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ORIGINAL PAPER

Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (Pseudonocardiaceae, Actinomycetales): a test of two symbiont-association models

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Abstract We reconstruct the phylogenetic relationships within the bacterial genus *Pseudonocardia* to evaluate two models explaining how and why *Pseudonocardia* bacteria colonize the microbial communities on the integument of fungus-gardening ant species (Attini, Formicidae). The traditional Coevolution-Codivergence model views the integument-colonizing *Pseudonocardia* as mutualistic microbes that are largely vertically transmitted between ant generations and that supply antibiotics that specifically suppress the garden pathogen *Escovopsis*. The more recent Acquisition model views *Pseudonocardia* as part of a larger integumental microbe community that frequently colonizes the ant integument from environmental sources (e.g., soil, plant material). Under this latter model, ant-associated *Pseudonocardia* may have diverse ecological roles on the ant integument (possibly ranging from pathogenic, to commensal, to mutualistic) and are not necessarily related to *Escovopsis* suppression. We test distinct predictions of these two models regarding the phylogenetic proximity of ant-associated and environmental *Pseudonocardia*. We amassed 16S-rRNA gene sequence information for 87 attine-associated and 238 environmental *Pseudonocardia*, aligned the sequences with the help of RNA secondary structure modeling, and reconstructed phylogenetic relationships using a maximum-likelihood approach. We present 16S-rRNA secondary structure models of representative *Pseudonocardia* species to improve sequence alignments and identify sequencing errors. Our phylogenetic analyses reveal close affinities and even identical sequence matches between environmental *Pseudonocardia* and ant-associated *Pseudonocardia*, as well as nesting of environmental *Pseudonocardia* in subgroups that were previously thought to be specialized to associate only with attine ants. The great majority of ant-associated *Pseudonocardia* are closely related to autotrophic *Pseudonocardia* and are placed in a large subgroup of *Pseudonocardia* that is known essentially only from cultured isolates (rather than cloned 16S sequences). The preponderance of the known ant-associated *Pseudonocardia* in this latter clade of culturable lineages may not necessarily reflect abundance of these *Pseudonocardia* types on the ants, but isolation

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biases when screening for *Pseudonocardia* (e.g., preferential isolation of autotrophic *Pseudonocardia* with minimum-nutrient media). The accumulated phylogenetic patterns and the possibility of isolation biases in previous work further erode support for the traditional Coevolution-Codivergence model and calls for continued revision of our understanding how and why *Pseudonocardia* colonize the microbial communities on the integument of fungus-gardening ant species.

Keywords Attine ant-microbe symbiosis · Mutualism · Antibiotic · Secondary rRNA structure

Introduction

Fungus-growing ants (Attini, Formicidae) cultivate a basidiomycete fungus in gardens that the ant nurture with dead or fresh plant substrate. The main food source for the ants is the cultivated fungus, which is thought to comprise the main living biomass in attine gardens (Mueller et al. 2005). The gardens are actually mini-ecosystems of competing, commensal, and mutualistic microbes, including a diverse assembly of filamentous fungi, yeasts, and bacteria (Bacci et al. 1995; Carreiro et al. 1997; Currie et al. 1999a; Santos et al. 2004; Mueller et al. 2005; Rodrigues et al. 2008; Rodrigues et al. 2009). The interaction network of these microbes is engineered in part by the physical manipulations and secretions of the ants (Santos et al. 2004; Mueller et al. 2005). Significant populations of microbes, including actinomycetes, also colonize the integument and the alimentary canal of the farming ants (the ant microbiome), as is probably true for most arthropods (Goodfellow and Williams 1983; Dattagupta et al. 2009 and references therein). A diversity of integumental actinomycete bacteria can be readily isolated from most attine ants with minimum-nutrient media favoring autotrophic bacterial growth, including bacteria in the genus *Pseudonocardia* (Cafaro and Currie 2005; Currie et al. 2006; Mueller et al. 2008; Fernández-Marín et al. 2009; Sen et al. 2009). As an extreme case, workers of the attine ant *Mycocepurus smithii* can be colonized by significant populations of several species of *Pseudonocardia*, one species of the closely related genus *Amycolatopsis*, and many other actinomycete and non-actinomycete bacteria (Sen et al. 2009).

At the time of the discovery of actinomycete growth on the attine integument 10 years ago, it was thought that the ants promote the growth of antibiotic-secreting actinomycetes on their integument as a specific defense to suppress the garden parasite *Escovopsis* (Hypocreales, Ascomycota) (Currie et al. 1999a, b; Currie 2001). The almost simultaneous discovery of the parasite *Escovopsis* and the integumental actinomycete growth led to the immediate speculation that *Escovopsis* and the growth must somehow be ecologically linked. Specifically, Currie et al. (1999b) and Currie (2001) argued that attine ants sustain growth of the actinomycete *Pseudonocardia* as mutualists on their integument to specifically combat *Escovopsis* parasites; that the mutualistic *Pseudonocardia* have been engaged in a co-evolutionary arms race with *Escovopsis* since the origin of the attine ant-fungus mutualism 50 million years ago; and that *Escovopsis* largely failed to evolve effective resistance against *Pseudonocardia* because of some unknown disadvantage in the co-evolutionary arms race (see also recent reviews discussing the *Escovopsis-Pseudonocardia* arms race; Suen and Currie 2008; Poulsen et al. 2009).

The original report and a string of follow-up studies seemed to support this ant-*Escovopsis-Pseudonocardia* co-evolution model, including (a) derived cuticular structures that seem designed to facilitate actinomycete colonization of the worker integument (Currie et al. 2006); (b) glandular structures underlying areas of significant actinomycete accumulation on the integument, suggesting that the ants may manipulate microbial growth through secretions (Currie et al. 2006); (c) greater susceptibility of gardens to *Escovopsis* infection if the integumental accretions are scraped off from workers (a procedure also likely to stress or injure ants) (Currie et al. 2003); (d) apparent clade-to-clade correspondences between ant, *Escovopsis*, and *Pseudonocardia* phylogenies, supporting the possibility of tight co-diversification in an ancient ant-*Pseudonocardia* association (Cafaro and Currie 2005; Poulsen et al. 2009); (e) marginal (but not strong) inhibition of *Escovopsis* by a peptide secreted in high concentrations by a *Pseudonocardia* isolate in vitro (Oh et al. 2009); (f) a growth-enhancing effect on the fungal cultivar by an unidentified actinomycete isolated from an ant integument (Currie et al. 1999b);

and (g) specific antibiosis of a single *Pseudonocardia* isolate against *Escovopsis* but not against 17 non-*Escovopsis* test fungi (Currie et al. 1999b). Taken together, the accumulating evidence seemed to support ant-*Escovopsis*-*Pseudonocardia* co-evolution and the possibility of a 50-million-year-old *Pseudonocardia*-*Escovopsis* arms race.

The image of co-evolved, pesticide-secreting, integumental microbes of attine ants seemed so convincing that the National Science Teacher Association (NSTA) and several science museums in the USA began promoting the attine-*Pseudonocardia*-*Escovopsis* association as an indisputable example of co-evolution (Diamond 2006). To visualize the evidence of co-evolved, specific antibiosis against *Escovopsis*, a hands-on exercise developed by NSTA asks biology students to measure growth of two garden pathogens, *Escovopsis* and *Trichoderma,* growing under the influence of *Pseudonocardia* secretions. Because the growth tracks on paper show that *Pseudonocardia* strongly inhibits *Escovopsis* but not *Trichoderma* (a finding originally reported in Currie et al. 1999b), *Pseudonocardia* apparently exhibits specialized antibiosis against *Escovopsis*, because, if *Pseudonocardia* "developed a substance that would kill only [*Escovopsis*], then that would support the idea that the two organisms had coevolved" (Diamond 2006, page 95; see www.nsta.org/pdfs/virus/Virus-Activity3.pdf).

A series of recent studies have eroded much of the key evidence that was thought to support the original formulation of specific antibiosis and ant-*Pseudonocardia*-*Escovopsis* co-evolution. For example, Kost et al. (2007) and Sen et al. (2009) showed that *Pseudonocardia* from the integument of attine ants have generalized, broad-spectrum antibiotic activities, comparable to the activities of environmental (free-living) actinomycete bacteria, and that therefore the attine-associated *Pseudonocardia* are not antibiotically specialized and evolutionarily derived as postulated by Currie et al. (1999b). Moreover, antibiotics secreted in vitro by ant-associated *Pseudonocardia* strongly suppress or kill the cultivated fungi from the corresponding nests (Sen et al. 2009), contrary to the original report of a growth-enhancing effect of *Pseudonocardia* on the cultivated fungi (Currie et al. 1999b). Even more unfortunate was a great delay before other research groups could replicate some of the original experiments. Because of a regrettable oversight, the original report did not specify the microbial methods for isolating *Pseudonocardia* from attine ants (Currie et al. 1999b), and it was not until 2005, with the first publications of the exact methods to isolate *Pseudonocardia* from ants (Cafaro and Currie 2005), that other research groups began to evaluate the earlier findings. Reviewing the history of ant-actinomycete research in a recent commentary, Boomsma and Aanen (2009) concluded that there exists a need for more careful hypothesis testing to prevent oversimplified evolutionary conclusions, and that a key message from ten years of ant-*Pseudonocardia* research may be the reminder to scientists at large "to continuously re-evaluate what we know for a fact and what we merely infer".

Following Boomsma and Aanen's (2009) call for more careful hypothesis testing to avoid premature conclusions of adaptive and co-evolved antibiotic design (*sensu* Gould and Lewontin 1979), we expand here on a test of phylogenetic predictions that was first explored by Mueller et al. (2008), testing for close phylogenetic affinities between ant-associated and environmental *Pseudonocardia* lineages. This same test can now be applied to a much larger dataset, because four times the sequence information has become available at GenBank for *Pseudonocardia*. Our test specifically juxtaposes critical predictions of the traditional ant-*Pseudonocardia*-*Escovopsis* Coevolution-Codivergence model (Currie et al. 1999b; Poulsen et al. 2009) and predictions of the alternate Acquisition model (Kost et al. 2007; Mueller et al. 2008). These two models represent extreme viewpoints, but they make different predictions regarding the phylogenetic proximity of ant-associated and environmental *Pseudonocardia*.

Predictions of the traditional Coevolution-Codivergence model

The Coevolution-Codivergence model assumes that the diversity of attine ants associate only with a limited number of *Pseudonocardia* lineages, and that these few lineages have co-diverged with corresponding ant lineages during a long association spanning millions of years (Cafaro and Currie 2005; Suen and Currie 2008; Poulsen et al. 2007; Poulsen et al. 2009). These predictions rest on two main assumptions; first that each attine colony associates with only a single *Pseudonocardia* strain; and second, that *Pseudonocardia* lineages are largely vertically transmitted

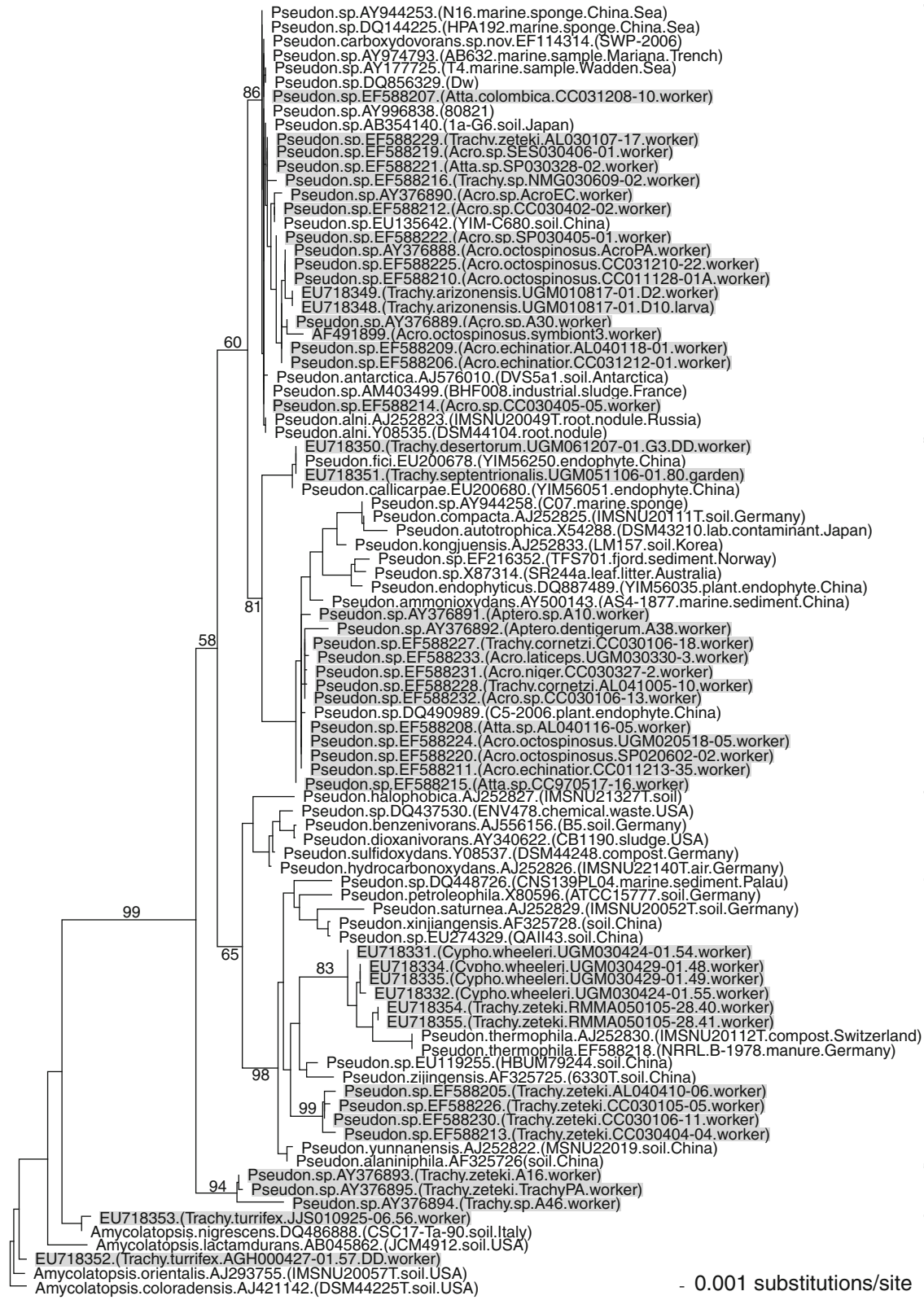
across many ant generations, punctuated by occasional horizontal transfer between ant lineages, and punctuated very rarely by de novo acquisition of *Pseudonocardia* from environmental sources (Cafaro and Currie 2005; Poulsen et al. 2007; Poulsen et al. 2009; Caldera et al. 2009). The traditional *Coevolution-Codivergence* model emphasizes *rare* acquisition from environmental sources, *long-term* vertical transmission of *Pseudonocardia* associates across many ant generations, and *strong* co-evolutionary interactions between *Pseudonocardia* and *Escovopsis*.

