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A global survey of the bacteria within earthworm nephridia

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ABSTRACT

Earthworms comprise 16 described families in the Crassiclitellata plus a few other minor groups. Microscopy studies of the early 20th century detected bacteria within the excretory organs, the nephridia, of species within a few of these families. More recent evidence for the consistent and specific association of bacteria with nephridia within the Lumbricidae has been well documented, but the presence and identity of nephridial bacteria among the rest of the Crassiclitellata families had not been explored. The study presented here aimed to identify members of Crassiclitellata families that harbor bacteria in their nephridia, and identify these bacteria based on 16S rRNA gene sequences. Eleven earthworm families were surveyed from countries of six continents, and two island nations. The results revealed members of four bacterial orders commonly occurred within nephridia of genera within nine Crassiclitellata families. Members of the bacterial phyla Bacteroidetes (order Sphingobacteriales), Betaproteobacteria (order Burkholderiales; family Comamonadaceae), and Alphaproteobacteria (orders Rhodospirillales and Rhizobiales) were detected in the nephridia of basal Crassiclitellata, as well as in derived families. Earthworm genera with meronephridia, multiple small nephridia per segment, lacked bacteria, whereas bacteria were often detected in holonephridia, single pairs of large nephridia with a distinct morphology and external excretory pore. The Acanthodrilidae members, a large derived family of earthworms, did not appear to possess nephridial bacteria regardless of nephridial form. Although earthworms from a variety of habitat types were sampled, there were no clear correlations of lifestyle with symbiont types, with the exception of the aquatic earthworms that contained bacteria unrelated to those in any other earthworms. The findings support an evolutionarily long association of bacteria within the Crassiclitellata, and suggest a contribution to nitrogen conservation for the earthworms.

1. Introduction

1.1. Context and background

Bacterial partnerships with animals and plants are widespread and confer physiological, biochemical and chemical advantages of many forms to their hosts. Microbial partnerships can range from highly diverse, such as gut microbiota, to single host-specific species, as in *Rhizobium*-legume and *Vibrio-Euprymna* (bobtail squid) systems (Hirsch and McFall-Ngai, 2000; McFall-Ngai, 2002; Ruby, 2008). Earthworms are no exception, with the earliest documentation of bacterial cells in the earthworm excretory organs, the nephridia, by Stanislas Maziarski, 1903 (Maziarski, 1903). The earthworms, commonly named for their similar body size and ecological function, comprise the Crassiclitellata (Jamieson, 1988), plus the non-crassiclitellate families Moniligastridae, Syngenodrilidae and Alluroididae within the Clitellata. The Clitellata are united by the common feature of a band of tissue, the clitellum, that produces materials for egg capsules during reproduction.

The nephridia process coelomic fluid for osmoregulation and waste excretion (Ramsay, 1949a, 1949b; Laverack, 1963), and vary in size, form and number per segment. Holonephridia are large, paired in each segment, and attached to the body wall and septa. They appear in the basal Crassiclitellata and in its sister-group Moniligastridae (James and Davidson, 2012; Fig. 1). Holonephridia have three main loops that include a tubule system of the first loop leading into a second loop with enlarged ampulla, and finally the bladder (if present) that excretes through a pore to the exterior (Fig. 2). The ampulla of holonephridia is the known location of symbiotic bacteria. Enteroic holonephridia excrete their waste into the hindgut, rather than to the exterior. Meronephridia are small, loop structures that occur in multiples in each segment, anywhere from less than ten to hundreds per segment. The presence of bacteria within the different morphotypes of nephridia will be addressed in the study presented here.

Prior work has established that many members of the earthworm family Lumbricidae harbor more than one type of nephridial symbiont, and that at least one bacterial genus, *Verminephrobacter*

