock River (RR), with distance upriver in kilometres as measured by the central channel (RR 0 = river mouth), or mainstem Stn 1 (38.3068° N, 76.2838° W) or Stn 12 (37.6483° N, 76.2742° W). Fish age was determined by either otolith or scale analysis. 'Disease severity' is explained in 'Materials and methods'; the presence (+) or absence (-) of acid-fast bacteria (AF) is given in parentheses

Isolate name	Density (\log_{10})	Co-isolate (acid-fast)	Fish collection date (mm/dd/yy)	Collection location	Fish age (yr)	Fork length (mm)	Sex	Disease severity (AF +/-)
<i>M. simiae</i>-related Group A								
324-844	2.0	Sc	4/2/07	RR 72	5	520	M	0 (-)
324-569	1.7	None	11/14/05	Mainstem (12)	2	319	F	0 (-)
324-137	1.8	Sc	10/16/06	RR 72	4	494	M	0 (-)
324-815	2.5	Sc	4/2/07	RR 72	5	479	M	1 (+)
324-635	2.1	None	4/6/06	RR 72	2	380	F	1 (-)
324-669	1.7	None	4/11/10	RR 72	3	432	M	1 (-)
<i>M. simiae</i>-related Group B								
164-251	2.2	None	3/21/05	Mainstem (1)	2	217	M	1 (+)
324-575	3.0	Mp	11/14/05	Mainstem (12)	2	299	F	1 (+)
324-692	1.6	None	4/10/06	RR 72	4	531	M	1 (+)
324-953	3.2	Ms	4/5/07	RR 72	5	562	M	1 (-)
Fast-growing								
324-016	4.8	NP	10/12/06	RR 72	3	423	M	1 (+)
324-668	3.4	Sc, Ms*	4/10/06	RR 72	4	516	M	1 (+)
<i>M. marinum</i>								
453-022	8.2	None	5/8/07	RR 0	3	476	M	2 (+)
324-958	5.8	None	4/5/07	RR 72	4	468	M	2 (+)
Misc. slow-growing								
324-166	6.5	Ms*	10/20/06	RR 0	4	541	M	2 (+)
453-019	5.6	None	5/8/07	RR 0	5	567	M	0 (-)
324-648	2.1	None	4/6/06	RR 72	2	385	M	1 (-)

(AY859686), at 98.2%. Isolate 324-668 was capable of growth at 37°C and was the only photochromogenic study isolate to possess the cytosine insertion at *Escherichia coli* position 184 characteristic of thermotolerant fast-growing mycobacteria (Tortoli 2003). 16S rRNA variable region B displayed a typical insertion-free (short-helix) sequence. Two deposited 16S rRNA gene sequences from Chesapeake Bay fishes grouped with isolate 324-668, although without bootstrap support (Fig. 2). These sequences, from *Mycobacterium* spp. FL04-42-151SpA (EU619845) and FL04-42-158A (EU619872) demonstrated 96.5% and 98.8% identity with 324-668, respectively. Isolate 324-668 was present at a density of $2.5 \times 10^3 g^{-1}$ splenic tissue in a polyinfection with *M. shottsii* and a scotochromogenic *Mycobacterium* (Table 2). Minor granulomatous inflammation and acid-fast bacteria were present.

A majority (10/17) of photochromogenic study isolates clustered in the global phylogeny with the '*Mycobacterium simiae*-related' group described by Tortoli (2003). These isolates formed 2 groups in the global phylogeny, with high (>85%) bootstrap support for the proximal node including all group isolates. We refer here to these groups as *M. simiae*-related groups 'A' and 'B' (Fig. 1).

