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ABSTRACT.—The giant earthworm, Rhinodrilus priollii Righi 1967, is among the largest terrestrial invertebrates known worldwide, reaching lengths >2 m. To investigate the evolutionary history of the species and aspects of their reproductive biology, we collected R. priollii specimens from several field sites in central Amazonia. Phylogenetic analyses of 16 individuals using a fragment of cytochrome c oxidase subunit 1 (CO1) identified seven haplotypes that diverged between 2–8%. Population structures indicate episodes of gene flow between populations and their divergence within the past 1–2 million years. Histological examination of clitella from sexually mature specimens identified cocoon secretory cells throughout the dorsal and dorsoventral epidermis. Unlike previously described secretory cells, those in R. priollii contained granules with a proteinaceous core covered by external glycosylation. Further, collagenous matrices formed the bulk of swollen clitella while albumin-secreting cells were noticeably absent, collectively suggesting a mechanism of cocoon production somewhat different from that described in other clitellate megadriles.

INTRODUCTION

Among ~6000 species of formally recognized terrestrial megadriles (Blakemore, 2009), relatively few (~20) attain adult lengths exceeding 1 m. These atypically large earthworms remain a scientific curiosity in terms of their ecology, physiology, behavior, and evolution. Indeed, their large size poses challenges related to nutritional requirements, oxygen transport, predation, burrowing, reproduction, and cocoon construction. Nevertheless, giant earthworms have successfully and independently colonized a variety of habitats worldwide. Examples include the Giant Gippsland Earthworm, Megascolides australis McCoy 1878 of southern Australia (up to ~2 m; Van Praagh, 1992), Megascolex mekongianus Cognetti 1922 from Vietnam (up to 2.9 m; Blakemore et al., 2005), Microchaetus microchaetus Rapp 1849 from South Africa (up to 1.8 m; Plisko, 1999), Tonoscolex birmanicus Gates 1926 from

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Burma (up to ~3 m in some reports; Gates, 1972), Glossoscolex giganteus Leuckart 1835/6 from South America (up to ~2.7 m; Beddard, 1895), Celeriella gigantean Benham 1906 from New Zealand (up to 1.4 m; Lee, 1959), Driloleirus americanus Smith 1897 from Washington State, North America (up to 1 m; in Blakemore et al., 2005), and several Rhinodrilus species from South America, including the subject of this study, Rhinodrilus priolii Righi 1967.

More than 20 species of Rhinodrilus (family Glossoscolecidae) have been described, many of which are native to South America (James and Brown, 2006). One of the largest species in the genus, R. priolii, appears to be endemic to the Amazon Basin, but otherwise its geographic range and other aspects of its biology remain mostly unexplored. In this report, we present baseline data on the biogeography of R. priolii in central Amazonia, and examine cellular and morphological aspects of its atypically large clitellum.

**Materials and Methods**

**Specimens**

*Rhinodrilus priolii* Righi 1967 specimens were collected between Sep. – Dec. 2008, from field sites near Manaus, Brazil. Worms were maintained in 500–1000 L fiberglass storage vessels, containing 2–3 cm of ground soil and forest litter from the collection site. Containers were kept moist with occasional water sprinkling, covered, and placed in a shady area (e.g., under forest canopy). Under such conditions, specimens survived up to several months at low densities (e.g., 2–5 worms per container); higher densities significantly reduced survival time.

**Histoogy**

Specimens were fixed in the field with 10% formalin for 1–3 d, then transferred to 70% EtOH for long-term storage. Following an ascending EtOH series to xylene, worm fragments were embedded in Paraplast X-tra (McCormick Scientific) and sectioned at 5–7 μm on a Spencer “820” microtome. Sections were stained with Masson’s trichrome according to Sheehan and Hrapchak (1980). Images were captured on a Canon Rebel XT coupled to a Zeiss Universal with a PL 232 mm 28 adaptor (Perspective Image LLC, Beaverton, OR).

**DNA Extraction and Amplification**

Tissue samples (~30 mg of muscle scraped from inside the cuticle) from postmortem specimens (fixed in 70% EtOH only) were removed with a scalpel, and genomic DNA was extracted using an E.Z.N.A. Tissue Isolation Kit (Omega Bio-Tek). Mitochondrial cytochrome c oxidase subunit I (CO1) was amplified from genomic DNA using universal primers LCO and HCO as described (Folmer et al., 1994), amplifying a ~600 bp fragment.

