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A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: A case for 16S, *gyrB*, and *glnA*

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ABSTRACT

Photorhabdus spp., the only known bioluminescent terrestrial bacteria are well known for their symbiotic association with heterorhabditid nematodes. This association, along with their ability to kill insects, has aroused interest in the evolutionary relationships within this bacterial group. Currently, three species are recognized within the genus *Photorhabdus*; *P. temperata* and *P. luminescens*, which are endosymbionts of *Heterorhabditis* spp., and *P. asymbiotica*, which has been isolated from human wounds and has recently been shown to also have a heterorhabditid nematode vector. To examine phylogenetic relationships among these taxa, we utilize total evidence Bayesian, likelihood, and parsimony based analyses of three genetic loci (16S rRNA gene, *gyrB*, and *glnA*) to construct a robust evolutionary hypothesis for the genus *Photorhabdus*. Here we use this phylogeny to evaluate existing specific and sub-specific taxonomic statements within the genus, identify previously undescribed *Photorhabdus* strains, test the utility of 16S rRNA gene, *gyrB*, and *glnA* in resolving various levels of relationships within the genus, and, finally, to investigate the evolution of bioluminescence. The genes examined produced the most robust phylogenetic hypothesis to date for the genus *Photorhabdus*, as indicated by strong bootstrap and posterior probability values at previously unresolved or poorly resolved nodes. We show that *glnA* is particularly useful in resolving specific and intra-specific relationships poorly resolved in other studies. We conclude that *P. asymbiotica* is the sister group to *P. luminescens* and that the new strains HIT and JUN should be given a new group designation within *P. asymbiotica*. Furthermore, we reveal a pattern of decline in bioluminescent intensity through the evolution of *Photorhabdus*, suggesting that this may be a trait acquired and maintained under previous ecological (aquatic) selection pressures that is now gradually being lost in its terrestrial environment.

1. Introduction

Photorhabdus spp., the only terrestrial bacteria known to exhibit bioluminescence (Gerrard et al., 2003) are motile, gram negative bacteria which are gut endosymbionts in juveniles of entomopathogenic nematodes from the genus *Heterorhabditis*. The close symbiotic relationship between *Photorhabdus* and *Heterorhabditis* has gained much attention due to their ability to work together to kill their insect host. Upon locating a suitable insect host, *Heterorhabditis* penetrates through natural openings (mouth, anus, spiracles) (Boemare, 2002), or directly into the hemocoel of the larval insect via the integument (Akhurst and Dunphy, 1993; Forst et al., 1997; Poinar, 1990), subsequently releasing bacteria into the hemolymph (Forst et al., 1997). Once in the hemolymph, *Photorhabdus* begins multiplying, simultaneously releasing toxins virulent enough to kill the insect within 24 h (Ciche and Ensign, 2003; Forst et al., 1997). All *Photorhabdus* strains are considered highly entomopathogenic, with an LD_{50} of <100 cells per insect (Boemare, 2002). Following death of the insect and consumption of all available nutrients, *Photorhabdus* and *Heterorhabditis* re-assimilate, leaving the dead insect in search of another insect host (Forst and Nealson, 1996).

One of the most unique characteristics of *Photorhabdus* is its bioluminescent capabilities. *Photorhabdus* spp. are the only

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terrestrial natural bioluminescent bacteria, though other aquatic bioluminescent bacteria do exist (i.e. *Vibrio harveyi*, *Vibrio fischeri*, *Vibrio cholerae*, *Photobacterium leiognathi*, *Photobacterium phosphoreum*, *Shewanella hanedai*). In *Photorhabdus* and other bioluminescent bacteria, *lux* genes are responsible for the production of light (Baldwin et al., 1989; Friedland and Hastings, 1967; Kuwabara et al., 1965), and these *lux* genes are organized into an operon that varies in the organization of genes from one bacterium to the next (Kasai et al., 2007; Meighen and Szittner, 1992; O'Kane and Prasher, 1992). Much speculation exists as to why *Photorhabdus* bioluminesces. Some hypotheses that have been proposed as to the functional significance of light production in *Photorhabdus* include a distraction mechanism, a molecular oxygen sink, an attractant, and a signal that synchronizes symbiosis (Waterfield et al., 2009). Alternatively, as suggested by Peat and Adams (2008), production of light in *Photorhabdus* may not have any real function and instead represents a trait present in an aquatic ancestor that is now being lost upon colonization of a terrestrial environment via the nematode vector or is a remnant of a horizontal gene transfer event that has not had sufficient evolutionary time to disappear. Though to thoroughly evaluate hypotheses of bioluminescence evolution in *Photorhabdus*, a robust phylogenetic hypothesis of the genus is required.

Initially classified as *Xenorhabdus luminescens*, the genus *Photorhabdus* would later be proposed by Boemare et al. (1993) based on the examination of phenotypic characters and DNA relatedness studies. Specific and sub-specific taxonomic designations within the genus *Photorhabdus* are based on phenotypic data including morphological, biochemical and physiologic characters (Akhurst et al., 1996; Fischer-Le Saux et al., 1999), DNA–DNA hybridization (Akhurst et al., 1996; Farmer et al., 1989), sequencing of a portion of 16S rRNA gene (Liu et al., 1997), sequencing of the complete 16S region of rRNA (Fischer-Le Saux et al., 1999), sequencing of the *gyrB* gene (Akhurst et al., 2004), and a multilocus sequence typing analysis of *recA*, *gyrB*, *dnaN*, *gltX* (Tailliez et al., 2009). Akhurst et al. (1996) concluded that phenotypic data alone could only separate two groups of *Photorhabdus,* the symbionts and the clinical strains. Clinical infections with *Photorhabdus* have been reported in a number of humans within the United States (Farmer et al., 1989) and Australia (Gerrard et al., 2003; Peel et al., 1999).