Within Group A, isolates 324-635 and 324-669 were 100% similar at all loci, and 324-815 and 324-569 differed by 1 position in the *rpoB* gene. All *M. simiae*-related Group A isolates shared 100% similarity at the 16S rRNA gene locus. *M. simiae*-related Group B isolates, consisting of 164-251, 324-953, 324-575 and 324-692, differed from each other and from Group A isolates at all loci. All 10 isolates in *M. simiae*-related Groups A and B had an uninserted helix 18 in the 16S rRNA gene, but variable region A, particularly *E. coli* positions 189 to 195, was consistent within groups. Group A isolates had the sequence 5'-ACG AGG C-3' at this position, which is consistent with *M. stomatepia* (HM022202) and '*M. sherrisii*' (AY353699), as well as deposited sequences from 3 Chesapeake Bay isolates, i.e. *Mycobacterium* spp. strains L30 (EU541618), FL04-60-11A (EU619875) and FL04-60-260B (EU619864). With the exception of 164-251 (5'-ATT TAG C-3'), *M. simiae*-related Group B isolates had the sequence 5'-ACT TAG C-3' at this position. This sequence at this position appears to be unique among the mycobacteria, with the exception of a deposited sequence from an uncultured soil isolate (GU172203; uncultured bacterium clone DSM-R31). Densities of *M. simiae*-related Group A and B isolates

recovered from striped bass spleens were generally low, ranging from 4.0×10^1 to 1.6×10^3 g⁻¹ spleen. Some (5/10) isolates were present in monoinfections, and polyinfections with *M. pseudoshottsii* and *M. shottsii* as well as uncharacterized scotochromogens were observed. In the case of monoinfections, granulomatous inflammation was observed in 4/5 instances; however, disease severity was minor. Acid-fast bacteria were observed in 2/5 monoinfections (Table 2). In addition to the 10 study isolates, 15 deposited sequences from *Mycobacterium* spp. isolated from Chesapeake Bay fishes were grouped with the *M. simiae*-related mycobacteria in the 16S rRNA phylogeny including these sequences (Fig. 2).

Two of the 17 study isolates, 453-022 and 324-958, were 100% similar to *Mycobacterium marinum* ATCC 927 at all 3 loci tested. Isolates 453-022 and 324-958 are separated from *M. marinum* in the global alignment due to a 2 bp difference from the *M. marinum* sequence deposited by Devulder et al. (2005), which was from strain CIP104528. Both isolates were phenotypically consistent with *M. marinum* (Lévy-Frédault & Portaels 1992), but differed in pyrazinamidase activity. In both cases, these isolates were recovered as a monoinfection from striped bass spleen, and were present in high densities of 1.6×10^8 g⁻¹ and 6.3×10^5 g⁻¹ spleen, respectively. Moderate granulomatous inflammation and large numbers of acid-fast bacteria were present in spleens from both fish (Table 2).

The phylogenetic placement of isolates 324-166 and 453-019 was ambiguous. Both were definitively placed within the slow-growing mycobacteria by a 12 bp insertion in helix 18 (Tortoli 2003). These isolates clustered together with high bootstrap support in the global phylogeny (Fig. 1); however, 16S rRNA gene phylogeny, including other Chesapeake Bay isolates, placed the former with *M. gordonae* and the latter in a group with members of the *M. marinum* clade and the *M. tuberculosis* clade. BLAST analysis was not particularly helpful in resolving the position of these isolates, as the highest similarity returned was 98.9% to the 16S rRNA gene of *M. tuberculosis* H37Ra (CP000611) for isolate 453-019 and 98.9% to the 16S rRNA gene of *M. gordonae* for isolate 324-166. The 16S rRNA phylogeny including Chesapeake Bay-derived sequences grouped isolate 324-166 with the 16S rRNA gene for the striped bass isolates FL04-5-253F (EU619842), FL04-23-N47C (EU619839), and FL04-60-213LA (EU619841), although without bootstrap support (Fig. 2). Isolate 453-019 was a sole, high-density (4.0×10^5 g⁻¹ spleen) isolate, and no granulomatous inflammation or acid-fast bacteria were noted. Isolate 324-166 was found at high density (3.2×10^6 g⁻¹ spleen) in a polyinfection with high-density *M. shottsii*, and moderate granulomatous inflammation with acid-fast bacteria was present (Table 2).