Prediction of the alternate Acquisition model

The Acquisition model assumes continual colonization of the attine integument by diverse microbes (including *Pseudonocardia*), microbial turnover on the integument, and regular loss (purging) of some integumental microbes. In essence, the Acquisition model assumes a microbiome on the ant integument that may parallel the complex surface microbiome of other arthropods (e.g., Dattagupta et al. 2009 and references therein). Specifically, the Acquisition model assumes that (i) a great diversity of microbes inhabits the ant integument; (ii) specific microbes colonize the ants predictably because the ants encounter these microbes inevitably in their particular environment or in their interactions with the gardening substrate; (iii) some of these integumental microbes may be transmitted through foundress queens between generations, but long-term vertical transmission across many ant generations is limited because of continual microbial turnover on the ant integument; (iv) not all *Pseudonocardia* strains on the ant integument are mutualists (some may be commensal, some may even be pathogenic); (v) antibiotic effects against *Escovopsis* may be incidental byproducts—but not necessarily co-evolved adaptations—of microbial evolution occurring *before* colonization of the ant integument or occurring in situ on the integument under microbe-microbe competition for limited resources (Kost et al. 2007; Mueller et al. 2008; Sen et al. 2009). The Acquisition model emphasizes *frequent* acquisition of *Pseudonocardia* from environmental sources, limited vertical transmission of *Pseudonocardia* associates across many ant generations, and *low* potential or absence of *Pseudonocardia-Escovopsis* co-evolution.

Using 16S rRNA gene sequence information from 45 ant-associated and 41 environmental *Pseudonocardia*, Mueller et al. (2008) recently began to reconstruct the phylogenetic relationships between these two types of *Pseudonocardia* within the genus. This previous study identified a number of ant-associated *Pseudonocardia* that are sequence-identical at the 16S gene to environmental *Pseudonocardia*, and documented that well-supported clades include closely related environmental and ant-associated *Pseudonocardia* that differ in only a few base pairs (Fig. 1). However, phylogenetic analyses also revealed some *Pseudonocardia* clades that appeared derived and that consisted exclusively of *Pseudonocardia* isolated from ants (Fig. 1). These derived *Pseudonocardia* clades could be ant-specific either because (a) these *Pseudonocardia* were anciently acquired by the ants from environmental sources and since then had been exclusively associated with attine ants; or (b) the clades appear only ant-specific because free-living *Pseudonocardia* are undersampled. This later explanation predicts that more comprehensive sampling of free-living *Pseudonocardia* will eventually show that unknown environmental strains exist that are nested within the putative ant-specific clades or that are very closely related to them. Because over 500 *Pseudonocardia* sequences have accumulated to date at GenBank (compared to 85 sequences in the analysis of Mueller et al. 2008), the above predictions of the Acquisition model can now be tested with a more comprehensive sampling of *Pseudonocardia* diversity. Additionally, we show

Fig. 1 Phylogeny of *Pseudonocardia* reconstructed by Mueller et al. (2008) from 16S rRNA gene sequence information. This reconstruction was based on a limited sample of 85 *Pseudonocardia* sequences available at GenBank in September 2007 and excluded sequence information in regions of unambiguous alignment (i.e., the reconstruction was not based on the 16S secondary-structure modeling employed in the present study). The clade labels on the far right correspond to the labels in Fig. 2a and b of the present study (Clade 1&2 was called "compacta-group" and Clade 3 was called "alni-group" in Mueller et al. 2008). This previous analysis revealed close affinities between many ant-associated (*shaded*) and environmental *Pseudonocardia* (*unshaded*), and predicted that future surveys will discover environmental *Pseudonocardia* nesting in groups appearing "ant-specific" at that time. This prediction is tested in the phylogenetic reconstruction shown in Fig. 2a and b, which is based on four times the sequence information that has become available for *Pseudonocardia* since the study by Mueller et al. (2008) ▶


Pseudon.sp.AY944253.(N16.marine.sponge.China.Sea)
Pseudon.sp.DQ144225.(HPA192.marine.sponge.China.Sea)
Pseudon.carboxydovorans.sp.nov.EF114314.(SWP-2006)
Pseudon.sp.AY974793.(AB632.marine.sample.Mariana.Trench)
Pseudon.sp.AY177725.(T4.marine.sample.Wadden.Sea)
Pseudon.sp.DQ856329.(Dw)
Pseudon.sp.EF588207.(Atta.colombica.CC031208-10.worker)
Pseudon.sp.AY996838.(80821)
Pseudon.sp.AB354140.(1a-G6.soil.Japan)
Pseudon.sp.EF588229.(Trachy.zeteki.AL030107-17.worker)
Pseudon.sp.EF588219.(Acro.sp.SES030406-01.worker)
Pseudon.sp.EF588221.(Atta.sp.SP030328-02.worker)
Pseudon.sp.EF588216.(Trachy.sp.NMG030609-02.worker)
Pseudon.sp.AY376890.(Acro.sp.AcroEC.worker)
Pseudon.sp.EF588212.(Acro.sp.CC030402-02.worker)
Pseudon.sp.EU135642.(YIM-C680.soil.China)
Pseudon.sp.EF588222.(Acro.sp.SP030405-01.worker)
Pseudon.sp.AY376888.(Acro.octospinosus.AcroPA.worker)
Pseudon.sp.EF588225.(Acro.octospinosus.CC031210-22.worker)
Pseudon.sp.EF588210.(Acro.octospinosus.CC011128-01A.worker)
EU718349.(Trachy.arizonensis.UGM010817-01.D2.worker)
EU718348.(Trachy.arizonensis.UGM010817-01.D10.larva)
Pseudon.sp.AY376889.(Acro.sp.A30.worker)
AF491899.(Acro.octospinosus.symbiont3.worker)
Pseudon.sp.EF588209.(Acro.echinatior.AL040118-01.worker)
Pseudon.sp.EF588206.(Acro.echinatior.CC031212-01.worker)
Pseudon.antarctica.AJ576010.(DVS5a1.soil.Antarctica)
Pseudon.sp.AM403499.(BHF008.industrial.sludge.France)
Pseudon.sp.EF588214.(Acro.sp.CC030405-05.worker)
Pseudon.alni.AJ252823.(IMSNU20049T.root.nodule.Russia)
Pseudon.alni.Y08535.(DSM44104.root.nodule)
EU718350.(Trachy.desertorum.UGM061207-01.G3.DD.worker)
Pseudon.fici.EU200678.(YIM56250.endophyte.China)
EU718351.(Trachy.septentrionalis.UGM051106-01.80.garden)
Pseudon.callicarpae.EU200680.(YIM56051.endophyte.China)
Pseudon.sp.AY944258.(C07.marine.sponge)
Pseudon.compacta.AJ252825.(IMSNU20111T.soil.Germany)
Pseudon.autotrophica.X54288.(DSM43210.lab.contaminant.Japan)
Pseudon.kongjuensis.AJ252833.(LM157.soil.Korea)
Pseudon.sp.EF216352.(TFS701.fjord.sediment.Norway)
Pseudon.sp.X87314.(SR244a.leaf.litter.Australia)
Pseudon.endophyticus.DQ887489.(YIM56035.plant.endophyte.China)
Pseudon.ammonioxydans.AY500143.(AS4-1877.marine.sediment.China)
Pseudon.sp.AY376891.(Aptero.sp.A10.worker)
Pseudon.sp.AY376892.(Aptero.dentigerum.A38.worker)
Pseudon.sp.EF588227.(Trachy.cornetzi.CC030106-18.worker)
Pseudon.sp.EF588233.(Acro.laticeps.UGM030330-3.worker)
Pseudon.sp.EF588231.(Acro.niger.CC030327-2.worker)
Pseudon.sp.EF588228.(Trachy.cornetzi.AL041005-10.worker)
Pseudon.sp.EF588232.(Acro.sp.CC030106-13.worker)
Pseudon.sp.DQ490989.(C5-2006.plant.endophyte.China)
Pseudon.sp.EF588208.(Atta.sp.AL040116-05.worker)
Pseudon.sp.EF588224.(Acro.octospinosus.UGM020518-05.worker)
Pseudon.sp.EF588220.(Acro.octospinosus.SP020602-02.worker)
Pseudon.sp.EF588211.(Acro.echinatior.CC011213-35.worker)
Pseudon.sp.EF588215.(Atta.sp.CC970517-16.worker)
Pseudon.halophobica.AJ252827.(IMSNU21327T.soil)
Pseudon.sp.DQ437530.(ENV478.chemical.waste.USA)
Pseudon.benzenivorans.AJ556156.(B5.soil.Germany)
Pseudon.dioxanivorans.AY340622.(CB1190.sludge.USA)
Pseudon.sulfidoxydans.Y08537.(DSM44248.compost.Germany)
Pseudon.hydrocarbonoxydans.AJ252826.(IMSNU22140T.air.Germany)
Pseudon.sp.DQ448726.(CNS139PL04.marine.sediment.Palau)
Pseudon.petroleophila.X80596.(ATCC15777.soil.Germany)
Pseudon.saturnea.AJ252829.(IMSNU20052T.soil.Germany)
Pseudon.xinjiangensis.AF325728.(soil.China)
Pseudon.sp.EU274329.(QAII43.soil.China)
EU718331.(Cypho.wheeleri.UGM030424-01.54.worker)
EU718334.(Cypho.wheeleri.UGM030429-01.48.worker)
EU718335.(Cypho.wheeleri.UGM030429-01.49.worker)
EU718332.(Cypho.wheeleri.UGM030424-01.55.worker)
EU718354.(Trachy.zeteki.RMMA050105-28.40.worker)
EU718355.(Trachy.zeteki.RMMA050105-28.41.worker)
Pseudon.thermophila.AJ252830.(IMSNU20112T.compost.Switzerland)
Pseudon.thermophila.EF588218.(NRRL.B-1978.manure.Germany)
Pseudon.sp.EU119255.(HBUM79244.soil.China)
Pseudon.zijingensis.AF325725.(6330T.soil.China)
Pseudon.sp.EF588205.(Trachy.zeteki.AL040410-06.worker)
Pseudon.sp.EF588226.(Trachy.zeteki.CC030105-05.worker)
Pseudon.sp.EF588230.(Trachy.zeteki.CC030106-11.worker)
Pseudon.sp.EF588213.(Trachy.zeteki.CC030404-04.worker)
Pseudon.yunnanensis.AJ252822.(MSNU22019.soil.China)
Pseudon.alaniniphila.AF325726(soil.China)
Pseudon.sp.AY376893.(Trachy.zeteki.A16.worker)
Pseudon.sp.AY376895.(Trachy.zeteki.TrachyPA.worker)
Pseudon.sp.AY376894.(Trachy.sp.A46.worker)
EU718353.(Trachy.turrifex.JJS010925-06.56.worker)
Amycolatopsis.nigrescens.DQ486888.(CSC17-Ta-90.soil.Italy)
Amycolatopsis.lactamdurans.AB045862.(JCM4912.soil.USA)
EU718352.(Trachy.turrifex.AGH000427-01.57.DD.worker)
Amycolatopsis.orientalis.AJ293755.(IMSNU20057T.soil.USA)
Amycolatopsis.coloradensis.AJ421142.(DSM44225T.soil.USA)
86
60
81
58
99
65
83
98
99
94
Clade 3
Clades 1 & 2
Clades 6-9
Clade 4
0.001 substitutions/site

here how 16S rRNA secondary structure modeling of *Pseudonocardia* can identify sequencing errors and help resolve ambiguities in alignments for phylogenetic analyses.

Methods

Utility of the 16S rRNA gene

Our phylogenetic analysis takes advantage of the exponentially increasing number of 16S rRNA gene sequences that have become available at the NCBI GenBank (http://www.ncbi.nlm.nih.gov/nuccore) for reconstruction of phylogenetic relationships. Some of these 16S sequences were generated for taxonomic descriptions of *Pseudonocardia* species (summarized in Huang and Goodfellow 2011), but the majority of the *Pseudonocardia* sequences derived from environmental surveys characterizing general bacterial diversity with either culture-dependent methods (e.g., specialized isolation media) or culture-independent methods (e.g., cloning and sequencing of 16S amplicons; direct pyrosequencing of 16S amplicons). Reliance on only the 16S gene in our phylogenetic analyses has disadvantages because this single gene does not fully resolve all relationships between closely related taxa, because bacterial genomes can contain several 16S genes that differ minimally in sequence (although this is currently unknown for *Pseudonocardia*), because of potential artifacts in multitemplate PCR on whole microbial communities in environmental surveys (Acinas et al. 2005), and because of the possibility of lateral gene transfer among bacteria (although lateral gene transfer is thought to be very unlikely for an isolated 16S gene because of the integrated, central role of this gene within a cell's genomic network; Woese 2000). Despite these potential disadvantages, our 16S-based approach is justified for three reasons. First, the amount of 16S information available from environmental surveys exceeds by several orders of magnitude that of any other gene, and the most comprehensive sample on *Pseudonocardia* diversity from diverse sources is critical to address close phylogenetic affinities between environmental and ant-associated *Pseudonocardia* lineages. Second, the main subgroups of *Pseudonocardia* identified in our phylogenetic reconstruction using only 16S information essentially matches the groupings inferred also by four protein-coding housekeeping genes also available at GenBank (UG Mueller, unpublished), showing that 16S and these four housekeeping genes have recorded parallel evolutionary histories that have not been eroded by lateral gene transfer. Third, our analysis does not aim to resolve relationship at all levels between all lineages of *Pseudonocardia*, but aims to identify closely-related lineages (i.e., we are less interested in resolving basal relationships between major subgroups within the genus) and, most importantly, aims to test for nesting of previously unknown environmental lineages within known clades of *Pseudonocardia* that currently are believed to be specialized to associate only with attine ants.