**DNA Sequencing and Editing**

PCR products were excised from 1% agarose gels and prepared for sequencing using GeneClean (MP Biomedicals, LLC). DNA sequencing was conducted by GeneWiz Inc. (South Plainfield, New Jersey) with forward and reverse PCR primers. Sequences were viewed and manually adjusted in ChromasPro (Technelysium, Queensland, Australia), and aligned with CLUSTALW (Thompson et al., 1994) to determine overlapping regions suitable for phylogenetic analyses. GenBank accession numbers for new COI sequences are listed in corresponding Figure legends.

**Phylogeny**

Maximum-likelihood (ML) analyses were performed for all DNA comparisons, using the pipeline sequence MUSCLE (Edgar, 2004) to align corresponding sequences from multiple
individuals or homologous DNA across species, Gblocks (Castresana, 2000) for alignment curation, PhyML (Guindon and Gascuel, 2003) for tree building and TreeDyn (Chevenet et al., 2006) for tree drawing, as configured in the Phylogeny.fr platform (Dereeper et al., 2008). The aLRT statistical test (approximation of the standard Likelihood Ratio Test; Anisimova and Grascuel, 2006) embedded in PhyML determined branch support values. Default settings were used for all parameters.

**Results**

Specimens of *Rhinodrilus* (Fig. 1) were collected from four geographic locations proximal to Manaus, Brazil: Universidade Federal do Amazonas (UFAM) experimental farm (02°38’44.35”S, 60°02’44.20”W), Instituto Nacional de Pesquisas Amazonia (INPA) field station (02°37’32.94”S, 60°02’38.72”W), RPPN field site near Encontro das Aguas

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**Fig. 1.—Live *Rhinodrilus priolii* specimen (~2 m) held by co-author M. Garcia**
and a private farm (02°54′33″S, 59°56′34″W)—all below elevation 100 m (Fig. 2). In total, ~50 specimens were collected, most of which (~30) were from the UFAM experimental farm. Worms were generally not observed during daylight hours except during persistent heavy rains, upon which they surfaced at densities up to ~100 per km². Under such conditions, they moved actively atop the forest litter for the duration of rainfall and several hours thereafter, leaving characteristic linear trails in soft sediment (e.g., dirt roads). Occasionally, worms were observed being carried passively in transient runoff streams during particularly heavy rainfall. Worms also surfaced during the evening based on remnant tracks, even in the absence of rainfall (though track numbers were much fewer under dry conditions). Specimens ranging in length between 1–2 m in an extended position were common in undisturbed areas (e.g., primary growth forest); developed areas, including nature reserves within urban areas, appeared to lack any significant numbers of R. priollii individuals. The largest specimens were collected at the UFAM field size, reaching lengths over 2 m (up to ~220 cm when extended).

**PHYLOGENY**

Gene fragments from CO1 were successfully amplified from 16 Rhinodrilus priollii individuals representing seven haplotypes (Table 1); seven specimens collected from the PM field site identified two haplotypes that were likely Rhinodrilus contortus Cernosvitov 1938, based on geographic and morphological criteria.

Comparisons of representative CO1 sequences indicated that haplotypes occurred within and between populations (Fig. 3). The two major Rhinodrilus priollii haplotype lineages were 7–8% divergent in the CO1 locus, and contained representatives from multiple populations suggesting recent episodes of gene flow. Two R. contortus haplotypes formed a third clade ~18% divergent from R. priollii CO1 sequences.

**CLITELLAR MORPHOLOGY**

The clitellum of Rhinodrilus priollii specimens was pronounced in mature individuals (up to ~3 cm in diameter), forming a saddle-like structure around the dorso-lateral aspect of the worm. Histological sections through clitella in mature individuals revealed thick, collagenous layers (2–3 mm) separating outer, circular muscle fibers and the epidermis (Fig. 4). Collagen appeared to be supported by a proteinaceous scaffolding (Fig. 5A), and was absent in comparable body wall sections or clitella from immature individuals (Fig. 5B). Thicker regions of the clitellum (i.e., more dorsal) contained two collagenous layers demarcated by a relatively thin, proteinaceous boundary (Fig. 6).