Isolate 324-648 did not group closely with other study isolates, but was placed with *M. terrae* in the global phylogeny. Sequence identity with *M. terrae* strain ATCC 15755 (DQ058407) was 97.1%, although higher identity (98.7%) was observed with a sequence reported for *M. terrae* strain M64 (GQ184162). Sequence identity of 100% was noted with the 16S rRNA gene of *Mycobacterium* sp. GN-9188 (FJ497240), a human clinical isolate from Greece. Isolate 324-648 was obtained as a low-density, sole isolate (1.3×10^2 g⁻¹ spleen), and minor granulomatous inflammation was noted with no acid-fast bacteria observed. Coloration of this isolate was unique, as it was pale pink, whereas all other isolates were pale to golden yellow. Placement of this isolate via the 16S rRNA phylogeny, including deposited Chesapeake Bay-derived sequences, was problematic in that the isolate was placed with the fast-growing *M. fallax* as well as *Mycobacterium* spp. strains 06FL-1-2A (EU619852) and 06FL-1-5B-1 (EU619837) (Fig. 2). While *M. fallax* has an uninserted short helix in the 16S variable region B, isolate 324-648, as well as *Mycobacterium* spp. strains 06FL-1-2A and 06FL-1-5B-1, have insertions in this region, clearly marking them genetically as slow-growing mycobacteria. Interestingly, while *M. fallax* is included in the same clade as the slow-growing *M. terrae* when isolate 324-648 is included in the 16S rRNA phylogeny excluding additional Chesapeake Bay isolates, *M. fallax* and *M. terrae* are placed in separate clades when isolate 324-648 is omitted.

DISCUSSION

Two isolates in the present study clustered genetically with fast-growing mycobacteria, but were not closely similar to either *M. chelonae* or *M. fortuitum*. Isolate 324-016 was clustered with *M. frederiksbergense* and related organisms by global and individual gene phylogenies. Sequence identity with *M. frederiksbergense* was 99.5% at the 16S rRNA locus, and higher identity (99.8%) was seen to *Mycobacterium* sp. IK1-18P (AB461006), isolated from soybeans. This grouping of mycobacteria contains the soil isolates *M. frederiksbergense* (Willumsen et al. 2001), *M. neoaurum*, *M. hodleri* and *M. diernhoferi* (Tsukamura et al. 1983), of which *M. neoaurum* has been implicated in human disease (Davison et al. 1988, Morimoto et al. 2007) and has been described from Chinook salmon *Oncorhynchus tshawytscha*, although this identification was made from phenotypic characters alone (Backman et al. 1990). Members of this group of mycobacteria can degrade polycyclic hydrocarbons (Kleespies et al. 1996, Willumsen et al. 2001) and are thus considered to be highly adapted to the environ-

mental niche, although human infections with *M. neoaurum* and striped bass infection with isolate 324-016 in the present study indicate that, like many other 'environmental' mycobacteria, members of this group are capable of opportunistic infection. The phylogenetic position of the second isolate in the fast-growing group, 324-668, was not well resolved. The closest 16S rRNA gene matches were to environmental isolates or environmental DNA, while the most closely similar recognized species was *M. moriokaense*, an isolate from human and soil sources (Tsukamura et al. 1986). This isolate did demonstrate affinity with the thermotolerant fast-growing mycobacteria, based on a characteristic cytosine insertion in helix 10 of the 16S rRNA.

The numerically dominant group of photochromogenic mycobacteria in the present study was clustered with the '*Mycobacterium simiae*-related' group described by Tortoli (2003), including *M. lentiflavum*, *M. simiae*, *M. triplex*, *M. genavense*, *M. palustre*, *M. interjectum*, *M. intermedium* and related species. While considered the most homogeneous grouping in *Mycobacterium* spp. based on the 16S rRNA sequence (Tortoli 2003), this group demonstrates considerable diversity in terms of phenotype and reservoirs. While several species in this group are known only from human clinical samples, others (e.g. *M. palustre*, *M. genavense* and *M. simiae*) have been detected in water and non-human animals (Schroder et al. 1992, Portaels et al. 1996, Torkko et al. 2002, Conger et al. 2004), and *M. lentiflavum*-like 16S rRNA sequences have been obtained from soil by PCR (Mendum et al. 2000). Mycobacteria belonging to the *M. simiae*-related group have also been described from a number of other finfishes (Herbst et al. 2001, Levi et al. 2003, Whipps et al. 2003, Poort et al. 2006). Whereas the diversity of *M. marinum*-group organisms infecting poikilotherms has received much attention in recent years (Ranger et al. 2006, Yip et al. 2007), the diversity of *M. simiae*-related mycobacteria is considerably less well studied.