Selection of sequence information

To compile a comprehensive sample of 16S sequences for *Pseudonocardia*, we obtained all 456 sequences listed under *Pseudonocardia* by 1. November 2009 at the Ribosomal Database Project (RDP; Center for Microbial Ecology, Michigan State University; http://rdp.cme.msu.edu/treebuilder/treeing.spr). This list included all taxa classified under *Pseudonocardia* at the NCBI Taxonomy Browser (sequences obtained from isolated cultures; www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1847), as well as additional *Pseudonocardia* sequences from environmental surveys deposited at the NCBI Core Nucleotide Database (CND; www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore). To find additional sequences not listed at the RDP (e.g., very recently deposited sequences at CND), we also searched the CND by BLASTing full sequence information (>1400 bp) of *Pseudonocardia carboxydivorans, callicarpae, ammonioxydans, halophobica, spinosispora, petroleophila, thermophila*, and *zijingensis*. These *Pseudonocardia* species were chosen because they span the phylogenetic diversity of the genus, and because they are closely related to *Pseudonocardia* lineages previously isolated from attine ants (Mueller et al. 2008). Because BLASTing of longer sequences can miss shorter sequences deposited at the CND, we also BLASTed, for each of these eight species, 16S-sequence segments of about 300–500 bp length that spanned one or two of the V2, V3, V4, or V9 regions of the 16S gene. These regions were chosen because they represent the most variable regions of 16S within the genus *Pseudonocardia*. We added to our dataset two

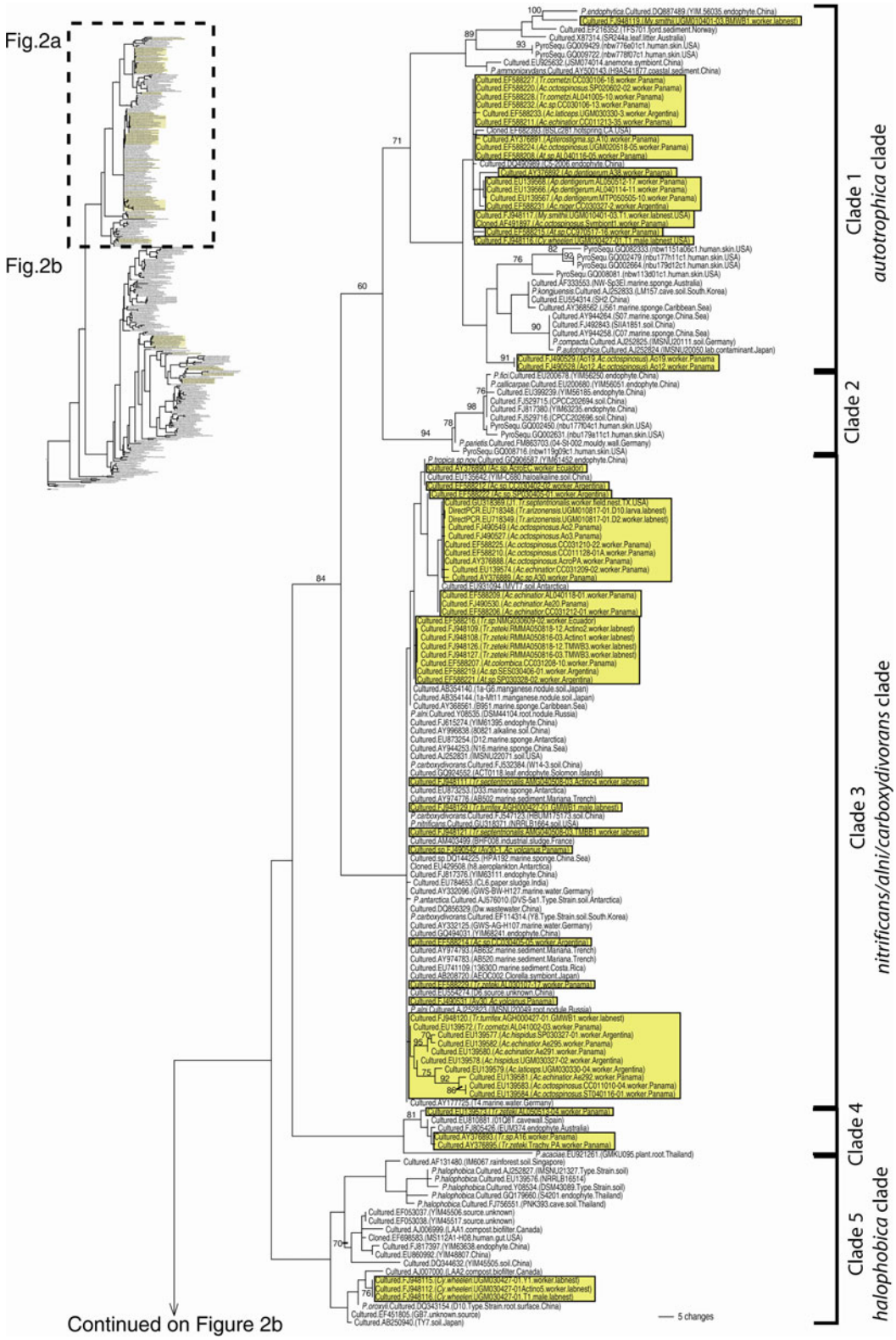
sequences (GU318369, GU318370) of *Pseudonocardia* isolated in an ongoing survey of actinomycete diversity in nests of the attine ant *Trachymyrmex septentrionalis* (Miller, Sen, Ishak, Mueller unpublished), and 16S sequences for *P. compacta* (NRRL B-16170; GU318372) and *P. nitrificans* (NRRL B-1664; GU318371; strains courtesy of David Labeda at the ARS Culture and Patent Culture Collections, United States Department of Agriculture, Peoria, Illinois). This search strategy accumulated a total of 519 sequences.

We excluded from this dataset those sequences actually belonging to other pseudonocardiaceous genera (e.g., some sequences currently listed at the RDP under *Pseudonocardia* actually belong into *Crossiella*, such as NCBI accessions FN297974, EU299989, FN297967, EF447067, EF447073, EF447078, EF188338, EF447079; sequence AJ871440 belongs into the genus *Actinostreptospora*; etc.), and we excluded poor-quality sequences flagged as such at the RDP. Second, to ensure sufficient overlap across phylogenetically informative regions of the 16S gene, we excluded all sequences shorter than 500 bp in length. This criterion of 500 bp minimum length also excluded the short sequences generated with next-generation sequencing platforms (e.g., 454 pyrosequencing) that are currently deposited at the NCBI Short Read Archive (www.ncbi.nlm.nih.gov/sites/entrez?db=sra). Third, we excluded all sequences that did not span both the V3 and the V4 regions. These two variable regions were targeted for three reasons: first, the great majority of partial 16S sequences available for *Pseudonocardia* included these two regions; second, these two regions are among the most variable 16S regions differentiating species in the genus *Pseudonocardia*; third, we believed it is important for tree stability in the phylogenetic analysis that all taxa overlapped in at least two contiguous variable regions (i.e., V3&V4). This criterion of V3&V4 sharing of all taxa means that these regions may have greater influence on the inferred phylogenetic relationships than other, similarly variable regions (e.g., V9). We excluded identical sequences that had been obtained in the same environmental survey, such as duplicate sequences from a large marine sediment survey from the Mariana Trench (AY974775-AY974796; retaining only AY974776, AY974783, and AY974793 in our analysis), and duplicate sequences from a large soil survey in Czechoslovakia (EF506948-EF507159; retaining from these only 13 non-identical *Pseudonocardia* sequences). Exclusion of these duplicate taxa does not affect any of our conclusions, but reduced computation time in our analyses. After exclusion of redundant taxa, our dataset included 325 *Pseudonocardia* taxa.

In the final dataset of 325 sequences, 67.4% of the *Pseudonocardia* sequences had been obtained by culture-dependent methods, 25.8% from cloning of 16S sequences amplified from microbial communities, 2.2% from direct PCR, and 4.6% from 454-pyrosequencing of 16S amplicons (Fig. 2a, b). Also from these 325 sequences, 26.8% of the sequences had been obtained from attine ants, 9.2% from plant material (e.g., endophytes, root bacteria), 3.4% from marine organisms (e.g., sponge symbionts), 5.2% from human or mouse skin, 31.7% from soil, 1.5% from rock surfaces, 5.2% from marine sediments, 5.5% from industrial sources (e.g., sludge, biofilters), and 11.4% from other sources (e.g., art paintings, house dust, air, etc.). Two *Kibdelosporangium*, two *Crossiella*, two *Actinokineospora*, and three *Amycolatopsis* were included as outgroups (Fig. 2b), covering pseudonocardiaceous genera most closely related to the genus *Pseudonocardia* (Huang and Goodfellow 2011; UGM unpublished).

On 30. November 2009, 16S sequences from an additional 24 ant-associated *Pseudonocardia* were released at GenBank (accessions EU283917-EU283940; Poulsen Cafaro, Erhardt, Little, Gerardo, Currie, unpublished, *Host-pathogen dynamics in an ancient antibiotic system*). While it was too late to

Fig. 2 Phylogeny of *Pseudonocardia* reconstructed for 325 ▶ taxa under the maximum-likelihood criterion from 16S rRNA gene sequence information. Ant-associated *Pseudonocardia* are boxed (shaded background); environmental *Pseudonocardia* are unboxed. Attine-associated lineages are closely related to soil-dwelling or endophytic *Pseudonocardia* lineages, indicating frequent recruitment of free-living *Pseudonocardia* into association with ants. Support values are inferred from 100 ML bootstrap pseudoreplicates. Each taxon label gives the method of characterization (sequencing of 16S amplicons from *cultured* isolates; sequencing of *cloned* 16S amplicons from environmental surveys; *pyrosequencing* of 16S amplicons; *direct PCR* from ants) and the GenBank accession, followed in parentheses by the ant species name from which a bacterium was sampled, the ant collection ID (e.g., UGM010817-01), the source from which the sequence was obtained (worker, garden, soil, plant, etc.), and the collection location. The attine ant genera are abbreviated as: Ac. = *Acromyrmex*; Ap. = *Apterostigma*; At. = *Atta*; Cy. = *Cyphomyrmex*; My. = *Mycocepurus*; Tr. = *Trachymyrmex*



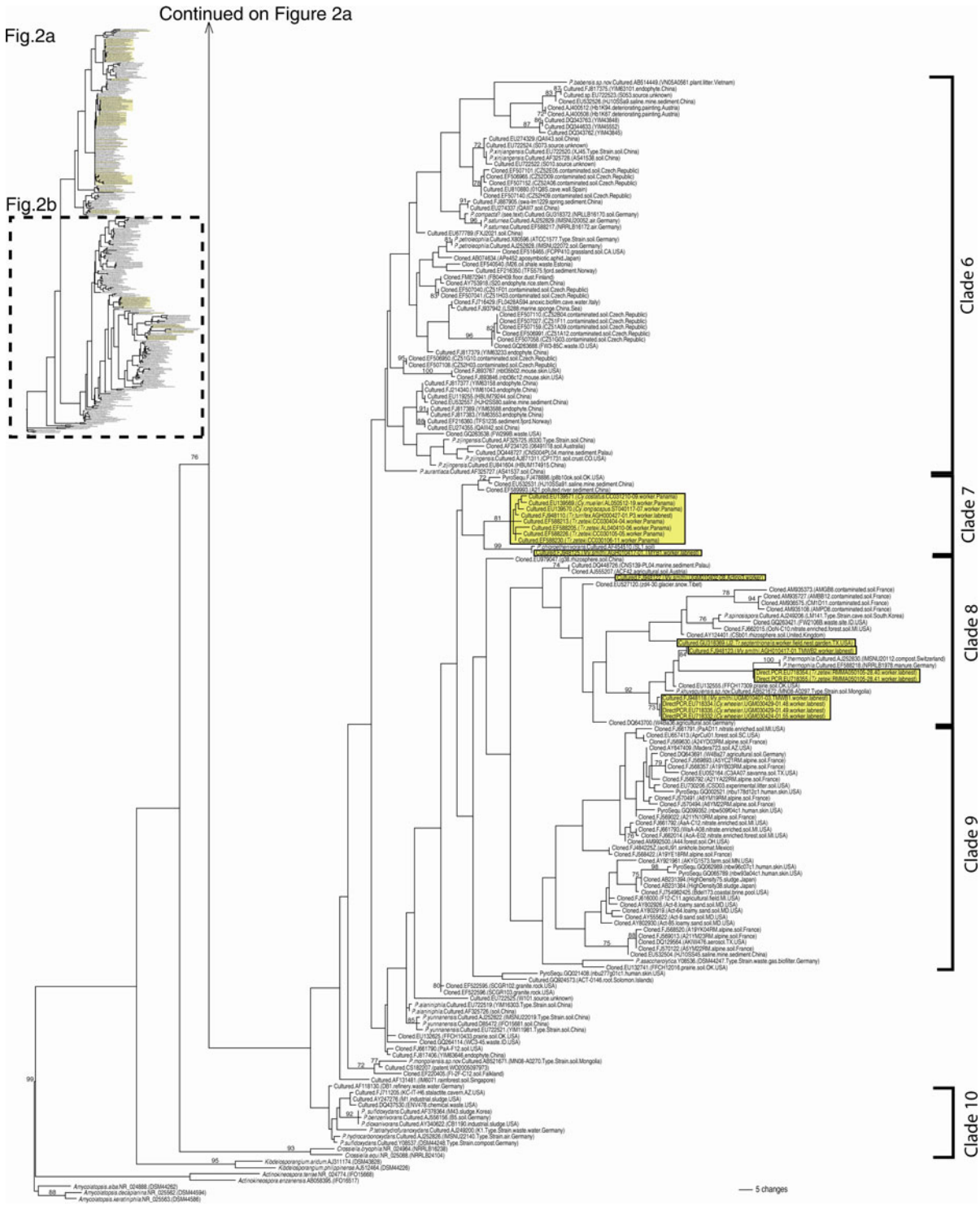


Fig. 2 continued

incorporate these sequences in our phylogenetic analyses, comparison of these new sequences with those in our phylogenetic reconstruction permitted precise placement of the new sequences into our phylogenetic framework (UG Mueller unpublished). Placement of these new sequences into the phylogenetic context (Fig. 2) does not change any of the conclusions emphasized below.