Liu et al. (1997) developed a phylogeny of *Photorhabdus* and another closely related bacterial endosymbiont of nematodes, *Xenorhabdus.* The study used 13 *Photorhabdus* isolates, most of which had no species designation. Based upon maximum likelihood analysis of a portion of 16S rRNA gene gene, Liu et al. showed four well supported major clades within the one recognized clade, supporting the possibility that more than one species of *Photorhabdus* exists. Through a polyphasic approach utilizing 16S rRNA gene phylogenetic inference, phenotypic characterization, and DNA–DNA hybridization data, Fischer-Le Saux et al. (1999) proposed the existence of three separate species of *Photorhabdus*: *P. luminescens*, *P. temperata*, and *P. asymbiotica*. Furthermore, the study went on to propose the existence of three subspecies within *P. luminescens*. A second polyphasic approach utilizing phenotypic characterization, DNA–DNA hybridization, and two molecular markers, *gyrB* and 16S rRNA genes proposed the separation of *P. asymbiotica* into two subspecies (Akhurst et al., 2004). Hazir et al. (2004) used riboprint analysis, metabolic properties, and a distance analysis of 16S rRNA gene, to propose two new subspecies (*P. luminescens* ssp. *thracensis* and *P. luminescens* ssp. *kayaii*). More recently, the subspecies *P. temperata cinerea* has been proposed based on *gyrB* data (Toth and Lakatos, 2008) and Tailliez et al. (2009) used *recA*, *gyrB*, *dnaN*, *gltX* to propose four new subspecies (*P. luminescens caribbeanensis*, *P. luminescens hainanensis*, *P. temperata khanii*, and *P. temperata tasmaniensis*) and the renaming of another (*P. temperata thracensis*).

Historically, 16S rRNA gene has been the marker of choice when classifying/naming *Photorhabdus* species and subspecies. It has been suggested that *Photorhabdus* species may be subjected to a higher evolutionary rate than that of its sister taxon *Xenorhabdus* (Rainey et al., 1995) based on analysis of 16S rRNA gene data. Conversely, Tailliez et al. (2009) suggest that *Xenorhabdus* evolves at a faster rate than *Photorhabdus* based on the analysis of dN/dS ratios for five different genes (*rplB*, *recA*, *gyrB*, *dnaN*, and *gltX*). While the 16S rRNA gene has been shown as useful in identifying many bacteria to the generic level (Fukushima et al., 2002; Wang et al., 1994), evidence of potential lateral gene transfer of the 16S rRNA gene exists in numerous bacterial genera including *Photorhabdus* (Tailliez et al., 2009), giving the 16S rRNA gene the potential to confound bacterial species relationships rather than resolving them, especially when using 16S alone. As such, other genes, particularly protein coding genes, may provide a clearer representation of the species relationships within the genus *Photorhabdus*. One such marker, *gyrB*, encodes the subunit B protein of DNA gyrase. DNA gyrase functions in the regulation of supercoiling of double stranded DNA. This enzyme is ubiquitous among all bacterial species (Yamamoto and Harayama, 1995). Previous studies have indicated that *gyrB* might prove to be more useful in identifying bacteria to the species level due to its higher rates of molecular evolution (Fukushima et al., 2002; Yamamoto and Harayama, 1995, 1998). Akhurst et al. (2004) utilized the *gyrB* gene to propose the split of the species *P. asymbiotica* into two subspecies, as well as confirm the presence of three species within the genus. Another gene that has shown promise in resolving relationships within the genus *Photorhabdus* is *glnA*, a gene that codes for the glutamine synthetase enzyme (Tullius et al., 2003). Gerrard et al. (2006) conducted a neighbor joining analysis using concatenated *gyrB* and *glnA* datasets to successfully confirm the identity of a *Photorhabdus asymbiotica* strain isolated from a nematode, though support for the most basal node and many terminal nodes in their phylogeny were extremely low. Additionally, a similar tree was utilized by Waterfield et al. (2008) to illustrate the locations of *Photorhabdus* strains of interest in their study.

To date, most single gene and all total evidence phylogenetic analyses of molecular data for the genus *Photorhabdus* have utilized distance based methods of phylogenetic reconstruction (i.e. neighbor joining). Neighbor joining and other distance based methods are useful in that they can build a phylogenetic tree very rapidly, though their use of overall similarity (phenetics) to build phylogenetic trees wastes potentially informative character data (Farris, 1981) while lacking the ability to distinguish between homology and homoplasy (Siebert, 1992). Furthermore, observed distances between sequences do not accurately reflect the evolutionary distances between them, and as such sequences may appear more closely related than they actually are (Holder and Lewis, 2003). Analyses of single genes, while providing a good depiction of the gene tree, often do not accurately reflect the species tree for a given organism (for further discussions see (Maddison, 1997; Pamilo and Nei, 1988)). To thoroughly investigate the phylogenetic relationships within the genus *Photorhabdus,* we advocate an approach whereby a combined simultaneous analysis of multiple molecular datasets is conducted utilizing more rigorous methods of phylogenetic reconstruction including parsimony, Bayesian, and likelihood analyses. Using these non-phenetic based methods to conduct simultaneous analyses of multiple molecular datasets should provide more power in resolving issues of species delineation within the genus *Photorhabdus* by allowing phylogenetic signal to emphasize itself over phylogenetic noise (De Queiroz, 1993). Additionally, when conducting model based analyses of concatenated datasets, we also advocate the use of mixed models, as a single model is usually inadequate to account for the differing histories of multiple genes (Bull et al., 1993; Huelsenbeck