With the exceptions of *M. marinum* isolates 324-958 and 453-022, none of the isolates described in the present study was closer than 99.0% identical at any locus to previously deposited mycobacterial sequences from Chesapeake Bay isolates, further demonstrating the unrecognized diversity present among *Mycobacterium* isolates from this environment. Examination of the partial 16S rRNA gene phylogeny of all study isolates and deposited sequences, however, does demonstrate some interesting trends. Most notable is the large number of isolates in the *M. simiae*-related group, with 10 of 17 photochromogenic isolates from the present study and 15 of 45 previously deposited sequences. It is therefore apparent

that fishes in Chesapeake Bay are capable of harbouring a large diversity of *Mycobacterium* spp. belonging to this taxonomic grouping, and that the prevalence and potential pathogenicity of these bacteria may be worthy of future study.

Isolates 324-016 and 324-668, which were genetically grouped with fast-growers in the present study, were grouped with 1 and 2 additional deposited sequences, respectively. In comparison, only 2 deposited sequences from *Mycobacterium* spp. strains FL04-68-5LA (EU619843) and FL04-58-32A (EU619844) were grouped closely with the classical fish pathogen *M. fortuitum* and none with *M. chelonae*/*M. abscessus*. This contrasts with most other reports of fast-growing mycobacterial infections in fishes, which typically implicate *M. fortuitum* or *M. chelonae*, instead involving mycobacteria from groups primarily associated with hydrocarbon degradation in contaminated soils.

Within study isolates genetically identified as slow-growers, 3 in particular (324-166, 324-648 and 453-019) were not well placed taxonomically, with inconsistent grouping and/or no bootstrap support at individual gene loci. All 3 isolates did, however, group with sequences from other Chesapeake Bay isolates (Fig. 2). It is worth noting that, of the 17 photochromogenic study isolates, only 4 (324-166 and 453-019, *M. marinum* isolates 453-022 and 324-958) were separated from another sequence derived from a Chesapeake Bay isolate by more than 1 node in the 16S rRNA phylogeny, and only 453-019 was more than 2 nodes distant from a grouping of these sequences. This would seem to indicate that despite the great diversity of *Mycobacterium* spp. that infect Chesapeake Bay fishes, the major groupings to which these species and strains belong are beginning to be described.

It should be carefully noted that isolates described in the present study represent single isolations from individual animals, and consequently, no information is conveyed as to their prevalence and distribution. Further, while some isolates were obtained in monocultures, and granulomatous inflammation was present in some of these cases, this is not a fulfilment of Koch's postulates and little can be said about their pathogenicity or virulence. The ability of bacterial and parasitic agents other than *Mycobacterium* spp. to elicit granulomatous inflammation is well-known, and we do not assume here that all observed granulomata were due to *Mycobacterium*. This situation is further complicated in the case of polyinfections with known pathogens such as *Mycobacterium shottsii*. *M. simiae*-group isolates, in particular, were typically found at low densities in the spleen and did not appear to be associated with severe pathology; however, whether they are capable of producing significant disease under conditions of host stress is unknown.

Acknowledgements. Funding to D.T.G. and A.M.H. for multi-locus sequence analysis was provided by the Old Dominion University Undergraduate Research Grant program. Isolates from the Rappahannock River, Virginia, were collected during projects funded by NOAA (Chesapeake Bay Office) and Virginia Marine Resources Commission through the Wallop-Breaux-funded striped bass monitoring and assessment program. We thank Dr. J. Hoenig, P. Sadler and D. Gonzales for assistance with these projects. Samples from the mainstem Chesapeake Bay were collected using the Chesapeake Bay Multispecies Monitoring and Assessment Program (Ches-MMAP) and funding from United States Geologic Survey Cooperative Research Agreement 02ERAG0071. We thank Dr. R. Latour, C. Bonzek and J. Gartland for use of the Ches-MMAP platform and Drs. C. Ottinger and F. Panek for their participation in the USGS agreement. The authors also thank M. Korngiebel-Rosique and M. Colasanto for laboratory assistance.

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Editorial responsibility: David Bruno,
Aberdeen, UK

Submitted: November 2, 2010; Accepted: February 25, 2011
Proofs received from author(s): May 26, 2011