Granulated secretory cells were observed throughout the dorsal and dorsolateral epidermis, along the length of the clitellum. Cell bodies were typically anchored in the outer collagenous layer, extending elaborate tubules to the epidermal surface (Fig. 7A). Granules were packed within the cell body and tubules, often displaying an azocarmine-staining core (red) surrounded by an alcian blue-staining surface (blue; Fig. 7B).

**DISCUSSION**

Our behavioral observations of Rhinodrilus priollii Righi 1967 are consistent with other megadrile species, in that worms were more active during evening hours, and surface during heavy rainfall. The cause of surfacing during rainfall has been debated, and may be related to increased carbon dioxide levels, burrow flooding and/or mating behavior (Edwards, 2004). That no R. priollii specimens were observed at peak daylight hours suggests they are efficient burrowers, even in the relatively hard, root-dense soil typical of central Amazonia.
Once might reasonably assume that the climatic regime and nutrient-poor soil characteristic of equatorial Amazonia would not be suitable for a large, burrowing annelid. However, the large size of individuals (300–400 g) likely acts as a thermal mass that prolongs survival in exposed areas; for example, relatively small specimens of *Rhinodrilus priollii* and other worm species were often found desiccated on dirt roads, while large specimens were not. More importantly perhaps is that lack of soil nutrients may necessitate ingestion and processing of large soil quantities to meet nutritional requirements. Indeed, dissected specimens were filled with soil debris, though gut: body ratios appear comparable to common earthworms, (*e.g.*, *Lumbricus terrestris*, *Eisenia fetida*). Gigantism in worms may, in principle, represent an evolutionary advantage in nutrient-deficient soils by permitting enhanced nutrient absorption due to a longer gut, but experimental support for this notion is lacking (*see* below).

**CLITELLM MORPHOLOGY**

In addition to their unusual length, a striking morphological feature of mature *Rhinodrilus priollii* specimens is their saddle-like clitellum which can measure up to ~3 cm in diameter, almost twice the diameter of a typical midbody segment. Histological sections revealed the underlying source of this biomass, namely collagenous layers that separated outer muscle layers from the epidermis. These collagenous matrices are likely transient structures associated with reproduction, based on their absence in clitella of immature specimens or midbody segments. Note that comparable collagenous material has not been described in other earthworm clitella; rather, elongated albumen-secreting cells appear in a similar position and are major contributors to clitellum swelling (Grove, 1925; Lufty, 1965). Possibly, *R. priollii* specimens examined in this study had recently secreted cocoons thus explaining the absence of albumen-secreting cells, though the abundance of granulated Type II/III-like cells throughout the clitellar epidermis does not support this hypothesis (Grove and Cowley, 1927; Sayers *et al.*, 2009). Alternatively, albumenotrophic glands may be positioned elsewhere in the reproductive system (*e.g.*, prostates; Omodeo, 2000). A detailed examination of secreted *R. priollii* cocoons and reproductive structures may reveal the basis of this apparent morphological difference.

**TABLE 1.—Description of field sites and number of *Rhinodrilus* specimens examined**

<table>
<thead>
<tr>
<th>Field site</th>
<th>Geographic coordinates</th>
<th>Number examined</th>
<th>CO1 haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instituto Nacional de Pesquisas Amazonia (INPA)</td>
<td>02°37′33″S 60°02′39″W</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>RPPN (near Encontro das Aguas)</td>
<td>03°06′53″S 59°54′17″W</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Universidade Federal do Amazonas (UFAM) experimental farm</td>
<td>02°38′44″S 60°02′44″W</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Private farm (PF)</td>
<td>02°54′33″S 59°56′34″W</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

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*Fig. 2.—Map showing field site locations near Manaus, Brazil. Major roads, BR174 and AM-010, are indicated. INPA – Instituto Nacional de Pesquisas Amazonia; UFAM – Universidade Federal do Amazonas experimental farm; PF – private farm; RPPN – proximal to Encontro das Aguas. North is up*
Fig. 3.—Phylogeny of cytochrome c oxidase subunit 1 (CO1) haplotypes. Two major Rhinodrilus priolii lineages were resolved (INPA 1/ UFAM 1 and remaining haplotypes) that diverged by ~8% at the CO1 locus. Specimens collected at PF (private farm) were designated as *R. contortus* Cernosvitov 1938. Numbers in parentheses indicate sample size for each haplotype. The tree was rooted with *Enchytraeus albidus* CO1 (GenBank accession GU453370.1). Accession numbers for remaining sequences are: INPA1-3 (JF501460–JF501462); UFAM1-3 (JF501463–JF501465); PF1,2 (JF501458, JF501459); RPPN (GU014169).