Alignment and character selection

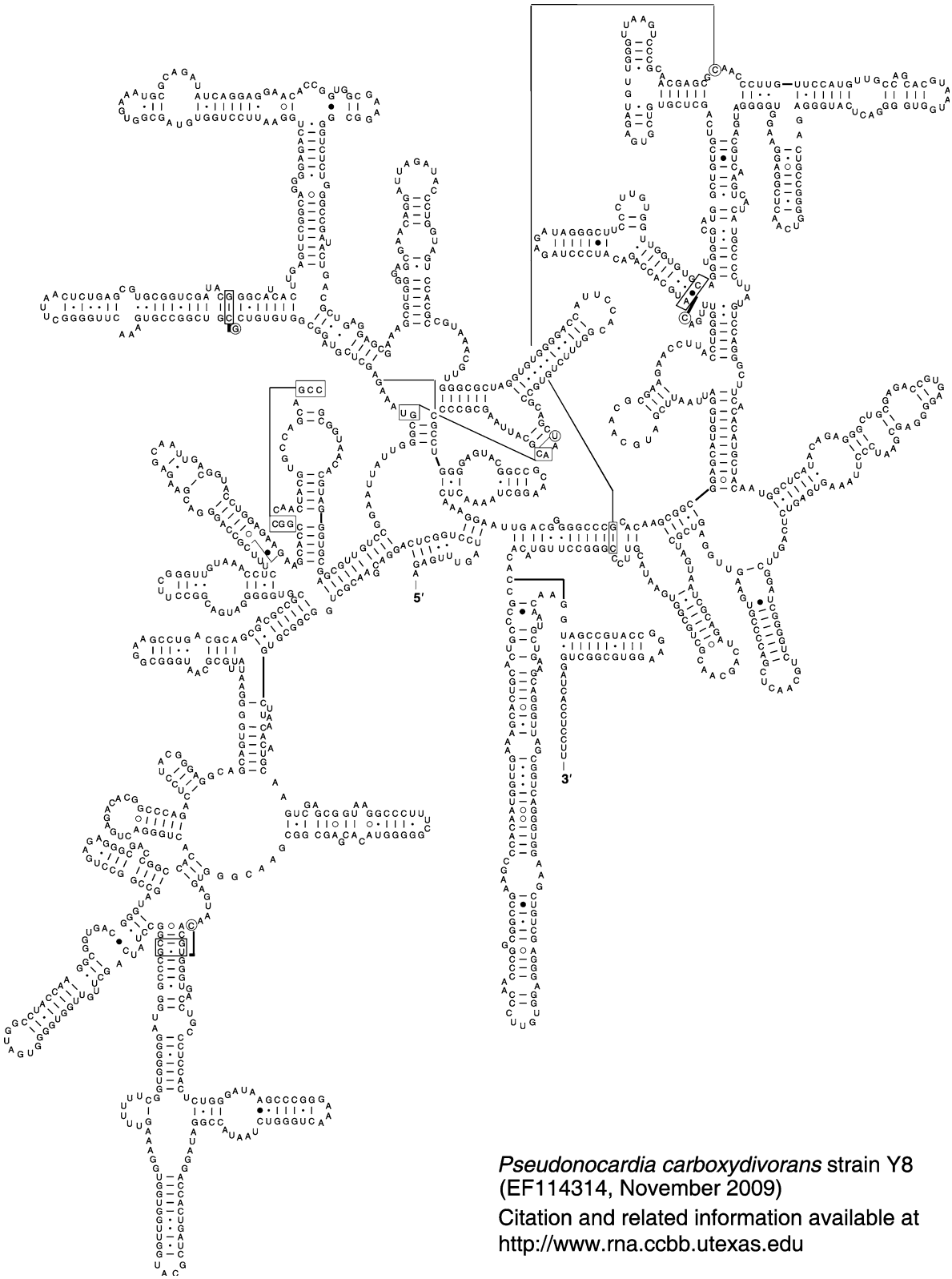
Sequences were aligned in MacClade version 4.06 (Maddison and Maddison 2000). The initial alignment of 1521 characters was adjusted manually to correct obvious alignment errors. To validate and adjust our initial alignment, we used the sequence alignment editor AE2 (Olsen et al. 1992) to manually align characters according to similar positioning in the RNA secondary structure. Regions of the 16S rRNA sequence that have significant sequence similarity can be aligned solely based on this identity. However, regions of the alignment that do not contain sufficient sequence similarity require additional constraints to accurately place nucleotides in columns that represent a similar part of the overall rRNA higher-order structure. The secondary structure diagrams were drawn with the interactive graphics program XRNA (B Weiser, H Noller unpublished).

Inspection of our alignment revealed that sequences from ant-associated *Pseudonocardia* and *P. halophobica* (accessions EU139566.1-EU139584.1; these sequences were later replaced after completion of our analyses and once we had pointed out sequencing errors; see Supplemental Information) submitted by Poulsen et al. (2007) contained highly unusual character scorings, some of them at positions within the 16S gene that are thought to be invariable or highly conserved across all Eubacteria (see Supplemental Information). The secondary structure analysis of these suspect 16S sequences revealed that these unusual characters are likely sequencing artifacts. These unusual characters were furthermore rendered suspect because they also appeared in a sequence deposited by Poulsen et al. (2007) for *P. halophobica* (EU139576.1, from strain NRRL B-16514), whereas all other sequences deposited at GenBank for *P. halophobica* (AJ252827 = strain IMSNU21327; Z14111 = strain DSM43089^T; Y08534 = strain DSM43089^T) do not show the unusual character scorings appearing in Poulsen et al. 2007 (see Figures S3 and S7 in the Supplemental Information for secondary-structure models of the *P. halophobica* 16S rRNA sequences with and without the sequencing errors). We therefore excluded from our phylogenetic analyses 11 suspect characters introduced by the Poulsen et al. (2007) sequences (these characters did not vary among all the other taxa, and exclusion therefore did not affect phylogenetic inferences about the remaining taxa). However, we were unsure about the error of four additional suspect characters in the Poulsen et al. (2007) sequences, which therefore were retained in our phylogenetic analyses and were expected to render the corresponding taxa as phylogenetically derived. The derived phylogenetic positions of some of the sequences from Poulsen et al. (2007) (EU139566-EU139584) in Fig. 2 therefore may be an artifact of uncorrected sequence-scoring errors.

16S RNA secondary structure modeling

To facilitate future 16S RNA alignments for phylogenetic reconstruction, we generated secondary structure models of 16S rRNA for five *Pseudonocardia* species spanning the diversity of the genus: *P. carboxydivorans* (GenBank accession EF114314; type strain Y8; Fig. 3), *P. kongjuensis* (AJ252833; type strain LM 157), *P. halophobica* (AJ252827; type strain IMSNU 21327), *P. spinosispora* (AJ249206; type strain LM 141), and *P. yunnanensis* (AJ252822; type strain IMSNU 22019) (see Supplemental Information). The 16S rRNA secondary structures were predicted with comparative methods for a secondary structure that is common to all of the known 16S rRNA sequences, using Covariation analysis (Woese et al. 1980, 1983; Gutell et al. 1985, 1992, 1994; Cannone et al. 2002). We are confident that the 16S rRNA secondary structure predictions are accurate, because, in a previous analysis, approximately 98% of the predicted model base pairs were present in the high-resolution crystal structure of the ribosomal subunit (Gutell et al.

Fig. 3 16S rRNA secondary structure model for *P. carboxydivorans* (GenBank accession EF114314; type strain Y8). The Supplemental Information presents additional secondary structure models for four additional species within the genus *Pseudonocardia* (*P. kongjuensis*, *P. halophobica*, *P. spinosispora*, *P. yunnanensis*). Canonical base pairs (G:C, A:U) are shown with tick marks, wobble (G:U) base pairs with small closed circles, A:G base pairs with large open circles, and all other non-canonical base pairs with large closed circles ▶



Pseudonocardia carboxydivorans strain Y8
(EF114314, November 2009)

Citation and related information available at
http://www.rna.ccbb.utexas.edu

2002). Bacterial 16S rRNA secondary structure diagrams for the modeled *Pseudonocardia* species are available at the Comparative RNA Web Site http://www.rna.ccbb.utexas.edu/DAT/3C/Structure/index.php, http://www.rna.ccbb.utexas.edu/SIM/4D/TOL/.

Phylogenetic analyses

Our final alignment utilized all characters of the 16S gene, but excluded the 11 suspect characters from Poulsen et al. (2007; see above) and excluded 72 characters from the conserved region at the beginning and end of the 16S gene because the great majority of the sequences were missing character information in these end regions. Our final alignment included 1436 characters (289 informative, 77 autapomorphic, and 1070 invariable characters). Phylogenetic relationships were inferred under the maximum likelihood (ML) criterion using the General Time Reversible (GTR + I + G) model of nucleotide substitution (Garli version 0.951; Zwickl 2006; www.zo.utexas.edu/faculty/antisense/garli/Garli.html). The appropriate model of nucleotide substitution was selected using the Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall 1998). We performed twenty separate likelihood searches to explore likelihood space more thoroughly. Branch support was calculated by performing bootstrap analyses with 100 repetitions. Figure 2 reports the best phylogenetic topology under the ML criterion (lowest negative log-likelihood score) among the twenty ML searches.

Results and discussion

Sub-groupings within the genus *Pseudonocardia*

The maximum-likelihood (ML) reconstruction shown in Fig. 2a and b places 87 known ant-associated *Pseudonocardia* within the larger phylogenetic context of 238 environmental *Pseudonocardia* obtained from diverse sources (e.g., soil, marine sediment, plants, marine invertebrates, mammal skin, industrial material, etc.). We recover the main sub-groupings of *Pseudonocardia* that already emerged in the 16S analyses of Lee et al. (2000) and Mueller et al. (2008), but our increased taxon sampling tentatively identifies at least 10 subgroups within the genus (labeled as Clades 1–10 in Fig. 2a, b). The genus *Pseudonocardia* appears to be split basally into two main groups, one clade comprised of five subgroups that contain the great majority of the known ant-associated *Pseudonocardia* (Fig. 2a); and a second, less satisfactorily resolved clade comprised of at least 5 subgroups (Fig. 2b). However, this basal split into these two main groups is only weakly supported in our ML analyses, as Clades 4 and 5 (shown in Fig. 2a) exhibited also affinities with the other main group (shown in Fig. 2b) in some of the less-likely ML reconstructions. Only few subgroups within the genus received adequate support in the ML bootstrap analyses, but two subgroups with acceptable support values (Clade 1 and a larger clade comprised of Clades 1–3; Fig. 2a) contain the great majority of the ant-associated *Pseudonocardia*. Clearly, information from additional genes will be necessary to resolve all subgroupings and affiliations with confidence. Fortunately, Wen-Jun Li's Lab at Yunnan University, China, is currently compiling sequence information of four protein-coding genes (*rpoB*, *fusA*, *gyrB*, and *lepA*) for a multilocus phylogenetic analysis of key species in the genus *Pseudonocardia*. This framework should be useful in future studies to place the ant-associated *Pseudonocardia* into the subgroups of the genus. Preliminary analyses (UG Mueller unpublished) of the sequence information available at GenBank for these four protein-coding genes also revealed the monophyly of Clade 1 and the monophyly of the larger clade comprised of Clades 1–3, as shown in Fig. 2a.

Nesting of environmental *Pseudonocardia* lineages within clades previously thought to be ant-specific

Our analysis confirms the prediction of the Acquisition model that there exist previously unknown environmental *Pseudonocardia* that insert themselves into clades that originally were thought to be "ant-specific". For example, Cafaro and Currie (2005) had identified a three-taxon clade of *Pseudonocardia* (isolated from *Trachymyrmex* ants from Panama) that had emerged also as phylogenetically unique and "ant-specific" in the phylogenetic analysis by Mueller et al. (2008; Fig. 1); however, we found two environmental *Pseudonocardia* nested within that group of allegedly *Trachymyrmex*-specific *Pseudonocardia* (Clade 4 in

Fig. 2a). One of these is an endophytic *Pseudonocardia* that was isolated from the surface-sterilized root of an *Eucalyptus* tree in Australia (GenBank accession FJ805426; 99.8% sequence-identical to AY376893 isolated from a *Trachymyrmex* worker from Panama), the second *Pseudonocardia* was isolated in a survey of microbial growth that degraded prehistoric cave paintings in Spain (Stomeo et al. 2008, 2009; EU810881; 99.3% sequence-identical to AY376893 isolated from a *Trachymyrmex* worker). The nesting of these two environmental *Pseudonocardia* inside a clade of ant-associated *Pseudonocardia* is less compatible with the phylogenetically derived position of ant-associated *Pseudonocardia* predicted by the Coevolution-Codivergence Hypothesis. Moreover, representatives of Clade 4 have so far been isolated only six times (four times from *Trachymyrmex* ants, twice from other sources; details in Supplemental Information) and they have so far not been detected in any culture-independent screen. This suggests that it may be sometimes inherently difficult to find environmental *Pseudonocardia* that are nested within ant-specific clades, either because these environmental *Pseudonocardia* are difficult to isolate, or they are rare, or a combination of both factors. Because of this inherent difficulty of detection, the absence of evidence for environmental *Pseudonocardia* that are nested within ant-associated *Pseudonocardia* clades should not be interpreted as evidence for absence, and therefore also not as evidence in support for the Coevolution-Codivergence hypothesis (e.g., Cafaro and Currie 2005; Poulsen et al. 2009).

Figure 2a and 2b reveal additional cases where environmental *Pseudonocardia* nest within clades that were previously thought to be largely ant-specific. For example, for the main group of ant-associated *Pseudonocardia* (Clade 3 in Fig. 2a), only one environmental counterpart was known previously (Fig. 1), whereas now a number of environmental affiliates are known (sequence identical or near sequence identical at >99% similarity), such as the endophytic *P. tropica* sp. nov. from China, EU931094 from Antarctic soil, *P. carboxydivorans* from soils in China and Korea, *P. nitrificans* from soil in the USA, etc. (Fig. 2a). For the second main group of ant-associated *Pseudonocardia* (Clade 1 in Fig. 2a), one environmental affiliate was known previously (Fig. 1) whereas now three environmental affiliates are known (Fig. 2a), including the endophytic *P. endophytica* from China, DQ490989 from an endophyte in China, and EF682939 from a hotspring in the USA. Another interesting case is a conglomerate of ant-associated *Pseudonocardia* from three species of lab-reared attine ants, whose *Pseudonocardia* have close affinities with *P. khuvsgulensis* sp. nov. from soil in Mongolia, *P. thermophila* from compost in Germany, and the cloned EU132555 from prairie soil in the USA (Fig. 2b). Overall, the phylogenetic patterns conform with the prediction of the Acquisition model that, because the attine integument is frequently colonized by bacteria from the environment, ant-associated *Pseudonocardia* have close phylogenetic links to diverse environmental *Pseudonocardia*. As we predicted before (Mueller et al. 2008), future studies are likely to uncover many additional close links between environmental counterparts and ant-associated *Pseudonocardia*, especially if more environmental *Pseudonocardia* are surveyed across the Neotropics (the realm of attine ants) and if more of the neglected basal attine ant lineages are surveyed for their integumental microbes.