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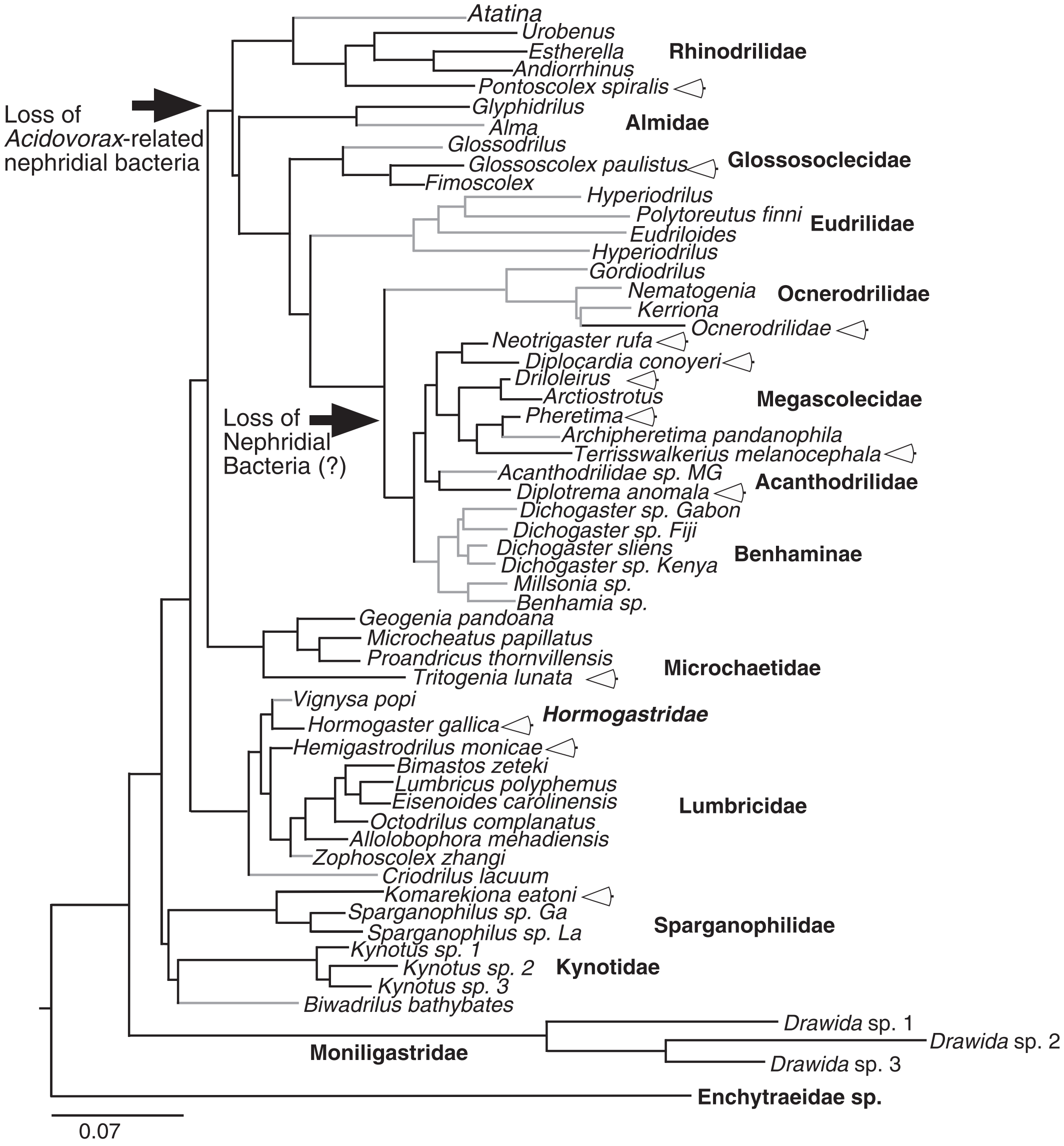

Fig. 1. Crassiclitellata family tree adapted from James and Davidson (2012), based on a partitioned 18s, 28s, 16s ribosomoal RNA gene sequence Bayesian analysis. Branch lengths are drawn proportional to the expected number of substitutions per site as measured with the scale bar. Grey branches indicate taxa within family were screened for bacterial symbionts, and black indicates specimen are in the collection but have not yet been screened for nephridial bacteria. Open arrows indicate genera that were negative for bacteria in nephridia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Pinel et al., 2008), has a long evolutionary relationship with their host lumbricid species (Schramm et al., 2003; Lund et al., 2010a; Davidson et al., 2010). The phylogenetic patterns of *Verminephrobacter* (Betaproteobacteria) and their Lumbricidae host species suggest these bacteria appeared in an ancestral lumbricid and have been co-diversifying with the worms ever since (Lund et al., 2010a). In addition to the phylogenetic evidence for the longevity of the association, *Verminephrobacter* is vertically transmitted to the next generation by mechanisms carefully orchestrated with earthworm embryo development (Davidson and Stahl, 2006, 2008). In addition, Davidson and Lund observed more than one type of bacteria by fluorescent in situ hybridization (FISH) to bacterial cells in the nephridia and egg capsules (Davidson et al., 2010; Lund et al., 2010a). Novel *Flexibacter* spp. and members of a novel Microbacteriaceae genus also consistently appear in the nephridia and egg capsules of *Eisenia fetida* (Davidson et al., 2010). The significance of nephridial bacteria for earthworm success remains unknown. A plausible hypothesis that the nephridial bacteria contribute nitrogen conservation was partially supported by the finding that *Aporrectodea tuberculata* fed a nitrogen-poor diet showed reduced reproductive success if cured of nephridial bacteria (Lund et al., 2010b). The long evolutionary history and the established mechanisms for maintaining the symbiont from one generation to the next suggest these bacteria do have a significant role in earthworm ecology.

1.2. Main objectives of the study presented here

The purpose of this study was to determine which members of the Crassiclitellata harbor nephridial bacteria, and to identify these bacteria. Earthworms are found on most continents and influence soil quality and communities. The success of these soil organisms, and their influence on soil chemistry, are potentially influenced by bacteria harbored in their nephridia. Are the bacterial symbionts detected in the Lumbricidae widespread among the Crassiclitellata, or an isolated acquisition of the Lumbricidae? The observations of Knop suggested there are other earthworm families with nephridial symbionts (Knop, 1926). Furthermore, leeches, also members of