Fig. 4.—Representative transverse section of the mid-clitellar region in a sexually mature *Rhinodrilus priolii* specimen. Collagenous connective tissue comprised the bulk of the biomass between epidermal and muscle layers. One half of the complete section is shown (arrow indicates the dorsal midline). Scale bar 1 mm.
In principle, collagenous layers observed in the *Rhinodrilus priollii* clitellum may have dual functions. First, the volumetric expansion of the clitellum leads to a linear increase in epidermal surface area, thus providing additional physical space for cocoon secretory glands. Secondly, the diameter of the secreted cocoon sheath will consequently be larger than the body, thus facilitating cocoon deposition via its characteristic sliding over smaller, anterior segments to remove the cocoon sheath (see Stephenson, 1930; Coleman and Shain, 2009).

Secretory glands observed in peripheral regions of the collagenous layer and epidermis were morphologically similar to Type II/III cells reported previously in the leech, *Theromyzon tessulatum* (Sayers et al., 2009). In contrast to those cells, however, *Rhinodrilus priollii* glands displayed granules that stained with both alcian-blue (outer granule) and azocarmine (inner granule) in Masson’s stain, suggesting a proteinaceous core surrounded by a glycosylated external layer. In *T. tessulatum*, Type II cells (glycosylated) and Type III cells (proteinaceous) give rise to opercula and cocoon membrane structures, respectively (Sayers et al., 2009). Thus, the apparent fusion of Type II and Type III granules observed in *R. priollii* suggests a different mechanism of cocoon construction that will require further investigation. Note that histological staining of comparable cells in other species using hematoxylin and eosin (H&E) and other stains did not resolve the morphologically indistinguishable Type II and Type III cells (blue and red in Masson’s stain, respectively; Sayers et al., 2009), and thus meaningful comparisons are difficult to make between *R. priollii*...
and cocoon secretory cells reported in older literature (e.g., Grove and Cowley, 1927; Defretin and Demailly, 1953; Lufty, 1965; Fleming and Baron, 1982).

**EVOLUTIONARY CONSIDERATIONS**

Using CO1 molecular clock variance values (e.g., Knowlton et al., 1993; Chang et al., 2008), the relative depth of *Rhinodrilus priollii* populations based on CO1 haplotype divergence (~8%) suggests they have occupied the Amazon Basin over the past 1–2 million years. *Rhinodrilus priollii* individuals are capable of moving considerable distances based on deep structures detected.

Fig. 6.—Dorsal regions of the *Rhinodrilus priollii* clitellum contained multiple collagenous layers. In addition to proteinaceous scaffolding (arrowheads), a thicker proteinaceous boundary (arrow) separated collagenous (Col) layers. CM – circular muscle, LM – longitudinal muscle. Scale bar 500 μm.
within populations (see Fig. 3). Active dispersal by crawling is the most obvious mechanism by which _R. priollii_ populations have expanded, but passive dispersal by transient run-off streams (as observed in this study), and possibly in seasonal or permanent waterways, cannot be excluded as an important dispersal mechanism. Taken together, the current geographic range of _R. priollii_ is likely to extend well beyond the area surveyed here, and additional sampling may reveal an ancestry considerably older than what has been determined here.

Giant earthworms have arisen independently and on multiple occasions, based on their higher-ranked taxonomic positions (e.g., Moniligasteridae, Microchaetidae, Glossoscolecidae, Megascolecidae; Blakemore _et al._, 2005). Further, they inhabit various environments, climatic regimes and geographic regions (e.g., Australia; Vietnam; South Africa, North America, South America, etc.), leaving open the question as to what underlying mechanism(s) or environmental factor(s), if any, lead to gigantism. In other animals (e.g., insects, mammals), gigantism has been explained as a consequence of elevated growth hormone levels (Nijhout, 1998; Maheshwari _et al._, 2000), a phenotype that is relatively rare within Animalia. By analogy, stochastic variation in growth hormone levels across the Clitellata as a consequence of random genetic variance may explain the occasional and punctuated (Eldredge and Gould, 1972) appearance of giant earthworm species in a variety of habitats worldwide.

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