Single *Pseudonocardia* lineages colonize the integument of a wide diversity of attine ants

In previous analyses (Cafaro and Currie 2005; Zhang et al. 2007; Mueller et al. 2008), Clade 3 appeared to contain *Pseudonocardia* that had been isolated mostly from *Atta* and *Acromyrmex* leafcutter ants. This near exclusive association between leafcutter ants and Clade 3 *Pseudonocardia* could be interpreted as evidence for co-diversification between the leafcutter ant clade and Clade 3 *Pseudonocardia* (sensu Poulsen et al. 2009). However, the accumulated evidence now documents also the presence of Clade 3 *Pseudonocardia* in the microbial communities of the integument of many *Trachymyrmex* ants (Fig. 2a), and it is possible that a more thorough survey of basal attine lineages could reveal associations of Clade 3 *Pseudonocardia* with ant lineages across the entire diversity of attine ants. As a parallel case, Sen et al. (2009) recently isolated from Clade 1 *Pseudonocardia* from laboratory nests of the lower-attine species *Mycocepurus smithii* (Fig. 2a), showing that more comprehensive sampling uncovered the first exceptions to the original "specificity" of Clade 1 *Pseudonocardia* on leafcutter and *Apterostigma* ants. Likewise, a group of closely related Clade 7 *Pseudonocardia* isolated from three species of

Cyphomyrmex (*costatus*, *muelleri*, *longiscapus*) was interpreted by Poulsen et al. (2007, 2009) as possibly *Cyphomyrmex*-specific, but our phylogenetic analyses now reveal *Pseudonocardia* from Clade 7 that had been isolated from diverse *Trachymyrmex* ant species (Zhang et al. 2007; Sen et al. 2009; Fig. 2b). The isolation of Clade 7 *Pseudonocardia* from *Trachymyrmex* laboratory colonies by Sen et al. (2009) could potentially mean that the screened *Trachymyrmex* workers were colonized by *Pseudonocardia* derived from *Cyphomyrmex* species that had been kept in the same lab room for several years. If so, this then would document how readily *Pseudonocardia* can colonize the integument of *Trachymyrmex* species from other sources. We conclude, therefore, that tests for ant-*Pseudonocardia* specificities and co-diversification should avoid conclusions based on small sample sizes (which too readily can underestimate the true diversity of *Pseudonocardia* associates). Rather, tests for specificities should first carefully document the full diversity of *Pseudonocardia* that colonize the integument of a single ant and different workers from the same colony, evaluate potential isolation biases (e.g., preferential isolation of autotrophic *Pseudonocardia* with minimum-nutrient medium; see below), and rule out cross-contamination from environmental sources.

Culturable versus unculturable *Pseudonocardia*

Perhaps the most striking difference between the two main *Pseudonocardia* clades in Fig. 2a and b is that 99% of the sequences in Fig. 2a (Clades 1–5) derived from *Pseudonocardia* that had been cultured, whereas the sequences in Fig. 2b derived from a mix of cultured and uncultured *Pseudonocardia* (cloned, pyrosequenced). In fact, the sizable Clade 9 in Fig. 2b consists entirely of uncultured *Pseudonocardia*, with the exception of the cultured *P. asaccharolytica*. Absence or underrepresentation of cultured *Pseudonocardia* in a clade suggests that these types of bacteria may be inherently difficult to isolate and were therefore not captured with the culture-dependent methods used so far. In contrast, the near absence of cloned *Pseudonocardia* in Clades 1–5 (Fig. 2a) suggests potential cloning biases, for example due to disruption of *E. coli* growth by the 16S sequences inserted into the *E. coli* genome during cloning (e.g., Hamady and Knight 2009, and references therein). Interestingly, the great majority of ant-associated *Pseudonocardia* characterized to date belong to two clades (Clade 1 and Clade 3) that are heavily biased towards culturable *Pseudonocardia* (this bias is also true for all the environmental *Pseudonocardia* in these clades; Fig. 2a), suggesting that these kind of *Pseudonocardia* are readily captured with the isolation methods used in the surveys so far, whereas the less culturable *Pseudonocardia* (i.e., from Clades 6–8) may have been missed by the same surveys.

The only study that compared culture-dependent and culture-independent methods for surveying ant-associated *Pseudonocardia* was Sen et al. (2009), who documented that, whereas the standard culture-dependent method of Cafaro and Currie (2005) yielded only a single *Pseudonocardia* type from workers of the ant *Trachymyrmex zeteki*, a culture-independent pyrosequencing survey of *T. zeteki* workers revealed the presence of several species from different *Pseudonocardia* subgroups. Taken together, the phylogenetic distribution of the cultured and uncultured *Pseudonocardia* (Fig. 2a versus 2b) and the preliminary findings of Sen et al. (2009) suggest that the standard isolation method of attine-associated *Pseudonocardia* with minimum-carbon, chitin medium (Cafaro and Currie 2005) may underestimate the true *Pseudonocardia* diversity present on attine ants. If so, the isolation of only one kind of *Pseudonocardia* from the integument of a particular ant (e.g., a *Pseudonocardia* from Clades 1 and 3) should therefore not be taken as evidence that this kind of *Pseudonocardia* is the only *Pseudonocardia* species in the microbial community on the ant integument. We therefore caution that previous studies that relied on minimum-nutrient isolation methods might have underestimated the true diversity of *Pseudonocardia* in the microbial communities growing on the integument of attine ants.

Colonization of the ant integument by *Pseudonocardia* from plant and soil sources

As already noted by Mueller et al. (2008), many ant-associated *Pseudonocardia* are closely related to *Pseudonocardia* from soil and plant material (e.g., endophytic *Pseudonocardia*), but several additional close affinities now emerge from our larger phylogenetic analysis. For example, ant-associated *Pseudonocardia* that have sequences identical to

P. carboxydivorans, P. tropica sp. nov., and *P. endophytica* are now known (Fig. 2a), all of which have been cultured as endophytes from plant material. Likewise, environmental *Pseudonocardia* from soil can be sequence-identical in 16S to attine-associated *Pseudonocardia* (Fig. 2a). Both soil-dwelling and endophytic *Pseudonocardia* therefore remain possible sources of *Pseudonocardia* that colonize the attine integument. This calls for experimental evaluation of the colonization rate of the attine integument by microbes from such environmental sources, which may cause fast or slow microbial turnover on the integument depending on the nature of the resident microbial community, the nature of the colonizing microbes, and the general disturbance in the nest. Such studies will require in-depth screening of the entire microbial community on the integument, as well as understanding of any biases of culture-dependent and culture-independent *Pseudonocardia* screens (see above *Culturable versus unculturable Pseudonocardia*).

Pseudonocardia symbiosis beyond fungus-growing ants

Pseudonocardia has been found not only in association with fungus-growing ants, but also in association with a wide diversity of other organisms, including plants, marine sponges, an anemone, a *Clorella* alga, an aphid, and mammals (human gut; human and mouse skin) (Fig. 2). Excluding the 87 ant-associated *Pseudonocardia* in Fig. 2, 12.6% of the surveyed *Pseudonocardia* derived from plants (endophytes), 4.6% from marine invertebrates, 0.4% from terrestrial arthropods (27% when including ants), and 7% from mammal skin (human, mouse). The absence or rarity of *Pseudonocardia* in other well-characterized microbial communities (e.g., human gut, termite gut, ungulate rumen) indicates that *Pseudonocardia* are not omnipresent and capable of life in all symbiotic niches. However, the frequent isolation from marine sponges and plants is intriguing and suggests that *Pseudonocardia* evolution may have been shaped by symbiosis with a wide array of organisms.

Autotrophic versus heterotrophic *Pseudonocardia* on the ant integument

Many *Pseudonocardia* isolated from attine ants are closely related to autotrophic *Pseudonocardia*, such as *P. carboxydivorans*, *P. autotrophica, P. nitrificans* (Fig. 2a), suggesting the possibility that the integumental *Pseudonocardia* of attine ants may also include autotrophs that are not limited in growth (or are less limited) by nutrients supplied by the ants, as assumed by the Coevolution-Codivergence hypothesis. The traditional formulation of the Coevolution-Codivergence hypothesis assumes that the ants have precise control over growth (upregulation of *Pseudonocardia* growth to combat an acute *Escovopsis* infection of the garden; Currie et al. 2003); such control may be less possible if *Pseudonocardia* are autotrophs that can thrive in nutrient-poor niches. The possibility of autotrophic *Pseudonocardia* on the ant integument could explain why it is relatively easy to isolate slow-growing *Pseudonocardia* with minimum-nutrient chitin medium (e.g., preferential selection of autotrophs), perhaps with similar biochemical properties as the closely related *P. carboxydivorans* that can fix carbon from carbon-monoxide (Park et al. 2008). While one could speculate that removal of toxic carbon-monoxide could be a potential function of *Pseudonocardia* in the interior of nests of fungus-growing ants, it is equally possible that the anoxic conditions in the bottom of fungus gardens promotes non-adaptive proliferation of autotrophic *Pseudonocardia* (e.g., on the immobilized *Acromyrmex* workers at the bottom of fungus gardens, as documented by Currie et al. 2003).

Phylogenetic position of *Pseudonocardia compacta* and *P. nitrificans*

Our new 16S sequence of *P. nitrificans* (strain NRRL B-1664) places this species into a clade with *P. alni, P. carboxydivorans*, *P. antarctica*, and *P. tropica* sp. nov. (Clade 3, Fig. 2a). Our *P. nitrificans* sequence (GU318371) is identical to AJ252831 (= strain IMSNU 22071 = *P. nitrificans*; Lee et al. 2000), which currently is reported at GenBank as an undescribed *Pseudonocardia*. We believe that these two sequences are of much better quality than sequence X55609 (=strain IFAM 379 = *P. nitrificans*), which has been used in previous phylogenetic analyses (Warwick et al. 1994; McVeigh et al. 1994; Huang and Goodfellow 2011) but whose phylogenetic placement in these analyses is now dubious because of the poor sequence quality of X55609. More surprising was the placement of our 16S sequence of *P. compacta* (NRRL B-16170;

GU318372) close to *P. saturnea* (Fig. 2b), that is, in a different subgroup of *Pseudonocardia* than accession AJ252825 (=IMSNU 20111; Fig. 2a) of *P. compacta* that is generally used in phylogenetic analyses of the genus. Interestingly, the placement of *P. compacta* in the vicinity of *P. saturnea* conforms approximately with the placement of *P. compacta* (DSM 43592) when using information from four protein-coding genes available at GenBank (EU722624, EU722594, EU722569, EU722535; UG Mueller unpublished observation), suggesting the possibility of strain mix-up between culture collections. [Note inserted at proof stage: We recently obtained a strain labeled *P. saturnea* (NRRL B-16172) from the ARS Culture and Patent Culture Collections (USDA, Peoria, Illinois) and the 16S sequence of this strain was identical to the sequence of *P. compacta* (AJ252825; IMSNU 20111^{T}) (H Ishak and UG Mueller unpublished). Because we had received the strain labeled NRRL B-16170 (believed to be *P. compacta*, but sequencing to *P. saturnea*) and the strain labeled NRRL B-16172 (believed to be *P. saturnea*, but sequencing to *P. compacta*) in two separate shipments from the ARS-USDA culture collection, and because we completed sequencing of NRRL B-16170 before we received NRRL B-16172, it seems that these two strains were mixed up prior to shipment from the ARS-USDA culture collection to our lab.] To resolve these issues, it will be necessary to re-sequence the type strains of *P. compacta*, *P. nitrificans,* and *P. saturnea* for 16S and other informative genes.

Conclusion

The two models juxtaposed in our analyses – the Coevolution-Codivergence model and the Acquisition model—represent two extreme viewpoints. The traditional Coevolution-Codivergence model stresses mutualism and long-term ant-*Pseudonocardia-Escovopsis* co-evolution and co-divergence, the more recent Acquisition model stresses diversity in ecological association and close links between ant-associated and environmental *Pseudonocardia*. These two models make distinct predictions regarding the phylogenetic proximity of ant-associated and environmental *Pseudonocardia*. Our expanded phylogenetic analyses conform more with the predictions of the Acquisition model, adding to recent antibiotic assays and phylogenetic analyses (Kost et al. 2007; Mueller et al. 2008; Haeder et al. 2009; Sen et al. 2009) that had started to question the plausibility of ant-*Pseudonocardia-Escovopsis* co-evolution and co-divergence. Specifically, our phylogenetic analyses reveal previously unknown cases of 16S-sequence-identical ant-associated and environmental *Pseudonocardia* (from soil and plants), and environmental *Pseudonocardia* that are nested within clades previously thought to contain only specialized, ant-associated *Pseudonocardia*. These phylogenetic patterns indicate frequent and continuous colonization of the attine integument by *Pseudonocardia* from environmental sources, microbial turnover on the ant integument over evolutionary time, and consequently disruption of long-term ant-*Pseudonocardia* associations. Such processes greatly reduce the potential for ant-*Pseudonocardia* co-evolution and for adaptive modification of *Pseudonocardia* to serve the fitness interests of the ants. Inadequate inclusion of environmental *Pseudonocardia* in previous analyses appears to have led to the premature identification of "ant-specific" *Pseudonocardia* clades. More sampling of environmental *Pseudonocardia* is needed to fully address the ecological links between ant-associated and environmental *Pseudonocardia*. Likewise needed are genomic and biochemical studies that evaluate genetic and phenotypic differences (or absence of differences) between ant-associated and environmental *Pseudonocardia*, particularly in the Neotropics where only few environmental bacterial surveys have been carried out to date. Because previous culture-dependent methods characterizing *Pseudonocardia* diversity on attine ants may have been biased (see *Culturable and unculturable Pseudonocardia*), future studies should also attempt to develop unbiased methods to characterize the true microbial diversity on attine ants. We encourage studies that test for both non-adaptive and adaptive roles of integumental microbes in carefully designed experiments, and we concur with the recent assessment by Boomsma and Aanen (2009) that the traditional Coevolution-Codivergence model greatly oversimplifies the nature and ecological diversity of ant-*Pseudonocardia* associations.

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actinomycete ecology, systematics, and evolution. We thank Linda Kinkel, Liz Wellington, and Linquan Bai for the invitation to give a plenary talk at the 2009 ISBA Congress in Shanghai, for which we developed many ideas presented here. We thank two anonymous reviewers for constructive comments on the manuscript. The research was supported by NSF awards DEB-0919519, DEB-0639879, and DEB-0110073 to UGM; and NIH award R01GM067317 to RRG.

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Supplemental Information

Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (Pseudonocardiaceae, Actinomycetales): a test of two symbiont-association models

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Identification of Sequencing Errors using 16S rRNA Secondary-Structure Modeling

We modeled 16S rRNA secondary structure for five representative *Pseudonocardia* species, including *P. carboxydivorans* (GenBank accession EF114314; type strain Y8; Figure S1), *P. kongjuensis* (AJ252833; type strain LM-157; Figure S2), *P. halophobica* (AJ252827; type strain IMSNU-21327^T; Figure S3), *P. spinosispora* (AJ249206; type strain LM-141^T; Figure S4), and *P. yunnanensis* (AJ252822; type strain IMSNU-22019^T; Figure S5), following the secondary-structure modeling strategy described in the Methods of the main article. The five *Pseudonocardia* species were chosen because these species span the diversity of the genus, because for each species almost the entire 16S sequence was available (over 1500 base pairs), and because these sequences seemed free of sequencing errors when interpreting the respective 16S rRNA secondary structure.

Inspection of our 16S alignment revealed that sequences from ant-associated *Pseudonocardia* and *P. halophobica* (accessions EU139566.1-EU139584.1) submitted by Poulsen et al. (2007) on 05. September 2007 contained highly unusual character scorings, some of them at positions within the 16S rRNA gene that are thought to be invariable or highly conserved across all Eubacteria (Cannone et al. 2002). An example of such an unusual 16S rRNA secondary structure is shown in Figure S6 (EU139577.1). The unusual characters were furthermore rendered suspect because these suspect characters also appeared in a sequence deposited by Poulsen et al. (2007) for *P. halophobica* (EU139576.1, from strain NRRL B-16514; Figure S7), whereas all other sequences deposited at GenBank for *P.halophobica* (AJ252827 = strain IMSNU21327; Z14111 = strain DSM43089^T; Y08534 = strain DSM43089^T) do not show the unusual character scorings of Poulsen et al. (2007) (compare the 16S rRNA secondary structures for *P. halophobica* in Figures S3 and S7). Note that strain NRRL B-16514 used by Poulsen et al. (2007) is derived from the type strain DSM43089^T; these two strains are therefore expected to have the same 16S rRNA gene sequence. Since submission of the present manuscript, we sequenced strain NRRL B-16514 (sent to us in November 2009 by Dr. David Labeda, ARS Culture and Patent Culture Collections, United States Department of Agriculture, Peoria, Illinois). Our 16S sequence for NRRL B-16514 does not show the unusual character scorings appearing in EU139576.1, which was derived from the same accession NRRL B-16514; rather, our character scoring is identical to the sequence of *P. halophobica* AJ252827, with only one single nucleotide difference in the repeat number of a G-mononucleotide repeat in the V1-region.

Of all the 16S rRNA sequences generated to date, only the sequences of Poulsen et al. (2007) showed the unusual character scoring appearing in Figures S6 and S7, suggesting either errors in sequence editing in that particular project, or some unusual modification of the sequences during the submission process to Genbank. We predicted that the unusual characters of Poulsen et al. (2007) would render the relevant sequences as artificially derived in a phylogenetic analysis (i.e., the sequences with errors would be subtended by long branches), perhaps even unite some of these sequences in artificial monophyletic clades. This was indeed the case (Figures S8a and S8b; the derived characters created artificial monophyletic clades uniting taxa based on shared sequence errors, as well as distorted general phylogenetic relationships).

We therefore excluded from our phylogenetic analyses 11 suspect characters introduced by the EU139566.1-EU139584.1 accessions. These characters did not vary among all the other taxa, and exclusion therefore does not affect phylogenetic inferences about the remaining taxa. However, we were unsure about the error of at least four additional suspect characters, which therefore remained included in our phylogenetic analyses and were expected to possibly render the corresponding taxa as phylogenetically derived. The derived phylogenetic positions of some of the sequences from Poulsen et al. (2007) (EU139566.1-EU139584.1) in Figures 2a and 2b therefore may be an artifact of such uncorrected sequencing errors.

Note added after acceptance of our manuscript:

In late December 2009, we communicated the possibility of sequence errors in the analyses of Poulsen et al. (2007) to the authors, who promptly corrected the most-suspect characters in their sequences deposited originally in September 2007 at GenBank (these replacement sequences are deposited as EU139566.2 - EU139584.2 at the NCBI Nucleotide Collection; note the ".2" ending of these corrected sequences, distinguishing them from the original submissions identified by a ".1" ending). Because the replacement sequences still showed the four additional suspect character-scorings that we discuss in the preceding paragraph, we requested to confirm these sequence scorings by inspection of the relevant sequencing chromatograms. The authors kindly sent us on 4. February 2010 a set of chromatograms (see Supplement 2) which they believed would validate the sequence-scoring of the replacement sequences released at Genbank on 5. January 2010. Inspection of these chromatograms revealed problems in the sequencing chromatograms sent to us (e.g., forward sequence of *Pseudonocardia* and reverse sequence of *Tsukamurella* compiled into a single sequence and edited to yield a *Pseudonocardia* consensus sequence; both forward and reverse sequence of *Tsukamurella* edited to yield a consensus sequence of *Pseudonocardia*; chromatograms for specific *Pseudonocardia* isolates that were inconsistent with either the respective original sequence submissions and also inconsistent with the respective corrected sequences deposited on 5. January 2010 to replace the original sequences). When pointing out the problems of this first set of chromatograms, the authors attributed the problems to mixup of files in databases, and they kindly sent us on 12. February 2010 a second set of chromatograms (see Supplement 3) to replace the first set of chromatograms. Inspection of this second set of chromatograms revealed two additional problems: (a) duplication of primary chromatogram reads that were labeled as if they had been derived from different sequencing runs (i.e., exactly duplicated chromatogram reads, including identical sequencing artifacts and identical background noise, in chromatogram files labeled as if they had been derived from different sequencing runs from different bacteria); and most puzzling (b) original base calls (primary nucleotide calls generated by the automated sequencer, preceding any manual editing) that did not match the associated chromatogram peaks. Across most sequence reads, the apparently original base calls were consistent with a *Pseudonocardia* sequence, but inconsistent with the associated chromatogram peaks indicating *Tsukamurella*. The authors attributed the problems of the second set of chromatograms to file mixup. We agree that some of the inconsistencies can be explained by file mixup (i.e., the duplication of chromatograms, including exact duplication of sequencing artifacts and background noise, but labeling as two different sequencing runs). On the other hand, we believe that the editing of *Tsukamurella* reads in the first set of chromatograms, and the changes to the original base calls in the second set of chromatograms, yielding consensus sequences of *Pseudonocardia* in both the first and the second sets of chromatograms, is less compatible with an explanation of file mixup. It is unclear whether the changes to the original base calls preceding manual editing (Supplement 3) and the particular sequence editing (Supplements 2 & 3) could have generated the 11+4 unusual character scorings mentioned above, as well as additional sequence artifacts not discussed here. At the present time of correcting the proofs of our manuscript, we have not been able to examine any chromatograms that verified the character scoring of the sequences of Poulsen et al. (2007). We thank the authors for their generous help to understand their sequence scorings.

Caption for Figures S1-S7

16S rRNA secondary structure models for representative *Pseudonocardia* species (Figures S1-S5), including two examples of suspect 16S rRNA sequences of Poulsen et al. (2007) (Figures S6 &S7). Canonical base pairs (G:C, A:U) are shown with tick marks, wobble (G:U) base pairs with small closed circles, A:G base pairs with large open circles, and all other non-canonical base pairs with large closed circles.

Figure S1. Secondary structure of 16S rRNA of *P. carboxydivorans* (EF114314; type strain Y8).

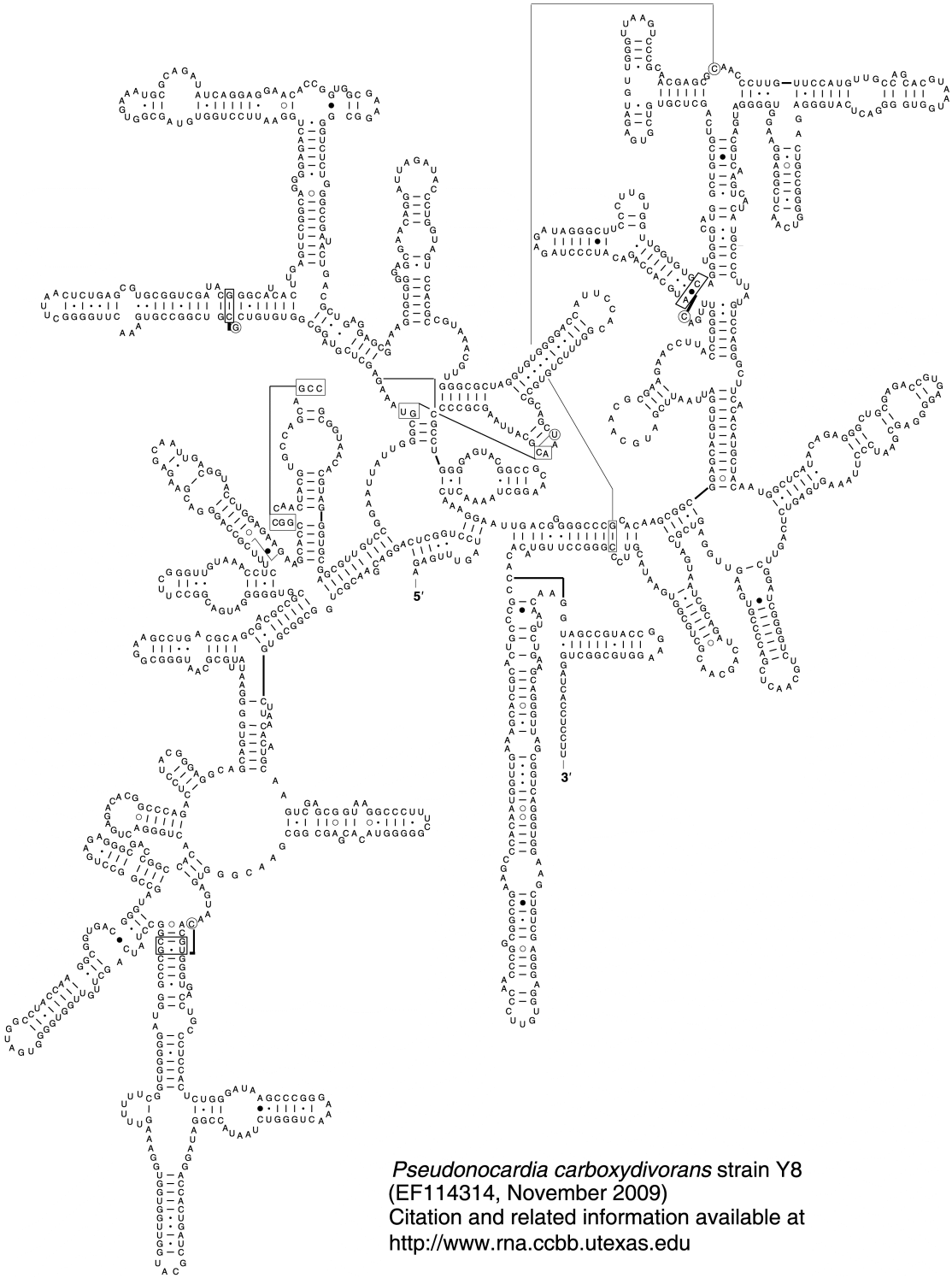


Figure S2. Secondary structure of 16S rRNA of *P. kongjuensis* (AJ252833; type strain LM 157).

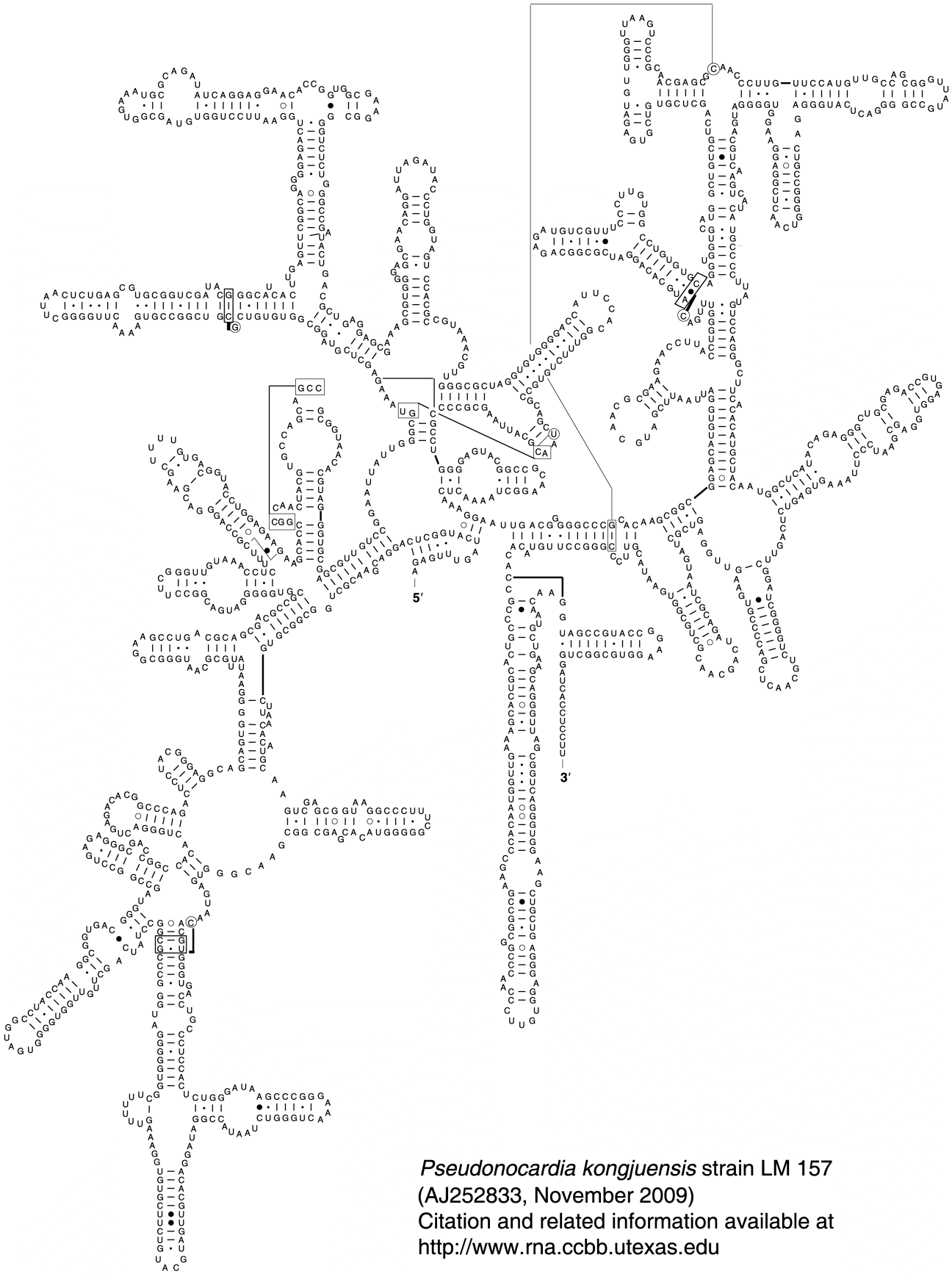


Figure S3. Secondary structure of 16S rRNA of *P. halophobica* (AJ252827; type strain IMSNU-21327^{T}).

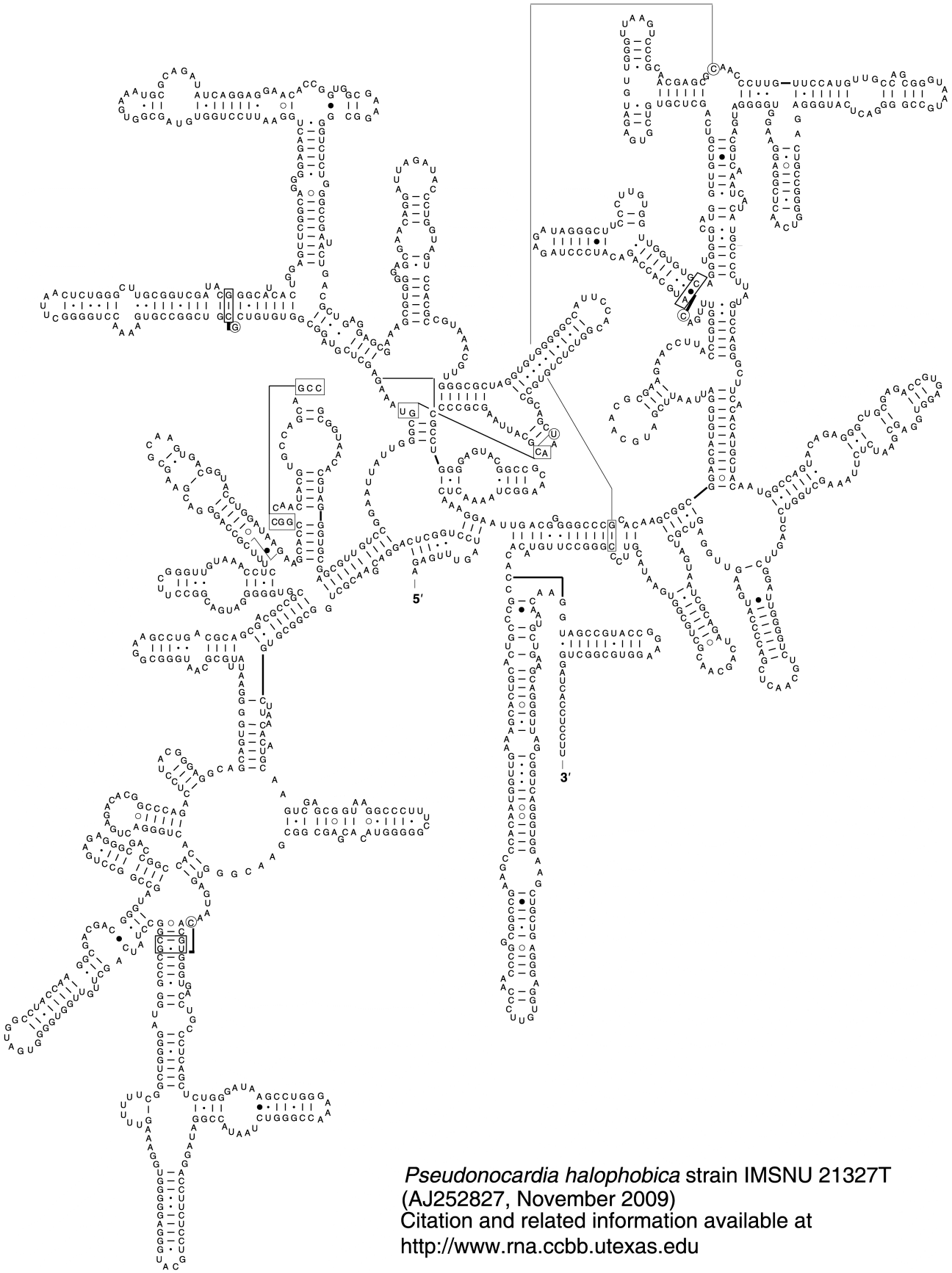


Figure S4. Secondary structure of 16S rRNA of *P. spinosispora* (AJ249206; type strain LM 141^{T}).

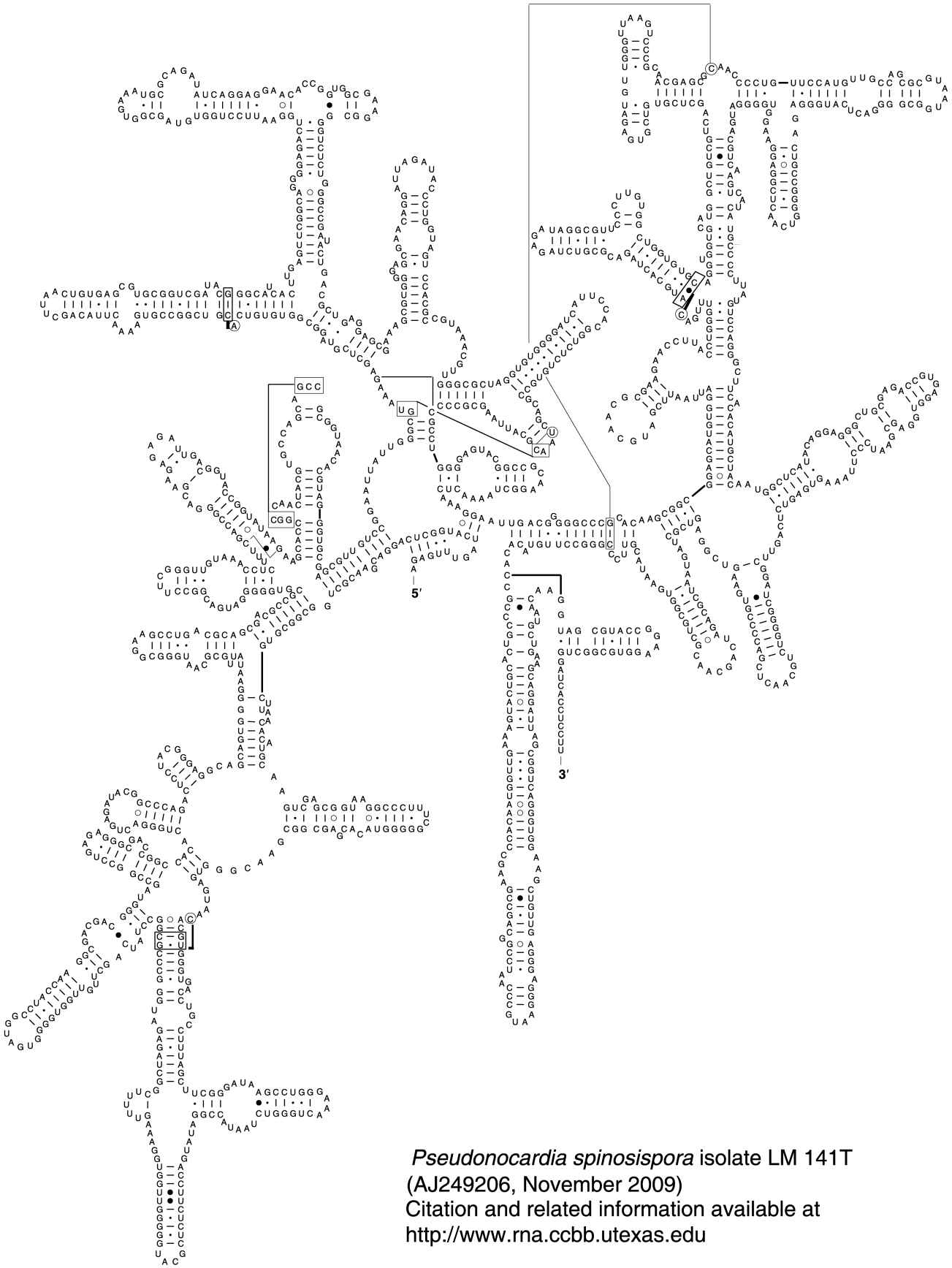


Figure S5. Secondary structure of 16S rRNA of *P. yunnanensis* (AJ252822; type strain IMSNU-22019^{T}).

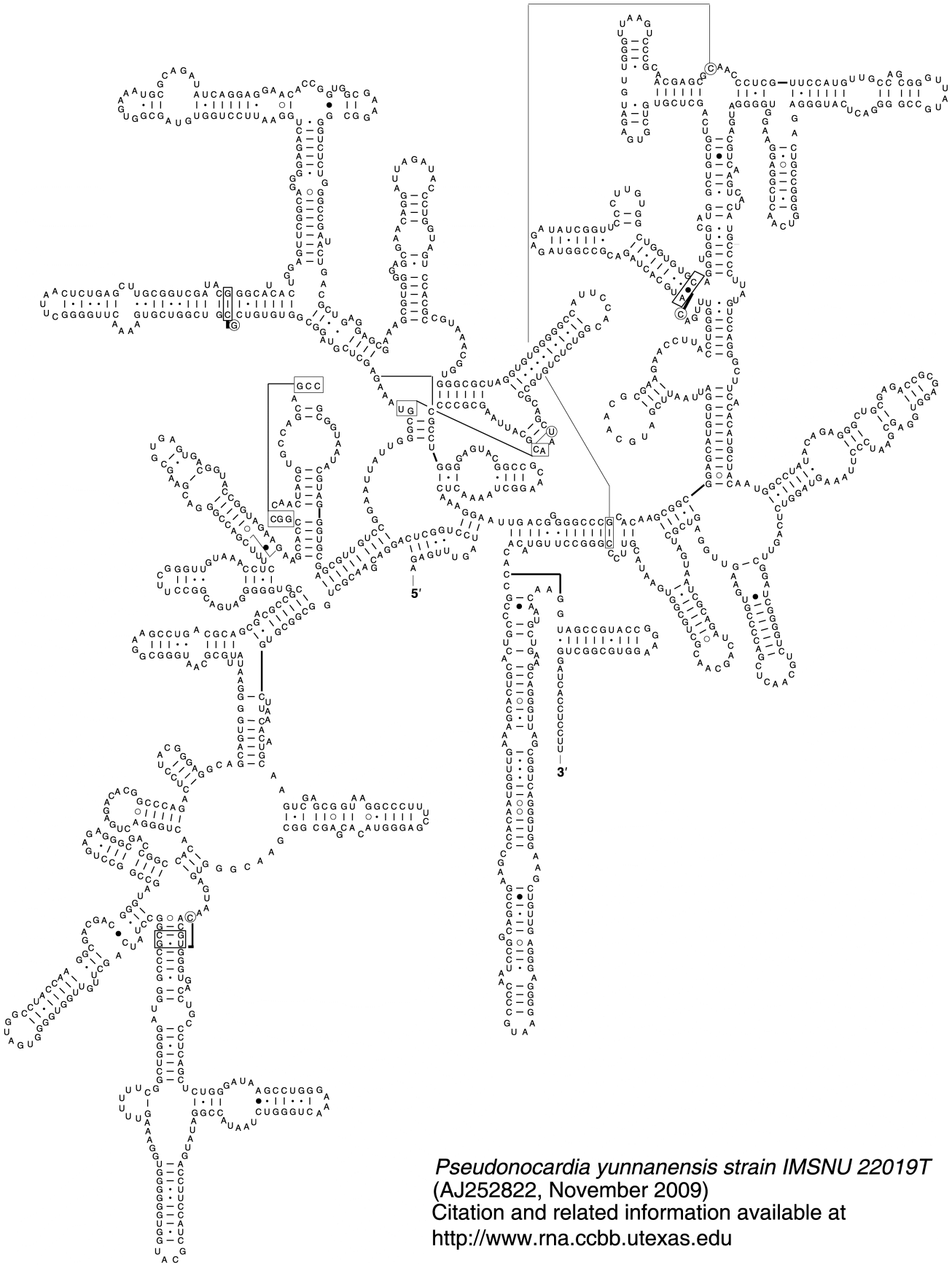


Figure S6. Inferred secondary structure of 16S rRNA of *Pseudonocardia* sp. SP030327-01 (EU139577.1) isolated from an attine worker and submitted by Poulsen et al. (2007). The comparison of this secondary structure with those shown in Figure S1-S5 identifies at least 11 sequencing errors in conserved regions of EU139577.1 and the other sequences submitted by Poulsen et al. (2007). See also Figure S7 and explanation in above text.

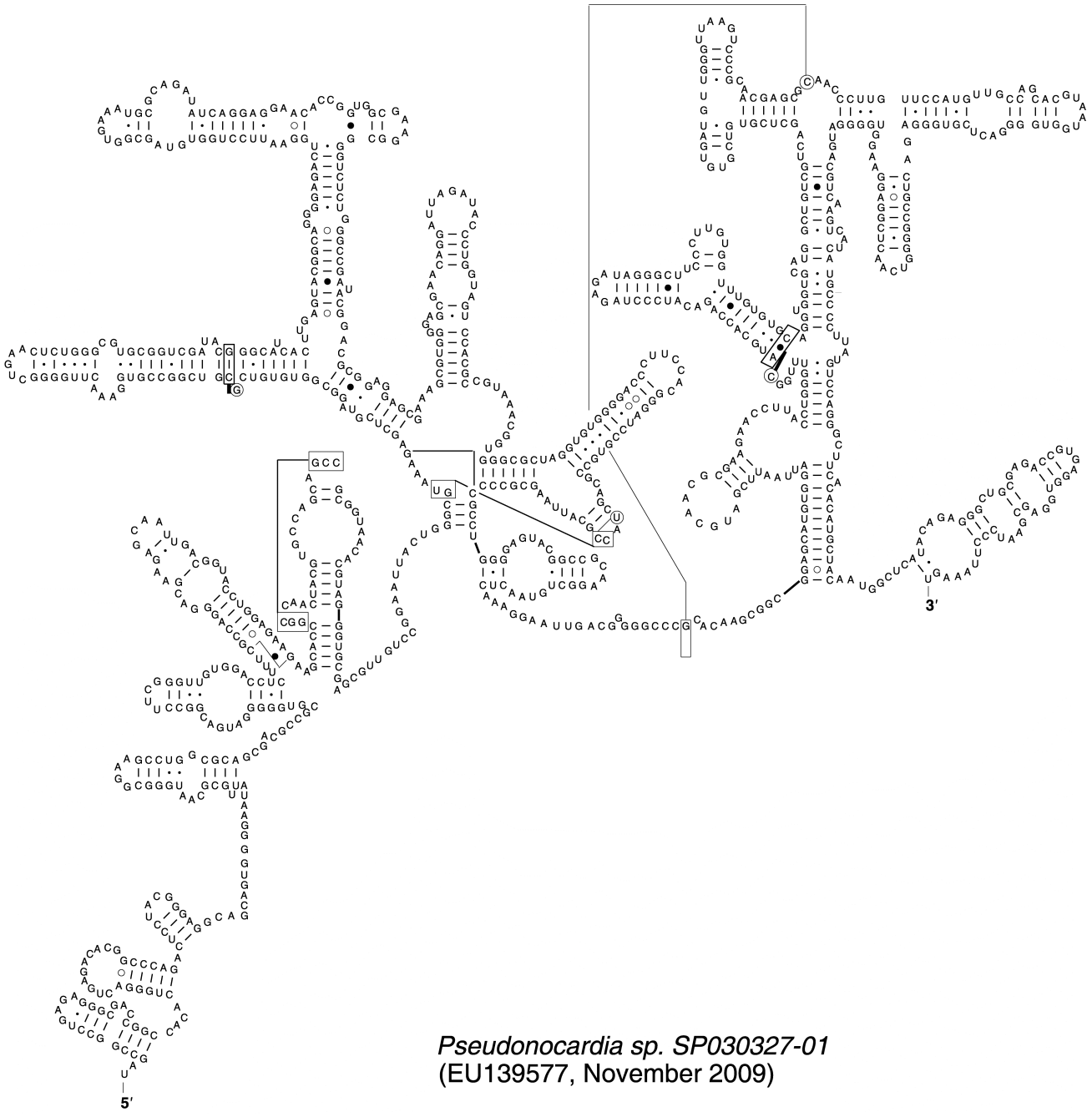


Figure S7. Inferred secondary structure of 16S rRNA of *P. halophobica* (EU139576.1, from strain NRRL B-16514) submitted by Poulsen et al. (2007). The comparison of this secondary structure with the one of *P. halophobica* in Figure S3 identifies at least 11 sequencing errors in conserved regions of EU139576.1 and the other sequences submitted by Poulsen et al. (2007). See also Figure S6 and explanation in above text.

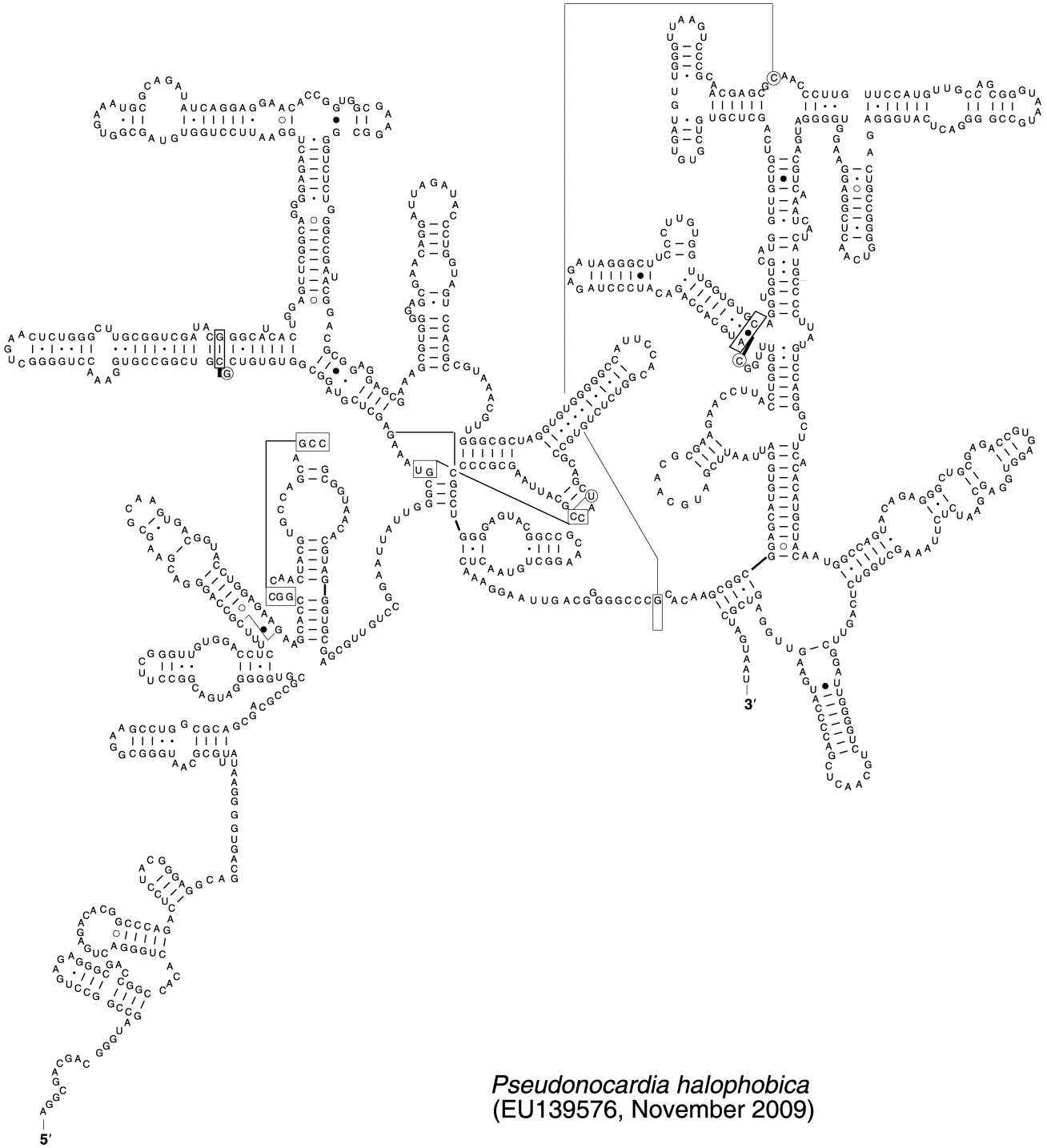
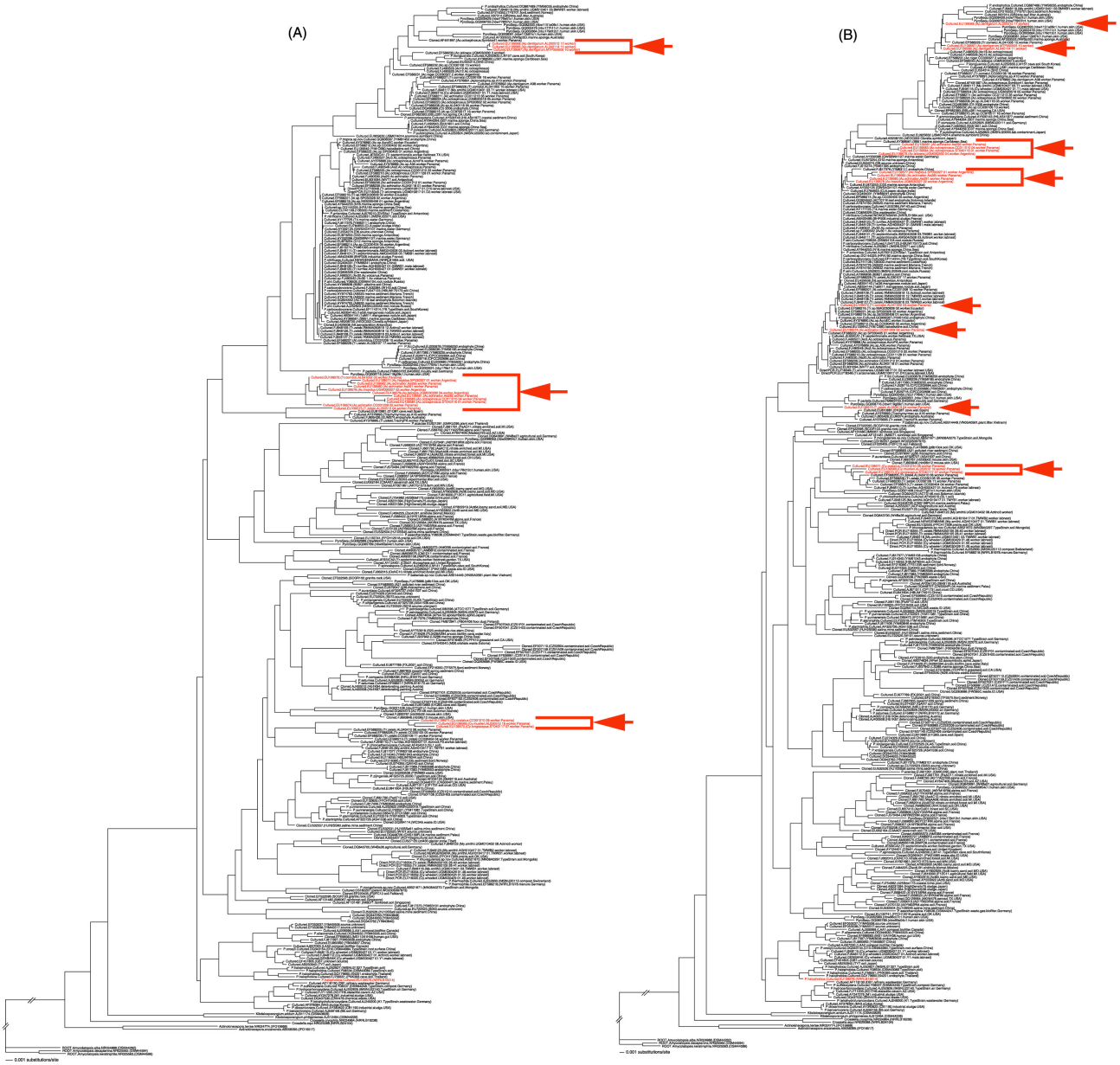


Figure S8. Comparison of neighbor-joining (NJ) trees inferred from two 16S rRNA gene alignments that either (A) included the 11 sequencing errors of Poulsen et al. (2007) (see above text for explanation), or (B) excluded these sequencing errors. Sequences derived from Poulsen et al. (2007) are highlighted in red. Comparison of the two tree topologies reveals that, as predicted, the sequences from Poulsen et al. (2007) appear artificially derived with the errors included [see longer branches subtending these taxa, including the *P. halophobica* sequence of Poulsen et al. (2007) that groups with other *P. halophobica* sequences at the bottom of the phylogenetic reconstructions]. Moreover, in several cases in the left (uncorrected) reconstruction, the suspect sequences are grouped into artificial monophyletic clades united by shared sequencing errors [compare how the sequencing errors artificially cluster the taxa of Poulsen et al. (2007) in the left reconstruction and overall distort the phylogenetic relationships among the main *Pseudonocardia* groups].



Isolates belonging to Clade 4 of *Pseudonocardia* (see Figure 2a in main article)

Cafaro & Currie (2005) had identified a three-taxon clade of *Pseudonocardia* (isolated from *Trachymyrmex* ants from Panama) that had emerged as phylogenetically unique and "ant-specific" clade in the phylogenetic analysis by Mueller et al. (2008; see Clade 4 in Figure 1 in main article). Our expanded phylogenetic analyses identify now two environmental *Pseudonocardia* nested within that *Pseudonocardia* clade, which was formerly hypothesized to be ant-specific (see Clade 4 in Figure 2a in main article). One of these environmental isolates is an endophytic *Pseudonocardia* that was isolated from a *Eucalyptus* tree in Australia (GenBank accession FJ805426; 99.8% sequence-identical to AY376893 isolated from a *Trachymyrmex* worker from Panama), the second environmental *Pseudonocardia* was isolated in a survey of microbial growth degrading prehistoric cave paintings in Spain (Stomeo et al. 2008, 2009; EU810881; 99.3% sequence-identical to AY376893 isolated from a *Trachymyrmex* worker). Representatives of Clade 4 have therefore been found only six times so far (four times from *Trachymyrmex* ants, twice from the two environmental sources mentioned above; see below table) and they have so far not been detected in any culture-independent screen. Note that sample AY376894 (isolate A46, from Cafaro & Currie 2005) does not appear in Clade 4 in Figure 2a because our secondary-structure rRNA modeling identified exceptionally many errors in this sequence. The unreliable sequence AY376894 was therefore excluded from our final alignment; consequently, only five taxa appear in Clade 4 in Figure 2a. In preliminary neighbor-joining and maximum-likelihood analyses, sequence AY376894 always grouped with the other five sequences, and sequence AY376894 therefore can be placed with confidence into Clade 4 of *Pseudonocardia* (Figure 2a).

GenBank Accession #	Isolate ID#, Source, and Country of Collection
AY376893	isolate A16 cultured from worker ant *Trachymyrmex*.sp. in Panama
AY376894	isolate A46 cultured from worker ant *Trachymyrmex*.sp. in Panama
AY376895	isolate TrachyPA cultured from worker ant *Trachymyrmex zeteki* in Panama
EU139573	isolate AL050513-04 cultured from worker ant *Trachymyrmex zeteki* in Panama
EU810881	isolate 01Q8T cultured from prehistoric painting on cave wall in Spain
FJ805426	isolate EUM374 cultured as endophyte from *Eucalyptus* tree in Australia

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